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TARGETED DISRUPTION OF THE RIβ AND RIIβ REGULATORY SUBUNITS OF THE cAMP-DEPENDENT PROTEIN KINASE (PKA) IN MICE: PHYSIOLOGICAL AND NEUROBIOLOGICAL DEFECTS

by

Eugene Paul Brandon

A dissertation submitted in partial fulfillment of the requirements for the degree of

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University of Washington

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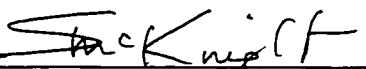
Approved by 
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Abstract

TARGETED DISRUPTION OF THE RIβ AND RIIβ REGULATORY SUBUNITS OF THE CAMP-DEPENDENT PROTEIN KINASE (PKA) IN MICE: PHYSIOLOGICAL AND NEUROBIOLOGICAL DEFECTS

by Eugene Paul Brandon

Chairperson of Supervisory Committee:
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The cAMP-dependent protein kinase (PKA) mediates the intracellular responses to myriad extracellular signals in eukaryotic organisms. Although the functions of this enzyme have been extensively studied, little is known about the roles of specific isoforms of PKA. In order to determine the roles of the regulatory subunits RIβ and RIIβ, gene targeting technology was utilized to establish mice that carry null alleles of either the RIβ or the RIIβ genes.

Previous experiments had suggested important roles for PKA in forms of hippocampal synaptic plasticity that may underlie learning in mammals. Both long-term potentiation (LTP) and long-term depression (LTD) were analyzed in the hippocampal slices prepared from mice lacking the RIβ subunit. No defects were observed in LTP at the Schaffer collateral-CA1 synapse, but a dramatic defect was observed in homosynaptic LTD, suggesting an important role for RIβ and PKA in this phenomenon.

PKA is the downstream effector of several neurotransmitters including dopamine. The dopaminergic projections to the mammalian striatum are important in regulation of motor function including coordination and locomotion. Because RIIβ is expressed at high levels in the striatum, it was hypothesized that this subunit might play a specialized role in dopaminergic signaling. Mice lacking RIIβ were found to have defects in both rotarod performance (a measure of coordination) and activation of locomotion by dopaminergic agonists. Moreover, these mice had changes in both expression of the gene encoding the neuropeptide dynorphin and the induction of the

immediate early gene c-fos by amphetamine in the striatum. These results demonstrate that the Type IIβ PKA is a direct mediator of gene induction in response to a dopaminergic agent, and suggest that Type IIβ PKA is responsible for the fine tuning of motor behavior.

Lastly, mice lacking RIIβ were found to have reduced adiposity and increased metabolism as measured by oxygen consumption and body temperature. These findings are believed to reflect a functional switch from Type IIβ PKA to Type Iα PKA in the adipocytes of these mutants. Collectively, these results begin to elucidate some of the specific roles of the different PKA regulatory subunits in mice.

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List of Non-Standard Abbreviations

AKAP	A-kinase anchoring protein
ATP	adenosine triphosphate
BAT	brown adipose tissue
bp	base pairs
cAMP	cyclic-3',5'-adenosine monophosphate
DA	dopamine
ES	embryonic stem
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HFS	high frequency stimulus
kb	kilobase pairs
KD	kilodaltons
LFS	low frequency stimulus
LTD	long-term depression
LTP	long-term potentiation
MSN	medium spiny neuron
PKA; cAPK	protein kinase A
UCP	uncoupling protein
WAT	white adipose tissue

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I would like to express my appreciation to both G. Stanley McKnight and Rejean L. Idzerda for their guidance and support throughout my time in the laboratory. Additionally I would like to thank the technicians in the laboratory whose help facilitated these projects immensely, including Thong Su, Kirstin A. Gerhold, Kelly M. Millett, and Mona Belyamani. Lastly, I am greatly indebted to the many collaborators who performed extensive analysis on the mutant mice and contributed invaluable intellectual input including: Ming Qi, David E. Cummings, Kouros Motamed, Josep Planas, Paul S. Amieux, Kimberly A. Burton, Yan-You Huang, Min Zhuo, Roussadan Bourtchouladze, Eric R. Kandel, Sheree F. Logue, Jeanne M. Wehner, Monique R. Adams, Elena Chartoff, Alan S. Unis, Daniel M. Dorsa, Joshua A. Gordon, Takao K. Hensch, Michael P. Stryker, Annika Malmberg, Allan Basbaum, Suzanne Appleyard, Charles Chavkin, Carol A. Robbins, and Philip A. Schwartzkroin.

Dedication

To my parents, Ann M. and D. David Brandon,
and my brother, Seth D. Brandon (who took care of me through graduate school)

Background

The protein kinase A family. Activation of second-messenger coupled cell surface receptors is a common mechanism by which the presence of an extracellular hormone or neurotransmitter is transduced into an intracellular change. One of the principle second messengers generated by these receptors in multicellular eukaryotic organisms is cyclic-3',5'-adenosine monophosphate, or cAMP. Cyclic AMP can directly activate ion channels in some types of cells, but a more ubiquitous mechanism of cAMP signaling is through activation of the cAMP-dependent protein kinase, also known as protein kinase A, or PKA. Each PKA molecule is a tetrameric holoenzyme that consists of two homodimeric regulatory (R) subunits each bound to a catalytic (C) subunit. When cAMP binds to the regulatory subunits, the catalytic subunits are released, and are then activated to perform the kinase function, which is covalent phosphorylation of specific protein substrates on serine and threonine residues. Reversible covalent phosphorylation is one of the most important mechanisms by which cellular, and therefore organismal, physiology is regulated (Krebs, 1989).

PKA actually exists as a family of proteins, and in mammals there are four known regulatory subunits (RIα, RIβ, RIIα, RIIβ), and two catalytic subunits (Cα, Cβ), each encoded by a unique gene (McKnight et al., 1988). The Type of PKA holoenzyme is defined by its regulatory subunits (eg. RIIβ-containing PKA is called Type IIβ PKA). In some cases, multiple isoforms of the subunits are believed to be derived by differential splicing of the transcripts, the alternative isoforms of the Cβ gene being the best characterized (Wiemann et al., 1991; Qi et al., 1996). The evolutionary conservation of multiple isoforms of PKA raises the very interesting question: do the various PKA subunits subserve unique functions, and if so, what are those specific functions? That different extracellular ligands utilizing the

common pathway of cAMP and PKA can effect different intracellular responses suggests that a unique set of substrates may be phosphorylated in response to each ligand. Yet many proteins expressed within any given cell contain the common PKA target sequence motif of -R-R-X-S/T-. It is possible that the multifarious substrate proteins are differentially phosphorylated with specific spatial and temporal constraints, and that these constraints are dictated by the various isozymes of PKA (Doskeland et al., 1993; Walsh and Van Patten, 1994). One very intriguing possibility is that the specific isoforms of PKA have unique properties, and that they are differentially activated (or inhibited) by each hormone or neurotransmitter.

Some unique properties of the different isoforms of PKA have already been discerned. For example, the Type I (those containing RI subunits) and Type II (those containing RII subunits) PKA holoenzymes show differential association with the A-kinase anchoring proteins (AKAPs) that are believed to dictate the subcellular localization of the PKA holoenzyme (Rubin, 1994). In fact all of the AKAPs that have been described to date preferentially bind RII subunits, but there is evidence that Type I PKA also has specific subcellular distribution (Skalhegg et al., 1994), likely due to the association of the RI subunits with as yet unknown kinase anchoring proteins. Thus, the specific subcellular distribution of the various isozymes of PKA in association with certain receptor/signaling complexes and/or substrates may play an important role in determining the specificity of the intracellular effects of extracellular signals.

The different isoforms of PKA may also have unique activation properties. For example, it has been shown that RIβ-containing Type I PKA is activated at a lower threshold of cAMP than RIα-containing Type I PKA (Cadd et al., 1990). Additionally Cβ-containing Type II holoenzyme has a lower threshold of activation than Cα-containing holoenzyme (Gamm et al., 1996). And Type I PKA appears to have a

lower threshold than Type II PKA (Cummings et al., 1996). Thus, the complement of subunits in any given holoenzyme may play an important role in determining the specific activation kinetics of that molecule.

Another possible mechanism to dictate the specificity of the intracellular cAMP signal might be the differential ability of the various catalytic subunits to phosphorylate various substrates. While no evidence for this mechanism has been discovered so far, it remains as a reasonable possibility (Walsh and Van Patten, 1994).

One of the primary goals of the research described herein was to determine if the specific regulatory subunits of PKA subserve specific functions. By rendering the genes for RIβ and RIIβ non-functional through gene targeting technology, we were able to find unique functions in the whole animal. A logical progression of this research is to analyze the effects of loss of specific isozymes of PKA at the cellular level, by performing experiments on primary cells in culture prepared from mice lacking the specific regulatory subunits. Ultimately, the role a specific subunit in a specific function should be tested by *replacement* of that subunit with another subunit at essentially the same cellular concentration. This will require technology that is still fairly early in its development. Nevertheless, we have discovered biochemical, physiological, and behavioral functions in the mouse that require specific regulatory subunits of PKA, as described below.

Roles for PKA in learning. PKA has been shown to play an important role in learning in the invertebrate organisms Aplysia and Drosophila. The Aplysia is a simple marine invertebrate with a fairly well defined, and easily studied nervous system. It shows rudimentary forms of learning such as sensitization and habituation. Because these behaviors remain observable in the partially dissected preparation (with the nervous system exposed), this animal has been an excellent resource for the study of the pharmacology, biochemistry, and neurophysiology that underlies learning. Studies along these lines have found that PKA is involved in short-term learning by means of its

activation by serotonin and consequent phosphorylation of a presynaptic potassium channel (Kandel and Schwartz, 1982; Shuster et al., 1985). Additionally, a role for PKA in longer-term memory has also been described, and this function is attributed to degradation of the regulatory subunits of PKA, and a consequent increase in basal PKA tone and phosphorylation of PKA substrates (Bergold et al., 1992). The changes in behavior are associated with changes in synaptic strength, and thus molecular mechanisms underlying the two have been correlated as well. The longest lasting memories in this organism are believed to require gene induction events, some of which are mediated through PKA phosphorylation of the CREB transcription factor, and activation of promoters containing CRE sequences (Dash et al., 1990; Byrne et al., 1991; Kaang et al., 1993; Bailey et al., 1994).

The common fruit fly Drosophila has also proved to be very useful in understanding molecular mechanisms of learning. While the neuroanatomy in this organism is not as simple as that of the Aplysia, and is still being worked out, it has the advantage of having a well understood genome, and genetic experiments in this organism have provided additional insights into the mnemonic processes. Following induced random mutagenesis, flies were screened for learning defects, and several critical loci identified. Interestingly, upon characterization these turned out to encode enzymes in the cAMP pathway, including an adenylyl cyclase (rutabaga) and a phosphodiesterase (dunce) (Dudai, 1986). No PKA mutants were discovered in these original screens, and in retrospect the reason may have been that PKA null flies might expire during embryogenesis (Jiang and Struhl, 1995). However, more recent experiments with transgenic flies expressing either dominant inhibitory PKA proteins (Drain et al., 1991) or modifications in the catalytic subunit of PKA (Skoulakis et al., 1993) have revealed an important role for this enzyme in learning in Drosophila as well.

In contrast to the abundance of data suggesting an important role for PKA in learning in invertebrates, very little has been

determined regarding a role in mammals. This has largely been due to the limitations of pharmacological manipulation of the mammalian central nervous system, and the fact that reverse genetic technologies have only recently been developed. However, genetic manipulation of related serine/threonine protein kinases has recently been performed, and has suggested that these enzymes are important in this function.

Specifically, mice lacking an isoform of the calcium-calmodulin-dependent protein kinase (αCaMKII) have been developed, and these mice have a specific defect in spatial learning (Silva et al., 1992a). Mice overexpressing αCaMKII also have defects in spatial learning, suggesting specific regulation of this enzyme is critical for its function in this regard (Bach et al., 1995). Mice lacking an isoform of the phorbol ester/calcium-dependent protein kinase (PKCγ) also have shown a defect in spatial learning (Abeliovich et al., 1993b).

Although a role for PKA has not been demonstrated, both upstream and downstream components of the cAMP signaling pathway have been shown to be important in mammalian learning. Once again, using gene disruption technology, mice lacking a specific isoform of adenylyl cyclase (ACI), the enzyme which generates the cAMP that activates PKA, were shown to have a subtle defect, perhaps best attributed to spatial memory (Wu et al., 1995). Mice lacking two isoforms of one of the most thoroughly studied substrates of PKA, the transcription factor CREB, also were found to have a defect, specifically in long-term memory (Bourtchuladze et al., 1994).

Thus, as described in the Conclusions chapter below, the mice lacking the RIβ and RIIβ subunits of PKA were tested in various learning paradigms. To date, no defects in learning have been found in these mice. This does not preclude a role for PKA in mammalian learning, as the genetic ablations of specific subunits may not disrupt the overall PKA levels to a sufficient degree to disrupt this important function of the organism. Behavioral analysis of mice expressing a transgene encoding the dominant negative regulatory subunit of PKA

(R_{AB}) should prove to be rather informative (Clegg et al., 1987; Nguyen et al., 1995).

Roles for PKA in synaptic plasticity. It is widely believed that memories are encoded in the central nervous system as modifications in synaptic strengths, although this hypothesis remains largely unproved. The hippocampus is a brain structure believed to be critical in the formation of memories, especially spatial and/or declarative memories, in mammals (Squire, 1987). Thus, many neuroscientists believe that understanding the molecular mechanisms underlying the modification of synaptic strengths in the hippocampal circuitry should provide insights into the molecular mechanisms underlying mammalian learning.

The two principle forms of synaptic modification that have been studied in the hippocampus, both in the slice preparation and in vivo, are termed long-term potentiation (LTP) and long-term depression (LTD). There are several variants of each of these phenomena, and they are induced by different stimulation paradigms at different synapses. Generally LTP is a use-dependent increase in synaptic efficacy induced by high frequency stimulation (HFS) of the presynaptic axons, and LTD is a use-dependent decrease induced by low frequency stimulation (LFS). LTP has been broken down into different phases, most commonly as an early phase of long-term potentiation (E-LTP or STP) and a late phase (L-LTP), and these different phases have unique biochemical properties. It is hypothesized that in behaving animals, learning may occur in the hippocampus through both LTP-like increases and LTD-like decreases in synaptic strengths (Stevens, 1996).

L-LTP at the Schaffer collateral-CA1 synapse in the hippocampus is believed to require PKA activity, while E-LTP appears to be independent of PKA activity (Frey et al., 1993; Matthies and Reymann, 1993; Huang and Kandel, 1994). In contrast to this pharmacological dissociation of the phases of LTP at the Schaffer

collateral-CA1 synapse, in mossy fiber-CA3 LTP, both phases appear to require PKA activity (Huang et al., 1994).

Unlike its role in LTP, very little has been determined about the role of PKA in LTD. It has been determined that phosphatases play an important role in LTD (Mulkey et al., 1993). Additionally, the phosphatase inhibitor that is activated by PKA-dependent phosphorylation (I1) is believed to be important in this phenomenon, so it would not be unexpected to find a role for PKA in LTD (Mulkey et al., 1994).

In conjunction with determination of the roles of the various PKA subunits in learning, we sought to determine the roles in hippocampal synaptic plasticity. In this way, the results between the two functions could be associated or dissociated, providing another test of the relationship between the two. Previously, analyses of this sort have provided correlations between defects in learning and defects in hippocampal synaptic plasticity, supporting the hypothesis that LTP- and LTD-like phenomena may reflect the biochemical changes that underlie learning in mammals. As described in Chapter II and the Conclusions chapter below, the RIβ mutants did not show this association, and begin to challenge the relevance of these in vitro phenomena as worthwhile models for understanding the biochemistry that actually underlies learning.

PKA in neuropharmacology. As mentioned above, PKA is directly downstream of receptors that respond to extracellular ligands. The members of the large family of seven transmembrane receptors are each coupled to G-proteins that when activated by ligand-bound receptor consequently exchange GDP for GTP, and in turn activate various second-messenger generating enzymes. Among the second messenger generating enzymes is adenylyl cyclase, which transforms ATP into cAMP. G_s-type G-proteins activate adenylyl cyclase, while G_i-type G-proteins are inhibitory to this enzyme. Thus, receptors known to be G_s coupled will activate PKA, and in the nervous system these include adenosine receptors A2a and A2b, norepinephrine

receptors β1 and β2, dopamine receptors D1 and D5, serotonin receptors 5HT4, 5HT6, and 5HT7, as well as some of the receptors for the neuropeptides corticotropin releasing factor (CRF), vasopressin, and vasoactive intestinal peptide (VIP). G_i coupled receptors will inhibit PKA activity, and these include A1 adenosine receptors, α2 norepinephrine receptors, CB1 cannabinoid receptors, D2, D3, and D4 dopamine receptors, several of the metabotropic glutamate receptors, several of the serotonin receptors, M2 and M4 muscarinic acetylcholine receptors, μ and δ opioid receptors, as well as receptors for the neuropeptides somatostatin and neuropeptide Y. Thus, pharmacological experimentation with mice lacking the various PKA subunits provides a fertile ground for research into the specific roles of the PKA isoforms in signal transduction of any of these neurotransmitters or neurohormones as discussed above. For example, it is largely unknown if any of the various PKA isoforms are more important than others in signaling through any of these receptors in the nervous system. Is it possible that the opioid morphine requires Type IIβ PKA but not Type Iβ PKA for its effects? Would mice lacking RIIβ be unresponsive to morphine? Studies described here begin to elucidate some of the differential roles of the PKA isozymes in neuropharmacology.

Targeted disruption technology. As recently as ten years ago, the idea of creating mouse strains carrying specifically engineered mutations in their genomes remained as fantasy. It was the marriage of targeted mutagenesis through homologous recombination (Smithies et al., 1985) in cultured cells, and the ability to maintain in culture multipotent embryonic stem cells that could eventually contribute to the germ line of chimeric mice (Bradley et al., 1984) that facilitated this novel approach to mammalian genetics (Capecchi, 1989). Since the first description of successful germ-line transmission of a specifically targeted mutation (Thompson et al., 1989), the technique has had a tremendous impact on biomedical research (Brandon et al., 1995b). Despite the great cost involved in generating and maintaining

null mutants by gene targeting, use of this technology has become surprisingly commonplace today. While in many cases the initial observations from knockout mice open up more questions than they answer, follow-up studies of knockout mice, or primary cells derived from these mice, should prove to be increasingly informative.

Research Aims

While PKA has been studied extensively over the last few decades, very little is known about the roles of specific isozymes of PKA (determined by their complement of regulatory and catalytic subunits) in mammalian physiology. To determine the necessity of various isozymes, targeted disruption of the genes encoding specific subunits of PKA was performed. Mice with "knockouts" of either RIβ or RIIβ were established and analyzed. These mice represent a novel tool for research directed toward understanding the roles of PKA, and specific subunits of PKA, in myriad mammalian functions. Also described here are some of the first analyses which begin to provide clues as to these various functions.

1. Targeted disruption of the genes encoding RIβ and RIIβ. Embryonic stem cells were derived to facilitate the establishment of mice carrying targeted mutations in the various PKA subunits. The derivation of the REK "parental" ES cell lines from 129 strain blastocysts and the targeting of both the RIβ and RIIβ genes in these cells are described in Chapter I.

2. Electrophysiological and behavioral analysis of RIβ mutant mice. To further understand the role of PKA and to determine if a specific requirement for RIβ exists in hippocampal synaptic plasticity, mice carrying a targeted disruption of the gene encoding this subunit were established. Germ-line transmission of the cells described in Chapter I to create the novel strain of $RI\beta^{-/-}$ mice is described in Chapter II. Additionally, electrophysiological analysis, including hippocampal LTP and LTD was performed, also described in

Chapter II. Behavioral assessment has also been carried out, and is described in the Conclusions chapter.

3. Neuropharmacological and behavioral analysis of RIIβ mutant mice. To begin to determine the roles of the RIIβ subunit of PKA in the whole animal, mice carrying a targeted disruption of the gene encoding this subunit were established. Germ-line transmission of the cells described in Chapter I to create the novel strain of $RII\beta^{-/-}$ mice is described in Chapter III. Like those carrying a targeted disruption in RIβ, these mice were found to be overtly normal. Because of the high expression of this subunit in the striatum, functions related to this structure were assessed. Motor coordination was determined with the rotarod apparatus. Locomotor activation by dopaminergic agents was also determined. To begin to correlate the defects at the level of the organism with molecular changes, gene expression studies were performed to determine the inducibility of the immediate early gene c-fos, and the expression of the gene encoding the striatal neuropeptide dynorphin. These studies are all described in Chapter III. Lastly, learning studies have been carried out on these mice as well, and these are discussed briefly in the Conclusions chapter.

4. Metabolic analysis of RIIβ mutant mice. In addition to the high expression of the RIIβ subunit in the nervous system, this subunit is also found at high levels in the adipose tissue of mice. The role of this subunit in this tissue was assessed through analysis of RIIβ knockout mice. As discussed in Chapter IV, these mice were found to have reduced adiposity, a compensatory increase in RIα in adipose tissue, and effectively a switch from Type I to Type II PKA. Consequently, the PKA in this tissue appears to be activated at lower thresholds of cAMP, and various downstream effects of PKA, including expression of uncoupling protein in brown adipose tissue, appear to be activated to a greater extent in the RIIβ mutant mice. The mice were also found to have an increased basal metabolic rate, suggesting an important role of Type IIβ PKA in maintaining normal homeostasis.

CHAPTER I

DERIVATION OF NOVEL EMBRYONIC STEM CELL LINES AND TARGETING OF CYCLIC AMP-DEPENDENT PROTEIN KINASE GENES

Introduction

Cyclic AMP-dependent protein kinase (cAPK) is the principle target of cAMP in most animal cells, and thus mediates cellular responses to hormones that affect levels of cAMP. In mice, the cAPK family of isozymes includes four regulatory subunits termed RIα, RIβ, RIIα, and RIIβ, and two catalytic subunits termed Cα and Cβ (McKnight et al., 1988). While information is available on the expression patterns and biochemistry of the various subunits, the question of whether the specific subunits play unique roles in intracellular signaling remains largely unanswered. To address this issue, we are creating mice that have null mutations in each of the genes encoding the cAPK subunits, and will assess the biochemistry, physiology, and behavior of these novel strains. To create the mice, we are utilizing the recently developed technology of whole animal gene targeting which involves introduction of genetically manipulated embryonic stem (ES) cells into the germ line of chimeric mice (Rossant and Joyner, 1989). This technology requires that the ES cells maintain the ability to contribute to the germ line of chimeric mice despite repeated passage and manipulation *in vitro*. In general, early passage ES cells contribute to the germ line more readily than late passage cells, probably due to the accumulation of chromosomal abnormalities that occurs in culture. Because it is advantageous to use early passage cells, we derived new ES cell lines to expedite the generation of mice that lack the various cAPK subunits.

We have used one of these cell lines to create sublines, and subsequently mouse strains, carrying null mutations in the cAPK

genes, RIβ and RIIβ. The resulting phenotypes are currently being analyzed. Here we describe the establishment of the cell lines and their frequency of contribution to the germ line of chimeric animals.

Experimental Procedures

Production of ES cell lines. ES cells were derived using previously published protocols (Robertson, 1987; Abbondanzo et al., 1993). 129Sv/J mice (The Jackson Laboratory, Bar Harbor, ME) were mated and blastocysts harvested from females approximately 3.5 days postcoitum were placed in tissue culture on a monolayer of fibroblast feeder cells. After 4 days in culture, the inner cell masses (ICMs) were physically dislodged from the surrounding trophoblast and feeder cells using micromanipulators and a mouth-controlled pipette. The ICMs were digested in microdrops of trypsin-EDTA solution containing 1% chicken serum for approximately 10-15 minutes at room temperature, triturated, and replated on a fresh confluent feeder layer. Colonies were picked from 6 to 18 days following the dissociation procedure, and transferred to feeder layers in multiwell dishes. These were inspected and those that had the characteristic ES cell morphology and continued to grow in culture were expanded onto larger monolayers. Four colonies (one picked 10 days and three picked 18 days following ICM dissociation) were designated as candidate ES cells, were maintained in culture, frozen in aliquots, and injected into blastocysts to determine germ line competence. The gender of the cell lines was determined by a DNA dot blot with a mouse Y chromosome-specific probe (generously provided by Richard D. Palmiter, University of Washington). Because chromosomal integrity is believed to be important for ES cells to contribute to the germ line, karyotype analysis of these cells was performed.

Gene targeting. Gene targeting was performed using standard techniques (Capecchi, 1989). Targeting vectors for RIβ and RIIβ were constructed from genomic fragments isolated from BALB/c and 129 strain genomic libraries, respectively. The RIβ vector has a neomycin

phosphotransferase cassette inserted in exon 3 and a thymidine kinase cassette outside the genomic fragment (Figure 1.1). The RIIβ targeting vector has the neomycin cassette substituted for 2 kb of genomic sequence, which includes exon 1 and the start site of translation. This vector does not have the negative selection marker. Newly derived ES cells were electroporated with each of these vectors, and recombinant cells were selected with 180-200 μg/ml G418 and 2 μM ganciclovir (RIβ) or G418 alone (RIIβ). Colonies were picked and isolated, and screened by genomic Southern blot (Ram'irez et al., 1992). Clones that had undergone the specific homologous recombination were injected into blastocysts which were then transferred to pseudopregnant foster mothers. Male chimeras with greater than 30% contribution of ES cells to their skin (as determined by coat color) were bred with C57BL/6 females to determine germ line contribution of ES cells.

Results and Discussion

Four pluripotent ES cell lines were isolated and named REK1, REK2, REK3, and REK4. It is of note that three of these were not picked from the cultures of dissociated ICM cells until 18 days after dissociation, rather than the 7-10 days recommended in the published protocols. No cells with the ES morphology were observed in the many other colonies picked, nor did these other colonies continue to proliferate. Each of the four ES cell lines contained some cells with non-ES morphology, ranging from approximately 5% to 40% of the total cells. While ES cells grow as indistinguishable members of a smooth colony, these contaminating non-ES cells were individually distinct, growing in a monolayer with a cobblestone-like appearance. The prevalence of these cells in subclones picked during the subsequent targeting procedure was more variable than in the parent cell line.

REK2 and REK3 were determined to be mostly euploid, XY cell lines by karyotype analysis (Table 1.1). REK1 and REK4 were found to be positive by dot blot analysis using a Y chromosome-specific probe. As expected, the XY cells masculinized injected XX blasts as shown by

a skewing of the sex ratio of chimeras toward male. Each of the cell lines produced chimeras, demonstrating they were indeed pluripotent ES cells. Because the 129Sv/J cells carry both the A^w (agouti) and *c* (albino) loci, which act in different cell types, chimeras had agouti coat color where the ES cells contributed to the follicular lineage, except where the ES cells contributed to the melanocyte lineage and albinism obscured the underlying follicular phenotype (Silvers, 1979). Weak positive correlations were seen between coat color chimerism and germ line chimerism (Table 1.2 and data not shown). Chimeras with greater than 30% of their coat color contributed by the ES cells were bred, and REK1, REK3, and REK4 were found to be germ-line competent by virtue of their ability to produce agouti offspring when bred with C57BL/6 females (Tables 1.1 and 1.2).

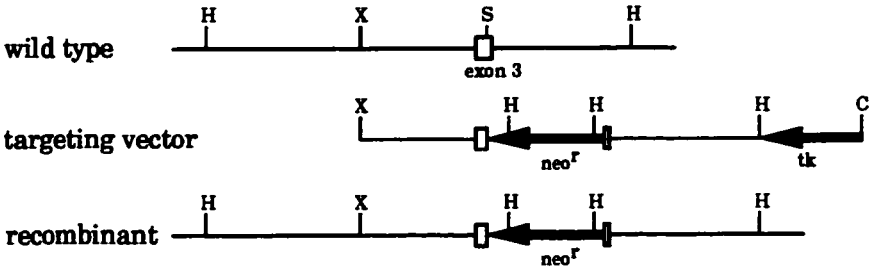
Concurrent with the determination of the germ line competence of the parental ES cell lines, gene targeting was carried out in the REK2 cell line, chosen based upon its karyotype and morphology. Six cell lines carrying the RIβ mutation were isolated from REK2 cells using the positive-negative selection strategy (Capecchi, 1989). These were used to make chimeric mice which were bred to create mice heterozygous for the mutation. Three of the cell lines showed germ-line competence in all of the high-percentage male chimeras bred (Tables 1.1 and 1.2). Using the RIIβ targeting vector and only positive (G418) selection, two cell lines carrying RIIβ mutations were derived from REK2 cells and one of these has passed through the germ line in three out of five high percentage chimeras bred (Tables 1.1 and 1.2).

The establishment of mouse strains carrying mutations in the cAPK genes allows us to study the function of the various subunits in multiple tissue and cell types. For example, because RIβ and RIIβ are both expressed at high levels in the nervous system, mutant mice may have altered neuronal biochemistry. Primary cultures of neuronal cells will be derived from mutant mice to determine if specific isozymes of cAPK are required for responses to neurotransmitters, hormones, and other stimuli. To determine whether synaptic connectivity and

plasticity are affected, neurophysiological paradigms will be tested in brain slice preparations. Because neuronal properties ultimately underlie behavior, the mice will be tested in behavioral paradigms as well. Thus, experiments at multiple levels may establish exciting new connections between molecular and cellular biology and whole animal physiology and behavior.

Figure 1.1. Schematic diagram of the RIβ and RIIβ genes targeted for mutation. Illustrated are the wild type endogenous allele, the targeting construct, and the predicted structure of the recombinant allele. In the RIβ targeting vector, the neomycin resistance gene interrupts the coding region of exon 3 at a Sgr AI site. For RIIβ, the neomycin resistance gene replaces a 2 kb Rsr I/Aat II fragment which includes exon 1 and the translational start site. Restriction site abbreviations: A (Aat II), E (Eco RI), H (Hind III), R (Rsr I), S (Sgr AI), and X (Xba I).

RIβ gene:



RIIβ gene:

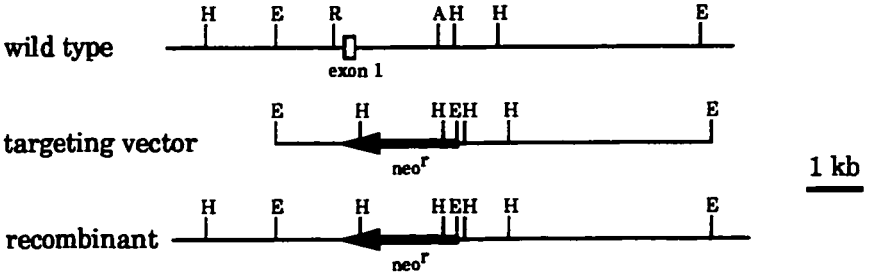


TABLE 1.1

Germ-line transmission of ES cell lines

Cell Line	Karyotype (early passage)	# Adult Chimeras ≥ 30%	% Male	Germ Line Chimeras/ Total Bred[a]
REK1	ND	7	86%	1/2
REK2	40, XY (60-100%)	5	80%	0/3
REK2-RIβ[b]	ND	15	73%	5/8
REK2-RIIβ[c]	ND	14	93%	3/7
REK3	40, XY (100%)	3	100%	2/2
REK4	ND	5	60%	1/3

[a] some chimeras have only recently begun breeding

[b] includes 6 independent cell lines

[c] includes 2 independent cell lines

TABLE 1.2

High Percentage Chimeras From Germ Line-Competent ES Cell Lines

Chimera	ES Cell Parent Line	Disrupted Line	% Chimerism (Agouti;Albino)	Agouti Pups/ Total Pups
REK1-1	REK1 p1	--	80;0	0/12
REK1-2	REK1 p1	--	95;5	20/20
REK2-10	REK2 p17	--	50;30	0/58
REK2-4	REK2 p9	--	75;20	0/80
REK2-9	REK2 p17	--	70;5	0/26
REK3-3	REK3 p12	--	70;5	25/62
REK3-4	REK3 p12	--	70;20	18/18
REK4-2	REK4 p5	--	60;1	0/47
REK4-4	REK4 p18	--	40;60	14/44
REK4-5	REK4 p18	--	40;10	0/31
RIβ A4-1	REK2 p7	A4 p6	55;2	3/52
RIβ B1-1	REK2 p7	B1 p4	60;40	3/3
RIβ B1-3	REK2 p7	B1 p4	95;2	5/33
RIβ B1-4	REK2 p7	B1 p4	60;0	15/30
RIβ C6-2	REK2 p7	C6 p7	30;0	5/29
RIIβ 4C9-1	REK2 p6	4C9 p4	40;1	2/14
RIIβ 4C9-10	REK2 p6	4C9 p8	85;5	0/5
RIIβ 4C9-3	REK2 p6	4C9 p4	90;5	9/26
RIIβ 4C9-4	REK2 p6	4C9 p4	40;0	1/2
RIIβ 4C9-9	REK2 p6	4C9 p8	90;5	0/11

CHAPTER II

HIPPOCAMPAL LONG-TERM DEPRESSION AND DEPOTENTIATION ARE DEFECTIVE IN MICE CARRYING A TARGETED DISRUPTION OF THE RIβ SUBUNIT OF PROTEIN KINASE A

Introduction

Little is known about the molecular mechanisms underlying homosynaptic long-term depression (LTD), an electrophysiological phenomenon thought to reflect some of the biochemical processes used in mammalian learning (Linden, 1994; Malenka, 1994; Bear and Abraham, 1996). Like long-term potentiation (LTP), LTD requires an increase in intraneuronal calcium through NMDA receptors (Dudek and Bear, 1992; Mulkey and Malenka, 1992). However, in LTD a low level of calcium influx is believed to preferentially activate phosphatases (Lisman, 1989; Mulkey et al., 1993; Kirkwood and Bear, 1994; Mulkey et al., 1994; O'Dell and Kandel, 1994) while in LTP a larger influx of calcium is thought to activate kinases, including PKA (indirectly) which is critical for the late phase of LTP (Frey et al., 1993; Matthies and Reymann, 1993; Huang and Kandel, 1994; Huang et al., 1994).

In addition to mediating use-dependent changes in synaptic efficacy, PKA may be important for certain forms of learning, and is specifically involved in the switch from short- to long-term memory (Frank and Greenberg, 1994). Since several different isoforms of PKA exist, the involvement of this kinase in synaptic plasticity and learning raises the question: Do different types of learning-related neuronal changes require specific regulatory or catalytic subunits? In the mouse, there are four different regulatory (R) subunits (RIα, RIβ, RIIα, RIIβ) that bind cAMP, and two catalytic (C) subunits (Cα, Cβ) that phosphorylate substrate proteins when released from the R subunits

upon cAMP binding. In the nervous system, RIβ appears to be specific to neurons (Massa et al., 1991) and is expressed in many regions including the neocortex, the pyramidal layer of the hippocampus, and the Purkinje and granular layers of the cerebellum (Cadd and McKnight, 1989). To further elucidate the role of PKA in synaptic plasticity, and to determine if specific subunits serve unique intracellular signaling functions, we generated mice carrying a null mutation in the RIβ subunit through homologous recombination in embryonic stem (ES) cells.

Experimental Procedures

Generation of mutant mice. The targeting of embryonic stem cells and establishment of chimeras carrying the RIβ mutation has been previously described (Brandon et al., 1995a). Three of the four REK2-derived cell lines and one of the four D3-derived cell lines that produced fertile chimeras contributed to the germ line. Mouse genotypes were determined by Southern blot.

RNA, protein, and kinase assay. RNA was prepared by the guanidine HCl method, and Northern blot analysis was performed essentially as described (McKnight et al., 1988). Filters were hybridized as described to a ^{32}P-antisense riboprobe synthesized from BglII-linearized RIβ cDNA (Clegg et al., 1988). Protein extracts were prepared by dounce homogenization in PBS containing 250 mM sucrose, 1 mM EGTA, 4 mM EDTA, 0.5% Triton X-100, 4 mM DTT, 2 μg/ml leupeptin, 3 μg/ml aprotinin, 0.2 mg/ml soybean trypsin inhibitor, and 1 mM AEBSF at 0°C followed by brief sonication and centrifugation. Western blots were probed with antiserum to murine RI subunits, RIIβ (generously provided by C. S. Rubin), RIIα (generously provided by J. D. Scott), or C subunits (generously provided by B. Hemmings). Signal was detected by enhanced chemiluminescence (ECL). Homogenates were used for kinase assays as described (Clegg et al., 1987).

Histologic analysis and determination of transgene expression. Adult males were intracardially perfused with 4% paraformaldehyde in 100 mM sodium phosphate, pH 7.4. Brains were removed, post-fixed for 2 hours, and embedded in paraffin. Coronal sections (6 μm) were cut and stained with cresyl violet. Staining of 20 μm sections of brains from pRIβ-lac mice for β-galactosidase activity was performed with X-gal (Gibco BRL) and counterstained in nuclear fast red as previously described (Rogers et al., 1992).

Electrophysiology. Mice were on a C57BL/6 x 129 mixed strain background, age 4-6 weeks for LTD and depotentiation experiments, or age 5-20 weeks for LTP and paired-pulse facilitation experiments. All experiments used age-matched controls and were performed blind to genotype. The details of stimulation and recording are as previously described (O'Dell and Kandel, 1994). Stimulus intensity was adjusted to produce a response of approximately 1 mV amplitude, with an initial slope of approximately -0.5 mV/msec). Tetanic stimulation to produce CA1 LTP was 100 Hz for 1 second, delivered either once or 4 times with an intertrain interval of 4 minutes. For paired-pulse experiments, stimulation was set to produce 25% maximal response, and facilitation of response at various interpulse intervals (25-400 msec) was measured. Stimulus to produce LTD at both synaptic loci tested was 1 Hz for 15 minutes. In experiments with picrotoxin, the concentrations of $CaCl_2$ and $MgSO_4$ were both 4.0 mM. For depotentiation experiments, the stimulus to produce LTP was 100 Hz for 1 second, delivered 2 times with an intertrain interval of 20 seconds. This was followed by a low frequency stimulus (LFS) of 5 Hz for 3 minutes to produce depotentiation.

Results

Targeted disruption of the RIβ gene yields viable homozygotes. Electroporation of the RIβ targeting vector (Figure 2.1A) into embryonic stem cells followed by positive-negative selection (Capecchi, 1989; Ram'irez et al., 1992) produced four germ line-

competent ES cell lines with a disruption in the RIβ gene (Brandon et al., 1995a). Heterozygous mice carrying the mutation were bred, and they produced wild type, heterozygous, and homozygous offspring at the expected 1:2:1 Mendelian ratio, indicating that the mutation caused no embryonic lethality (Figure 2.1B). RIβ$^{-/-}$ mice have shown normal growth profiles and viability. Both male and female RIβ$^{-/-}$ mice are fertile and can be interbred to produce viable offspring.

Total PKA activity remains the same in the RIβ knockout brain. Northern blots showed that heterozygotes had a reduction in RIβ mRNA, while homozygotes had no detectable mRNA at the size of normal RIβ mRNA, approximately 2.8 kb (Figure 2.1C). Western blot analysis demonstrates that in multiple regions of the brain RIβ protein is completely absent (Figure 2.1D).

Direct measurements of basal and cAMP-induced PKA activity in whole brain extracts from wild type and mutant mice were very similar (Figure 2.2). It is likely that kinase activity remained unchanged due to a compensatory increase in RIα subunit. By Western blot, the homozygotes showed a consistent increase in RIα protein compared with wild type controls in both neocortex and hippocampus (Amieux et al., manuscript in preparation). No significant changes in the quantity of any of the other PKA subunits were observed in either hippocampus or neocortex.

Histological analysis and identification of neurons capable of RIβ-lac transgene expression. Histological analysis of brain, adrenals, and testes revealed no abnormalities in the mutants. Of particular relevance, the gross neuroanatomy of the RIβ$^{-/-}$ hippocampus appears normal, as shown in the coronal sections in Figure 2.3. In the RIβ$^{-/-}$ mice, the development of neurons that would normally express RIβ might be impaired, leading to a compensatory replacement by neurons that express other regulatory subunits. In order to examine this possibility, RIβ null mutant mice were crossed with a previously established transgenic mouse line that expresses an RIβ promoter-driven β-galactosidase reporter gene (Rogers et al.,

1992). Examination of hippocampal sections from these mice revealed no differences in transgene expression in RIβ$^{-/-}$ as compared to RIβ$^{+/+}$ transgenic littermates, indicating that neurons that normally would have expressed RIβ are not preferentially lost during development in the RIβ$^{-/-}$ mice, and that the lack of RIβ protein does not dramatically affect expression from the RIβ promoter (Figure 2.3).

Mutant mice are deficient in LTD and depotentiation. In wild type mice, a conditioning LFS produced a significant depression of field EPSP, lasting at least 35 min (Figure 2.4A). In contrast, the mutant mice showed a short-term depression that returned to baseline by 20 minutes following the LFS. It has been proposed that LTD requires inhibitory interneuron activity (Yang et al., 1994). However, we find that in wild type slices the GABA receptor antagonist picrotoxin does not inhibit LTD induction (data not shown), indicating a lack of involvement of this inhibitory pathway in LTD. This suggests that the LTD defect in the mutants is inherent to the principal excitatory cells, and not secondary to a defect in inhibitory interneurons.

In addition to manifesting LTD, wild type slices show a related depression called depotentiation, whereby LTP, once induced, is abolished (Barrionuevo et al., 1980; Staubli and Lynch, 1990; Fujii et al., 1991; Larson et al., 1993; O'Dell and Kandel, 1994). Although slices from RIβ$^{-/-}$ mice showed the immediate phase of depotentiation, they rapidly returned to a potentiated response in less than 15 minutes (Figure 2.4B). Thus, the deficiency in maintaining LTD correlates with an inability to exhibit stable depotentiation in slices from the mutant mice.

Pharmacologic inhibitors of PKA block LTD and depotentiation. In order to test whether a decrease in PKA activity might account for the defects in LTD and depotentiation, we examined slices from wild type mice in the presence of KT5720, which blocks the enzymatic activity of the catalytic subunit of PKA. KT5720 blocked LTD entirely (Figure 2.4C), and also inhibited depotentiation (Figure

2.4D), confirming the requirement for PKA activity in both forms of synaptic depression.

RIβ$^{-/-}$ mice exhibit normal LTP and paired-pulse facilitation in CA1. Since PKA has been shown to be important in the late phase of LTP at the Schaffer collateral-CA1 pyramidal cell synapse (Frey et al., 1993; Matthies and Reymann, 1993), we examined LTP of this synapse in hippocampal slices from mutant animals as compared with wild type controls. Using a protocol that ensures robust potentiation (100 Hz stimulation for 1 second duration delivered 4 times at an intertrain interval of 4 minutes), a similar degree and time course of LTP was observed in wild type and mutant slices (Figure 2.5A). Likewise, no obvious difference in potentiation was seen when the stimulus was delivered only once (data not shown). These data suggest that the action of RIβ is fairly selective at this synapse and restricted to LTD and depotentiation.

To assure that RIβ deletion did not interfere with other aspects of synaptic transmission, we examined the maximal response that could be evoked at this synapse and found it to be similar between the two groups (7.9 ± 0.8 mV, n = 10 slices for mutants, compared to 9.1 ± 0.5 mV for wild type, n = 13 slices). Another measure of synaptic function, paired-pulse facilitation, whereby a temporally associated second stimulus pulse yields a larger postsynaptic response than the first due to the accumulation of calcium in the presynaptic terminal, was normal at this synapse at several interstimulus intervals (Figure 2.5B).

The LTD defect is also seen in the dentate gyrus of RIβ$^{-/-}$ mice. To examine the generality of the LTD deficit observed in the CA1 region, we turned to the perforant path-dentate granule cell synapse, where low-frequency stimulus-induced LTD has not previously been demonstrated in the hippocampal slice preparation. We found that the same LFS (1 Hz for 15 minutes) that produces LTD in the CA1 region also reliably produces homosynaptic LTD of the lateral perforant path-dentate granule cell synapse. Slices from RIβ$^{-/-}$

mice were deficient in this type of LTD as well (Figure 2.6), suggesting a similar molecular mechanism requiring RIβ underlies LTD at both synapses.

Discussion

The experiments described here demonstrate for the first time a significant role for PKA in use-dependent synaptic depression. Studies using the pharmacological inhibitor show that PKA catalytic activity is required, and the studies utilizing the RIβ null mutant mice show that the acitvity must be appropriately regulated, possibly by RIβ specifically. RIβ-containing holoenzyme has been shown to be particularly sensitive to cyclic nucleotide activation (Cadd et al., 1990; Solberg et al., 1994), and theoretically may also be subcellularly localized in the proximity of critical adnenylyl cyclases or target substrates, analagous to the observed localization of RII and RIα subunits (Johnson et al., 1994; Rosenmund et al., 1994; Rubin, 1994; Skalhegg et al., 1994). Either or both of these unique properties of RIβ might be essential for PKA mediation of LTD and depotentiation.

The surprising findings that PKA activation is required for LTD and that the RIβ null mutant mice cannot sustain LTD suggest that further refinement of the proposed mechanistic models of synaptic depression is necessary. The model for LTD proposed by Lisman (1989) and further elaborated by Malenka (1994) would predict that PKA inhibition should lead to activation of phosphatase 1 and therefore, a possible increase in LTD. We see the opposite result: a loss of LTD when PKA is either altered by genetic manipulation of kinase isoforms or inhibited pharmacologically.

Thus, our data suggest that a molecular explanation of LTD based on calcium influx must consider the myriad potential interactions between calcium and cAMP signaling cascades. Elevated calcium can lead to changes in cAMP concentrations via either the neuronally expressed calmodulin-dependent adenylyl cyclases (types I, III and VIII) (Xia et al., 1996) or the calmodulin-stimulated

phosphodiesterases (Yan et al., 1994). Calcium can also lead to the dephosphorylation of PKA substrates by activating phophatases such as calcineurin. For its part, PKA is known to phosphorylate the voltage-gated calcium channels (Hell et al., 1993) and glutamate receptors (Raymond et al., 1993b) that are expressed in neurons. Given these and other sites of crosstalk, it is premature to outline a detailed model. Perhaps the simplest idea is that LTD might result from the synergistic effects of modest increases in both intracellular calcium and cAMP, leading to simultaneous activation of calcineurin and the Type Iβ holoenzyme of PKA. By extension, LTP in the CA1 region might depend on robust activation of both calcium and cAMP dependent pathways. The Type Iβ holoenzyme would not be required for LTP as long as the stimulus raised cAMP to a threshold capable of activating other less sensitive PKA isoforms. Genetic manipulation of the various other regulatory and catalytic subunits of PKA using gene disruption and mutation in mice should further illuminate the role that the cAMP cascade plays in synaptic plasticity and provide mutant mice to correlate behavioral changes with electrophysiological defects.

Figure 2.1. Generation and analysis of RIβ$^{-/-}$ mice. **(A)** The RIβ locus and targeting vector. Targeting of the endogenous gene (above) by homologous recombination interrupts the coding region in exon 3 with the neomycin resistance cassette (neo^{r}). The fragment used to make radioactive probes for genomic Southern blots is indicated. **(B)** Genomic Southern blot of offspring from heterozygote crosses. Hind III digested DNA shows a wild type band of 8.4 kb (wt) and/or a mutant band of 5.8 kb (ko) when hybridized with the probe indicated in (A). Shown are wild type (lanes 10; 11), heterozygous (lanes 3-5; 7-9), and homozygous mutant (lanes 1; 2; 6) offspring. **(C)** Northern blot analysis of one homozygous mutant (-/-), two heterozygous (+/-), and two wild type (+/+) mice. The amount of total brain RNA loaded in each lane, and the migration of 28S and 18S ribosomal RNA bands are indicated. **(D)** Western blot analysis of wild type (+/+) and homozygous (-/-) mutant mice. Protein samples were prepared from the cerebellum (cbl), neocortex (ctx), and hippocampus (hpc), and 40 μg were loaded into each lane. Blots were probed with a polyclonal antiserum that identifies both RIα and RIβ protein.

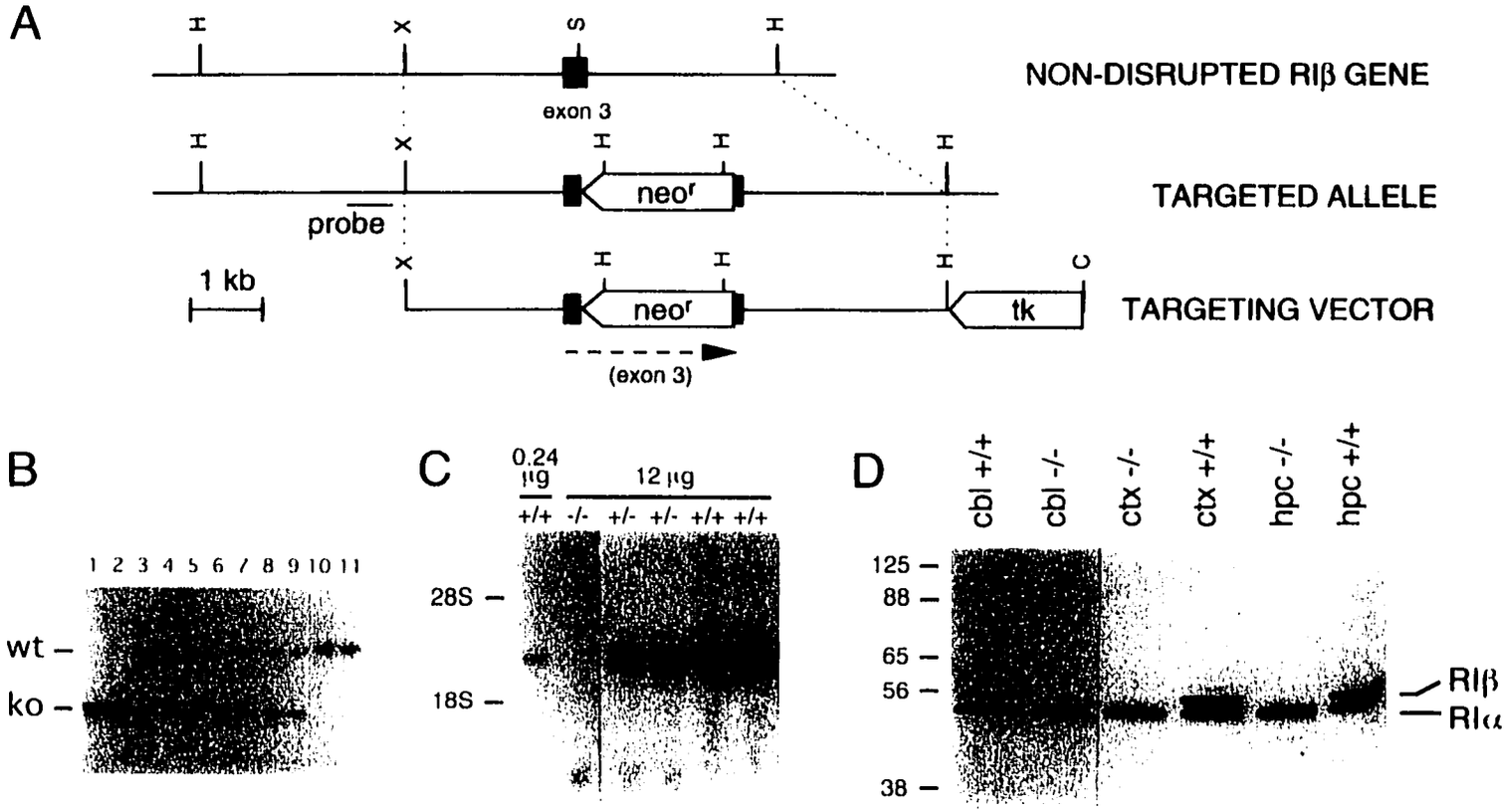

A
H
X
S
H
exon 3
NON-DISRUPTED RIβ GENE
neor
TARGETED ALLELE
probe
1 kb
tk
TARGETING VECTOR
(exon 3)
B
1 2 3 4 5 6 7 8 9 10 11
wt
ko
C
0.24 µg
12 µg
+/+ -/- +/- +/- +/+ +/+
28S
18S
D
cbl +/+
cbl -/-
ctx -/-
ctx +/+
hpc -/-
hpc +/+
125
88
65
56
38
RIβ
RIα

Figure 2.2. Kinase assay from wild type (+/+) and mutant (-/-) mice. Phosphorylation of a peptide substrate of PKA (Kemptide) was assayed using whole brain homogenates, in the presence or absence of 40 μg/ml protein kinase inhibitor peptide (PKI) or 5 μM cAMP. Results from one male (M) and one female (F) of each genotype are shown.

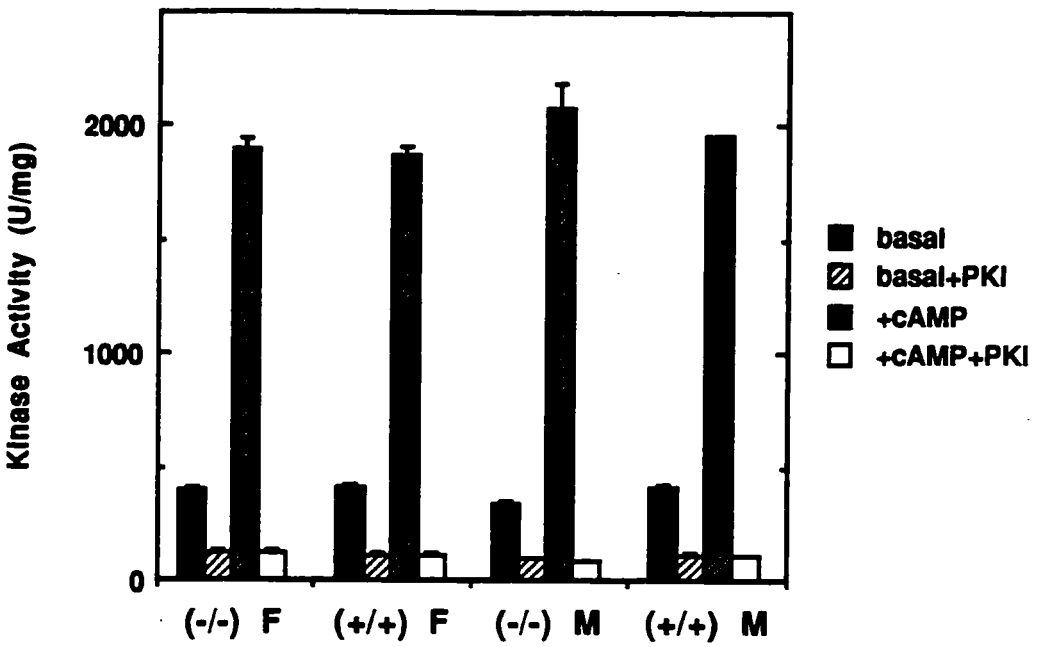

Kinase Activity (U/mg)
2000
1000
0
(-/-) F
(+/+) F
(-/-) M
(+/+) M
basal
basal+PKI
+cAMP
+cAMP+PKI

Figure 2.3. Histologic analysis and expression of pRIβ-lac transgene. Cresyl violet stained sections from a wild type and a mutant hippocampus are shown (top panels). Expression of a β-galactosidase transgene driven by the RIβ promoter in wild type and an RIβ mutant hippocampus is shown (bottom panels).

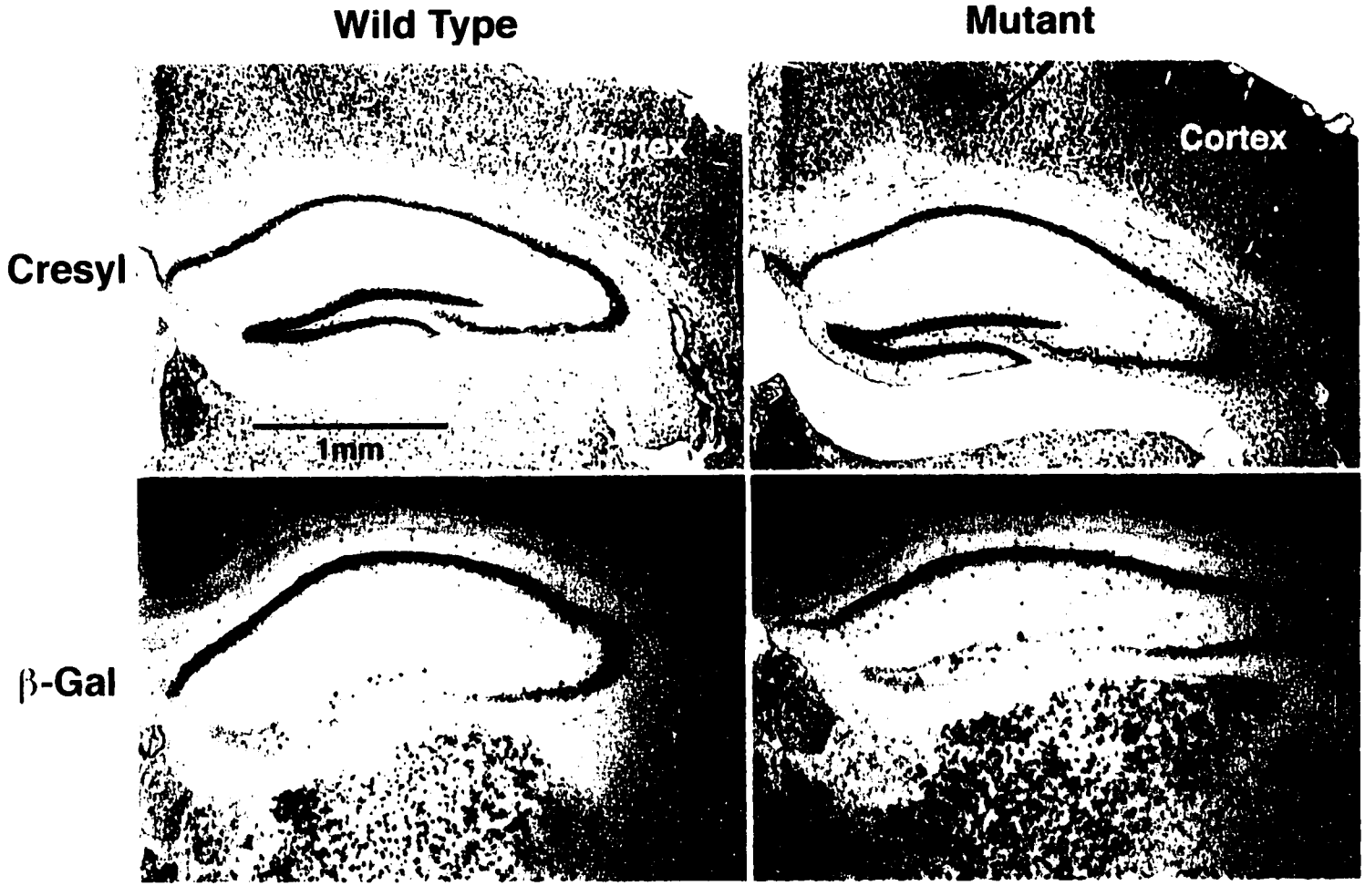

Wild Type
Mutant
Cresyl
β-Gal
Cortex
1mm

Figure 2.4. Synaptic depression is defective at the Schaffer collateral-CA1 pyramidal cell synapse in hippocampal slices from RIβ$^{-/-}$ mice, and in wild type slices treated with a PKA inhibitor. **(A)** LTD: Low frequency stimulus (LFS, bar) produced a reduction in the initial slope of the field EPSP in slices from wild types (open squares, 70.3 ± 8.7%, n = 9 slices from 5 mice, $p < 0.01$), but not mutants (closed squares, 97.1 ± 5.7%, n = 20 slices from 9 mice). EPSPs at 25-30 minutes following LFS were significantly different between genotypes, $p < 0.05$. **(B)** Depotentiation: Six minutes following a high frequency stimulus (HFS, double triangle) to produce LTP, slices were subjected to a low frequency stimulus train (LFS, bar). The field EPSPs from wild type slices (open squares) were depotentiated back to baseline 25-30 minutes following LFS (104.7 ± 14.0%, n = 7 slices from 5 mice) while mutant slices (closed squares) were not persistently depotentiated (155.1 ± 9.0%, n = 9 slices from 5 mice), and these values were significantly different ($p < 0.01$). Wild type slices not subjected to the low frequency train show normal LTP (open triangles). **(C)** Low frequency stimulation (LFS, bar) was delivered in the presence or absence of 1 μM KT5720. KT5720 (closed squares) did not significantly affect baseline responses (data not shown) but prevented LTD (105.3 ± 12.1%, n = 5 slices from 5 mice). **(D)** KT5720 also prevented depotentiation, as measured 25-30 minutes following LFS (176.1 ± 21.3%, n = 6 slices from 6 mice, $p < 0.05$ as compared to untreated slices). Error bars show standard error of the mean.

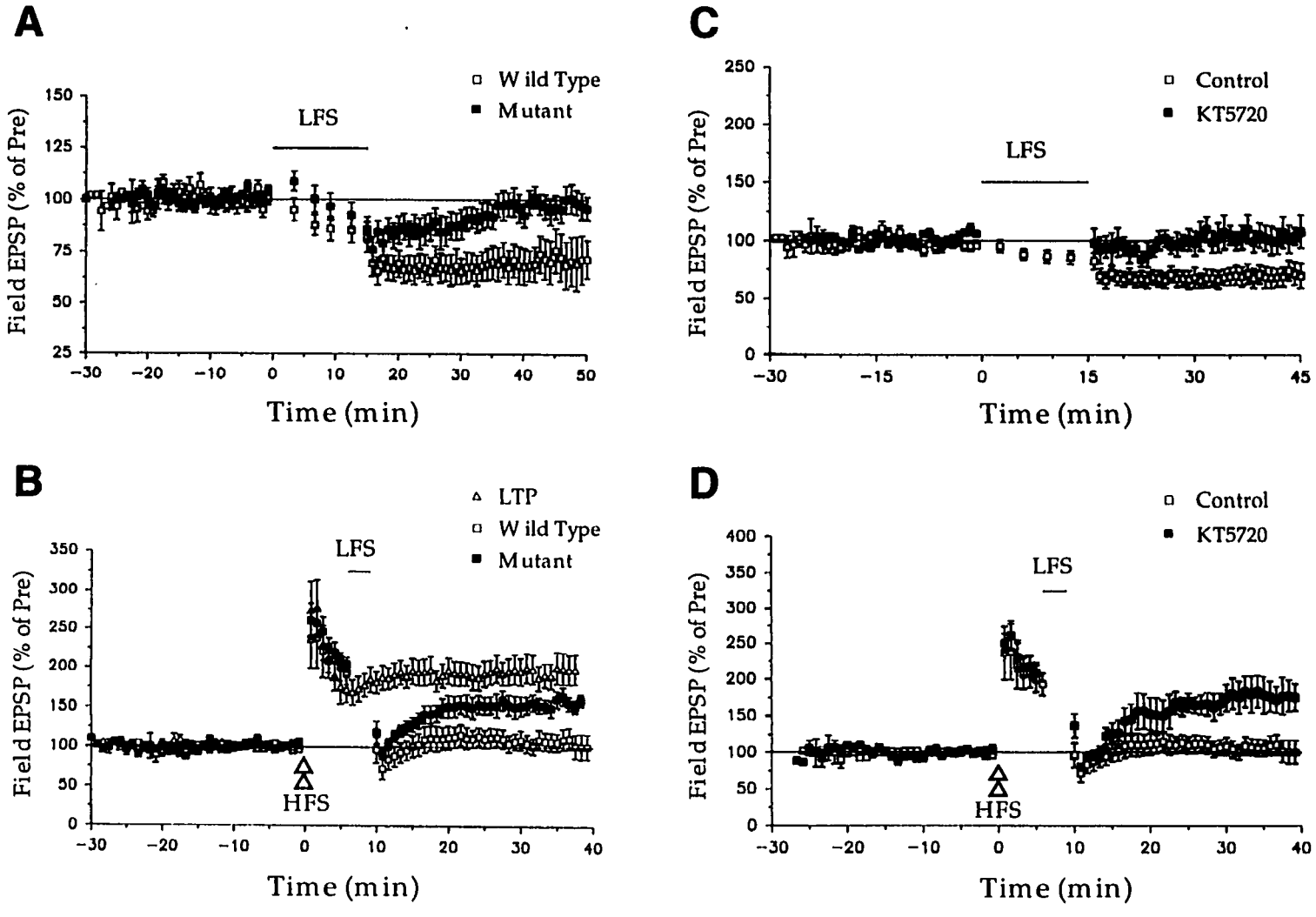

A
Wild Type
Mutant
LFS
Field EPSP (% of Pre)
Time (min)
B
LTP
Wild Type
Mutant
LFS
HFS
Field EPSP (% of Pre)
Time (min)
C
Control
KT5720
LFS
Field EPSP (% of Pre)
Time (min)
D
Control
KT5720
LFS
HFS
Field EPSP (% of Pre)
Time (min)

Figure 2.5. LTP and paired-pulse facilitation are normal at the Schaffer collateral-CA1 pyramidal cell synapse. **(A)** As long as 3 hours following high frequency stimulation (HFS, 4 triangles) the field EPSPs from wild type (open triangles, n = 8 slices from 7 mice) and mutant (closed squares, n = 9 slices from 9 mice) slices were not significantly different. **(B)** Wild type (open circles, n = 7 slices from 4 mice) and mutant (closed circles, n = 15 slices from 8 mice) slices also showed no significant difference in paired-pulse facilitation of the EPSP at various interpulse intervals. Error bars show standard error of the mean.

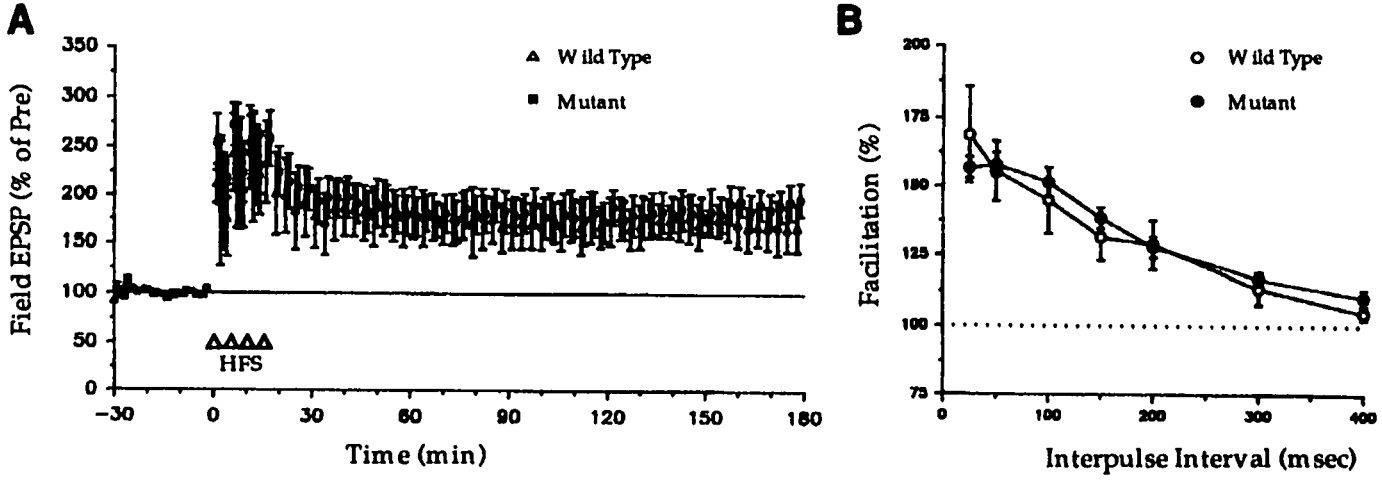
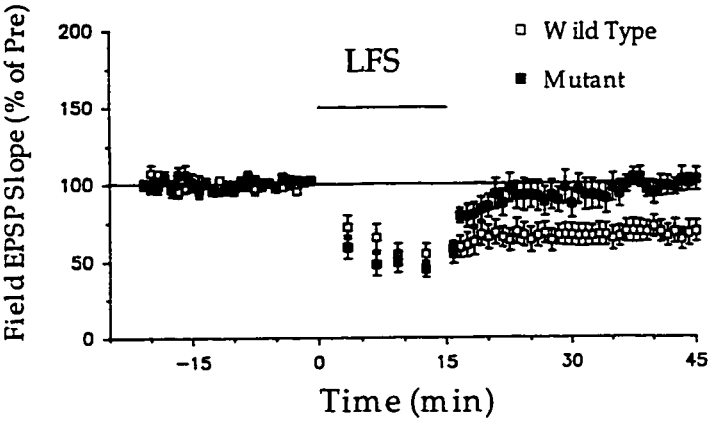

A
Field EPSP (% of Pre)
350
300
250
200
150
100
50
0
Wild Type
Mutant
HFS
−30
0
30
60
90
120
150
180
Time (min)
B
Facilitation (%)
200
175
150
125
100
75
Wild Type
Mutant
0
100
200
300
400
Interpulse Interval (msec)

Figure 2.6. LTD is absent at the lateral perforant path-dentate granule cell synapse. 25-30 minutes following a low frequency stimulus (LFS, bar), average EPSP from wild type slices (open squares) was reduced significantly from baseline (68.3 ± 6.8%, n = 12 slices from 6 mice, $p < 0.001$) while that from mutants (closed squares) was not (100.8 ± 6.3%, n = 12 slices from 7 mice). The EPSPs from wild type and mutant slices were significantly different 25-30 minutes following LFS ($p < 0.01$). Error bars show standard error of the mean.


Field EPSP Slope (% of Pre)
200
150
100
50
0
LFS
Wild Type
Mutant
−15
0
15
30
45
Time (min)

CHAPTER III

THE TYPE IIβ ISOZYME OF PROTEIN KINASE A IS CRITICAL IN MOTOR FUNCTION AND STRIATAL GENE EXPRESSION IN MICE

Introduction

The basal ganglia mediate motor output from the central nervous system, and have been implicated in several movement disorders including Parkinson's disease, Huntington's disease, and those associated with chronic antipsychotic treatment (Albin et al., 1989). The nigrostriatal dopaminergic projection plays a critical role in modulation of basal ganglia function, and is severely degenerated in Parkinson's disease. Evidence from several lines of research demonstrates that dopaminergic activity in the striatum (nucleus accumbens and caudoputamen) also modulates motor function in rats and mice, including both locomotion and coordination (Gage et al., 1983; Walsh and Wagner, 1992; Emerich et al., 1993; Zhou and Palmiter, 1995).

The psychomotor stimulants cocaine and amphetamine are believed to exert their locomotor activating properties primarily by increasing dopaminergic neurotransmission in the striatum. Amphetamine is believed to increase synaptic dopamine by facilitating redistribution of catecholamies from vesicles to the cytoplasm and then to the synaptic cleft via reverse action of the dopamine transporter. Amphetamine also blocks reuptake of dopamine by this transporter (Groves and Rebec, 1976; McMillen, 1983; Sulzer et al., 1995; Giros et al., 1996). The mesostriatal dopaminergic projection appears to be necessary for both basal as well as amphetamine enhanced locomotion in rodents (Thornburg and Moore, 1973; Creese and Iversen, 1975; Kelly and Iversen, 1976; Koob et al., 1981; Clarke et al., 1988). Activation of both D1- and D2-like dopamine receptors is required for

behavioral activation by amphetamine (Mailman et al., 1984; Ujike et al., 1989; Vezina and Stewart, 1989). Sensitization to amphetamine occurs with repeated administration (Robinson and Becker, 1986; Kalivas and Stewart, 1991), requires activation of D1- and D2-like dopamine receptors (Ujike et al., 1989; Vezina and Stewart, 1989), and has been found to be protein synthesis-dependent (Karler et al., 1993).

Recently mice have been developed that have alterations in their dopaminergic function. Mice genetically deficient in dopamine show extreme hypoactivity unless rescued with L-DOPA (Zhou and Palmiter, 1995). Conversely, mice lacking the dopamine transporter have constitutively higher levels of dopaminergic transmission (despite the organism's attempts to downregulate the other components of the signaling pathway), are hyperactive, and are unresponsive to the locomotor activating effects of amphetamine and cocaine (Giros et al., 1996). Mice lacking the various dopamine receptors have also been established. One line of mice lacking the D1 dopamine receptor (D1R) was found to have increased locomotor activity (Xu et al., 1994a; Xu et al., 1994b), although this was an unexpected phenotype based upon the effects D1R agonists and antagonists have on locomotion (Nestler, 1994). Curiously, an independently generated second line of D1R knockout mice was not reported to be hyperactive (Drago et al., 1994). Mice lacking the D3 dopamine receptor (D3R) have recently been developed, and these mice also show locomotor hyperactivity (Accili et al., 1996). In contrast, mice carrying a targeted disruption of the D2 dopamine receptor (D2R) have a hypolocomotor syndrome, including impaired performance on the rotarod task (Baik et al., 1995). Collectively, these various mutants demonstrate a critical role for dopaminergic signaling in the regulation of motor output in mice. Additionally, in several of these cases, changes in expression of striatal peptides that are believed to modulate motor output (such as dynorphin, substance P, and enkephalin) were correlated with the motor defects.

In the striatum, the D1 and D2 receptors are expressed for the most part in distinct populations of efferent neurons (Gerfen et al., 1990; Surmeier and Kitai, 1994; Hersch et al., 1995; LeMoine and Bloch, 1995). D1 receptors are expressed largely in projection neurons to the substantia nigra pars reticulata and entopeduncular nucleus, the "direct pathway" of striatal output. In contrast, D2 receptors are preferentially found in the "indirect pathway", that which projects to the globus pallidus. A temporally regulated balance of activity between the two pathways allows the regulation of normal movement (Gerfen, 1992). Activation of D1 and D2 receptors in the striatum modulates the activity of these two pathways and consequently, coordination of motor output.

D1 receptors act through G_S-type proteins to increase the production of cAMP by adenylyl cyclase, and thus activate the cAMP-dependent protein kinase (protein kinase A; PKA). Conversely, D2 receptors inhibit cyclase activity via G_i-type proteins, and in this way are believed to decrease PKA activity below "basal" levels (Stoof and Kebabian, 1981). Thus, PKA likely serves as a primary effector molecule for regulation of motor output activity by dopamine.

In the mouse, the PKA family consists of six distinct members, each a product of a unique gene. Each PKA holoenzyme consists of two homodimeric regulatory (R) subunits, and two catalytic (C) subunits; the type of holoenzyme is defined by its regulatory subunits (i.e. $RII\beta_2C_2$ is Type IIβ PKA). In situ analysis has suggested that of the regulatory subunits (RIα, RIβ, RIIα, and RIIβ), RIIβ has the highest expression in the striatal complex, including the caudoputamen, nucleus accumbens, and islands of Calleja (Cadd and McKnight, 1989). RIIβ protein is found in dendritic and perikaryal regions of the medium spiny neurons of the striatum (Ludvig et al., 1990; Glantz et al., 1992), suggesting possible roles in both immediate responses to dopaminergic transmission, and longer term gene induction events associated with cAMP signalling. A-kinase anchoring proteins (AKAPs) are coexpressed in striatal neurons and are likely essential for the specific

subcellular distribution of RIIβ (Glantz et al., 1992; Rubin, 1994; Faux and Scott, 1996).

Given the high expression of RIIβ mRNA in the striatum and the important role of PKA in the dopaminergic signal transduction pathways, we hypothesized that Type IIβ PKA comprises a major fraction of striatal PKA, and plays a critical role in modulation of motor output. Thus, to test these hypotheses, and to determine the essential roles of RIIβ in the whole animal, we disrupted the gene encoding this specific subunit of PKA in the mouse. The resulting knockout mice have a severe deficiency in PKA activity in their striatal neurons and also exhibit defects in motor function and in psychomotor stimulant-mediated induction of both gene expression and locomotor activity.

Experimental Procedures

Gene disruption. The disruption of the RIIβ gene in embryonic stem (ES) cells has been previously described, in the context of the establishment of novel parental ES cell lines (Brandon et al., 1995a). The cell line "4C9" carrying the expected targeted mutation produced several germ line competent chimeras, whose heterozygous offspring were bred to produce homozygous knockout mice and control wild type mice. All experiments described here were performed on either these F1 mice or descendants generated through their interbreeding; all experiments employed age and gender matched controls.

Southern blot analysis. Total nucleic acid was prepared from tail biopsies and digested with HindIII, separated on 1% agarose gels and blotted onto Hybond-N membrane (Amersham). Radioactive probe was prepared by random-primed labeling (Boehringer Mannheim) of an 800 base pair genomic RIIβ fragment 5' of the region of targeting vector homology. 50% of the agouti pups of chimeric mice were heterozygous for the mutation.

Northern blot analysis. Northern blot analysis was performed essentially as described (Mosley et al., 1989). Tissue was homogenized in 8M Guanidine/25 mM sodium acetate, and RNA was precipitated with 0.6 volumes ethanol. After centrifugation, the RNA pellet was resuspended in 8M Guanidine/25 mM sodium acetate, extracted with a 50:50 mixture of phenol and chloroform, and then re-precipitated with 0.6 volumes ethanol. RNAs were electrophoresed through agarose containing formaldehyde and transferred to Hybond-N filters. Filters were hybridized as described (Idzerda et al., 1990), to a ^{32}P-antisense riboprobe synthesized from a 357 base pair XbaI-XhoI cDNA fragment from the 3' end of the mouse RIIβ coding region.

Western blot analysis. Protein was prepared from various regions of the brain by dounce homogenization in lysis buffer (250 mM sucrose, 1 mM EGTA, 4 mM EDTA, 0.5% Triton X-100, 4 mM DTT, 2 μg/ml leupeptin, 3 μg/ml aprotinin, 0.2 mg/ml soybean trypsin inhibitor, and 1 mM AEBSF, in PBS) at 0°C followed by brief sonication. Supernatants were collected following centrifugation for 10 minutes at 12,000xG at 2°C, and protein concentration determined by Bradford assay. 40 μg of protein was run on 10% polyacrylamide gels, and transferred onto nitrocellulose (Schleicher & Schuell) on a mini-apparatus (BioRad). Blots were pretreated overnight at room temperature in blocking buffer (5% BSA, 0.2% Tween-20, 0.01% sodium azide), and probed with antisera to murine PKA subunits as described (Brandon et al., 1995c; Qi et al., 1996).

Kinase assay. Various regions of the brain were dissected from adult mice and homogenized as above for Western blots. Kinase activity was assayed as described (Clegg et al., 1987) using Kemptide (Kemp et al., 1977) as a substrate in the presence or absence of 5 μM cAMP, each in the presence or absence of 40 μg/ml PKI protein, and was expressed as units of activity (pmoles per minute) per mg protein.

Rotarod performance. The rotarod apparatus (Ugo Basile, Italy) used was 6 cm in diameter, with a ribbed plastic surface. Each lane was 5 cm wide. The apparatus was used in two different

accelerating modes, gradually increasing from either 4 to 30 rpm ("slow" speed) or 5 to 35 rpm ("fast" speed) over 5 minutes. Mice (age 6-56 weeks, gender and age matched) were placed on the apparatus and rotation was initiated within about five seconds. Latency to fall was automatically recorded. Trials were given within the last 4 hours of the light phase of the light-dark cycle (12 hours each), 10-25 minutes apart. Ten trials were given on the first day, and four on the second. Mice that stayed on the rotarod for more than 300 seconds were considered complete responders; their latency recorded as 300 seconds. At the slow speed only 6 of 17 wild type mice ever reached this criteria, and never did a mutant. At the fast speed only 1 of 11 mutants reached this criteria, and never did a wild type.

Locomotor activity. Locomotor activity was assessed in an automated circular open field arena (60 cm diameter) illuminated by a bright white light. The number of infrared photocell interupts in each perpendicular axis was recorded by a Commodore 64 computer. The arena was cleaned with 75% ethanol after each trial. Mice were placed in the darkened testing room 30 minutes prior to testing, injected intraperitoneally with drug or saline 15 minutes before testing, and placed in a holding cage until testing. Amphetamine and cocaine testing sessions were for 15 minutes; those for quinpirole and SKF-38393 were 30 minutes.

D1 agonist SKF 81297 behavioral assessment. Mice were individually housed for 24 hours prior to the experiment. Trials were given within the last 4 hours of the light phase of the light-dark cycle. Mice were weighed and injected intraperitoneally with either 5.0 mg/kg SKF 81297 or saline (vehicle). Food and water were removed form the cage. Each mouse was observed once per minute and its activity recorded (activities included locomotion, grooming, nibbling on bedding or feces, digging in bedding, and stationary/sleeping). The proportion of each type of activity observed from 15 minutes to 115 minutes following injection was determined and analyzed.

Gas chromatography for amphetamine. Each brain was weighed and homogenized in 3 ml of 0.1 PCA with the internal standard added directly to each homogenate. Samples were centrifuged at 4°C for 10 minutes, and supernatants were applied to pre-conditioned SPE C18 columns. Eluents were dried under nitrogen steam in N-Vap, resuspended in 50/50 buffer, and loaded onto the gas chromatographer.

Glucocorticoid analysis. Blood samples were taken, and plasma separated by centrifugation for analysis. The assay was performed using the rat corticosterone-^{3}H kit (ICN Biomedicals, Inc.).

Dopamine transporter assay. Striata were dissected from RIIβ knockout and control mice, homogenized, centrifuged, and P2 pellets resuspended. Resuspended pellets were incubated with various concentrations of D-amphetamine or saline, then with ^{3}H-dopamine. Samples were filtrated, and filters and flow through solution counted on a scintilation counter.

In situ hybridization. For quantitative analysis, images from autoradiograms were captured and densitometry determined using the MCID system. For both c-fos and prodynorphin quantification samples of the size indicated in figure 3.6.A were taken from coronal slices between the genu of the corpus collosum and the crossing of the anterior commisure and analyzed. An average value for each region was determined for each mouse (approximately 20 samples per region per mouse); the averages shown in figures 3.5.B and 3.6.B represent averages of the values determined for each mouse. For the data shown in figure 3.6.B, values from the septum of each slice were subtracted out before any averaging; this region shows no specific signal over cells on emulsion coated slides and thus can be considered non-specific background.

Immunohistochemistry. IHC for phospho-CREB like immunoreactivity was performed essentially as described (Cole et al., 1995). Mice were given sodium pentobarbital (130 mg/kg) 15 minutes following administration of D-amphetamine or saline, and perfused

with a 4% paraformaldehyde solution 5 minutes later. Brains were excised, post-fixed overnight at 4°C, sunk in sucrose (20% and 30%, each overnight at 4°C), frozen on dry ice, and sliced to 50 μm on a cryostat. Blocking (20% normal goat serum, 1% BSA, 0.3% triton X-100 in PBS) was for one hour at room temperature, and probing with the primary antibody to PCREB (1:1000 in 1% normal goat serum, 0.3% triton X-100 in PBS; generously provided by Dr. D. D. Ginty) was overnight at 4°C. Secondary antibody (1:40,000 goat anti-rabbit IgG) was for one hour at room temperature, followed by the ABC peroxidase kit (Vector Labs, Burlingame, CA). Each step except primary antibody was preceded by 3 ten minute washes with PBS.

Results

Mice lacking RIIβ appear normal and healthy. Gene targeting was carried out by standard techniques (Capecchi, 1989). The mutation eliminates the entire coding region of the first exon of the RIIβ gene, including the translation start site (figure 3.1A). Germ line competent chimeras were bred to produce heterozygous mice. Wild type, heterozyous, and homozygous mutant offspring from crosses of heterozygotes (figure 3.1B) were produced at the predicted Mendelian frequency, indicating no embryonic lethality is associated with the mutation. Homozygous mutant mice expressed no detectable mRNA for RIIβ, as determined by Northern blot analysis on whole brain (figure 3.1C) and in situ analysis of the brain (figure 3.1D). Figure 3.1D also illustrates the high expression of RIIβ message in the striatum of normal mice. In accordance with the lack of detectable RIIβ mRNA, no RIIβ protein is observed in the mutant brain (figure 3.2B).

RIIβ is expressed at high levels in adipose tissue, brain, and hematopoietic tissues (fetal liver and bone marrow) in the mouse. RIIβ is also expressed in reproductive cells, where it is regulated by hormones that activate PKA, suggesting an important reproductive function (Jahnsen et al., 1986; Oyen et al., 1988). However, RIIβ

mutant mice exhibit normal fertility, giving rise to normal size litters at a normal frequency. The mice are morphologically normal in all tissues examined except the adipose, where they show a reduction in fat accumulation (Cummings et al., 1996).

RIIβ is the major isoform of PKA in the striatum. While both in situ analysis of RIIβ mRNA expression in the brain (Cadd and McKnight, 1989), and immunohistochemistry determining the regional and subcellular distribution of RIIβ (Ludvig et al., 1990; Glantz et al., 1992) have been performed, the relative contribution of RIIβ holoenzyme to the total PKA complement in any given region of the brain has not previously been determined. Western blots comparing equal amounts of protein from various regions of the mouse brain show that RIIβ expression is highest in the striatum, lower in other regions of the brain, and is nearly undetectable in the cerebellum (figure 3.2A). Kinase assays were performed to determine the amount of PKA activity remaining in various brain regions of RIIβ knockout mice. In the whole brain, PKA activity was reduced by approximately 50%. PKA activity was reduced by approximately 65-85% in the striatum (figure 3.2A). Thus, Type IIβ PKA must comprise the majority of PKA in the striatum, as loss of RIIβ protein results in this concomitant loss of PKA activity. Other structures showed smaller reductions in PKA activity, and notably no change was observed in the cerebellum (figure 3.2A).

Previous studies suggest that loss of RIIβ might lead to compensatory changes in other PKA subunits. A compensatory increase in RIα has been seen in multiple systems where the PKA system has been perturbed (Amieux et al., manuscript in preparation), including the brown adipose tissue of the RIIβ mutants (Cummings et al., 1996), and the brain of RIβ null mutants (Brandon et al., 1995c). In the striatum of the RIIβ knockout mouse, this same compensation by RIα is observed, and interestingly a compensatory increase is also seen in the level of RIβ protein (figure 2B). This suggests a common mechanism of regulation of the two Type I subunits in the brain. As

predicted by the kinase assays, catalytic subunits of PKA (Cα and Cβ) are dramatically reduced in the mutant striatum (figure 2B).

RIIβ knockout mice are impaired in the rotarod task. Because loss of RIIβ produced such a dramatic reduction in striatal PKA, and because PKA is situated downstream of the striatal dopamine which is critical for motor coordination, we sought to determine whether motor output was disrupted in these mice. The rotarod task is an indicator of the ability of an animal to coordinate the movements required to stay on a rotating rod (Dunham and Miya, 1957). A few seconds after mice were placed on the rod, rotation was initiated and the rod then accelerated from 5 to 35 rpm over the course of 5 minutes. As shown in Figure 3.3A, most wild type mice had some difficulty with the task at first, but within a few trials improved their performance significantly. In contrast, RIIβ mutants usually showed some acquisition, but never attained the same competence with this task as the wild type mice. This suggests a disruption of the ability to coordinate motor output is effected by the absence of RIIβ. The defect in motor coordination is specific to the loss of the RIIβ subunit of PKA, as mice lacking the RIβ subunit showed no such decrement (figure 3.3B).

It is conceivable that the RIIβ knockout mice were actually collecting information to acquire the task, but were simply not able to incorporate the information without a consolidation period. This phenotype might be expected if the Type IIβ holoenzyme plays a significant role in short-term motor learning, but not in a long-term memory formation, i.e. that which might require novel gene expression or protein synthesis (DeZazzo and Tully, 1995). Thus, the mice were tested again a day later to determine if their latencies improved. Only slight improvement by the mutants was seen on the second day (figure 3.3A). With further testing, mice of both genotypes were found to maintain performance similar to that observed on day 2, with the latencies for mutants remaining consistently below those of wild type mice (data not shown).

In a second set of experiments the mice were tested at a less challenging rate of acceleration to determine if this might facilitate their acquisition. The wild type mice showed a more rapid acquisition and an increase in their average latency to fall at this lower rate, but the RIIβ mutants were still dramatically impaired (figure 3.3C).

RIIβ knockout mice are hyperresponsive to D-amphetamine. Acute administration of the indirect dopaminergic agonist D-amphetamine causes increased horizontal locomotion in mice. While wild type control mice showed fairly typical increases in locomotion, RIIβ mutants showed greater responsiveness to the locomotor-inducing effects of D-amphetamine (figure 3.4A). The effect at 2.5 mg/kg is shown; similar results were obtained at several different doses of amphetamine tested (1.0, 5.0 and 10.0 mg/kg, data not shown). Under some treatment regimens, repeated administration of D-amphetamine produces a sensitization phenomenon, whereby each sequential administration of drug results in a greater response than that seen previously (Robinson and Becker, 1986; Kalivas and Stewart, 1991). We found that while the wild type mice showed modest sensitization the RIIβ mutant mice showed significantly greater sensitization to D-amphetamine at several doses (figure 3.4A).

One simple explanation for the increased responsiveness to amphetamine seen in the RIIβ knockout mice could be altered pharmacokinetics (Camp et al., 1994). Amphetamine levels in the brains of mutant and wild type mice 15 minutes following drug (5.0 mg/kg) administration were analyzed by gas chromatography, and were found to be 6.85 ± 1.09 and 5.99 ± 1.03 mg/g ($n = 12$ mutants and 9 wild type, $p = 0.55$), respectively. Thus, no significant difference in brain amphetamine levels was observed, largely dismissing a pharmacokinetic explanation for the observed behavioral difference.

Hyperresponsiveness to amphetamine has also been seen in rodents with elevated glucocorticoid levels (Badiani et al., 1992; Deroche et al., 1992; Pauly et al., 1993; Angulo and McEwen, 1994). When stressed by amphetamine administration (5.0 mg/kg i.p.), RIIβ

mutant mice actually have lower glucocorticoid levels than wild type control mice (145.7 ± 20.6 for wild type mice, n = 15; 86.6 ± 18.0 for RIIβ mutants, n = 14; all 15 minutes following drug administration), so we believe that this system is not responsible for the increased responsiveness observed in the mutants.

Besides the obvious loss of PKA in the striatum, what other elements of the dopaminergic pathways might be altered in the mutant mice? As shown in Table 3.1, no significant difference in the level of dopamine was found in the striatum of the RIIβ knockout mice as determined by HPLC. They do however, have a 20% reduction in striatal D1-like receptors, as assessed by ^{125}I-SCH23982 binding. This difference is greatest (30% reduction) in the dorsolateral region of the caudoputamen. In contrast, no difference in D2-like receptors, as assessed by ^{125}I-sulpiride binding, was found. The function of the dopamine transporter was also assessed by determining dopamine uptake into synaptosomes. No differences were observed in either basal transporter function, or in the inhibition of dopamine uptake by amphetamine in synaptosomes from mutant mice.

Does the difference in amphetamine responsiveness reflect a specific defect in one of the dopaminergic signaling pathways? The responsiveness of mutant mice to D2- and D1-specific agonists was determined. While 2.5 mg/kg of the D2R agonist quinpirole activated locomotion in wild type mice, especially with repeated administration, no significant activation was observed in RIIβ mutants (figure 3.4B). Similar results were observed with other doses of quinpirole (0.25, 0.5, and 5.0 mg/kg, data not shown). In contrast, the D1R agonist SKF38393 activated locomotion in both mutant and wild type control mice at several doses (8.0 mg/kg is shown in figure 4B). Additionally, the D1R agonist SKF81297 increased grooming significantly in both genotypes (data not shown). Together these data suggest that D2R-mediated signaling is severely impaired in the mutant mice, but D1R-mediated signaling remains at least partially functional. Furthermore, the increased responsiveness to amphetamine does not appear to

reflect a simple increase in responsiveness to dopamine at either of these receptors.

The RIIβ subunit of PKA is specifically required for induction of c-fos in the dorsomedial striatum by D-amphetamine. To begin to ascertain whether gene regulation events might be involved in the altered sensitization of RIIβ mutant mice to amphetamine induced locomotion, the ability of amphetamine to induce c-fos mRNA in the RIIβ knockout striatum was assessed (Graybiel et al., 1990). The c-fos promoter contains a Ca/CRE element (Sheng et al., 1990), which is responsive to cAMP, and thus expression of c-fos provides a good indicator of endogenous PKA-mediated gene induction (Sassone-Corsi et al., 1988).

Basal levels of c-fos mRNA are low in the striata of both RIIβ knockout and wild type mice. One hour following administration of D-amphetamine, wild type mice show a significant induction of c-fos mRNA in the dorsomedial region of the striatum. In striking contrast, the RIIβ mutant mice almost entirely lack this induction (figure 3.5, A & B). Interestingly, mice lacking the RIβ subunit of PKA show normal induction of c-fos in this region, demonstrating the specificity of the defect. Hybridization signal for c-fos mRNA is high in the cingulate and piriform cortices even in vehicle treated mice, possibly due to injection induced stress, but is further increased in response to amphetamine. In contrast to the lack of responsiveness of c-fos in the dorsomedial striatum of RIIβ knockout mice, the induction of cortical c-fos appears to be normal in both RIIβ and RIβ mutants (figure 3.5B).

The CREB/ATF transcription factors are responsive to signalling through cAMP and PKA. When CREB is phosphorylated on serine 133 (ser133) by PKA, it can activate immediate early gene expression (Yamamoto et al., 1988; Sheng et al., 1991; Meyer and Habener, 1993). To determine if the RIIβ isoform of PKA is necessary for CREB phosphorylation in response to amphetamine, immunohistochemistry was performed using an antibody specific for the ser133 phosphorylated form of CREB (Ginty et al., 1993; Konradi et al., 1994).

Little or no signal was observed in the striata of saline treated animals. In contrast, significant induction of nuclear signal was observed in both wild type and RIIβ mutant striata 15 minutes following amphetamine administration (figure 3.5C). No obvious differences in the amount or distribution of staining were seen in the mutants as compared to wild type control mice.

A reduction of prodynorphin mRNA is observed in the mutants. Striatally expressed endogenous neuropeptides such as dynorphin, a κ-opiate receptor agonist, are believed to affect motor function and sensitization to psychostimulants (Thompson et al., 1990; Ukai et al., 1992; Angulo and McEwen, 1994; Heidbreder et al., 1995). Psychostimulants have been shown to induce mRNA encoding dynorphin in the striatum of the rat (Hurd and Herkenham, 1992; Steiner and Gerfen, 1993; Jaber et al., 1995; Wang et al., 1995). Additionally, amphetamine induction of this gene in rats is believed to largely be regulated by activation of CREB-like transcription factors (Douglass et al., 1994; Cole et al., 1995). In situ analysis for prodynorphin mRNA was performed on brains from mice treated chronically (once per day for five days, or twice per day for three days) with amphetamine or saline. No induction of prodynorphin mRNA by amphetamine was observed in either wild type or mutant dorsolateral striatum in these paradigms. However, prodynorphin mRNA was found to be at constitutively lower levels in the mutant striatum, particularly in this dorsolateral region, where the expression appears to be about 30% of normal (figure 3.6). Expression in the dorsomedial region is also reduced.

Discussion

Type IIβ, the major isoform of PKA in the striatum, regulates motor function. A great deal of the literature concerning the biology of basal ganglia function implicates specific molecular components of the dopamine-cAMP pathway, including specific isoforms of dopamine receptors, G-proteins, adenylyl cyclases,

phosphodiesterases, phosphatases, and phosphatase inhibitors. While it is generally assumed that PKA must be a critical player in the dopaminergic modulation of striatal function, a role for PKA has not been directly demonstrated previously, nor has a requirement for a specific isozyme of PKA been investigated. Demonstrated here is a critical role for the Type IIβ isozyme in striatal function at the molecular level, as well as roles in motor output at the organismal level.

Previous work has shown that RIIβ mRNA expression in the brain is highest in the striatum (Cadd and McKnight, 1989). Immunohistochemistry has shown that RIIβ protein is enriched in the striatum as well, and is localized to dendritic and perikaryal regions of neurons (Ludvig et al., 1990; Glantz et al., 1992). Because the protein is apparent in a vast majority if not all of the neurons in the striatum, it must be highly expressed in the GABAergic medium spiny projection neurons which constitute as many as 95% of the neurons found in this structure (Bolam and Bennett, 1995). Similarly, it can be inferred that both D1R and D2R expressing cells normally express the RIIβ subunit of PKA. Work presented here confirms by Western blot that in the brain RIIβ is expressed at the highest levels in the striatum. Moreover, genetic ablation of RIIβ protein causes the most severe PKA deficit in the striatum. These results indicate that the Type IIβ holoenzyme of PKA is normally the major isoform found in this brain structure.

Given the literature implicating striatally expressed PKA-coupled receptors in motor output, we predicted that the significant loss of striatal PKA in the RIIβ knockout mice might disrupt this function. Indeed, the mutants showed a significant impairment in the rotarod task, demonstrating the importance of PKA in this task, a role that has not been previously demonstrated. The defect observed in the rotarod task could be due to a variety of factors. Cerebellar defects have been shown to affect performance in this task (Lalonde et al., 1995), but given the lack of an appreciable reduction in PKA activity in

the RIIβ knockout cerebellum, we believe this is not the most likely locus of the defect. The findings that striatal manipulations can also affect rotarod performance (Emerich et al., 1993) and the dramatic defect in striatal PKA in the RIIβ mutant mice suggest the simplest interpretation of the data described here -- that dopamine signaling through Type IIβ PKA in the striatal complex regulates motor output. Dopamine metabolism has been found to be increased in the striatum during the performance of this task (Bertolucci et al., 1990), and we believe that in the mutants, the effectiveness of the endogenous dopaminergic signal is compromised leading to an inability to properly implement the movements required to stay on the rotarod. Alternatively, the mutants may have a limitation in their ability to learn the task, although most of them do show some acquisition. These mice have also been found to have no defects in several other learning tasks (S. F. Logue et al., unpublished observations). With further study, it might be possible to determine whether the precise defect is in learning the action sequences required for the task, or in performance per se (Graybiel, 1995).

Further evidence that RIIβ-containing PKA is critical in motor output comes from the experiments with exogenously introduced dopaminergic agents. Amphetamine acts by releasing endogenous dopamine in the striatum to effect locomotion; the RIIβ knockout mice show increased responsiveness. The D2 agonist quinpirole acts on presynaptic D2 receptors to inhibit release of dopamine, and on postsynaptic D2 receptors to modulate the response of the indirect pathway to glutamatergic input from the thalamocortical motor circuit. Locomotion induced by quinpirole is absent in the mutant mice. Because D1R-mediated signaling is required for quinpirole-induced locomotion (Walters et al., 1987), this result might simply be providing evidence for the role of RIIβ in G_S-type signaling (likely G_{olf} in the striatum, Herv'e et al., 1993), and not necessarily in G_i-coupled signaling. Alternatively, D2R-mediated signaling per se may be disrupted -- intracellular signaling through decreases in cAMP should

require a regulatory subunit of PKA. In concert with the the functional defects in striatal biochemistry, these behavioral results suggest a complex defect in striatal function in the RIIβ mutant mice, overtly expressed as alterations in motor output. We propose that Type IIβ PKA in medium spiny neurons plays an important role in dopaminergic modulation of corticostriatal input, especially in D2 expressing cells, possibly through phosphorylation of the ion channels that are responsive to glutamatergic input and/or membrane potential (see below).

Regulatory subunits serve multiple functions in the PKA system. It is becoming increasingly evident that the regulatory subunits of PKA subserve at least three distinct functions. First, the regulatory subunits have their "classic" role in *regulation* of PKA activity. It seems very likely that at least some of the phenotypic changes seen in the RIIβ mutant are due to a lack of PKA activity in critical intracellular signaling pathways. The lack of striatal c-fos induction in response to amphetamine strongly suggests that the signalling from the D1R to the nucleus is disrupted (Graybiel et al., 1990).

Second, the regulatory subunits are responsible for *localization* of the catalytic subunits. The Type II regulatory subunits contain critical amino acid residues near their N-termini that are required for binding to A-kinase anchoring proteins (AKAPs) (Rubin, 1994; Faux and Scott, 1996). While some of the phenotypic differences observed in the RIIβ knockout might be due to loss of PKA activity, others may be partially derived from a loss of specific subcellular localization of the enzyme. The Type I regulatory subunits that partially compensate for the loss of RIIβ would not be expected to achieve the same subcellular localization of PKA, as they have not been found to interact with the AKAPs that bind Type II subunits.

Third, regulatory subunits are important for *stabilization* of the catalytic subunits. One might have predicted that a knockout of a regulatory subunit would result in a loss of cAMP regulation of PKA

activity, and thus an increased basal phosphorylation of various substrates. However, the gross loss of PKA activity seen in the RIIβ mutant striatum indicates that this subunit plays a critical role in protecting the catalytic subunits from proteolytic degradation. Thus, one would hypothesize that PKA substrates would be less likely than normal to become phosphorylated in the mutant. The situation is complicated by the compensatory increase in Type I regulatory subunits (RIα and RIβ). This compensatory change has been shown to cause a complete shift from Type II to Type I PKA with little loss of C subunit in the brown adipose tissue of the RIIβ mutant (Cummings et al., 1996). However, in the striatum the compensation is only partial, preserving only 15 - 35% of the total PKA activity. This remaining PKA is likely to be largely Type I, which appears to be activated at lower cAMP levels than Type II holoenzyme (Cummings et al., 1996). In the context of increased sensitization expressed by the RIIβ mutant mice, and this role in catalytic subunit stabilization, it is interesting to reconsider the findings of Cunningham and Kelley (1993). These investigators found that infusion of cholera toxin into the ventral striatum, which would produce chronic activation of PKA, leads to hypersensitization to amphetamine. We suggest that the chronic activation of PKA may have allowed proteolytic degradation of C subunits, and actually decreased the amount of total PKA, analogous to the situation in the RIIβ mutants.

How does PKA fit into the proposed model of striatal function? Over the past several years a model of basal ganglia circuitry has developed that incorporates both behavioral and anatomical findings (Albin et al., 1989; DeLong, 1990; Graybiel, 1990; Gerfen, 1992). While it is generally agreed that the model is oversimplified, it does appear to be largely accurate (Graybiel, 1996), and can help to understand the roles of the various dopaminergic signaling components (including PKA) in striatal function, especially as the results from the various mutant mice are placed in the context of the model (figure 3.7). Essential features of the model for the

purposes of this discussion are as follows: (1) the activity of inhibitory GABAergic projection neurons from the substantia nigra pars reticulata (SNr) to the thalamic and other motor output areas is inversely correlated with motor activation, (2) the activity of the SNr projection neurons is coordinately regulated by the two types of GABAergic striatal projection neurons, (3) activity of the D1R expressing cells of the direct pathway results in inhibition of the SNr neurons, and facilitates movement by way of disinhibition of the neural activity in the motor output structures, and (4) conversely, activity of the D2R expressing cells of the indirect pathway ultimately results in activation of SNr neurons, and tends to inhibit movement.

Despite extensive studies, the functional role of dopamine on the activity of striatal projection neurons remains somewhat obscure, although it appears that dopamine likely modulates the ability of these cells to respond to other inputs, as opposed to simply evoking excitatory or inhibitory potentials itself (Bargas and Galarraga, 1995; Surmeier et al., 1995b). Together with the functional model, our results make a prediction regarding this issue. Specifically, the behavioral results using dopaminergic agonists and RIIβ mutant mice are consistent with the hypothesis that the tendency of the striatal projection neurons to fire is correlated with their activation state of PKA, as dictated by the dopaminergic receptor activation state. Given the normal expression pattern of RIIβ (Ludvig et al., 1990; Glantz et al., 1992), it is expected that the severely compromised PKA activity seen in the striatal homogenates from RIIβ mutants reflects a reduction in both D1R and D2R expressing striatal neurons. However, D1R expressing neurons appear to remain partially responsive to dopaminergic agents in the mutant mice, as behavioral responses to the D1R specific agonists SKF38393 and SKF81297 remain grossly normal; the residual activatable PKA in these neurons is likely sufficient to confer this responsiveness. In contrast, D2R expressing neurons may be entirely unresponsive to dopaminergic agents, possibly due to the loss of specifically localized regulatory subunits required for

G_i-coupled signaling, and a consequential decrease in the phosphorylation state of relevant substrates. In other words, the D2R cannot signal in the mutants because the PKA activation state and substrate phosphorylation state already mimic those that would be effected by activation of this receptor. This contention is supported by the lack of behavioral activation by the D2R agonist quinpirole in the RIIβ mutant mice. Amphetamine remains effective in the mutant mice, as the balance of activity of the two pathways will still tend toward the direct pathway, despite the reduction in total PKA, and the predicted reduction in activity of both of the pathways.

How might the activation state of PKA be reflected in the excitation state of the projection neurons? PKA is likely modulating the conductance of both ionotropic glutamate receptors, which would be directly transfer the signals from the cortex to the striatal cells, and voltage-gated ion channels which presumably transfer glutamatergic responses to a tendency of striatal projection neurons to fire action potentials. Several of the AMPA/kainate-type ionotropic glutamate receptors are expressed in striatal neurons (Martin et al., 1993; Chesselet et al., 1995), and some of these have previously been shown to increase their conductance when activated by PKA (Keller et al., 1992; Raymond et al., 1993a; Wang et al., 1993). Subcellular anchoring of Type II PKA may even be a critical factor in the modulation of glutamate responsiveness (Rosenmund et al., 1994). It should be noted that a direct study of striatal neurons showed that in the slice preparation dopamine generally decreased the response of AMPA/kainate-type receptors, whereas the conductance of NMDA-type ionotropic glutamate receptors tended to reflect the predicted PKA activation state of the cell (Cepeda et al., 1993). That is, D1R activation increased, while D2R activation decreased, NMDA-evoked excitations. In another study it was demonstrated that basal PKA activity is important in NMDA-type ionotropic glutamate receptor function, and that dephosphorylation decreases NMDA-evoked currents (Raman et al., 1996).

Regarding voltage-gated ion channels, studies indicate that PKA-mediated phosphorylation regulates the conductance of sodium, potassium, and calcium in neostriatal cells (Surmeier and Kitai, 1993; Schiffmann et al., 1995; Surmeier et al., 1995a). Analogous to the glutamate receptor modulation, AKAP-mediated subcellular localization of Type II PKA has been shown to be important in calcium channel modulation as well (Johnson et al., 1994). Striatal sodium channels are inhibited by PKA activating receptors, and N- and P- type calcium channels have been shown to be directly inhibited by PKA (Schiffmann et al., 1995; Surmeier et al., 1995a). In contrast, L-type calcium channels show increased conductance in response to D1R-mediated activation of PKA. Potassium channels have decreased conductance in response to PKA activation, and this may also increase the tendency of the neuron to fire (Surmeier and Kitai, 1993). Thus, if the hypothesis that the tendency of MSN's to fire is correlated with the PKA activation state (as dictated by the type of dopaminergic receptor activated) is correct, one would predict that the conductance states of potassium channels, L-type calcium channels, and NMDA-type glutamate receptors play a major role in determining the tendency to fire. Electrophysiological analysis of these responses in the mutant mice should prove extremely informative.

Dynorphin and motor output. The opioid peptide dynorphin is a high affinity agonist of the κ-type opiate receptors (Chavkin et al., 1982; Corbett et al., 1982). These receptors are expressed in the striatum and substantia nigra, both target regions for the dynorphin containing striatal medium spiny neurons (Mansour et al., 1995). In vivo microdialysis studies have shown that dynorphin analogs and κ-agonists inhibit dopamine release in the striatum (Di Chiara and Imperato, 1988; Spanagel et al., 1990). Moreover, dynorphin analogs and κ-agonists have been shown to inhibit behavioral effects of psychostimulants (Ukai et al., 1992; Heidbreder et al., 1995). It is reasonable to consider that the increased sensitization of the RIIβ mutant mice to amphetamine might be due in part to a loss of

dynorphinergic inhibition of dopamine release and/or motor output. Our data also suggest the very interesting and perhaps unexpected possibility that transcription factors induced by PKA in response to amphetamine, including c-fos in the dorsomedial striatum, actually *inhibit* the development of sensitization.

The relationship between phosphorylation of CREB and c-fos expression. Why is CREB still phosphorylated in response to amphetamine the RIIβ mutant striatum? Previous work has shown that in striatal cells, CREB is phosphorylated at ser133 in response to D1R stimulation; one might have predicted that this response is mediated through PKA (Konradi et al., 1994). Given the dramatic reduction in striatal PKA in the RIIβ mutant mice, but the observed phospho-CREB-like immunoreactivity in response to amphetamine, it can be concluded that (at least in the RIIβ mutants) this response is mediated either through a PKA holoenzyme other than Type IIβ, or another protein kinase entirely. CREB has been shown to induce transcription in response to phosphorylation at ser133 by various other protein kinases, including CaM kinases II, and IV (Dash et al., 1991; Sheng et al., 1991; Enslen et al., 1994; Matthews et al., 1994; Sun et al., 1994), and a ras-dependent protein kinase (Ginty et al., 1994).

Why is c-fos not induced despite the phosphorylation of CREB at the activating ser133? Previous studies have suggested that phosphorylation of CREB at this residue is necessary for activation of c-fos transcription (Sheng et al., 1991), and CREB has been shown to be specifically required for c-fos induction in the striatum by amphetamine (Konradi et al., 1994). However, it is becoming increasingly apparent that CREB phosphorylation is not sufficient for gene induction. Recent experiments using PC12 cells demonstrated that calcium-induced phosphorylation of CREB at ser133 is not sufficient for c-fos induction, and showed that a second PKA-dependent signal is required (Thompson et al., 1995). Additionally, experiments with transgenic mice that express β-galactosidase driven by c-fos promoters containing various mutations indicate that the Ca/CRE

element (which binds CREB) is not sufficient for c-fos induction by kainate in vivo (Robertson et al., 1995). Moreover, point mutations in other (non-Ca/CRE) regulatory elements prevent c-fos induction by db-cAMP, an activator of PKA, in primary cells. Interestingly, experiments with PC12 cells lacking Type II PKA showed a diminished activitation of the c-fos promoter by forskolin, specifically implicating Type II PKA in c-fos transcription (Sassone-Corsi et al., 1988; Velcich and Ziff, 1990). Thus, our results corroborate those from both the cultured cells and the transgenic mice, and in conjunction with those previous findings, it can be concluded that in multiple systems phosphorylation of CREB at ser133 is necessary, but not sufficient, for induction of c-fos transcription. Our findings also support the suggestion that a second signal is required for transcription of this gene, and likely for full function of the proposed "interdependent transcription complex" (Robertson et al., 1995). In the striatum of an amphetamine treated animal this second signal may be mediated through D1R activation of Type IIβ PKA, as the induction of c-fos that is absent in the mutants is believed to be dependent upon activation of this receptor (Graybiel et al., 1990). The constitutive reduction in prodynorphin mRNA in regions normally rich in Type IIβ PKA might also be a result of the loss of this second signal. One speculation is that the second signal is PKA-mediated phosphorylation of CREB binding protein (CBP) which links activating transcription factors to the basal transcription machinery (Brindle et al., 1995).

Further experimentation using the RIIβ mutant mice, especially when crossed with mice carrying mutations in other components of the dopaminergic signaling pathways, is expected to provide valuable new insights into the complex relationships between biochemistry, cellular and systemic neurophysiology, and behavior.

Figure 3.1. Targeted disruption of RIIβ. **(A)** Genomic locus, targeting vector, and predicted structure of targeted locus. The RIIβ targeting vector replaces the major transcription initiation start site and coding region of exon 1 of the RIIβ gene with a neomycin resistance cassette (neo). Restriction enzyme sites shown are: A, Aat II; E, Eco RI; H, Hind III; R, Rsr I. The probe fragment used to identify disrupted alleles in ES cells and mice is shown. **(B)** Genomic Southern blot of tail DNA from offspring of a cross of heterozygotes. DNA was digested with with Hind III and probed with the fragment shown in (A). The wild type allele appears as a 4.7 kb band, and the disrupted allele as a 3.0 kb band. Genotypes are indicated above each lane: wild type (+/+), heterozygous (+/-), and homozygous mutant (-/-). **(C)** Northern blot of brain RNA from wild type (+/+) and homozygous mutant (-/-) mice, probed with an antisense riboprobe specific for the RIIβ sequence. The migration of the normal RIIβ mRNA, 28S and 18S ribosomal bands is indicated. **(D)** In situ hybridization of wild type (wt) and homozygous mutant (RIIβ ko) brain slices using a probe for RIIβ mRNA. Expression is high in the cortex and striatum, and is low in the globus pallidus.

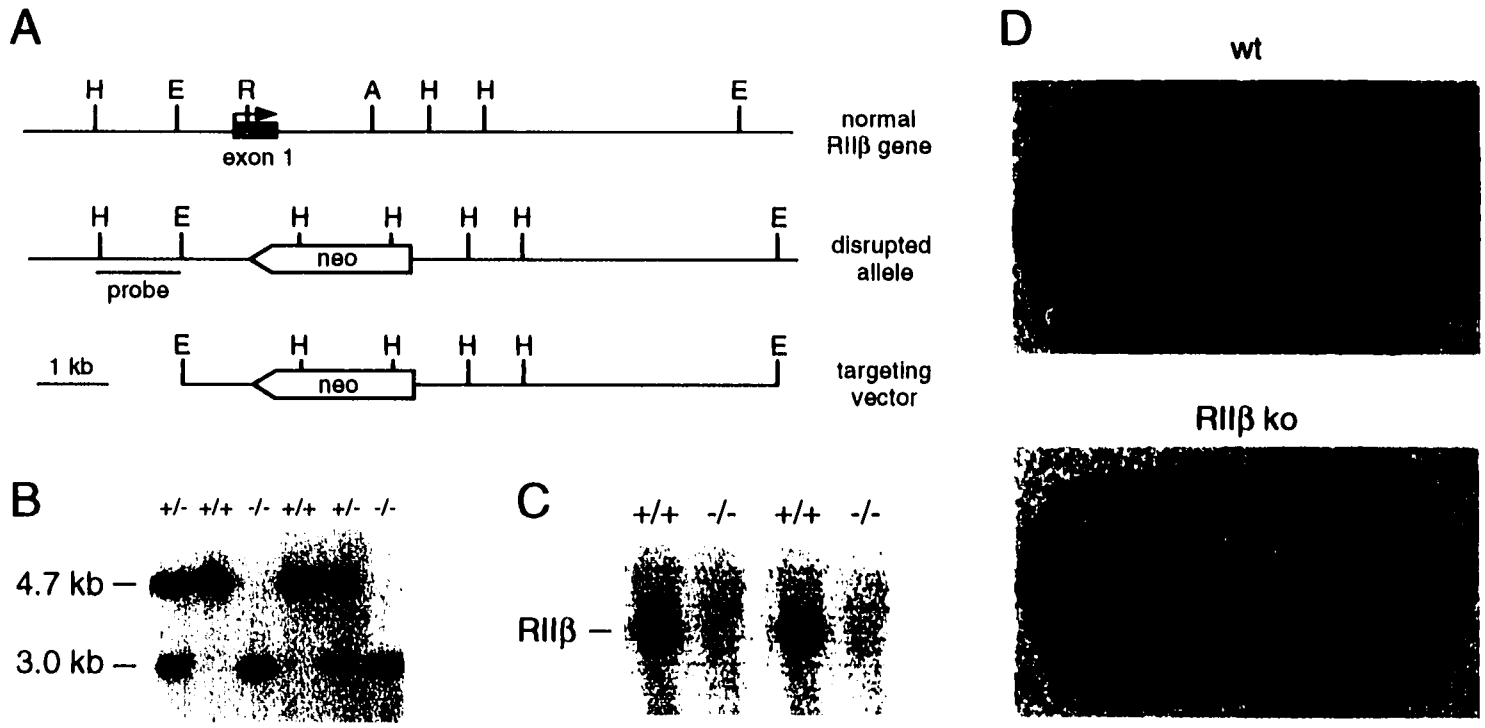

A
H
E
R
A
H
H
E
exon 1
normal
RIIβ gene
H
E
H
H
H
H
E
neo
probe
disrupted
allele
1 kb
E
H
H
H
H
E
neo
targeting
vector
B
+/- +/+ -/- +/+ +/- -/-
4.7 kb —
3.0 kb —
C
+/+ -/- +/+ -/-
RIIβ —
D
wt
RIIβ ko

Figure 3.2. RIIβ is the major PKA isoform in the striatum. **(A)** Western blot of several regions of wild type mouse brain indicates that RIIβ is expressed at the highest levels in the striatum, and lowest in the cerebellum, and at intermediate levels in various other regions of the brain. Below each lane is listed the percent PKA activity found in the mutant brain structure as compared to wild type control. Loss of RIIβ causes the most dramatic reduction in PKA activity in the striatum, and no appreciable loss in the cerebellum. Lanes, from left to right: cblm, cerebellum; bstm, brainstem; hpth, hypothalamus; hppc, hippocampus; cllc, colliculi; mdbr, midbrain; strm, striatum; nctx, neocortex. **(B)** Absence of RIIβ leads to compensation by R subunits and loss of C subunits. Western blots of striatal homogenates from three wild type (+/+) and three RIIβ knockout (-/-) mice were probed with antibodies to the various PKA subunits.

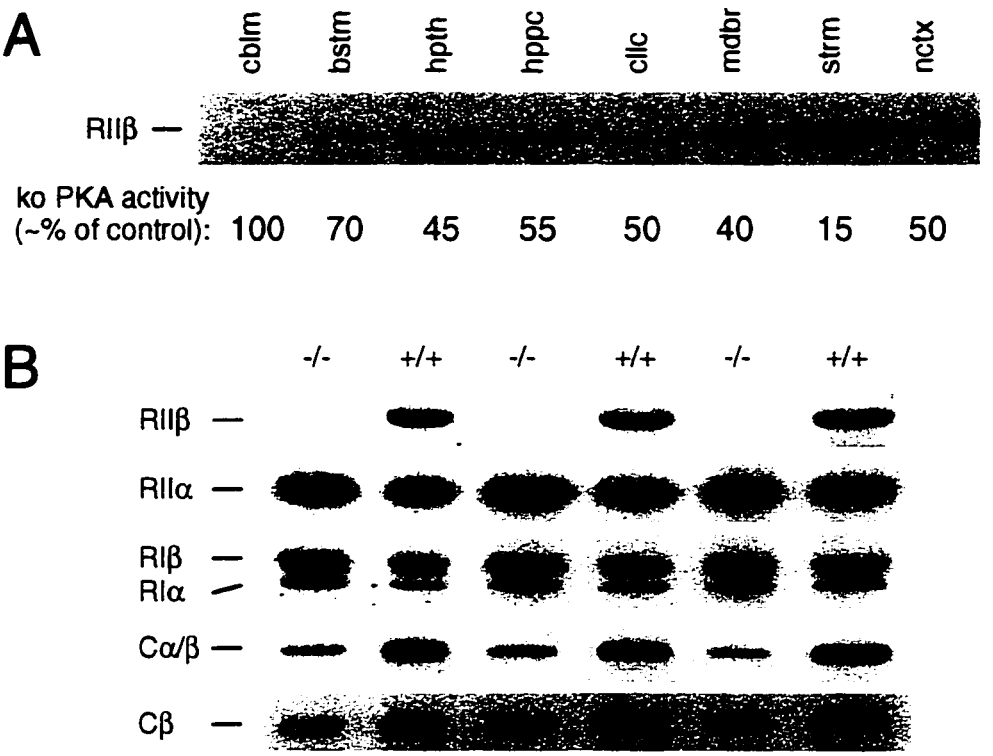

A
cblm
bstm
hpth
hppc
cllc
mdbr
strm
nctx
RIIβ —
ko PKA activity
(~% of control): 100 70 45 55 50 40 15 50
B
-/- +/+ -/- +/+ -/- +/+
RIIβ —
RIIα —
RIβ —
RIα
Cα/β —
Cβ —

Figure 3.3. Impairment of RIIβ knockout mice on the rotarod task. **(A)** RIIβ mutant (open squares; n = 11) and wild type control mice (closed squares; n = 13) were tested for their ability to stay on the accelerating rotarod. Ten trials were conducted on the first day and four on the second day (error bars represent standard error of the mean). ANOVA for repeated measures revealed a significant effect of trials 1-10 in both wild type ($F_{(9,108)} = 7.62$, $P < .001$) and RIIβ knockout mice ($F_{(9,90)} = 5.11$, $P < .001$), as well as a significant effect of genotype ($F_{(1,22)} = 14.07$, $P < .002$ on day 1; $F_{(1,22)} = 10.24$, $P < .005$ on day 2). **(B)** RIβ mutant mice (open circles; n = 6) were also tested and showed no significant differences from wild type control mice (closed circles; n = 12; $F_{(1,16)} = 0.16$, $P = 0.69$ on day 1; $F_{(1,16)} < .001$, $P > 0.99$ on day 2). **(C)** RIIβ mutant (open squares; n = 18) mice were tested at a lower rate of acceleration, and still were significantly impaired as compared to wild type control mice (closed squares; n = 17; $F_{(1,33)} = 60.76$, $P < .001$ on day 1; $F_{(1,33)} = 26.90$, $P < .001$ on day 2).

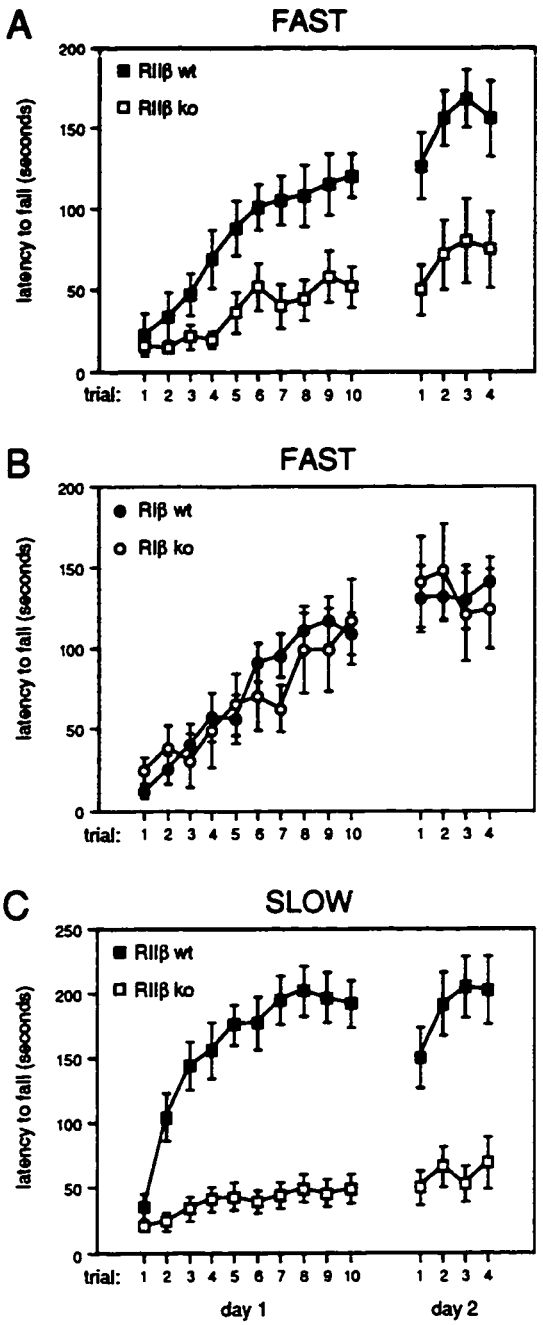

A
FAST
RIIβ wt
RIIβ ko
latency to fall (seconds)
200
150
100
50
0
trial: 1 2 3 4 5 6 7 8 9 10 1 2 3 4
B
FAST
RIβ wt
RIβ ko
latency to fall (seconds)
200
150
100
50
0
trial: 1 2 3 4 5 6 7 8 9 10 1 2 3 4
C
SLOW
RIIβ wt
RIIβ ko
latency to fall (seconds)
250
200
150
100
50
0
trial: 1 2 3 4 5 6 7 8 9 10 1 2 3 4
day 1
day 2

Figure 3.4. RIIβ mutants show altered behavioral responsiveness to various dopaminergic agents. **(A)** RIIβ knockout mice are hyperresponsive to the locomotor effects of D-amphetamine. Shown is data from wild type (solid bars; n = 5) and RIIβ mutant (stippled bars; n = 5) mice treated with saline or a 2.5 mg/kg dose of D-amphetamine (n = 6 wt and 6 ko). When comparing saline treatment to 1.0, 2.5, 5.0, and 10.0 mg/kg doses of amphetamine, repeated measures ANOVA revealed a significant effect of genotype ($F_{(1,42)} = 41.07$, $P < .001$). All error bars in this figure represent standard error of the mean. **(B)** RIIβ knockout mice are unresponsive to the locomotor effects of the D2 agonist quinpirole, but are responsive to the locomotor effects of the D1 agonist SKF38393. Shown is data from wild type (solid bars; n = 8) and RIIβ mutant (stippled bars; n = 11) mice treated with saline, a 2.5 mg/kg dose of quinpirole (n = 5 wt and 7 ko), or an 8.0 mg/kg dose of SKF38393 (n = 5 wt and 7 ko). When comparing saline treatment to 0.25, 0.5, 2.5, and 5.0 mg/kg doses of quinpirole, repeated measures ANOVA revealed a significant effect of genotype ($F_{(1,54)} = 44.21$, $P < .001$). When comparing saline treatment to 2.0, 4.0, 8.0, and 16.0 mg/kg doses of SKF38393, repeated measures ANOVA revealed a significant effect of dose ($F_{(4,56)} = 7.84$, $P < .001$), but no significant effect of genotype.

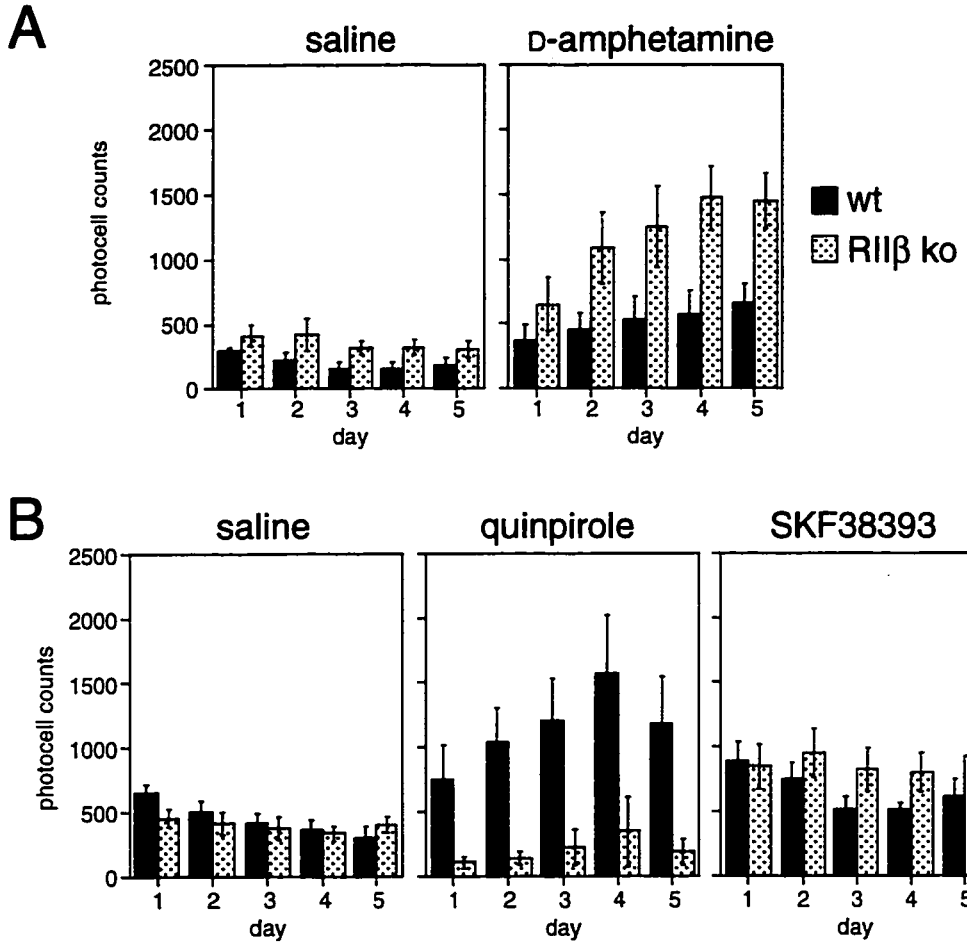

A
saline
D-amphetamine
photocell counts
2500
2000
1500
1000
500
0
1 2 3 4 5
day
wt
RIIβ ko
B
saline
quinpirole
SKF38393
photocell counts
day

Figure 3.5A. RIIβ knockout mice lack induction of c-fos mRNA in the dorsomedial striatum. In situ labeling for c-fos mRNA is shown as colorized (red) silver grains visualized using a confocal microscope in reflectance mode. These images are overlaid on transmitted light images (black and white) that show cresyl violet staining. Representative composite images demonstrate that c-fos mRNA expression is induced in wild type (top; wt) and RIβ knockout mice (bottom; RIβ ko) one hour following i.p. injection of 10 mg/kg D-amphetamine (right; amph), but is not induced in RIIβ mutants (middle; RIIβ ko). Images from saline treated animals of each genotype are shown on the left. Scale bar = 50 μm.

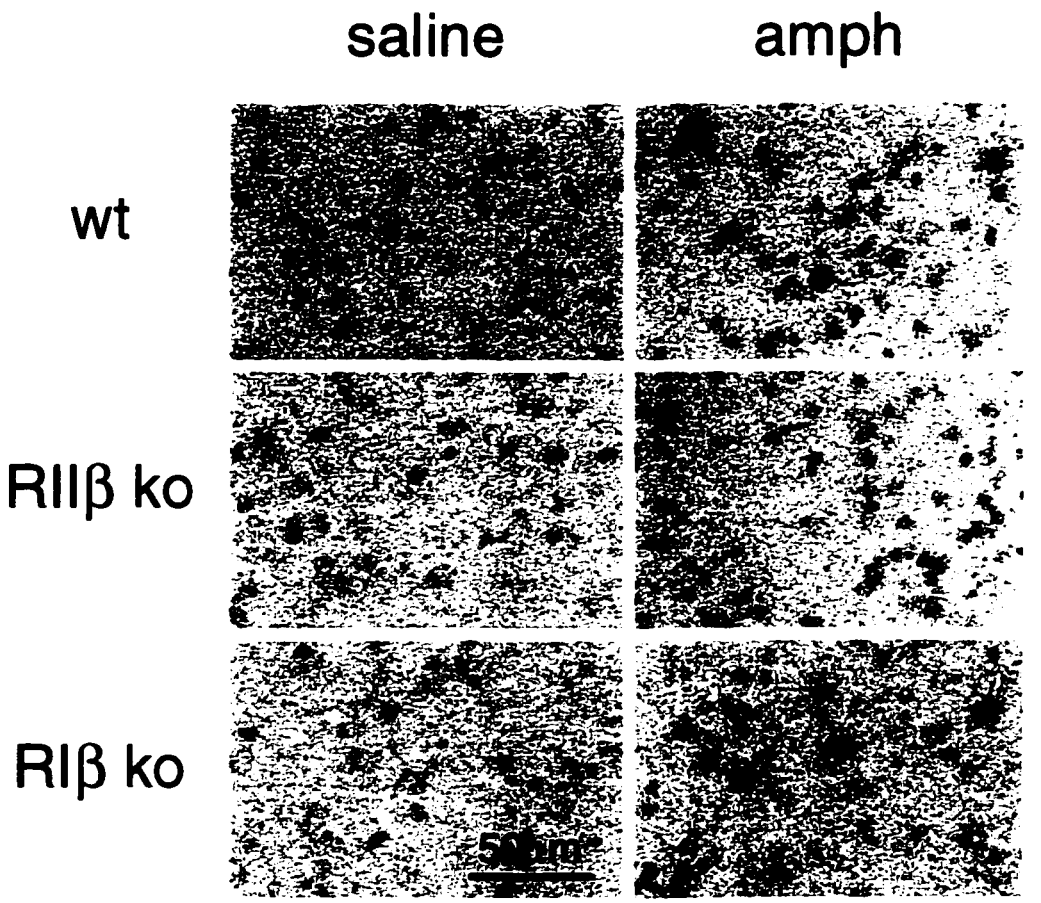

saline
amph
wt
RIIβ ko
RIβ ko

Figure 3.5B. Densitometry measurements from several regions of the forebrain of wild type (wt; solid bars), RIIβ knockout (RIIβ ko; stippled bars), or RIβ knockout (RIβ ko; shaded bars) mice treated with saline or 10 mg/kg D-amphetamine. RIIβ mutant mice do not induce c-fos in the dorsomedial (DM Str) region as compared to wild type mice ($p < .001$ by two tailed t-test). Other regions analyzed are ventrolateral striatum (VL Str), dorsolateral striatum (DL Str), cingulate cortex (Cing Cx), and piriform cortex (Piri Cx). Error bars represent standard deviation (n = 3-6 mice for each condition).

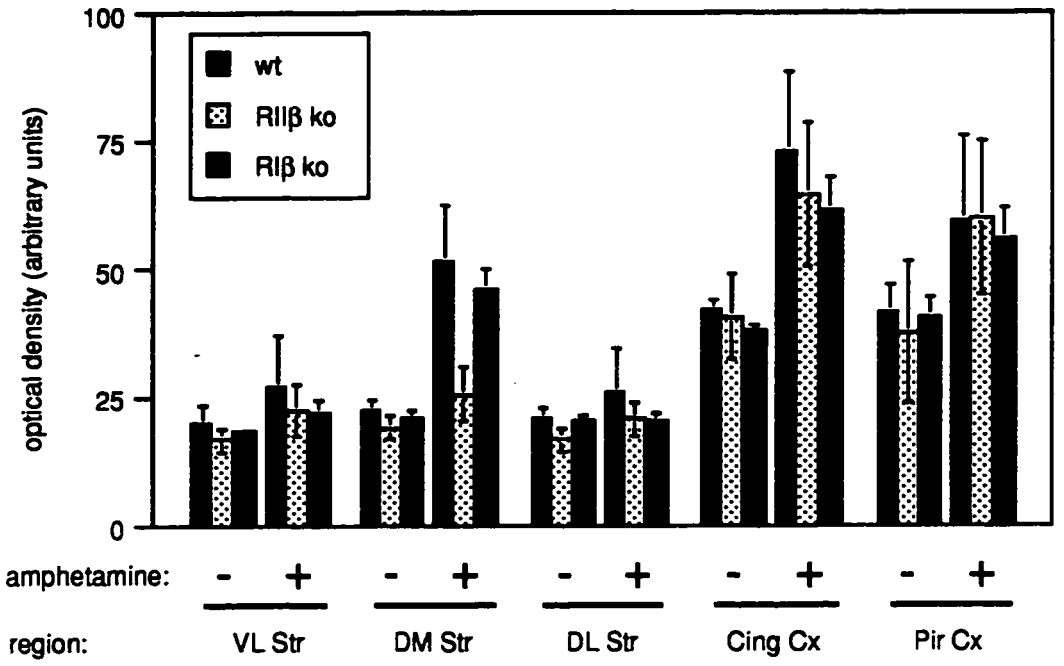

100
75
50
25
0
optical density (arbitrary units)
wt
RIIβ ko
RIβ ko
amphetamine:
- + - + - + - + - +
region:
VL Str
DM Str
DL Str
Cing Cx
Pir Cx

Figure 3.5C. RIIβ knockout mice phosphorylate CREB in response to amphetamine treatment. Representative images of phospho-CREB-like immunoreactivity observed in the striatum of wild type (wt) and RIIβ mutant (RIIβ ko) mice treated for 20 minutes with saline (top) or D-amphetamine (10 mg/kg; bottom) are shown. Bead-like signal is believed to be non-specific staining of blood vessels.

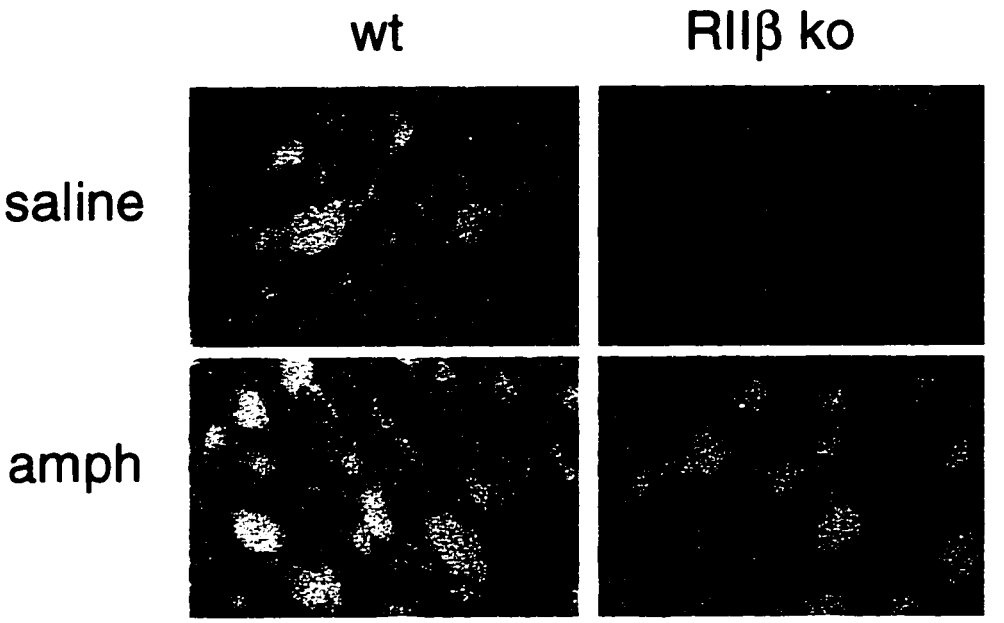

wt
RIIβ ko
saline
amph

Figure 3.6A. Dynorphin mRNA is reduced in RIIβ knockout mice. mRNA encoding prodynorphin is expressed in a grossly normal pattern in RIIβ mutant mice (right; RIIβ ko), but at reduced levels in the dorsolateral and dorsomedial regions. A section from a wild type (wt) brain is shown on the left for comparison. Ovals indicate the regions sampled for densitometry analysis shown in (3.6.B).

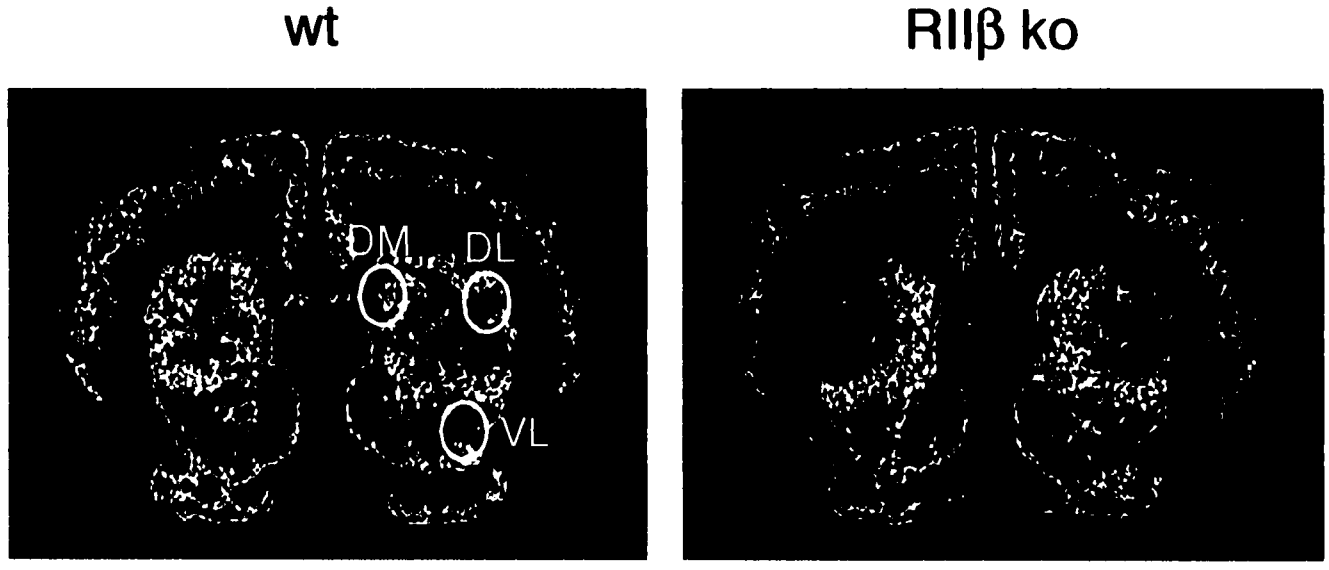

wt
RIIβ ko
DM
DL
VL

Figure 3.6B. Quantitative densitometry of three regions of the striatum from wild type (solid bars) and RIIβ mutant mice (stippled bars) shows that no signifcant difference is observed in the ventrolateral striatum, but reductions are seen in the dorsolateral ($p < .001$ by two tailed t-test) and dorsomedial ($p < .002$ by two tailed t-test) regions. Abbreviations: DL, dorsolateral; DM, dorsomedial; VL, ventrolateral.

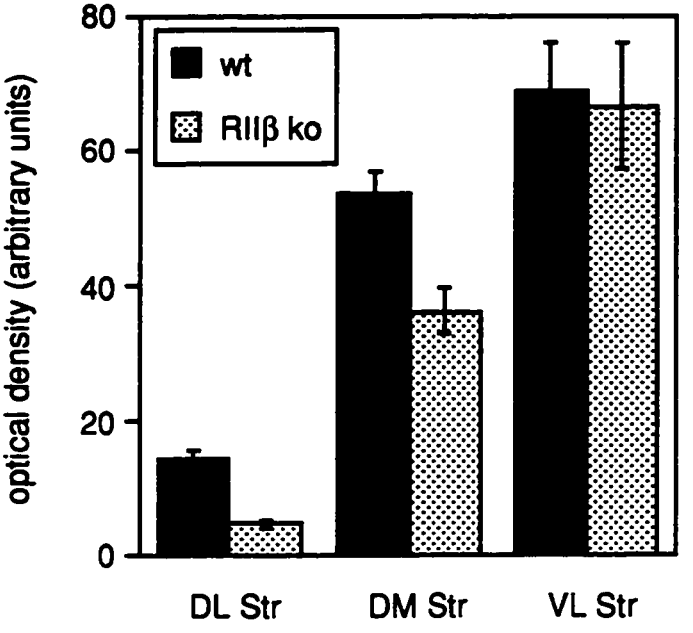
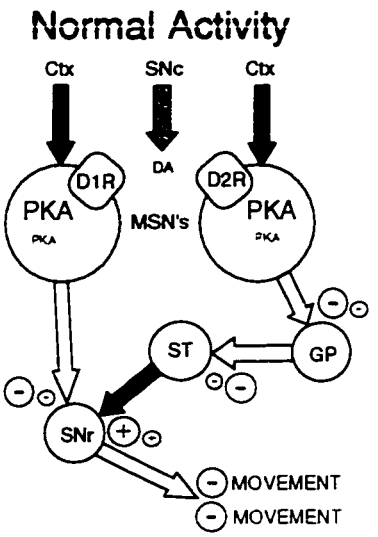
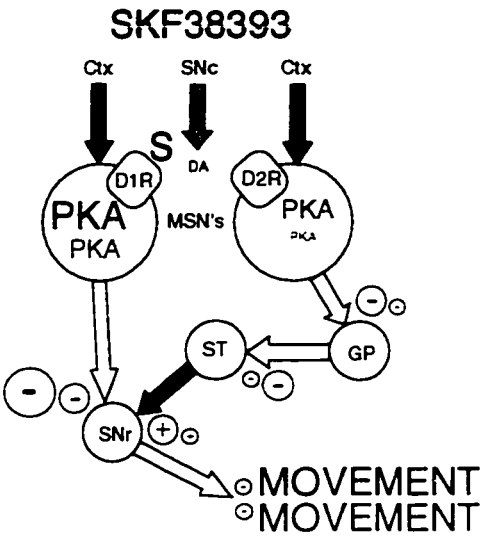
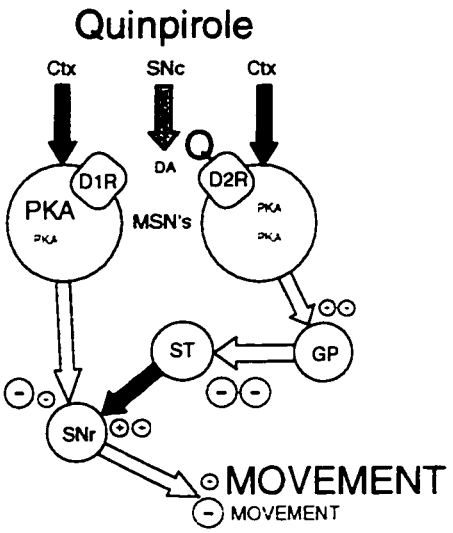
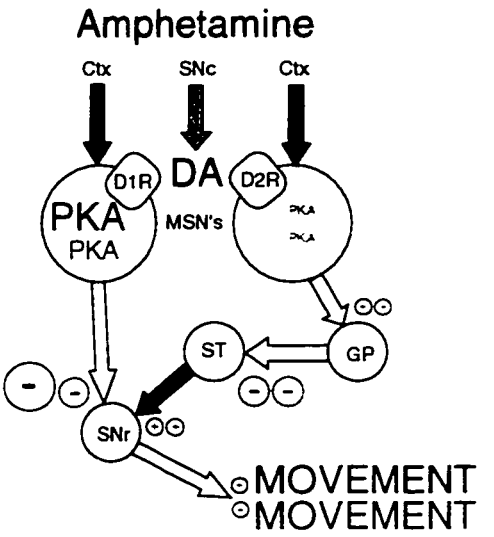

80
60
40
20
0
optical density (arbitrary units)
wt
RIIβ ko
DL Str
DM Str
VL Str

Figure 3.7. A proposed role for PKA in dopaminergic modulation of striatal projection neurons is suggested by the behavioral pharmacology of the RIIβ mutant mice. According to previous models, locomotor activation is inhibited by activity of GABAergic neurons that project from the substantia nigra pars reticulata (SNr) to central motor structures such as the thalamus (open arrows and "-" represent inhibitory projections; closed arrows and "+" represent excitatory projections; the size of these symbols represents the amount of neural activity of each projection). The activity of these SNr neurons is dually regulated by inhibitory inputs from the direct pathway and excitatory inputs from the indirect pathway. Activity of the two pathways is modulated by dopaminergic (DA) activation of the D1 (D1R) and D2 receptors (D2R) expressed by the medium spiny striatal projection neurons (MSN's) of the direct and indirect pathways, respectively. We propose that the tendency of the MSN's to fire is correlated with their activation state of PKA (represented by lettering size), and that the PKA activation state is increased by D1R activation, and decreased by D2R activation. The key features of the proposal are that relative to wild type mice (blue lettering) the RIIβ mutants (red lettering) have significantly less PKA activity in both types of MSN's, and that while some activation is still possible in D1R expressing cells, D2R expressing cells cannot further reduce their PKA activity (i.e. it is already at a minimum) in the mutants. Under normal circumstances (upper left), although mutant mice have significantly less PKA activity in both types of MSN's, the net balance of the inputs to the SNr cells remains the same; thus locomotion is similar between saline treated wild type and RIIβ knockout mice. When treated with the D1R agonist SKF38393 (upper right), both wild type and mutant mice increase their locomotor activity; both types of mice increase firing of the direct pathway relative to the indirect pathway. In contrast, RIIβ mutant mice are unresponsive to the locomotor activating effects of the D2R agonist quinpirole (lower left); in wild type mice this drug decreases the firing of the indirect pathway, but in the mutants G_i-coupled









Blue = Wild Type
Red = RIIβ Knockout

(Figure 3.7 continued)

signaling is ineffective. Lastly, the indirect dopaminergic agonist amphetamine (lower right) increases the activity of the direct pathway relative to the indirect pathway in both types of mice, and thus remains effective in the RIIβ mutants. Other abbreviations: Ctx, cortex; GP, globus pallidus; SNc, substantia nigra pars compacta; ST, subthalamic nucleus.

Table 1. Dopaminergic Components in RIIβ Knockout Striatum

Component	Wild Type	RIIβ mutant	Units
Dopamine	41.9 ± 5.6	38.5 ± 3.4	pg/mg
D1 Receptor (B_{max}):			
DL	50.4 ± 7.6	35.7 ± 2.1*	fmol/mg
DM	47.1 ± 7.2	39.9 ± 3.4	fmol/mg
VL	45.0 ± 6.7	38.0 ± 5.5	fmol/mg
VM	42.2 ± 6.5	38.4 ± 3.9	fmol/mg
D2 Receptor (B_{max}):			
DL	41.8 ± 2.7	36.5 ± 3.2	fmol/mg
DM	32.9 ± 1.8	28.2 ± 2.6	fmol/mg
VL	33.4 ± 1.1	32.9 ± 2.3	fmol/mg
VM	27.8 ± 1.1	26.4 ± 1.3	fmol/mg
Dopamine Transporter:			
DA uptake (V_{max})	101.8 ± 5.6	98.2 ± 1.1	mol/min/mg
Amph inhibition (IC_{50})	0.16 ± .07	0.19 ± .11	μM Amph

*p = 0.07

CHAPTER IV

GENETICALLY LEAN MICE RESULT FROM TARGETED DISRUPTION OF THE RIIβ SUBUNIT OF PROTEIN KINASE A

Introduction

Cyclic AMP is a key second messenger in the coordinated regulation of cellular metabolism (McKnight, 1991). Its effects are mediated by cAMP-dependent protein kinase (PKA), which is assembled from two regulatory (R) and two catalytic (C) subunits. In mice there are four R genes (RIα, RIβ, RIIα, and RIIβ) and two C genes (Cα and Cβ), expressed in tissue-specific patterns. The RIIβ isoform is abundant in brown and white adipose tissue and brain, with limited expression elsewhere. To elucidate its functions, we generated RIIβ knockout mice. Mutants appear healthy but have markedly diminished white adipose tissue despite normal food intake. They are protected against developing diet-induced obesity and fatty livers. Mutant brown adipose tissue exhibits a compensatory increase in RIα, which almost entirely replaces lost RIIβ, generating an isoform switch. The holoenzyme from mutant adipose tissue binds cAMP more avidly and is more easily activated than wild type enzyme. This causes induction of uncoupling protein and elevations of metabolic rate and body temperature, contributing to the lean phenotype. Our results demonstrate a unique role for the RIIβ holoenzyme in regulating energy balance and adiposity.

Experimental Procedures

Special diets. Specialized mouse food was provided by Research Diets, Inc., New Brunswick, NJ. By calories, the low fat diet contained 60% corn starch and 7% hydrogenated coconut oil, while the high fat diet contained 13% corn starch and 54% coconut oil. Both

diets contained 12% maltodextrin, 4% soybean oil, 16% casein, and identical vitamins and minerals. Hepatic triglycerides were measured with an enzymatic assay obtained from Boehringer Mannheim, Inc.

Western blot and kinase assay. BAT protein homogenates (40 mg/lane) were resolved by 10% SDS-PAGE and transferred to nitrocellulose. Coomassie Blue staining confirmed uniformity of protein loading. Immunoblotting was performed with polyclonal antisera raised against recombinant murine PKA subunits and diluted as follows: RIIβ 1:10000, RIIα 1:1000, RI 1:200, and C 1:2000. The anti-RI antisera recognizes both RIα and RIβ, which are resolvable using this gel system; the anti-C antibody recognizes both Cα and Cβ, which co-migrate. Anti-RIIβ and RIIα antisera were generously provided by J. Scott (Vollum Institute, Portland, OR), and anti-hamster UCP (1:2000) by L. Kozak (Jackson Labs, Bar Harbor, ME). Primary antibodies were visualized with HRP-coupled goat anti-rabbit Ig (1:40000) and ECL (Amersham). Ion exchange chromatography was performed as previously described (Clegg et al., 1988). Four mg of BAT protein were loaded onto DEAE columns and separated by HPLC with a linear salt gradient from 0 to 250 mM NaCl. Kinase activity of individual fractions was determined with Kemptide substrate as previously described (Clegg et al., 1987), in the presence or absence of 5 mM cAMP. Type I PKA elutes between 40 and 80 mM NaCl (fractions 20-25), and type II between 130 and 180 mM NaCl (fractions 31-35). Free C subunit elutes just before type I holoenzyme.

cAMP binding. cAMP binding capacity was measured by incubating 160 mg of BAT protein with H^3cAMP for 45 minutes at 37°C in 240 ml of buffer containing 20 mM Tris (pH 7.0), 0.5 mM IBMX, 1 mg/ml BSA, 10 mM Mg acetate, 5 mM NaF, 10 mM DTT, 200 mM ATP, and protease inhibitors. Proteins were precipitated with NH_4SO_4 as previously described (Doskeland and Ogreid, 1988). Non-specific binding measured in duplicate samples containing a 1000-fold excess of unlabelled cAMP was subtracted from total counts to determine specific binding.

Metabolic analysis. $\dot{V}O_2$ was measured from individual mice in an Oxymax chamber (Columbus Instruments) with an air flow of 1.0 L/min. Total $\dot{V}O_2$ was determined as the mean of 50 samples of cage air measured during the 2.2-hour sampling window shown in figure 4.4c. Body temperature was assessed with a rectal thermister (Yellow Springs Instruments).

Results and Discussion

RIIβ mutants are fertile, long-lived, and exhibit no overt abnormal phenotype. However, they have remarkably decreased white adipose tissue (WAT) mass. Weights of fat pads in three anatomic locations were about half as great in mutants as wild types (Fig. 4.1A). Magnetic resonance imaging (Fig. 4.1B) confirmed that the reduction in fat occurs bodywide. Whole-mouse magnetic resonance spectroscopy (Stein et al., 1995) and volume-of-distribution experiments with ^3H_2O showed that mutants have approximately 6% body fat, compared with 15% in wild types.

The knockouts are not cachectic. Overall weight is reduced ~10%, consistent with loss of fat as the only alteration in body composition (Fig. 4.1A). Leanness does not appear to result from decreased food intake or absorption. Mutants tend to be slightly hyperphagic (Fig. 4.1A), and show normal post-prandial triglyceride blood levels. Plasma cholesterol, free fatty acids, insulin, glucose and thyroid hormones are also unperturbed (data not shown). The reduction of WAT appears to arise principally from decreased triglyceride stores, rather than diminished adipocyte number, as revealed by the normal cellularity of fat pads (Fig. 4.1A) and reduced adipocyte size (Fig. 4.1C). There are no obvious histologic changes in tissues other than fat.

Mutants are protected against some of the adverse effects of consuming a high fat diet. After eating a 58% fat diet for four months, normal mice became obese while mutants remained lean (Fig. 4.2A).

Furthermore, wild types developed fatty livers while mutants did not (Fig. 4.2, B & C).

Mutants appear to be lean, at least in part, because of changes in PKA activity and gene expression in brown adipose tissue (BAT). BAT facilitates non-shivering thermogenesis during cold acclimation (Himms-Hagen, 1990) or chronic overeating (Rothwell and Stock, 1979). This is accomplished by uncoupling protein (UCP), a proton translocator that uncouples mitochondrial respiration from oxidative phosphorylation in BAT, so that energy derived from oxidation of fatty acids is dissipated as heat, rather than stored as ATP. UCP is induced by a cAMP-dependent mechanism following β-adrenergic stimulation (Kopeck'y et al., 1990).

RIIβ is the principal PKA regulatory subunit in BAT. It is expressed more abundantly there than in any other tissue (data not shown). Though there are no histologic changes in mutant BAT, loss of RIIβ protein is associated with a compensatory increase of the RIα isoform, normally scarce in this tissue (Fig. 4.3A). RIβ is not expressed in BAT, and RIIα is barely detectable in both wild types and mutants. Thus, elimination of RIIβ transforms the PKA in BAT from a predominantly RII-containing (type II) holoenzyme to a predominantly RI type. HPLC separation of type I and II PKA confirms this isoform switch (Fig. 4.3B). The increased RIα compensates almost entirely for lost RIIβ, such that overall cAMP binding capacity (a reflection of total R subunit quantity) is reduced only approximately 10% (Fig. 4.4A). Total C subunit is decreased by roughly one third based on kinase activity (Fig. 4.4B) and Western analysis (Fig. 4.3A). Thus it appears that an R:C ratio of at least 1:1 is maintained in mutant BAT. These changes in RIα and Cα reflect altered rates of protein degradation (Amieux et al., manuscript in preparation).

In BAT, the largely type I PKA in mutants binds cAMP more avidly than does the type II PKA predominating in wild types, with Kd's of 140 and 380 nM, respectively (Fig. 4.4A). Mutant PKA holoenzyme is consequently more readily activated by cAMP than is

wild type enzyme (Ka's of 80 and 350 nM, respectively, Fig. 4.4B). Accordingly, basal PKA activity is elevated approximately five fold in mutant BAT (Fig. 4.4B, insert). cAMP levels in wild type and mutant BAT showed no differences that might be attributed to extrinsic (e.g., sympathetic) stimulation (data not shown). These data suggest that the changes in PKA activity result from the type II to type I isoform switch, rather than from a gross deregulation of C subunits due to either lost R subunits or increased cAMP.

The elevated basal PKA activity leads to a 4-5 fold increase in UCP (Fig. 4.3A), by inducing UCP mRNA (data not shown). Figs. 4.4 C & D show that knockouts have an elevated metabolic rate (assessed by oxygen consumption, which closely reflects energy expenditure (Jequier and Felber, 1987), and a 0.8°C increase in body temperature (Fig. 4.4E). These findings suggest that excess UCP in BAT, arising from increased basal PKA tone, renders RIIβ mutant mice metabolically inefficient, causing food calories to be wasted as heat and depleting fat stores. Furthermore, lipoprotein lipase in BAT, whose expression is also PKA-dependent (Carneheim et al., 1988), is overexpressed, possibly helping mutant BAT to compete with WAT for dietary triglycerides, to be burned rather than stored.

After BAT, RIIβ is most highly expressed in WAT, where it is also the principal PKA regulatory isoform (data not shown). In this tissue, PKA transduces signals from catecholamines and glucagon to stimulate lipolysis and inhibit lipogenesis. Like BAT, RIIβ mutant WAT displays an increase in RIα, a type II to I isoform switch, and elevated basal PKA activity despite moderately decreased total PKA activity and C subunit protein (data not shown). These changes are associated with a small increase in lipolysis (assessed by glycerol release from cultured adipocytes), which may contribute to the lean phenotype (data not shown). However, serum glycerol was similar in wild type and mutant mice. Since both BAT and WAT are affected in RIIβ mutants, we are unable to determine their relative contributions to the lean phenotype without generating a tissue-specific RIIβ defect.

Major advances in understanding body weight regulation have resulted from the study of genetically obese rodent strains (Zhang et al., 1994; Chen et al., 1996; Chua et al., 1996; Lee et al., 1996; Noben-Trauth et al., 1996) , of which there are at least twelve (Friedman and Leibel, 1992; Lowell et al., 1993; Tecott et al., 1995). RIIβ mutants are one of only a few animal models of genetic leanness (Kozak et al., 1991; Schneider et al., 1993; Katz et al., 1995). Several obese rodent strains differ from RIIβ mutants in that they have reduced UCP and thermogenically inactive BAT (Himms-Hagen, 1990). It will be interesting to see if the RIIβ knockout can rescue an obese phenotype when two mutant strains are interbred.

RIIβ mutants have markedly reduced leptin mRNA and plasma levels (data not shown), corroborating leptin's proposed role as an adiposity indicator (Zhang et al., 1994). Leptin is also thought to be a satiety factor (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). However, only mild hyperphagia, insufficient to maintain normal adiposity, is seen in RIIβ mutants despite reduced leptin. A change in their body weight regulatory system could exist that prevents them from developing fully compensatory hyperphagia. It has been proposed that BAT might produce an appetite-suppressing signal (Flier, 1995), based on the unexpected finding that BAT-deficient mice develop hyperphagia (Lowell et al., 1993). It is possible that such a factor is induced in the overactive BAT of RIIβ mutants, inhibiting them from eating enough to maintain normal lipid stores.

One way the brain is thought to regulate adiposity is by modulating sympathetic stimulation of PKA in BAT to control facultative energy expenditure through UCP (Himms-Hagen, 1990). RIIβ mutants provide evidence for this phenomenon, since their elevated basal PKA activity in BAT is associated with increased UCP, excess energy expenditure, and leanness. In contrast, mice made deficient in BAT with a toxigenic transgene develop an abnormally efficient metabolism and obesity (Lowell et al., 1993). Chronic

sympathetic stimulation normally causes BAT hypertrophy (Lafontan and Berlan, 1993). However, wild type and mutant interscapular BAT showed no differences in weight (65 ± 17 *vs.* 60 ± 17 mg, respectively) or DNA content (119 ± 7 *vs.* 123 ± 5 mg), and mutant tissue had only slightly increased protein content (8.8 ± .5 *vs.* 10.9 ± 1.2 mg). In mice, adrenergic stimulation of β_1 receptors is believed to be the principal mediator of BAT hypertrophy, while β_3 receptors are probably most important for UCP induction (Lafontan and Berlan, 1993). PKA changes in RIIβ mutant BAT mimic β_3 more than β_1 stimulation.

Chronic activation of PKA in adipose tissue via β-adrenergic stimulation is being investigated as a mode of obesity therapy. Research has focused on agonists specific for β_3 receptors (Arner, 1995), which are adipose-specific (Lafontan and Berlan, 1993). Our results raise the possibility that RIIβ could also provide a target for anti-obesity drugs.

Figure 4.1. **(A)** RIIβ null mutant mice have reduced adipose tissue mass. Discrete white fat pads from three anatomic sites were dissected and weighed. "Reproductive" refers to the epididymal and parametrial pads of males and females, respectively. WAT cellularity was ascertained by DNA quantitation. Consumption of standard mouse chow (Harlan Teklad, Inc., Madison, WI) was measured over a four month period starting at six weeks of age. Results represent means ± s.e.m.

		Reproductive Fat Pad Weight (% body wgt)	Inguinal Fat Pad Weight (% body wgt)	Retroperitoneal Fat Pad Weight (% body wgt)	Reproductive Fat Pad Cellularity (cells/pad x 10-6)	Inguinal Fat Pad Cellularity (cells/pad x 10-6)	Retroperitoneal Fat Pad Cellularity (cells/pad x 10-6)	Body Weight (grams)	Food Intake (kcal/mouse/d)
MALE	Wild Type	1.29 ± .10	.96 ± .10	.29 ± .04	17.4 ± .9	18.6 ± 1.9	3.7 ± .5	25.2 ± .9	11.4 ± 2.7
	Mutant	.62 ± .04	.63 ± .04	.11 ± .01	15.4 ± 1.5	21.4 ± 2.6	3.4 ± .5	23.4 ± 1.1	12.4 ± 1.5
	% of Wild Type	48%	65%	37%	89%	115%	92%	93%	109%
		P ≤ .0001	P ≤ .002	P ≤ .0001	P = .14	P = .21	P = .31	P = .10	P = .39
FEMALE	Wild Type	2.25 ± .34	1.09 ± .03	0.23 ± .02	19.0 ± 2.0	21.6 ± 2.3	ND	22.4 ± 1.7	9.1 ± .4
	Mutant	.97 ± .13	.72 ± .04	.11 ± .02	17.4 ± 1.7	21.1 ± 2.8	ND	19.5 ± .8	10.9 ± .3
	% of Wild Type	43%	66%	48%	92%	98%		87%	120%
		P ≤ .0006	P ≤ .0001	P ≤ .001	P = .21	P = .44		P = .06	P = .01

Figure 4.1. **(B)** Fat-selective magnetic resonance images of wild type and mutant female mice. T1-weighted spin echo inversion-recovery sequences are shown. The differential magnetic relaxation properties of protons associated with lipid *vs.* aqueous tissues were used to highlight collections of adipose tissue, which appear white. Images are 3-mm thick axial body sections at the base of the hind legs. Similar results were obtained from images throughout the body. mf, mesenteric fat; sqf, subcutaneous fat; sc, spinal cord; h, proximal hind leg. **(C)** Representative histology of normal white adipose tissue (WAT), composed of large, polygonal cells with prominent triglyceride depots (clear spaces) and flattened nuclei. Comparable RIIβ mutant WAT reveals smaller, rounded adipocytes with diminished triglyceride stores, more eosinophillic aqueous cytoplasm, plump appearing nuclei, and scattered multivesicular cells. Both sections were formalin fixed, paraffin embedded, and stained with haematoxylin and eosin.

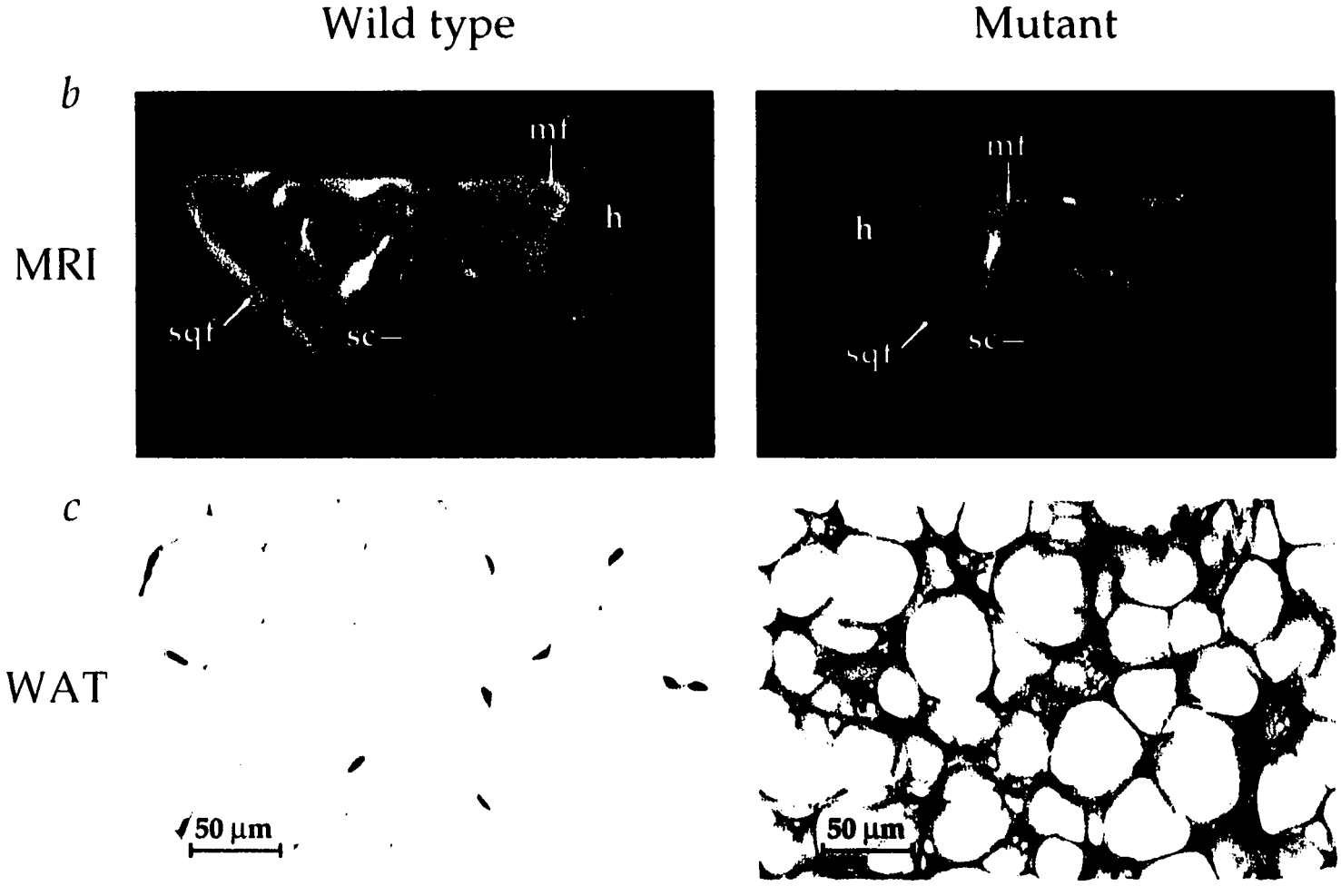

Wild type
Mutant
b
MRI
mf
h
sqf
sc
mf
h
sqf
sc
c
WAT
50 µm
50 µm

Figure 4.2. Effects of a high fat diet on RIIβ mutant mice. **(A)** Fat pad weights of 6 mice per group measured after four months on either an 11% or 58% lipid diet (Surwit et al., 1995). The combined weights of bilateral epididymal, inguinal, and retroperitoneal fat pads are expressed as a percentage of total body mass. On the high fat diet normal mice showed a marked increase in adiposity, with a 3.5-fold elevation of fat pad weight. In contrast, mutants were resistant to such changes in body composition, and only reached the level of fat pad mass found in wild types on a low fat diet. Interestingly, the overall body weights of both groups were only slightly increased on this diet. **(B)** Mutant mice are protected from developing fatty livers on the high fat diet. Hepatic triglycerides were quantitated in five wild type and five mutant mice. Values represent means ± s.e.m.; P = .001.

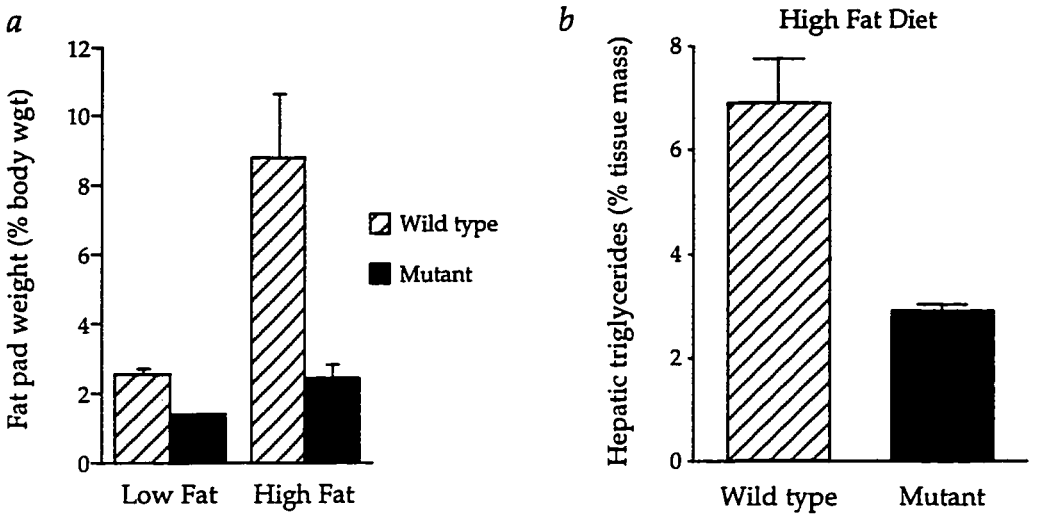
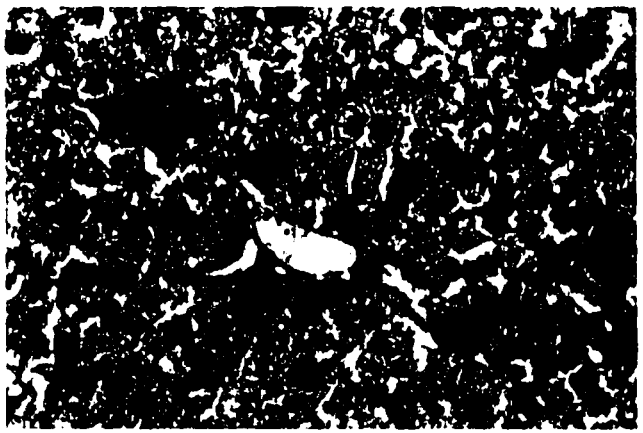

a
Fat pad weight (% body wgt)
12
10
8
6
4
2
0
Low Fat
High Fat
Wild type
Mutant
b
High Fat Diet
Hepatic triglycerides (% tissue mass)
8
6
4
2
0
Wild type
Mutant

Figure 4.2. (C) Haematoxylin and eosin stained liver sections from wild type and mutant mice are counterstained with oil-red-O to reveal abnormal lipid deposits (bright red) in hepatocytes of normal animals. This pathology is absent in mutants.

C

High Fat Diet

Wild type liver



Mutant liver

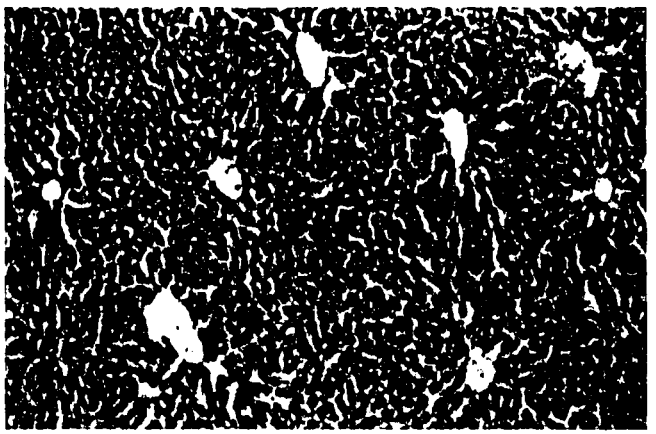


Figure 4.3. (A) Changes in protein levels and PKA activity in RIIβ mutant BAT. Western blots of BAT from wild type (+) and knockout (-) mice probed with the antibodies shown. Elimination of RIIβ in mutants is confirmed by the complete absence of RIIβ protein in BAT. The RIα isoform is markedly increased in mutants, while there is a small loss of C subunit. RIβ is not detectable in either wild type or mutant adipose tissue. RIIα is barely detectable, and is unaltered in mutants. UCP is increased 4-5 fold in mutants, as revealed by scanning densitometry.

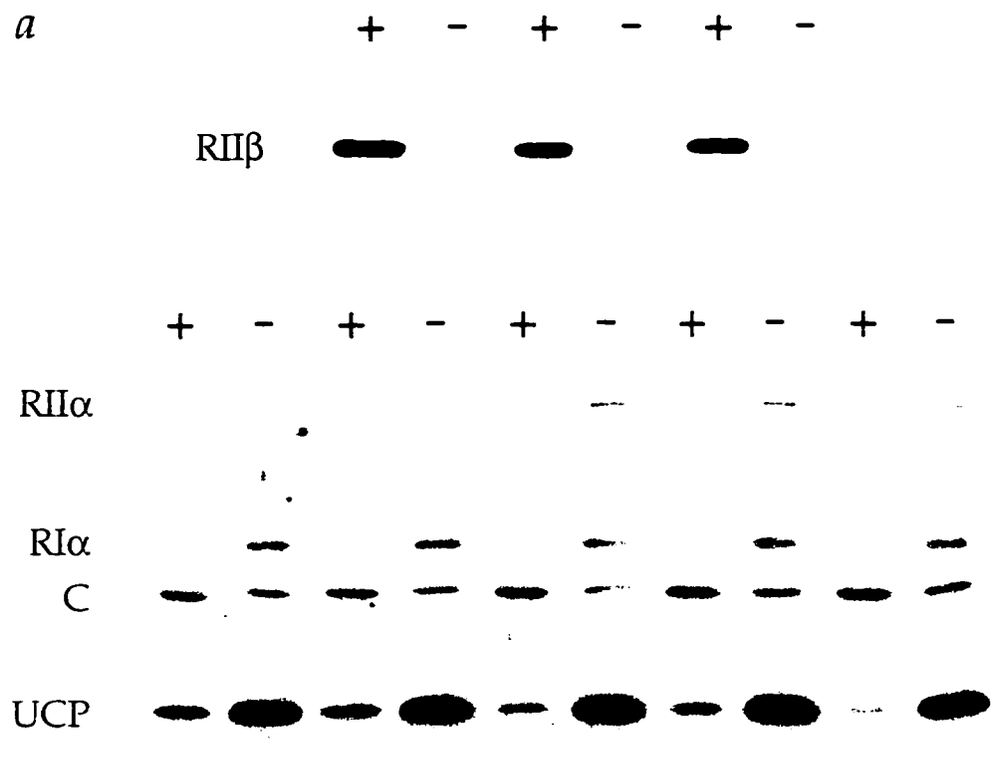

a
+ - + - + -
RIIβ
+ - + - + - + - + -
RIIα
RIα
C
UCP

Figure 4.3. (B) Mutant BAT displays an isoform switch from type II to type I PKA. Type I (fractions 20-25) and type II (fractions 31-35) PKA from BAT homogenates were resolved by ion exchange chromatography. Displayed are basal (-cAMP) and total activatable kinase activity (+cAMP).

b

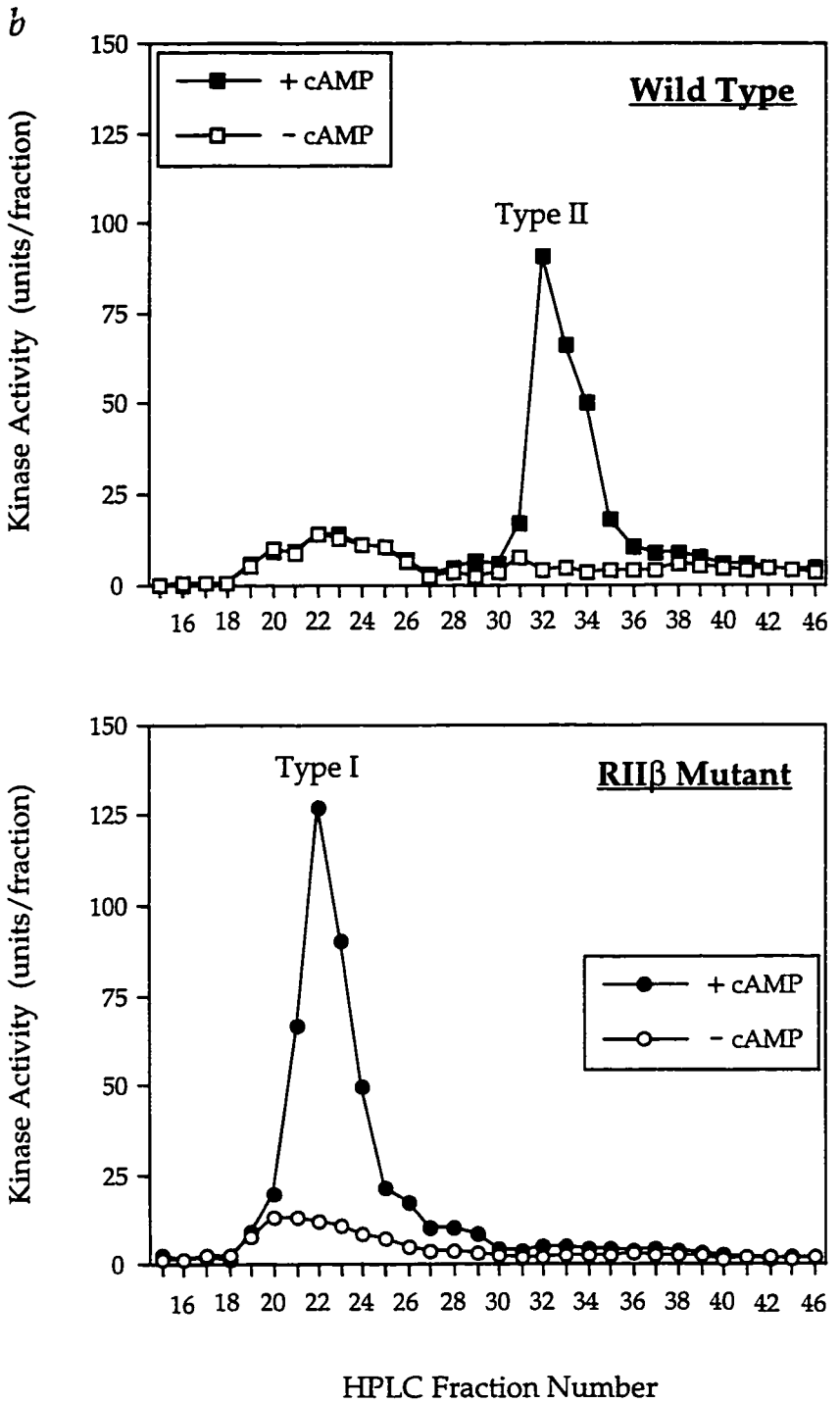
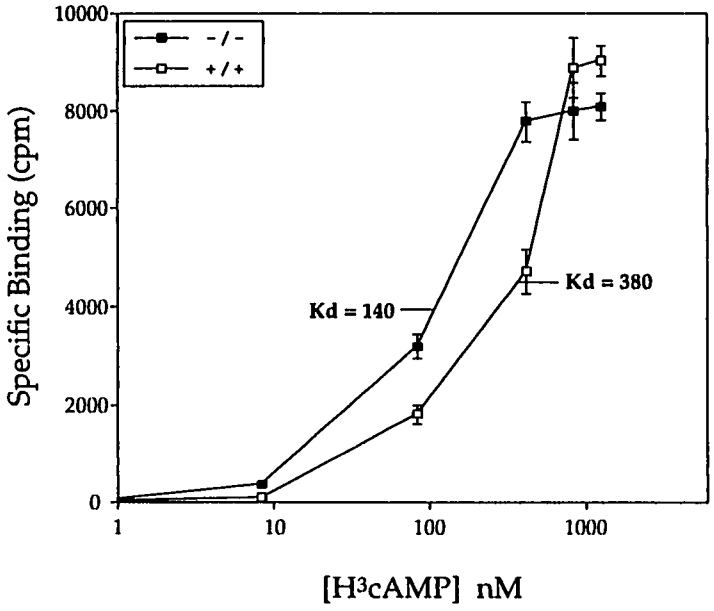

Wild Type
+ cAMP
- cAMP
Type II
Type I
RIIβ Mutant
Kinase Activity (units/fraction)
HPLC Fraction Number
0
25
50
75
100
125
150
16 18 20 22 24 26 28 30 32 34 36 38 40 42 46

Figure 4.4. **(A)** cAMP binding capacity in BAT. Total binding capacity at saturating cAMP concentrations is reduced only ~10% in RIIβ mutants. Half-maximal binding occurs at a lower cAMP concentration in mutants than wild types, reflecting greater R subunit avidity for cAMP. Results represent means ± s.e.m. from five different experiments, each comparing one pair of mice. **(B)** Mutant BAT PKA activates at lower cAMP concentrations than does wild type enzyme. Results represent means ± s.e.m. of triplicate samples from two mice. *Insert*: Mutant BAT has decreased total but increased basal PKA activity. Results represent means ± s.e.m. of four mice per group, each assayed in triplicate and expressed as % of total wild type activity. Total activatable PKA activity (+cAMP) is reduced by 30% in knockouts (P = .029), while basal PKA activity (-cAMP) is about five times higher (P = .0001). In liver, which does not express RIIβ, mutant and wild type PKA activities were similar (data not shown).

a


cAMP BINDING CAPACITY
Specific Binding (cpm)
10000
8000
6000
4000
2000
0
-/-
+/+
Kd = 140
Kd = 380
1
10
100
1000
[H³cAMP] nM

b

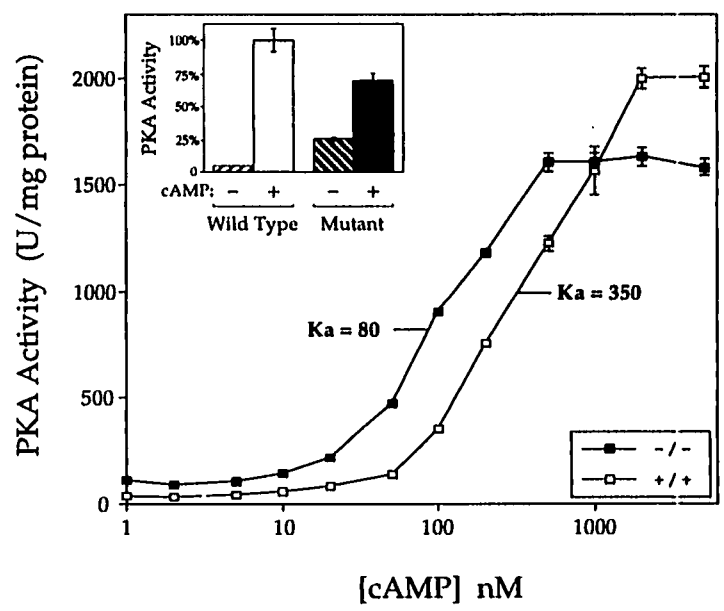
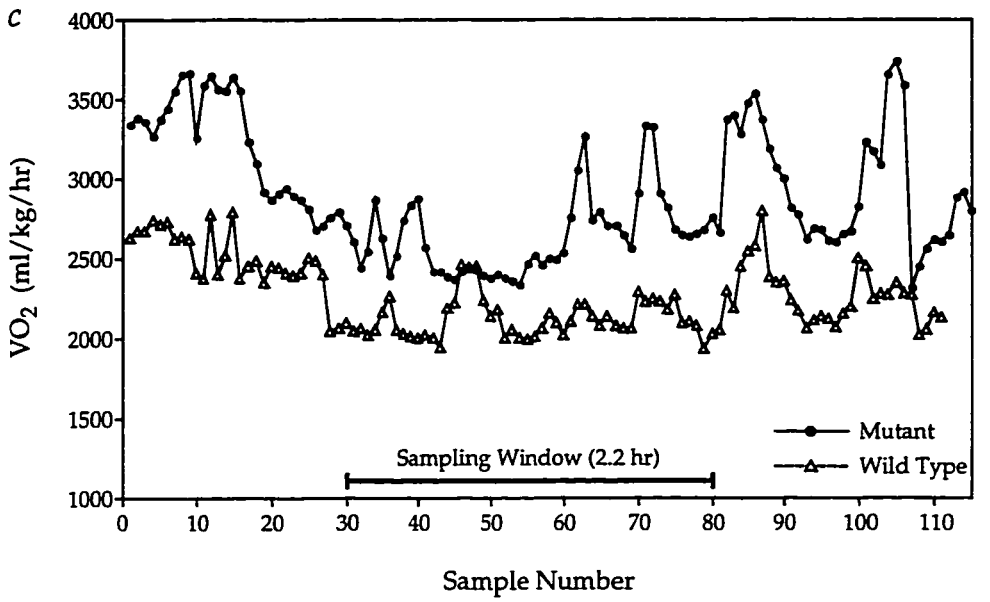
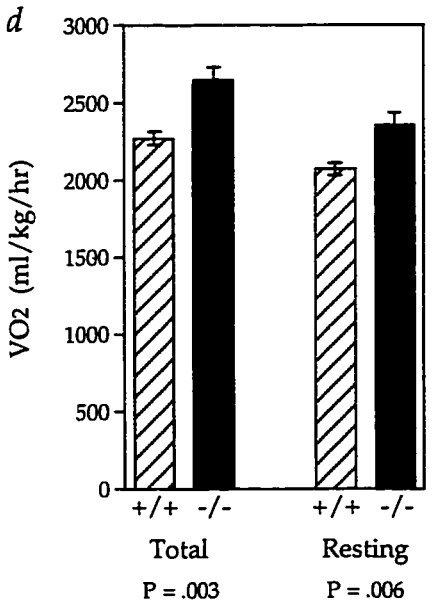
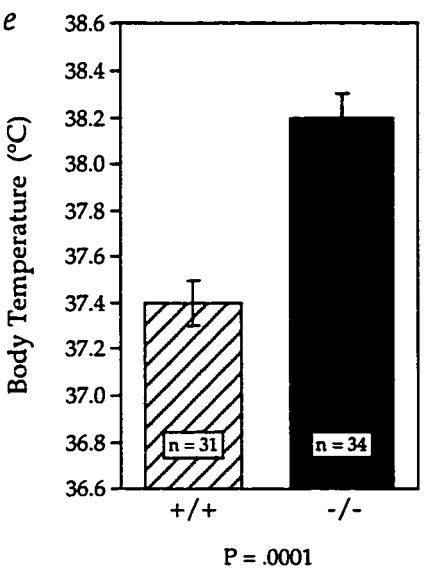

PKA ACTIVITY
PKA Activity (U/mg protein)
2000
1500
1000
500
0
PKA Activity
100%
75%
50%
25%
0
cAMP: - + - +
Wild Type
Mutant
Ka = 80
Ka = 350
-/-
+/+
1
10
100
1000
[cAMP] nM

Figure 4.4. RIIβ mutants display increased energy expenditure. **(C)** Representative oxygen consumption (VO_2) tracings from indirect calorimetry. VO_2 peaks correspond to periods of activity, while baseline values represent rest or sleep. **(D)** Average total and resting VO_2 of five wild type *vs.* five mutant mice. Resting VO_2 is increased in mutant mice, consistent with a higher basal metabolic rate. **(E)** Mutants have elevated body temperature.


c
4000
3500
3000
2500
2000
1500
1000
VO2 (ml/kg/hr)
Sampling Window (2.2 hr)
Mutant
Wild Type
0
10
20
30
40
50
60
70
80
90
100
110
Sample Number


d
3000
2500
2000
1500
1000
500
0
VO2 (ml/kg/hr)
+/+
-/-
+/+
-/-
Total
Resting
P = .003
P = .006


e
38.6
38.4
38.2
38.0
37.8
37.6
37.4
37.2
37.0
36.8
36.6
Body Temperature (°C)
n = 31
n = 34
+/+
-/-
P = .0001

CONCLUSIONS

Mice lacking specific PKA regulatory subunits are viable. One of the first questions regarding the roles of the various subunits of PKA was whether they would be required for viability. A review of hundreds of knockouts generated through 1994 determined that of these proteins, approximately one quarter were absolutely required for life (Brandon et al., 1995b). Because PKA is so ubiquitous in mammalian signal transduction, including having recently discovered roles in embryonic development (Jiang and Struhl, 1995; Hammerschmidt et al., 1996), it was a distinct possibility that the mice lacking RIβ or RIIβ would not survive. Quite to the contrary, these mice were found to not only survive, but to appear overtly normal. That is, gross inspection of the mice, including morphology and behavior, revealed no defects associated with these null mutations. Indeed, detailed analyses were required to uncover the specific requirements for these subunits in mammalian physiology.

One interesting set of findings that has emerged from the analyses of these mice is the ability of the PKA system to compensate for the loss of one of the subunits. In particular, the RIα subunit was found to be increased in several cases. Multiple structures in the brain of RIβ knockout mice showed an upregulation of RIα, as did both the striatum and the adipose tissues of RIIβ knockout mice (Brandon et al., 1995c; Cummings et al., 1996). Brain structures in RIβ knockouts showed no obvious change in total or basal PKA activity, suggesting the possibility that RIα had essentially replaced RIβ in these structures, although it clearly did not functionally compensate, as defects in hippocampal synaptic plasticity existed in these mice. Similarly, the adipose tissue in the RIIβ knockouts showed an approximately 80% replacement of Type IIβ PKA by Type Iα, but this replacement did not maintain the normal function of this tissue. In

contrast, although there was some increase in RIα levels in the RIIβ knockout striatum, the overall PKA levels were reduced by as much as 85% in this structure. Given that RIIβ expression is believed to be limited to the neural and not glial cells in this structure (Glantz et al., 1992), the actual reduction in neurons per se might be even greater than that found in the homogenate. These multiple cases of RIα compensation suggest that this subunit of PKA provides an effective "buffer" for PKA activity in multiple cell types. Thus it is interesting to note that of the six subunits of PKA for which knockout mice have now been developed, only the RIα knockouts suffer obvious embryonic lethality, indicating the importance of this PKA "buffering" function (P. S. Amieux et al., unpublished observations).

Previous work had suggested important roles for PKA in learning in the invertebrates Drosophila and Aplysia. Additionally, PKA is believed to be essential for various forms of hippocampal synaptic plasticity that are hypothesized to underlie learning in mammals. Generation of PKA knockout mice allowed us to address several interesting questions: (1) Is PKA important in mammalian learning, and if so, are specific subunits essential? (2) Are specific subunits of PKA critical for the various forms of synaptic plasticity that are hypothesized to reflect the biochemistry that underlies learning? (3) Can correlations be established between defects in synaptic plasticity and defects in learning?

Prior to our experiments, several mutant mice had been established that supported the existence of a correlation between hippocampal synaptic plasticity and spatial learning. These included mice with knockouts of αCaMKII (Silva et al., 1992a; Silva et al., 1992b), PKCγ (Abeliovich et al., 1993a; Abeliovich et al., 1993b), ACI (Wu et al., 1995), the tyrosine kinase fyn (Grant et al., 1992), and the mGluR1 metabotropic glutamate receptor (Aiba et al., 1994), as well as mice overexpressing αCaMKII (Bach et al., 1995; Mayford et al., 1995). In contrast to these examples that supported the hypothesis that the biochemistry that underlies the various forms of synaptic plasticity

also is used in learning, the PKA knockout mice have shown no such association. In fact, the RIβ mice appear to have normal learning and memory functions in three different tasks that are believed to require hippocampal function: the Morris water maze, the Barnes circular maze, and contextual fear conditioning (Huang et al., 1995). These findings begin to question the relationship between the plasticity phenomena, at least as measured under specific conditions in the hippocampal slice preparation, and spatial learning. Not only was LTD defective in the context of normal learning, but it was also determined that mossy fiber-CA3 LTP was defective in RIβ knockout mice as well. Additionally, it should be noted that mice lacking the $C\beta_1$ subunit of PKA also showed a very similar dissociation (Huang et al., 1995).

Since the discovery of this dissociation in the PKA knockout mice, other mutants have been shown to exhibit similar dissociations (Goda and Stevens, 1996). Specifically, mice lacking Thy-1, a neuronally expressed cell surface glycoprotein, showed a defect in LTP in the dentate gyrus of the hippocampus, but performed normally in the Morris water maze (Nosten-Bertrand et al., 1996). Conversely, mice carrying the Steel mutation (*Sl*), which encodes the ligand for the kit tyrosine kinase receptor, show a deficit in the Morris water maze, but normal mossy fiber-CA3 LTP (Motro et al., 1996). This finding is tempered however with the caveat that basal transmission at this synapse is defective in these mice. Nevertheless, the relationship between hippocampally mediated learning and hippocampal synaptic plasticity phenomena is likely not absolute, as demonstrated by these several recent analyses of mutant mice. In fact the PKA mutants may show the most straightforward of these dissociations, as basal synaptic transmission was found to be normal at multiple hippocampal synapses, and normal spatial learning was observed in multiple tasks evaluated. An interesting corollary to this dissociation has been discovered in the visual cortex of RIβ knockout mice, in that these mice show normal developmental responses to monocular deprivation, but

lack LTP in the visual cortex in several paradigms tested (J. A. Gordon et al., manuscript in preparation). However, one paradigm did yield normal LTP in the mutants. This finding actually points out one of the benefits of this approach -- that certain types of plasticity phenomena can be dissociated, and therefore hypothesized to not reflect the biochemical changes underlying the behavior in the intact animal, while others may be deemed more relevant as models for the whole animal process of interest.

Although the dissociation of the various forms of hippocampal synaptic plasticity from spatial learning processes suggests that these phenomena may not be valuable models for understanding the molecular events which underlie spatial learning, other interpretations of the data should be noted. For example, in the case of the RIβ knockout analysis, only plasticity in hippocampal slices was determined. It is conceivable that the slice preparation somehow brings out a defect that would not be observed in the intact hippocampus. However, the defective LTP observed in Thy-1 knockout mice was found in intact animals. Further analysis of LTP in RIβ knockouts in vivo should resolve this possibility. Another possibility is that hippocampal synaptic plasticity that can be measured in the slice preparation is actually required for spatial learning, but that the specific synapses that were analyzed are not the critical synapses for learning. This is the interpretation of the results that is discussed by Huang et al. (1995). Furthermore, the possibility also exists that although wild type mice may normally utilize the same biochemical changes at the same specific synapses in both in vitro hippocampal synaptic plasticity phenomena and spatial learning (for example PKA-mediated LTP at the mossy fiber-CA3 synapse), the various mutants may co-opt entirely novel biochemical or anatomical pathways for learning (for example bypassing this synapse and utilizing another not normally used for this function). Biological organisms have a remarkable capacity to adapt to various impediments, and perhaps nowhere has this been so apparent as in the knockout literature. In

fact, at least at the molecular level, this is one of the most direct effects of the knockouts of RIβ and RIIβ -- compensation by the RIα subunit, which presumably restores some degree of normal function. It would not be surprising to find that the mammalian brain can "devise" novel approaches to overcome a genetic defect, especially in a function as important as learning. Thus, compensatory changes in response to the genetic lesion may mask some of the functions normally subserved by the eliminated protein.

Similarly to the RIβ and Cβ1 knockout mice, RIIβ knockouts were found to have no defects in various spatial learning tasks, despite the fact that this subunit is also normally expressed in the hippocampus (S. F. Logue et al., unpublished observations). In fact, this was the only mutant of the three that showed a detectable decrease in hippocampal PKA activity. Preliminary data suggest that these mice may have defects in hippocampal synaptic plasticity as well, further extending the dissociation discussed above (Y.-Y. Huang et al., unpublished observations). In contrast to the lack of evidence for a "declarative" learning deficit in these mice (as modeled in rodents by hippocampally-mediated spatial learning), they may have a "procedural" or motor learning deficit (Squire, 1987). That is, although these mice do show some acquisition in the rotarod motor task, they never achieve the same competence with this task as wild type mice (or RIβ knockout mice). From the experiments described here, it is difficult to determine if these mice have a defect in performance per se, or a limitation in the ability to learn the task (Graybiel, 1995). If the deficit were in procedural learning, it would be interesting to correlate that defect with synaptic plasticity analysis in the striatum, as this is likely a critical structure in this type of learning (Kombian and Malenka, 1994; Calabresi et al., 1996).

It is likely that the RIIβ mutant mice have defects both in immediate responsiveness to endogenous neurotransmitters such as dopamine, and in plastic changes that not only underlie learning but in responses to repeated receptor stimulation (such as sensitization and

tolerance) as well. This is simplistically suggested by the finding that RIIβ, and therefore presumably Type IIβ PKA holoenzyme, is located in post-synaptic regions, where it may have a role in phosphorylating specific targets such as various ion channels that would regulate the immediate electrophysiological activity of neurons, and in perikaryal regions where it may localize catalytic subunits near the transcription machinery involved in longer-term changes in neuronal structure and function. Regarding this second role, it is interesting that repeated exposure to psychostimulants has been shown to cause long-term changes in PKA activity in the nucleus accumbens, presumably a reflection of changes in catalytic subunit levels, but that the levels of regulatory subunits have not been assessed in these analyses (Nestler et al., 1993). These analyses should prove especially informative with the RIIβ knockout mice, as further clues toward understanding the mechanisms underlying plastic changes in response to chronic drug exposure will likely be revealed.

Have the experiments described here determined any specific roles for the various subunits of PKA? This was one of the primary goals from the outset of this research. In fact, many of the experiments described here have not yet been performed with multiple PKA knockout strains. But in several instances multiple mutants were utilized. In the case of the rotarod task, it was found that the RIIβ subunit, but not the RIβ subunit, was required for normal performance. This requirement was recapitulated in the induction of c-fos in the dorsomedial striatum by amphetamine. As shown in figure 3.2, RIβ is highly expressed in the striatum, and thus, we have determined a specific role for RIIβ and not RIβ in these functions. In this context it is interesting to note that these two mutations had quite opposite effects on haloperidol induced transcription of the gene encoding the peptide neurotensin in the dorsolateral striatum. RIIβ knockout mice entirely lack this induction, while RIβ knockouts have both increased basal levels of this transcript as well as significantly higher induced levels as compared to wild type mice (M. R. Adams et

al., manuscript in preparation). In one last example, it has been found that while mice lacking RIβ have a subtle difference in tolerance to morphine, they generally show a normal range of responsiveness. In contrast, tested mice lacking RIIβ have virtually all died in response to an amount of this drug that causes very little morbidity in wild type and RIβ knockouts (S. Appleyard et al., unpublished observations).

Certainly, the establishment of these two null mutants provides only the starting point from which many questions can be addressed. And the cursory analyses described here actually open up many more biological questions than they answer. But they suggest many more experiments that should be increasingly more precise in their design and increasingly informative in their resolution. The RIβ and RIIβ knockout mice could provide a useful tool for mammalian neurobiology research for many years to come. In fact, the knockout mice in general provide a wonderful new approach in integrative research, that which can be queried and correlated from the level of biochemistry through cellular biology and systems physiology all the way to the behavior of the whole organism.

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