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An Old Neurotransmitter learns two new tricks: the role of serotonin receptors and other related GPCRs in regulating pancreatic islet development and novelty-related behaviors

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An old neurotransmitter learns two new tricks: the role of serotonin receptors and other related GPCRs in regulating pancreatic islet development and novelty-related behaviors.

by

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An Old neurotransmitter learns two new tricks: the role of serotonin receptors and other related GPCRs in regulating pancreatic islet development and novelty related behaviors.

by Miles Berger

Abstract

Serotonin modulates a wide array of neurobehavioral and physiologic processes in mammals, and it has been implicated in the pathophysiology of clinical disorders ranging from primary pulmonary hypertension to irritable bowel syndrome. Yet, the specific cellular and molecular mechanisms underlying the modulatory role of serotonin is still largely obscure, in part due to the multiple receptors and release sites for serotonin, and in part due to the lack of highly specific pharmacologic tools for these receptors. Indeed, serotonin signals thru some 15 receptors, 14 of which are G-Protein Coupled Receptors (GPCRs).

Here, I have used mouse genetic techniques to examine how 5-HT receptors and other similar GPCRs regulate two diverse biological processes: pancreatic islet development and novelty-related behavior. My results suggest that serotonin and Gi-coupled receptor signaling play a critical role in the former by regulating cell division during pancreatic development, and that serotonin and the 5-HT2C receptor play a critical role in the latter by regulating dopamine release in the dorsal striatum. Taken together, these results highlight the wide array of biological processes that are orchestrated by serotonin and its receptors, and they shed light on how evolution has co-opted an ancient neurotransmitter into regulating some of the most complex mammalian physiological and behavioral processes.

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Chapter I. GPCR's and Gi-signaling in mammalian biology

The largest gene family in the mammalian genome encodes a set of cell surface receptors that couple to heterotrimeric G proteins, thus known as G-protein coupled receptors (GPCRs). Indeed, ~3% of the entire mammalian genome encodes GPCRs (Lander ES et al, 2003), and GPCRs are expressed by all known mammalian cell types (Gilman AG, 1995). Early on, the realization that signals ranging from small molecules (like epinephrine and serotonin) to single photons could activate several different but structurally similar receptors (the beta 2 adrenergic receptor, the 5-HT1A receptor, and rhodopsin) led to the hypothesis that these receptors must signal thru common mechanisms, and that they must be part of a common gene family (Kobilka BK et al, 1987).

Recent work has confirmed this initially daring hypothesis, and has shown that GPCRs share a common structure of seven transmembrane spanning alpha helices connected by three intracellular and three extracellular loops, with the N terminus on the extracellular face of the membrane and the C terminus on the intracellular side (reviewed in Ridge KD et al, 2007). Extracellular stimuli ranging from photons to small peptides to steroid hormones activate these receptors by shifting the transmembrane alpha helices and exposing residues on the intracellular loops that activate heterotrimeric G proteins.

These heterotrimeric G proteins are themselves made up of alpha, beta and gamma subunits. Initially, the alpha subunit is bound by GDP, and exists in a tight complex with the beta-gamma subunits. Upon receptor activation, the alpha subunit then exchanges GDP for GTP, and then the GTP bound alpha subunit and the beta-gamma dimmer disassociate from each other and the receptor. The GTP-bound alpha subunit and the beta-gamma dimmer then activate or inhibit a variety of effector enzymes.

Eventually, the intrinsic GTPase activity of the alpha subunit hydrolyzes GTP into GDP, and the GDP-alpha subunit then tightly re-associates with the beta-gamma dimmer and the receptor, thereby terminating the signal.

The mammalian genome codes for G protein alpha subunits, five beta subunits and 12 gamma subunits, and these heterotrimers are loosely grouped into several classes based on what signaling pathways they activate (table 1). Although GPCR's also activate a variety of other effector enzymes and signaling cascades directly without G protein intermediates (Lefkowitz RJ, 2004), they have nonetheless been classified on the basis of which G protein family they activate. Thus, GPCR's are typically referred to as Gi, Gs or Gq coupled.

Among these classes, much work has focused on Gi-coupled receptors, particularly in neurons and other secretory cells. The study of Gi-coupled receptor signaling has long been facilitated by the availability of highly specific pharmacologic tools, such as the toxin of *bordatella pertussis*, otherwise known as pertussis toxin (PTX). PTX catalyzes the ADP ribosylation of G alpha i family members, which blocks receptor mediated guanine nucleotide exchange and thereby locks the G protein heterotrimer in the inactive state (Katada T et al, 1982).

In neurons, Gi-coupled receptors typically mediate inhibitory signals; somatodendritic Gi-coupled receptors such as the 5-HT1A receptor open GIRK channels via their beta-gamma subunits and thus hyperpolarize cell bodies and block action potential generation (Luscher C et al, 1997), while Gi-coupled receptors on axons and axon termini like the 5-HT1B receptor decrease neurotransmitter release at specific terminal sites (Knobelman DA, et al, 2001). Similarly, the activation of Gi-coupled receptors on enterochromaffin cells and other secretory cells usually inhibits hormone release from cytoplasmic vesicles (Zeng N, et al, 1998).

In addition to these well-described roles of Gi-coupled receptors in regulating vesicular release and cellular activity, recent work in invertebrates suggests that GPCR signaling may control cell division during embryonic development (Wang et al, 2005). However, little is known about what role, if any, Gi-coupled receptors and other GPCRs may play during mammalian embryogenesis and organ development. This knowledge gap probably due in part to the complex genetics and extensive labor required to manipulate gene expression with temporal control in mice, in addition to two other more general reasons I will discuss below. In the few studies on this topic, Gi-signaling has been reported to either decrease or increase cell division and proliferation depending on cell type being studied (Lahlou H et al, 2003; Shinohara H et al, 2004); clearly, much more data is needed.

There are two major reasons for the paucity of data in this area. First, most studies of cell, tissue and organ development have focused on understanding the cascades of transcription factors that specify cell fate and lineage commitment. Substantial work has been made in this area, and elegant models of these transcriptional cascades have been developed to explain the development of cell types ranging from CNS 5-HT neurons (Scott MM et al, 2005) to pancreatic beta cells (Gangaram-Paday ST et al, 2007). GPCRs have traditionally been viewed as a way for cells to respond to non cell autonomous cues from their neighbors, while most studies of development have focused on understanding the cell autonomous or intrinsic factors that mediate differentiation from pluripotent stem stem cells to increasingly specialized and differentiated cell types.

Second, most of our understanding of canonical GPCR signaling has come from studies in cultured cells; this approach allows for facile biochemical purification of relevant proteins and easy treatment of the cells with various small molecule signaling inhibitors and toxins, etc. However, while immortalized laboratory cell lines like HEK-293 cells may offer these significant advantages, they clearly cannot recapitulate the

nuanced signaling mechanisms and specialized features of terminally differentiated cell types *in vivo*. To study signaling thru entire receptor classes during mammalian development *in vivo* requires the ability to introduce relevant signaling inhibitors or activators into specific spatially restricted cell lineages during development. Furthermore, in some cases it is also helpful to have temporal control over the expression of the particular inhibitors/activators, which usually requires the use of time-consuming and expensive multi-transgene approaches in mice (Matthaei K, 2007).

These are significant hurdles. I believe they (in addition to the conceptual issues addressed above) explain why we do not fully understand how GPCRs and other signaling pathways act to modulate cell differentiation and replication during development, and I believe these same hurdles explain why we lack a full understanding of how GPCRs act in complicated neural circuits within the CNS. In chapter IV of this work, I will discuss one prototypical sub-family of GPCRs, those that recognize the neurotransmitter serotonin. These serotonergic GPCRs regulate a diverse array of biological functions, from the development of the cardiac valves to the control of bowel motility and the modulation of the emotional behavior; an illustration of how evolution has utilized a small coterie of receptors to allow one ancient simple, small molecule (5-HT) to direct an array of increasingly complex biological phenomena.

In chapter II, I will discuss how serotonin and its receptors may play a role in regulating two different cell types (pancreatic islets and CNS serotonin neurons), and how altered development of these two cell types could help explain the high co-morbidity between depression and diabetes. Upon this foundation, I will then discuss in chapter III my own work examining the role of 5-HT and Gi-coupled receptor signaling in pancreatic islet development, and I will discuss the importance of these findings for understanding the causes of type II diabetes and for potentially treating type I and II diabetes. In chapter V, I will then discuss my own work attempting to correlate behavioral

abnormalities in a line of mice that lack serotonin with altered activity of specific serotonin receptors, before concluding in chapter VI with a discussion of future directions for research on how serotonin receptors in particular and GPCRs in general modulate animal physiology and behavior.

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Chapter II. Depression and Diabetes- different diseases, common mechanisms?

Major depression and diabetes are two of the most significant diseases in the Western World, with a prevalence of 17% and 5% respectively in the United States and similar rates in other developed countries (Bloom B, 2004; Zimmet P et al, 2001). Each of these diseases presents with a unique pattern of symptoms and signs, namely depressed mood and neuro-vegetative signs in the former, and increased blood sugar in the latter (see table 1 for full diagnostic criteria and subtypes of each disorder).

Though these diagnostic criteria are entirely non-overlapping, there is nonetheless a high degree of co-morbidity between depression and diabetes. For example, a recent meta-analysis of the field found that the prevalence of depression in patients with diabetes is roughly twice as high as in control patient groups (Anderson RJ et al, 2001). This increased rate of depression among diabetics held true in both type 1 and type 2 diabetics, and the increased rate was seen in men and women.

Conversely, diabetes is seen in depressed patients at a higher frequency than in non-depressed controls), and treating depression may actually lead to improvements in glycemic control as well (Weber-Hamann B et al, 2006). This high degree of co-morbidity between these two diseases and the finding that treating one disease can improve the other leads to the question of whether they are somehow causally linked; i.e. could one disease somehow play a role in the etiology of the other, or could both be caused in part by a common underlying pathophysiology?

One way to approach this question is to ask whether one disease usually precedes the other (which would raise the possibility that the first disease somehow causes the second). However, the temporal relationship between these two illnesses is complex, and depends on the subtype of diabetes. Type 1 diabetes usually presents in adolescence thru the early twenties, while type II diabetes commonly presents in the fifth decade of life or later (although it can occur earlier); depressive episodes can occur at

almost any age, but major depressive disorder typically begins in the later teens or twenties. Thus, most type 1 diabetics who also have depression will have their first major depressive episode after they have already been diagnosed with diabetes, while most type II diabetics who also have depression will have had depressive episodes long before they go on to develop diabetes.

These are generalizations, though; some type 1 diabetics will develop depression before diabetes, and some type II diabetics will develop diabetes before having depression. Furthermore, these distinctions may be artificial, because both diabetes and depression are clinical diagnoses, and each may be associated with underlying trait abnormalities (or a prodromal period) before the disorder becomes clinically manifest; thus, the underlying prodromal or trait abnormalities of both disorders may both be present in an individual years before either disease manifests clinically. MDD is a clinical diagnosis made on the basis of behavioral symptoms; it is unclear whether there are core trait phenotypes that characterize individuals who will go on to develop MDD even before they first become depressed or between depressive episodes (Bhagwagar Z et al, 2004), or whether the entire phenotype exists only during the actual depressive episodes (Linkowski, 2003). In the case of diabetes, there is some evidence of underlying trait abnormalities in both type I and type II diabetes (Jahromi M et al, 2007; Stoeckli R et al, 2004) even before they meet clinical criteria for diabetes.

Thus, there is thus no general rule for which disease precedes the other overall (including both subtypes of diabetes), and the entire question may be irrelevant if both diseases are actually manifestations of trait abnormalities present long before either disease presents clinically. Taken together, this argues that neither depression nor diabetes “causes” the other in a simple, linear fashion. Rather, there are two other ways to explain this co-morbidity:

1. Perhaps each disorder could worsen any potential predisposition towards the cognate disorder. Thus, depression could exacerbate a pre-existing sub-clinical disorder of glucose homeostasis and lead to full blown diabetes, while diabetes could exacerbate a sub-clinical mood imbalance and lead to a full major depressive episode.
2. A common underlying problem could contribute to both disease processes, and either disorder could present first depending on other factors.

These hypotheses are not mutually exclusive, and some evidence exists in support of each. For the first hypothesis, there is data showing that depression is associated with insulin resistance (Winokur A et al, 1988), perhaps through increased HPA axis activity and the release of stress hormones such as cortisol and catecholamines that has been described in depression (McEwen BS, 2004). Similarly, simply being diagnosed with diabetes and having to carefully manage this illness is a significant stressor that could contribute to some patients developing depression (Adili F et al, 2006), while chronically poor glucose control and hyperglycemia can impair brain function and mood (Sommerfield AJ et al, 2004).

The second hypothesis is more difficult to evaluate in part because we do not fully understand the underlying etiology of depression or type I or II diabetes. Nonetheless, we do have clues about the pathophysiology and treatment of each of these disorders, and I will discuss these now.

The Pathophysiology and Treatment of Major Depression:

Multiple abnormalities have been described in depression, and it remains unclear whether this is truly a single disorder with one discrete underlying pathophysiogy, or a set of disorders with varying causes that nonetheless present with similar symptoms. The monoamine hypothesis is the most well established theory of depression (although

some recent work has moved in other directions as well; Duman RS et al, 2006); it holds that depression is caused by an imbalance or deficiency in one or more mono-amine neurotransmitters (such as serotonin, norepinephrine, or dopamine).

Several lines of evidence support this hypothesis. First, acutely depleting monoamines and serotonin in particular can worsen mood in some people (Ruhe HG et al, 2007), while treatment with drugs that chonically deplete monoamines like reserpine can cause depression and suicide in people (Carlsson A, 1976). Second, decreased monoamine metabolite levels have been found in the cerebro-spinal fluid (CSF) of at least a subset of patients with mood disorders (Bourne HR et al, 1967), although this abnormality may be more associated with impulsivity/suicidality than with depression per se (Mann JJ, 2003). Third, increasing monoamine neurotransmission (and serotonin in particular) effectively treats depression in about two thirds of patients (Mann JJ et al, 2005). Fourth, recent studies have identified mutations in several genes involved in monoamine synthesis and release that are associated with depression and mood disorders (reviewed in Berger M. et al, 2006). Taken together, these data strongly suggest that at least a subset of cases of depression may be caused in part by decreased serotonin (or other monoamine) neurotransmission, which I will refer to here as the “serotonin subtype” of depression.

Thus, although depressive symptoms are presumably mediated thru abnormal activity in multiple brain circuits (such as the VTA to NAcc “reward” circuit in anhedonia, hypothalamic circuits for neuro-vegetative symptoms, increased HPA axis reactivity, etc), a potential proximal cause of these abnormalities is disordered release of serotonin by neurons in the midbrain dorsal and median raphe nuclei. These neurons have elaborate processes that innervate virtually all of the other brain regions that have been implicated in depression by neuroimaging studies (Etkin A, 2007; Kalin NH et al, 1981), while polymorphic alleles of genes expressed by serotonin neurons can perturb brain

activity in the amygdala and increase anxiety (Fisher PM et al, 2006; Albert PR et al, 2004). Animal studies have similarly shown that deletion of the serotonin transporter or other serotonergic genes and lesions of the serotonin system can also perturb anatomically distant brain regions and cause behavioral abnormalities, which I will discuss in depth in chapter IV.

The Pathophysiology and Treatment of type I Diabetes:

Type I or insulin-dependant diabetes mellitus is characterized by a near or complete deficiency of insulin. In most cases, this is caused by an auto-immune attack upon the pancreatic islet cells (Pihoker C et al, 2005), although it can also be caused by genetic defects that disrupt islet development without inducing auto-immune attack (Stoffers DA et al, 1997). In the former cases, several genetic variants have been identified that are thought to increase the risk of T cell attack upon the islets, due to deficits in the development of peripheral and central tolerance induction by the thymus (Abel M et al, 2001). In at least some cases, the defects in immune tolerance that lead to destruction of the islets can also lead to destruction of other neuro-endocrine organs (Heino M et al, 2001; Anderson MS et al, 2002). A similar autoimmune attack upon the brain could theoretically also lead to depression (Irwin MR et al, 2007), somewhat similar to the autoimmune attack upon the CNS that cause Multiple Sclerosis (Pender MP et al, 2007), though this possibility remains more speculative than proven. Nonetheless, type I diabetes can be treated effectively with careful administration of exogenous insulin, suggesting that this truly is a disease of insulin deficiency, irrespective of the actual cause of the said deficiency.

The Pathophysiology and Treatment of type II Diabetes:

Type II diabetes is characterized by an insufficient supply of insulin to meet the demand/need of the body. Like any supply/demand imbalance, this can be due to a decreased supply, increased demand, or a combination of both. Practically, this means that type II diabetics make some insulin but perhaps less than normal. Many type II diabetics have obesity-induced insulin resistance, in which insulin does not exert its normal biological activity on target tissues. In these individuals, the pancreas may compensate to some extent by increasing the number of islets (known as islet hyperplasia), but it fails to compensate fully, and hyperglycemia results (Mahler RJ et al, 1987). Thus although type II diabetics do have insulin, they nonetheless have a pancreatic defect, in the sense that their pancreatic islets do not secrete enough insulin to maintain glucose homeostasis.

One of the main causes of insulin resistance is increased adiposity (Keller U, 2006), since a loss of just 8 kg is sufficient to cause a significant improvement in insulin sensitivity in moderately obese individuals (Su HY et al, 1995). This link is thought to be mediated by increased release of hormones like resistin by adipose tissue (Keller U, 2006), which block the effects of insulin on target tissues. However, many obese people do not develop diabetes despite this increased insulin resistance; in these individuals, islets hyperplasia is sufficient to compensate for the peripheral insulin resistance, and thus blood glucose remains in the normal physiologic range of 60-120 mg/dL.

Thus, the cause of most cases of type II diabetes is essentially the failure of the pancreatic islets to hypertrophy sufficiently and/or to secrete enough insulin to match peripheral metabolic demand. Viewed this way, the question becomes, why do the islets of some individuals fail to hypertrophy sufficiently? The answer is not totally clear, but accumulating evidence points to mutations in genes that encode proteins essential for pancreatic islet development, replication or maintenance.

Pancreatic Islet Development

Pancreatic development when the dorsal and the ventral pancreatic buds pouched out from the endodermal epithelium, around e9.5 in rodents. The ventral bud then rotates towards the dorsal bud, and they fuse as the epithelium invaginates. The pancreatic precursor cells then differentiate into committed endocrine and exocrine precursor cells (Fig 1); in particular, a specific cascade of transcription factors mediates the commitment to the endocrine lineage and subsequent differentiation into mature beta (insulin producing) and alpha (glucagon producing) cells (Fig 2). The commitment to the endocrine lineage is thought to happen when cells turn on the helix-loop-helix transcription factor neurogenin-3 at around e15.

Endocrine progenitor cells then switch off neurogenin-3 and turn on either insulin or glucagon, thus becoming committed to either beta or alpha cell fate, respectively. During the early post-natal period, there is a significant amount of islet cell replication, which produces the beta cell mass necessary for the pup to regulate its own blood glucose homeostasis (during pregnancy, this function is subserved by maternal insulin). This process of islet cell replication continues, albeit at a slower rate, in adult animals, and serves to replenish the pool of beta cells with new members as older cells undergo apoptosis (Bouwens L. et al, 2005). Indeed, some have theorized that a differential replication capacity of beta cells among people may explain why some individuals mount a sufficient beta cell hyperplasia response to meet increased insulin needs (Georgia S. et al, 2004), while other fail to mount such a response and thus develop type II diabetes.

The potential factors that could explain a differential beta cell replication potential between individuals remain unclear, though, partly because we lack a full understanding of beta cell replication in general. However, understanding the mechanisms of beta cell replication is currently an intense research focus, due to the hope that this

understanding will help us devise means to increase islet cell mass in type II diabetics and to expand embryonic stem cell derived islets for cellular transplantation therapies.

Serotonin neuron development:

CNS serotonin neurons are specified by a molecular cascade of transcription factors that is surprisingly similar to those that specific pancreatic islet cell fate (Fig 3). The differentiation of both cell types depends on nkx2.2 and nkx6.1 expression, and recent work by my colleagues in the Tecott and German laboratories has also shown that several other transcription factors (such as pdx1-1 and pet-1, respectively) previously implicated solely in pancreatic islet or serotonin neuron differentiation also play roles in the differentiation of the other cell type. Furthermore, defects in some of these transcription factors disturb both cell types: Nkx2.2 and Nkx6.1 each play a required role in both 5-HT neuron and beta cell development, since KO's of either of these genes show abnormalities of both cell types (Cheng L et al, 2003; Craven SE et al, 2004; Habener JF et al, 2005). This shared transcription specification pathway suggests that beta cells may have more in common with serotonin neurons than has been previously realized, perhaps at the level of adult cellular function as well.

Indeed, evidence exists in support of this hypothesis. Though these two cells are located far apart and derive from two different embryonic layers (ectoderm and endoderm, respectively), they share surprising commonalities. For example, both cell types engage in vesicular release, and thus express many of the same genes involved in this process such as synaptotagmins, synaptobrevin, etc (Iezzi M et al, 2005; McDonald PE et al, 2007). My colleagues in the German laboratory have also recently found evidence for this: beta cells also express the cellular machinery to synthesize and release serotonin, such as VMAT2, TPH2, the 5-HT1B receptor and the serotonin transporter (M. German, pers. communication).

Taken together, these similarities between suggest that the similarities between beta cells and serotonin neurons extend past development and into the adult biology and function of each cell type. These similarities are all the more interesting in light of the involvement of these cell types in depression and diabetes and the high co-morbidity between these two diseases. These findings imply that a common genetic, developmental or functional defect could perturb both of these cell types, and thus place an individual at higher risk of depression and diabetes.

However, this is merely an interesting conceptual hypothesis right now; to my knowledge, there have been no reports of a single genetic mutation in humans that increases the risk of depression and diabetes. One obstacle to investigating this unifying hypothesis is that we do not know the full set of shared genes that are expressed by both beta cells and 5-HT neurons. Microarray studies have identified genes expressed by adult beta cells, but such studies have yet to be reported on 5-HT neurons. Furthermore, if the shared gene(s) in question are expressed only during the development of these two cell types, then gene expression studies would need to be performed at the appropriate developmental time points.

To further the conceptual basis for this unifying hypothesis, my colleagues in Dr. Michael German's lab and I first began by examining the expression of serotonergic marker and receptor genes in pancreatic islets during development and adulthood. Surprisingly, we found that beta cells express many of the same serotonergic genes and receptors as CNS 5-HT neurons, with the sole exception of the 5-HT1A receptor. We then expressed the 5-HT1A receptor on developing islets, in part to ask whether the lack of 5-HT1A receptor expression is functionally important. Data from these and subsequent studies suggested to us that Gi-coupled receptors like 5-HT1A receptor play a critical role in regulating pancreatic islet development, a novel finding with implications

for both the pathophysiology and treatment of diabetes, and the subject of the following chapter.

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Figure 1

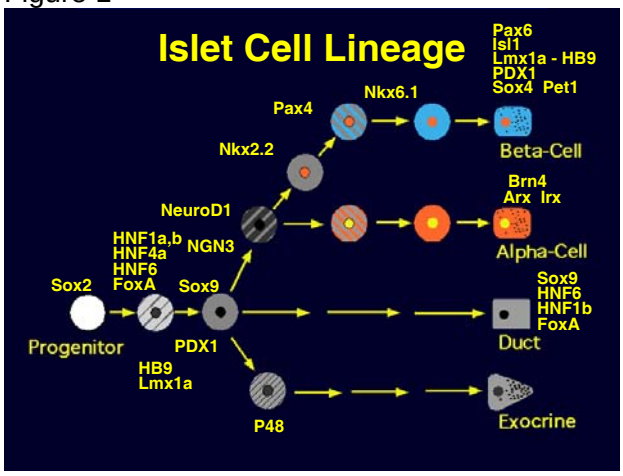


Figure 2

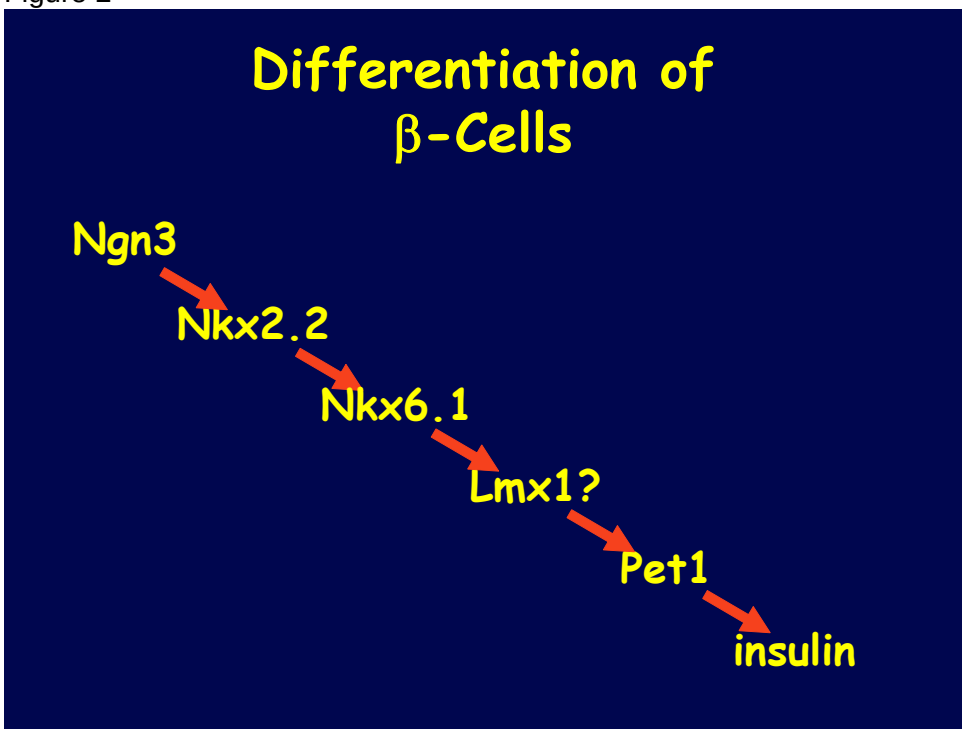
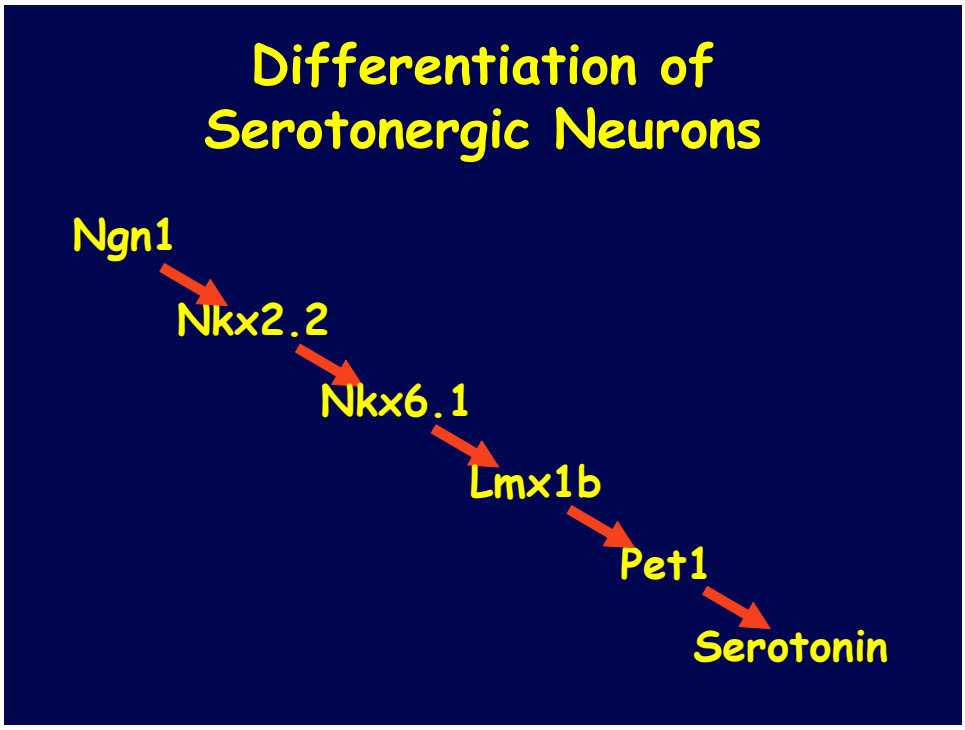

Differentiation of
β-Cells
Ngn3
Nkx2.2
Nkx6.1
Lmx1?
Pet1
insulin

Figure 3


Differentiation of
Serotonergic Neurons
Ngn1
Nkx2.2
Nkx6.1
Lmx1b
Pet1
Serotonin

Chapter III. Gi-signaling regulates pancreatic islet development, beta cell mass and adult glucose homeostasis

Running title: Gi signaling and pancreatic development

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1 Table

3 Figures

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Title **"Gi-coupled receptor signaling is a critical regulator of pancreatic islet development, beta cell mass and adult glucose homeostasis"**

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Abstract:

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Keywords: glucose homeostasis, development, pancreatic islet.

G_i-signaling regulates hormone/ neurotransmitter release by many cell types and plays a role in invertebrate development, but little is known about what role G_i-signaling might play in mammalian organ development. Here we show that over-expressing the G_i-coupled 5-HT1A receptor or the G_i-coupled Ro1 RASSL on developing pancreatic beta cells decreases cell replication and thus results in decreased adult beta cell mass and worsened glucose homeostasis. Conversely, inhibiting G_i-coupled receptor signaling by expressing pertussis toxin (PTX) in

developing pancreatic islets increases cell replication and protects animals against hyperglycemia later in life. Thus, G_i-coupled receptor signaling controls beta cell replication during early post-natal development, and thereby plays a critical role in regulating subsequent adult beta cell mass and blood glucose homeostasis. Inhibition of endogenous G_i-coupled receptors could promote the development of pancreatic islets for regenerative medicine.

Introduction:

G-protein coupled receptors (GPCRs) form the largest family of cell surface receptors in the human genome, accounting for ~3% of all human genes. Many GPCRs signal thru the G_i pathway to lower intracellular cAMP and activate other effector molecules, and G_i-signaling is known to play an important role in both neurons and endocrine cells in regulating neurotransmitter and hormone release, respectively (Sadja R et al, 2003; Sharp et al, 1996). Studies in invertebrate systems suggest that Gi-signaling can also regulate cell division during embryonic development (Wilkie TM et al, 2005) but little is known about what role G_i signaling might play in mammalian development, or how such developmental G_i signaling could influence adult cellular behavior and animal physiology.

Here, we have investigated this question in pancreatic beta cells using transgenic mice. First, we expressed the mouse *5-htr1a*, a G_i-coupled receptor under the control of the pet1 enhancer fragment (Scott MM et al, 2005; Ota et al, 2007) (supplemental figure 1A) in developing pancreatic islets. We identified two independent transgenic founder lines carrying this transgene (supplemental figure 1B). In the higher copy line, we noticed the presence of runted pups that appeared dehydrated and displayed polyuria. We thus measured blood sugar in these pups and found that they were extremely hyperglycemic (fig 1A). All of the transgenic pups displayed this runting phenotype and hyperglycemia, and quickly diverged from their wild type

littermates in weight (Supplemental fig 1C). Indeed, all of these transgenic runts died by approximately five weeks of age, and none of them ever achieved a weight of above 5 g.

Consistent with the severe hyperglycemia observed in these transgenic offspring, we also observed a striking absence of insulin or glucagon positive cells in the pancreas of these mice while exocrine tissue was preserved (data not shown). We attempted islet transplantations to restore normal glucose homeostasis and rescue the lethal phenotype in these animals: one transgenic animal survived the surgery and subsequently gained weight up to 16 grams (data not shown) and showed normal blood glucose measures, although subsequent surgeries were complicated by the small size and altered metabolic status of these animals.

Animals from our lower copy number epet1-1A line bred normally and displayed normal weight gain (supplemental fig 2A), though these mice showed a significantly higher plateau and took longer to return to baseline in a glucose tolerance test (Fig 1C), and significantly lower basal and glucose-stimulated insulin secretion (Fig 1D). To rule out any potential coincident decrease in peripheral insulin sensitivity, we conducted an insulin tolerance test and found a normal drop in blood glucose in both transgenic and wild type control animals (supplemental figure 2B). Taken together, these data argue the epet1-1A transgene causes a deficit in basal and glucose-stimulated insulin secretion without altering peripheral insulin receptor sensitivity.

To better understand how the epet1-1A transgene might impair pancreatic islet function, we determined the time course of transgene expression in the pancreas by qPCR. Transgene expression first appears in the pancreas at e14.5, peaks at around P1, and it declines to undetectable levels by P24 (data not shown). This suggests that the transgenic receptors impair pancreatic islet development during the late prenatal and early postnatal period, but that the transgene does not affect adult pancreatic function in real time. Consistent with this time course, we also found decreased beta cell mass in the transgenic animals at post-natal day 0 and post-natal day 7 (Fig 1E). We reasoned that this decreased beta cell mass could arise either from a decrease in the number of ngn3+ endocrine progrenitor cells, or from a decrease in the rate of

beta cell replication during this period. We found no difference in the number of ngn3+ cells at e15.5 between transgenics and wild type littermates (Fig 1F), while we saw fewer replicating beta cells at both P0 in these transgenics (Fig 1G).

These data demonstrate that the expression of the transgenic 5-HT1A receptor on developing pancreatic endocrine cells decreases the number of insulin and glucagon positive cells by lowering the rate of cell replication. We next wanted to examine whether these effects were due to the 5-HT1A receptor in particular versus whether such effects could be caused in general by overexpressing any G_i-coupled receptor on pancreatic islets. Thus, we expressed the G_i-coupled Ro1 RASSL on pancreatic islets until post-natal day 7 using the tetracycline transactivator system (Tet-On). As Fig 2A shows, expressing RO1 solely during beta cell development leads to worsened adult glucose homeostasis in adulthood, likely due to decreased glucose stimulated insulin release (Fig 2B). Since Ro1 has constitutive receptor activity but is insensitive to endogenous opiates (Baker AJ et al, 2001; Coward P et al, 1998), this shows that basal G_i-coupled receptor signaling during beta cell development is sufficient to suppress cell replication and to worsen adult glucose homeostasis.

These data suggest that increasing G_i signaling during pancreatic islet development can have detrimental effects on islet development and adult glucose homeostasis, but we wondered what role endogenous Gi coupled receptor signaling might play during this time period. To examine this question, we expressed pertussis toxin in beta cells by crossing ROSA-ptx knock-in mice to epet-cre transgenic mice (Regard J, Manuscript under review, JCI, 2007; Scott MM et al, 2005). These mice displayed a dramatic protection against hyperglycemia (Fig 3A) and significantly higher glucose-stimulated insulin secretion (Fig 3B), and higher beta cell mass at P7 (data not shown). We are currently examining whether this change in beta cell mass is due to altered beta cell replication at P0 or due to a change in the number of Ngn3+ precursor cells at e15.5. Taken together, these findings suggest that endogenous G_i coupled receptor signaling constrains beta cell mass during development.

However, the protected glucose tolerance phenotype in these mice could be due to these developmental changes or due to the blockade of Gi-coupled receptor signaling during adulthood in the epet-cre x ROSA-ptx mice. To differentiate between these possibilities, we selectively blocked G_i-coupled receptor signaling on developing beta cells using RIP-rtTA/tetO-ptx double transgenic mice. In these animals, we also saw increased glucose homeostasis accompanied by increased insulin release in a glucose tolerance test (Fig 3C and 3D), though of a lesser magnitude than that seen in Fig 3B.

This finding suggests that a tonic level of G_i-coupled receptor signaling constrains islet cell growth during development, but what are the specific G_i-coupled receptors that might mediate this constraining signal during development? To answer this question, we performed taqman qPCR for each of the non-olfactory mouse GPCR's in developing pancreatic islets. We found over 100 receptors expressed in these cells during this period of high beta cell replication; many of these receptors thus present potential novel targets for interventions to increase beta cell mass during development, and/or to promote differentiation of human embryonic stem cells into beta cells.

Taken together, these results show that G_i-coupled receptor signaling plays a critical role in regulating pancreatic islet development, beta cell mass and adult blood glucose homeostasis. These findings also have implications for understanding the potential pathophysiology of type II diabetes, for cellular transplantation therapies for both type 1 and type 2 diabetes, and more generally for understanding the role of GPCR signaling during development. Type II, or non-insulin dependant, diabetes patients are often overweight and typically have enough insulin to maintain normal blood sugar under fasting or non-fed conditions, but they have insufficient insulin release to meet the glycemic challenge of eating. Many overweight non-diabetic individuals display pancreatic islet hyperplasia that is sufficient to meet the enhanced insulin requirements caused by obesity-associated insulin resistance (Sesti G, 2002), so it is unclear why type II diabetics are unable to increase their islet cell numbers sufficiently to match glycemic

demands. One potential explanation is that these individuals may have less islet cells at baseline or since birth, giving them decreased potential to expand in size to keep up with rising glycemic loads. Our data suggest that increased G_i-coupled receptor activity could play a role in restraining islet cell numbers during development, and thus could predispose some individuals to type II diabetes via this mechanism.

Our data could also be used to improve cellular transplantation therapies for diabetes. Embryonic stem cells can be turned into insulin producing cells that could be transplanted into diabetics, thus enabling them to live without insulin injections; the major problem with this approach has been generating sufficient numbers of insulin positive cells. Our data suggest that blocking G_i-coupled receptor signaling could help increase the number of insulin positive cells derived from ES cells for such regenerative medical applications, and our expression data suggest several novel GPCRs that could be targeted by such interventions.

Finally, GPCRs have traditionally been thought of as regulators of adult cell function, though accumulating evidence in invertebrate systems suggests they may also regulate cell division during embryonic development (Wilkie TM et al, 2005). Our data suggest that GPCRs play a similar role in regulating cell division and embryonic development in mammalian organisms.

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Methods:

Animals. Mice were housed on a 12-hr light–dark cycle in controlled climate rooms (21.5-22.5 degrees C). C57Bl/6j control mice were obtained from Jackson laboratories. All mice had water and laboratory chow pellets available *ad libitum.* All procedures used were approved by institutional Animal Care and Use Committee and in accordance with University of California, San Francisco, regulations.

Cloning of the epet1-5-HT1A transgene: The entire 5-HT1A coding sequence and 3' untranslated region was cloned into the BamHI site of pUNIV-FLAG, to place the FLAG epitope tag and signal sequence onto the N terminus of the 5-HT1A receptor to create pFLAG-5-HT1A. Subsequently, pFLAG-5-HT1A was cut with ecoRV and the sticky ends were blunted with the use of T4 DNA polymerase. Ires-GFP was cut out of the plasmid pIRES-EGFP2 (Clontech) with ecoR1 and BamhI, and the sticky ends were blunted with T4 DNA polymerase as well. The IRES-EGFP insert was subsequently blunt end ligated into the FLAG tagged 5-HT1A plasmid, thus creating pFLAG-5-HT1A+iresGFP.

pFLAG-5-HT1A+iresGFP was then cut with NotI to liberate the 4 kb insert, which was then ligated into pBGZAMod3 (a generous gift of Jessica Lerch and Evan Deneris). This ligation was performed to fuse the beta globin promoter element onto the 5' end of the FLAG-tagged 5-HT1A–iresGFP coding sequence as well to flank the entire coding sequence with loxP sites to enable Cre recombinase mediated recombination *in vivo*, thus creating pBG-FLAG-5-HT1A-iresGFP. This plasmid was then cut with RsrII and the 4.5 kb fragment was purified by agarose gel electophoresis using the Quiagen gel extraction kit. This 4.5 kb fragment was then cloned into the RsrII site of the pBAC-ePet to generate the ePet-5-HT1A transgene shown in Fig 1B. Correct orientation of the inserted fragment was verified by diagnostic restriction digests as well as direct sequencing of the junctions. The final construct was also sequenced with 2x read coverage to verify the correct coding sequence.

This final construct was then cut with Not1 to excise the backbone portion and to linearize the transgene for pro-nuclear injection, and the correct length of the transgene was verified by pulse field electrophoresis. The transgene was then diluted in injection buffer (10 mM Tris, pH 7.4, and 0.25 mM EDTA) to 1 ng/ul, and injected into hybrid C57Bl6j/SJL pronuclei by the Stanford University transgenic core. The resulting pups were screened by PCR for the transgene as previously described (Scott et al, 2005).

RIP-rtTA/tetO-Ro1 and tetO-ptx mice- We thank Shimon Efrat for the generous gift of the RIP-rtTA mice. RIP-rtTA and all tetO transgenic mice were given food containing 200 mg/kg doxycycline (BioServ) during the indicated time periods. To derive the tetO-PTX mice, the cDNA sequence encoding S1-PTX was graciously obtained from Dr Eitan Reuveny (Weizmann Institute of Science, Rehovot, Israel) {Vivaudou, 1997 #17495}, subcloned into the tTA-inducible pUHG 10-3 plasmid (tetO-S1-PTX), and cut with the *ApaLI* and *NdeI* enzymes to isolate the coding region. This linear fragment was purified for pronuclear injection by gel agarose electrophoresis, electroelution, and Elutip filtration (Schleicher and Schuell, Dassel, Germany) and diluted in injection buffer (10 mM Tris, pH 7.4, and 0.25 mM EDTA) to 5 ng/ul. Pronuclear injections into FVB/N oocytes were performed according to standard protocols at the Gladstone Institutes Transgenic Core Facility (San Francisco, CA). Eight founder mice were identified by PCR analysis of genomic DNA isolated from tail tips (primers 5'-CCA TAG AAG ACA CCG GGA CCG-3' and 5'-GGA ACG TCC GGT CAG ATG GTC GA-3' resulting in a 288-bp fragment).

Glucose Tolerance Test- All mice were fasted overnite and placed in fresh cages to ensure that no food was available on the cage floor. The following morning, each mouse was injected with 2 mg/kg of glucose in saline via intraperitoneal injection. Glucose measurements were taken from tail blood at the indicated times, using a OneTouch Ultra Glucometer and test strips.

Insulin Tolerance Test- All mice were fasted overnite and placed in fresh cages to ensure that no food was available on the cage floor. The following morning, each mouse was injected with 0.75 units/kg of insulin, and glucose measurements were taken from tail blood as described above.

Pancreatic Histology- Adult animals were transcardiac perfused with 10 ml cold PBS and 10 ml 4% PFA, then the pancreas was dissected out and post-fixed in 4% PFA for 24 hours, washed 5

times in cold 1x PBS, once in 50% ethanol and then placed into 70% ethanol. Pancreatic tissue was then embedded in paraffin blocks and sectioned in 6 micron slices on a microtome, and stained as previously described (Lynn et al, 2007).

Serum insulin measurements- Blood was obtained from tail bleeds and placed into 1.5 ml heparanized tubes on ice to prevent clotting. The blood samples were then centrifuged at 15,000 RPM with a microcentrifuge, and the upper plasma layer was taken off for assaying of serum insulin levels. Insulin levels were measured by ELISA.

Pancreatic RNA isolation and Quantitative PCR-

Mice were anaesthetized with 4% avertin and lack of consciousness was verified by lack of response to a tail pinch stimulus. Subsequently, the pancreas was dissected out, and immediately placed in RNAlater (Ambion) on ice. The pancreas was minced in the RNAlater solution, and then was allowed to incubate in this solution overnight. Subsequently, the pancreatic pieces were pelleted by centrifugation for 2 min at 15,000 RPM at 4 degrees in a microcentrifuge, the supernatant was removed, and the pancreatic pieces were then suspended in 5 ml of Trizol and homogenized with a sonifier, and RNA was isolated by the trizol protocol. First-strand cDNA was prepared from 1–3 ug of total RNA with the Invitrogen SuperScript first-strand cDNA synthesis kit and oligo dT primers; taqman primer and probe sets were used as described (Regard J et al, 2007).

Figures:

Fig 1. a.. glucose in 925 bar graph

1b. islet pics from 925 line with insulin, glucagon staining

1c. GTT from epet1-1A 949 line

1d insulin values from epet1-1A 949 line GTT

1e. P7 beta cell mass in epet1-1A 949 transgenics

1f. Ngn3 staining at e15.5 in epet1-1A 949 transgenics

1g. P0 BRDU and insulin co-staining in the epet1-1A 949 transgenics, and replication rate graph

Fig 2. a. GTT in RIP-rtTA/tetO-Ro1 mice (with dox until P7)

b. insulin values from 2a.

Fig 3. a. ROSA-ptx x epet-cre GTT

b. insulin values from part 3A

c. RIP-rtTA/tetO ptx mice GTT (with dox until P7)

d. Insulin values from part 3e.

Table 1 A. gpcr array data in early post-natal pancreatic beta cells.

supplemental figures

1A epet1-1A tg diagram

1B.qPCR for copy number in e1A 925 vs 949 tg's

1C. weight curve in e1A 925 tg's

2a. weight/growth curve in line 949 transgenics vs wt's

2b. insulin tolerance test in 949 line transgenics vs wt's

Acknowledgements: We thank Greg Szot and Pavel Koudria for islet isolations.

Contributions: M. Berger conceived of this project, set up all mouse crosses, cloned the epet1-1A transgene and did all of the work with this transgenic line, performed all physiological studies,

contributed to the histologic studies, and wrote this manuscript. E. Deneris mentored M. Berger during the cloning of the epet1-5-HT1A transgene. B. Conklin contributed the tetO-ptx line as well as invaluable mentorship and encouragement. J. Regard and S. Coughlin contributed the ROSA-ptx mouse line, and J. Regard performed the qPCR array study in Table 1. H. Kim performed the insulin ELISA's. D. Scheel performed all histologic studies with M. Berger. P. Sharifnia, Y. Tang, L. Yu, P. Hoang and E. Mangir assisted M. Berger with physiological assays, mouse genotyping and colony maintenance, and statistical analysis; Y. Tang also performed the qPCR studies described here, with assistance from J. Wang. L. Tecott mentored M. Berger, tried to keep him in line, and oversaw all aspects of this project; M. German mentored M. Berger and oversaw the histologic studies of this project.

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Wilkie TM et al. New roles for Galpha and RGS proteins: communication continues despite pulling sisters apart. Curr Biol. 2005 Oct 25;15(20):R843-54.

Fig 1A

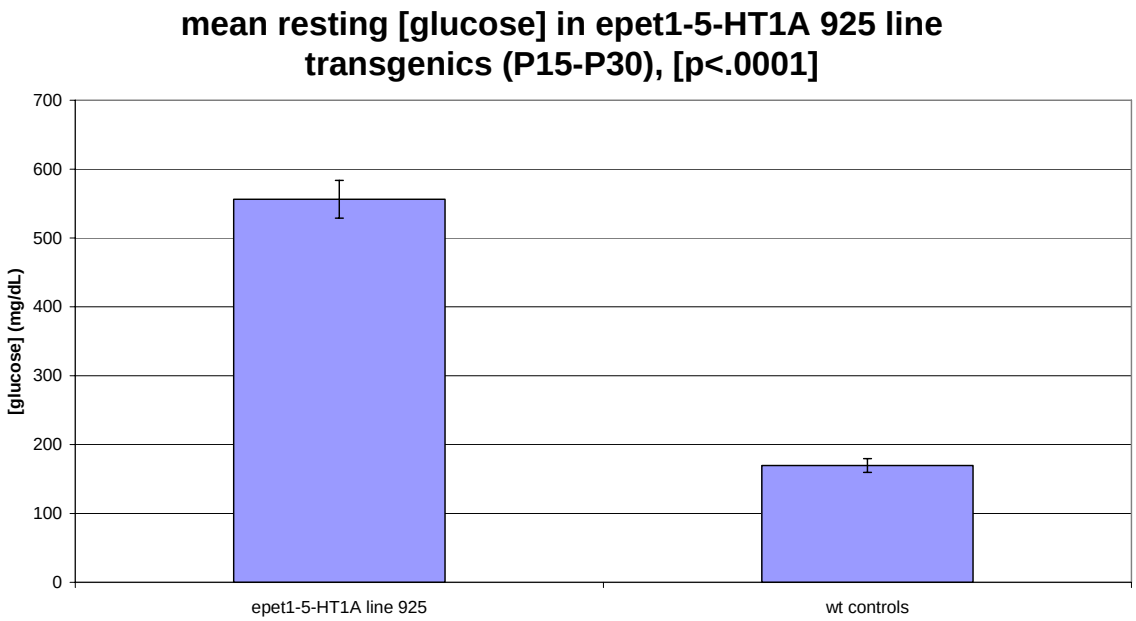

mean resting [glucose] in epet1-5-HT1A 925 line transgenics (P15-P30), [p<.0001]
[glucose] (mg/dL)
700
600
500
400
300
200
100
0
epet1-5-HT1A line 925
wt controls

Fig 1B

Fig 1C

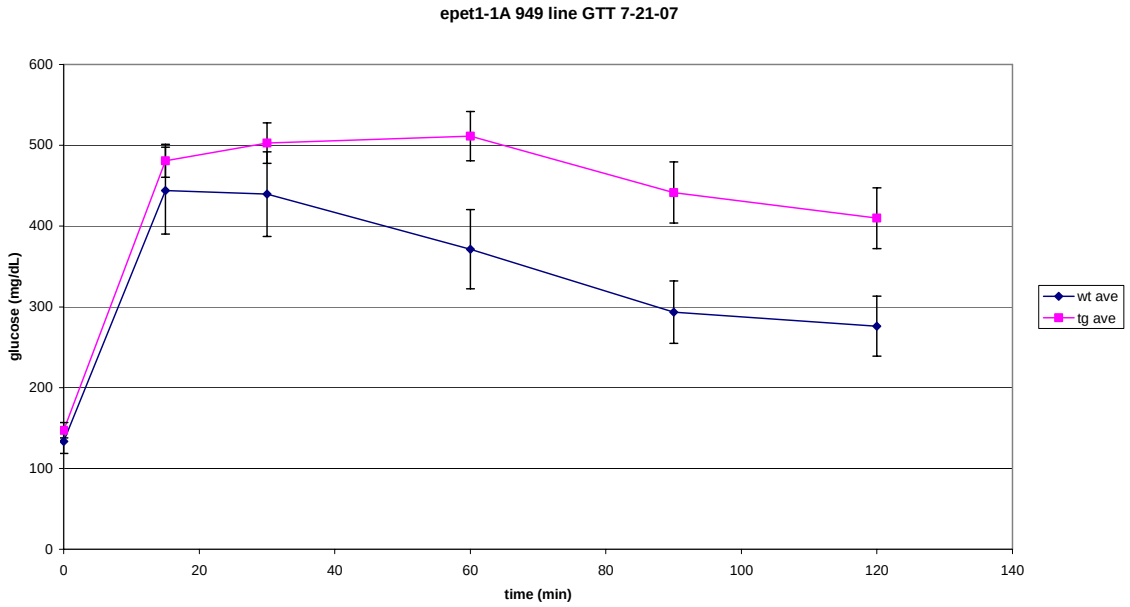

epet1-1A 949 line GTT 7-21-07
glucose (mg/dL)
600
500
400
300
200
100
0
0
20
40
60
80
100
120
140
time (min)
wt ave
tg ave

Fig 1D

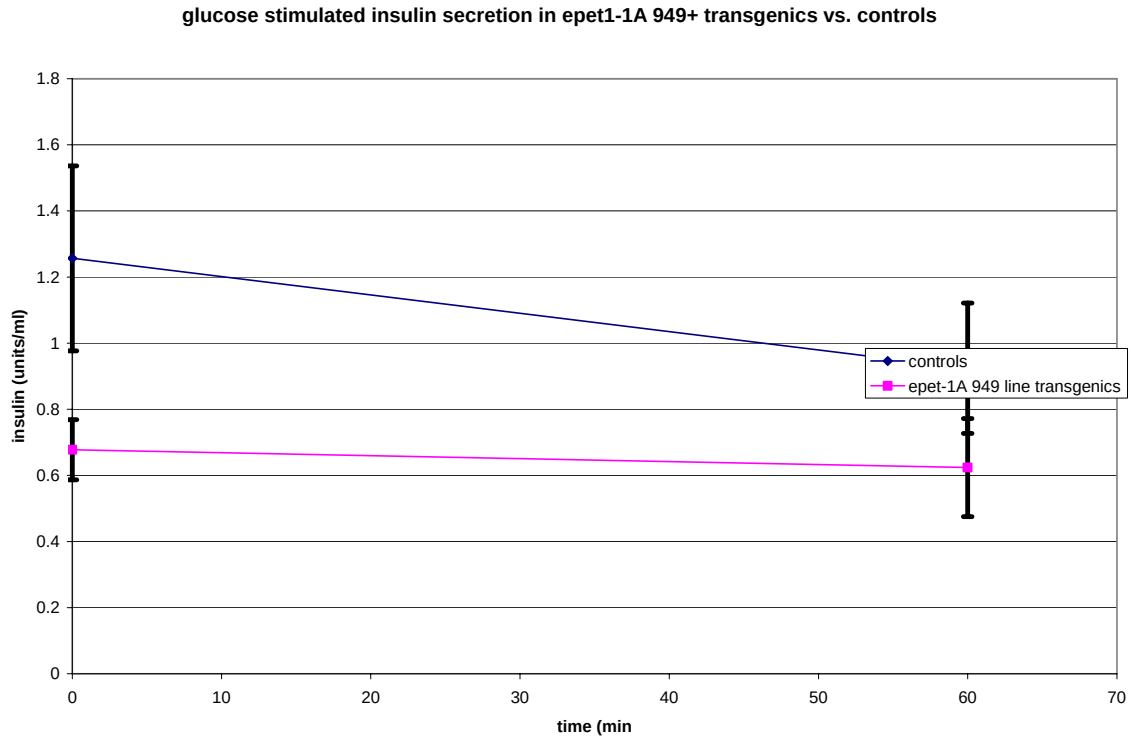
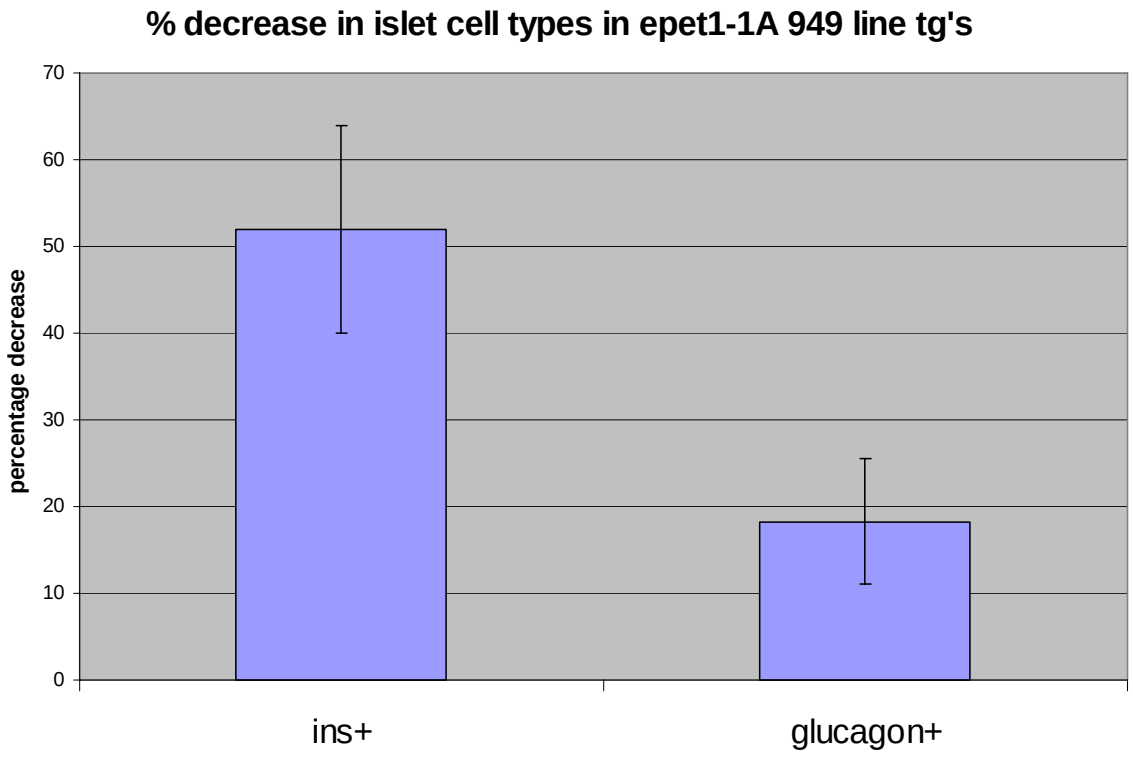
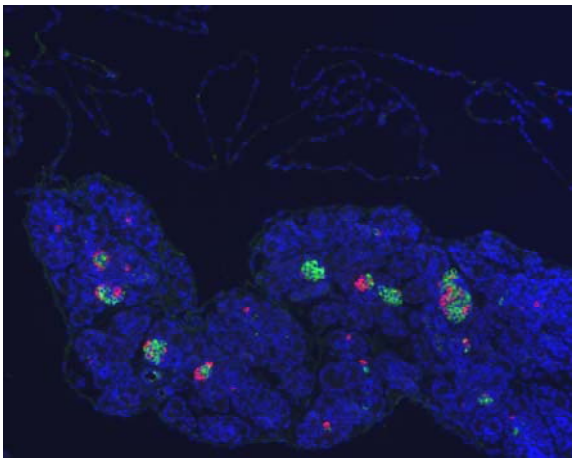

glucose stimulated insulin secretion in epet1-1A 949+ transgenics vs. controls
insulin (units/ml)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
time (min
controls
epet-1A 949 line transgenics

Fig 1E



e1A949+ P7 insulin- green glucagon- red



e1A949- P7 insulin- green glucagon- red

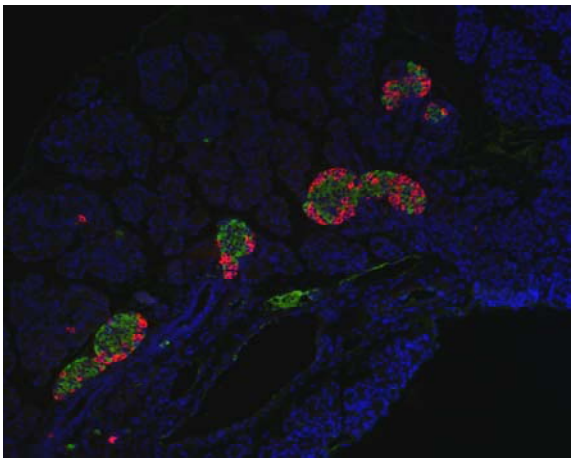


Fig 1F

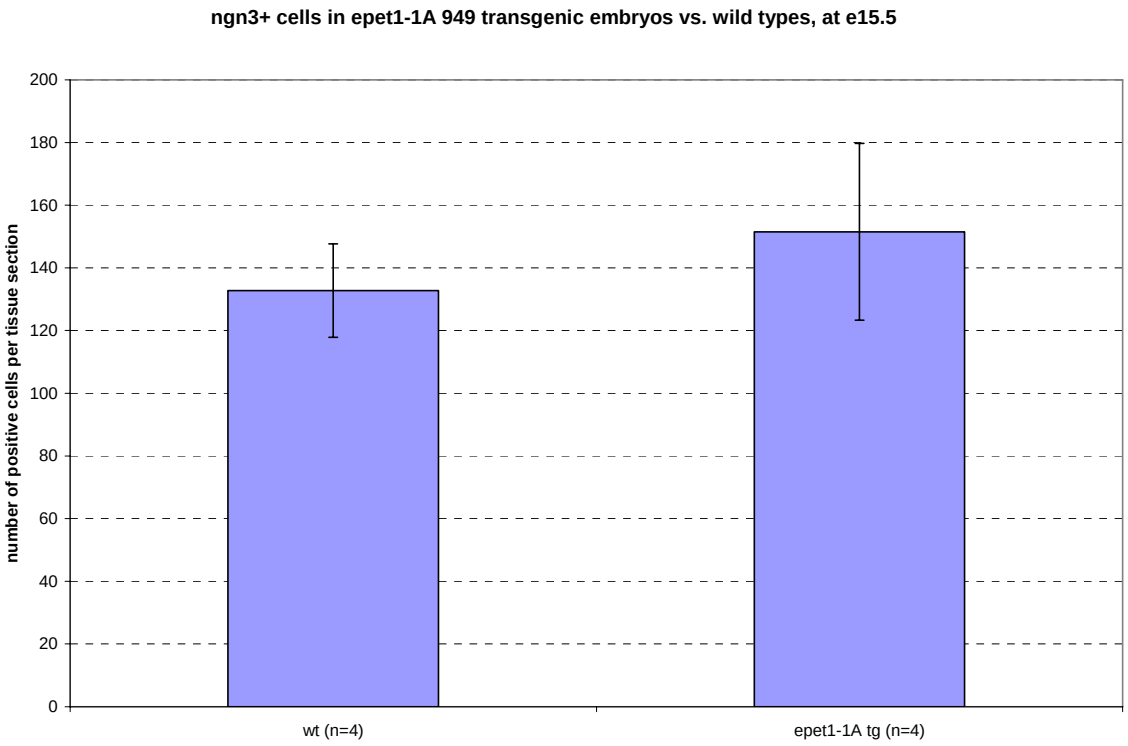
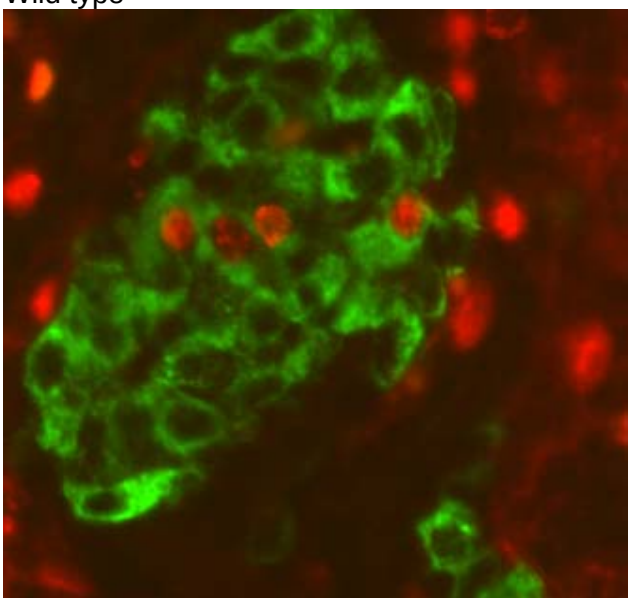

ngn3+ cells in epet1-1A 949 transgenic embryos vs. wild types, at e15.5
number of positive cells per tissue section
200
180
160
140
120
100
80
60
40
20
0
wt (n=4)
epet1-1A tg (n=4)

Fig 1G

Wild type



Epet1-1A 949+ transgenic

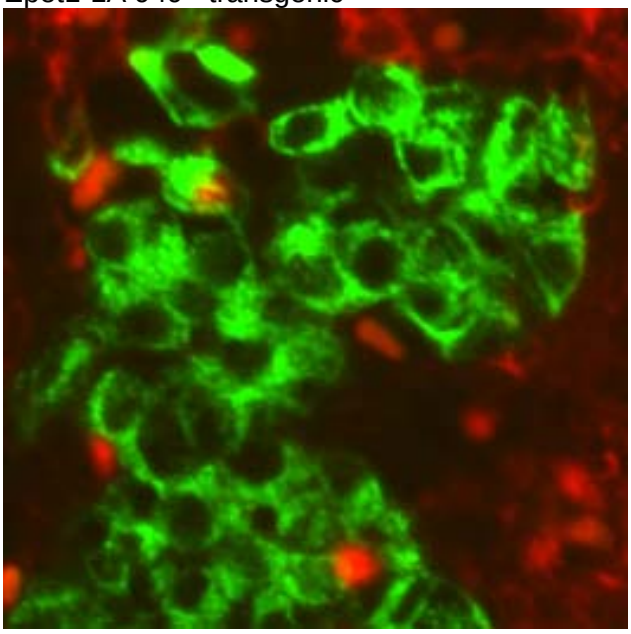


Fig 2A

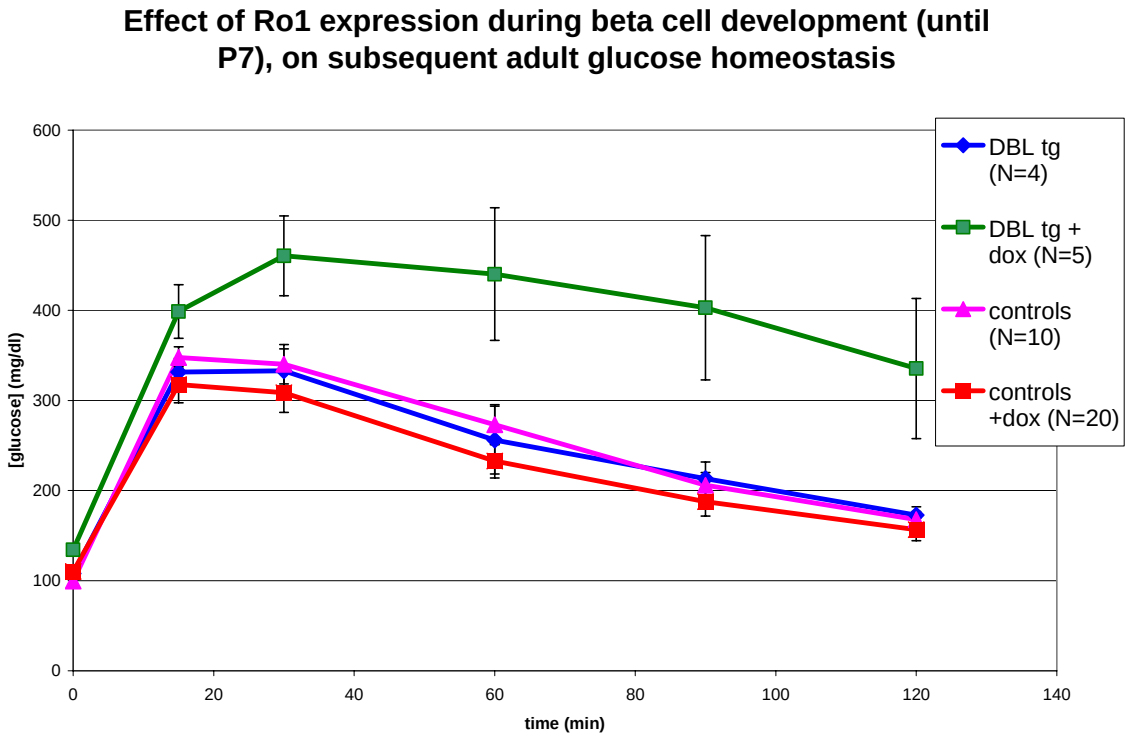

Effect of Ro1 expression during beta cell development (until P7), on subsequent adult glucose homeostasis
[glucose] (mg/dl)
time (min)
0
100
200
300
400
500
600
0
20
40
60
80
100
120
140
DBL tg (N=4)
DBL tg + dox (N=5)
controls (N=10)
controls +dox (N=20)

Fig 2B

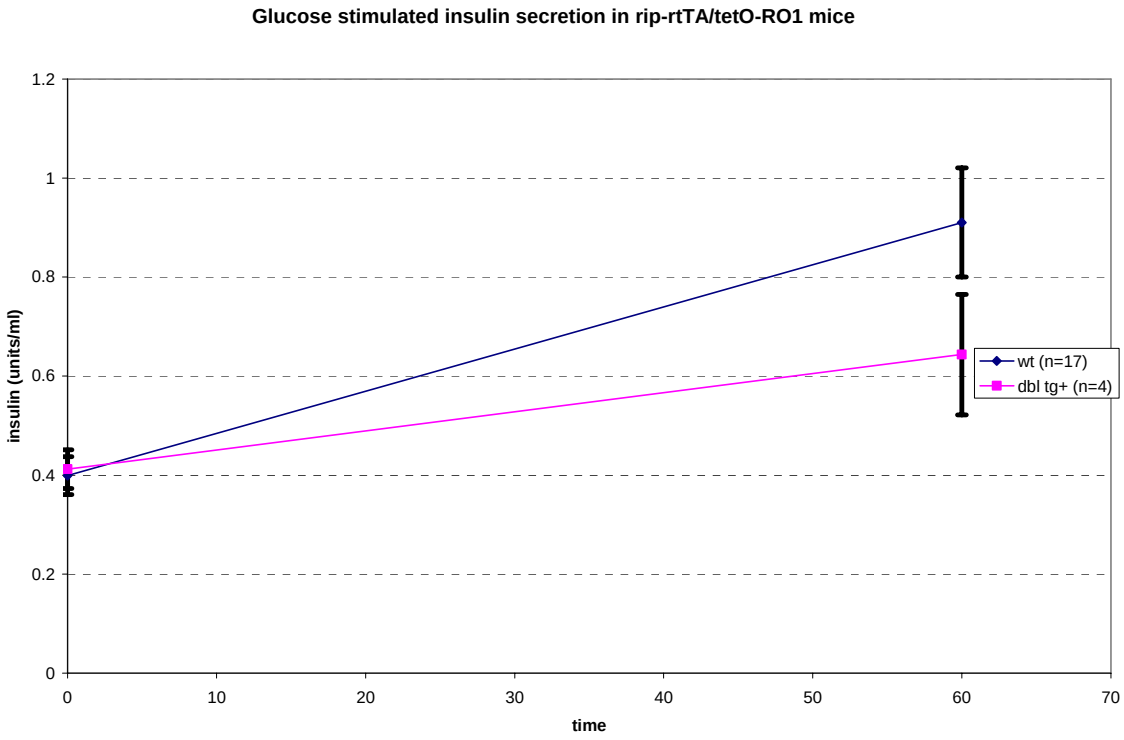

Glucose stimulated insulin secretion in rip-rtTA/tetO-RO1 mice
insulin (units/ml)
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
time
wt (n=17)
dbl tg+ (n=4)

Fig 3A

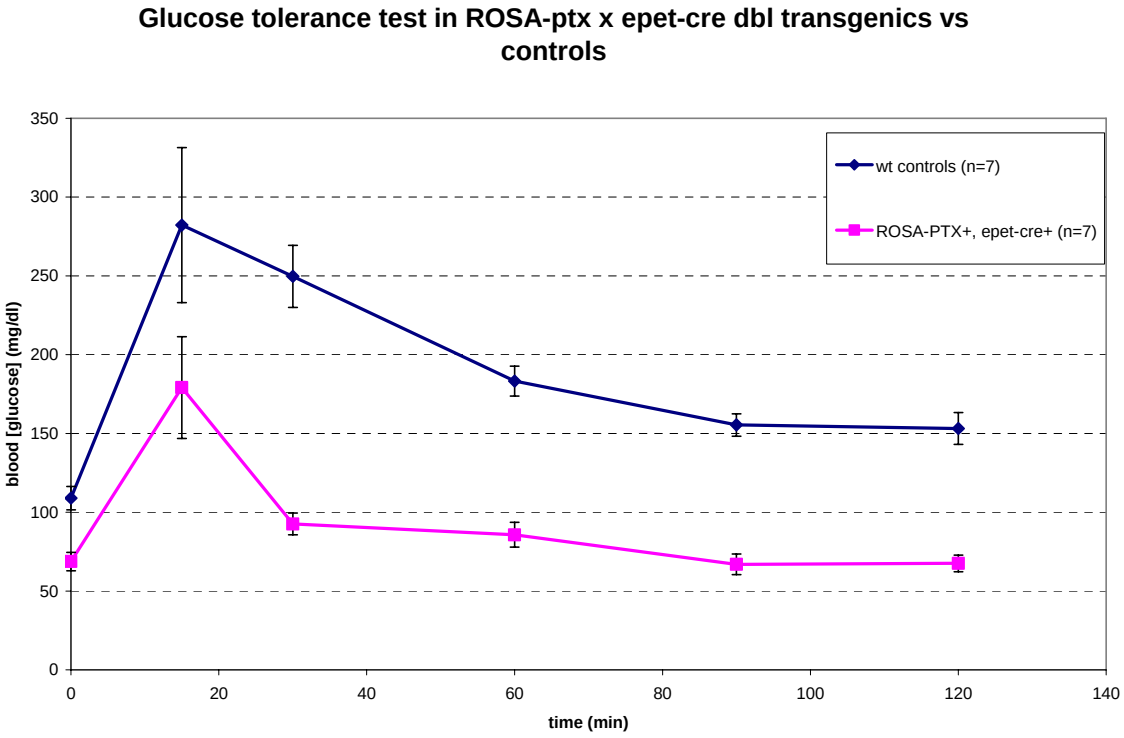

Glucose tolerance test in ROSA-ptx x epet-cre dbl transgenics vs controls
blood [glucose] (mg/dl)
350
300
250
200
150
100
50
0
0
20
40
60
80
100
120
140
time (min)
wt controls (n=7)
ROSA-PTX+, epet-cre+ (n=7)

Fig 3B

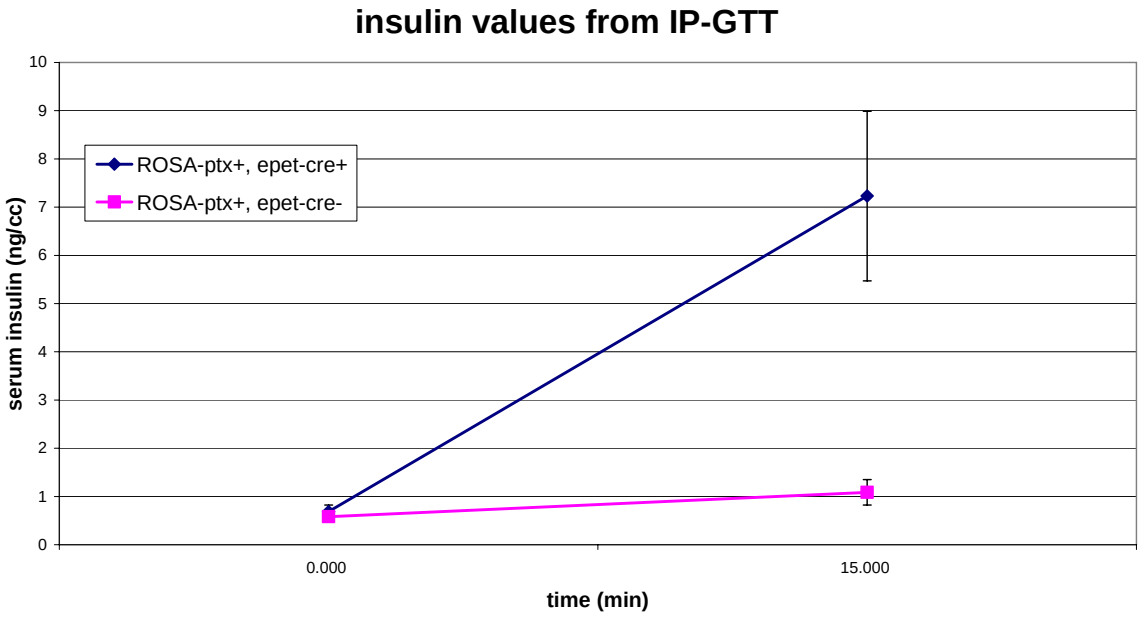


Fig 3C

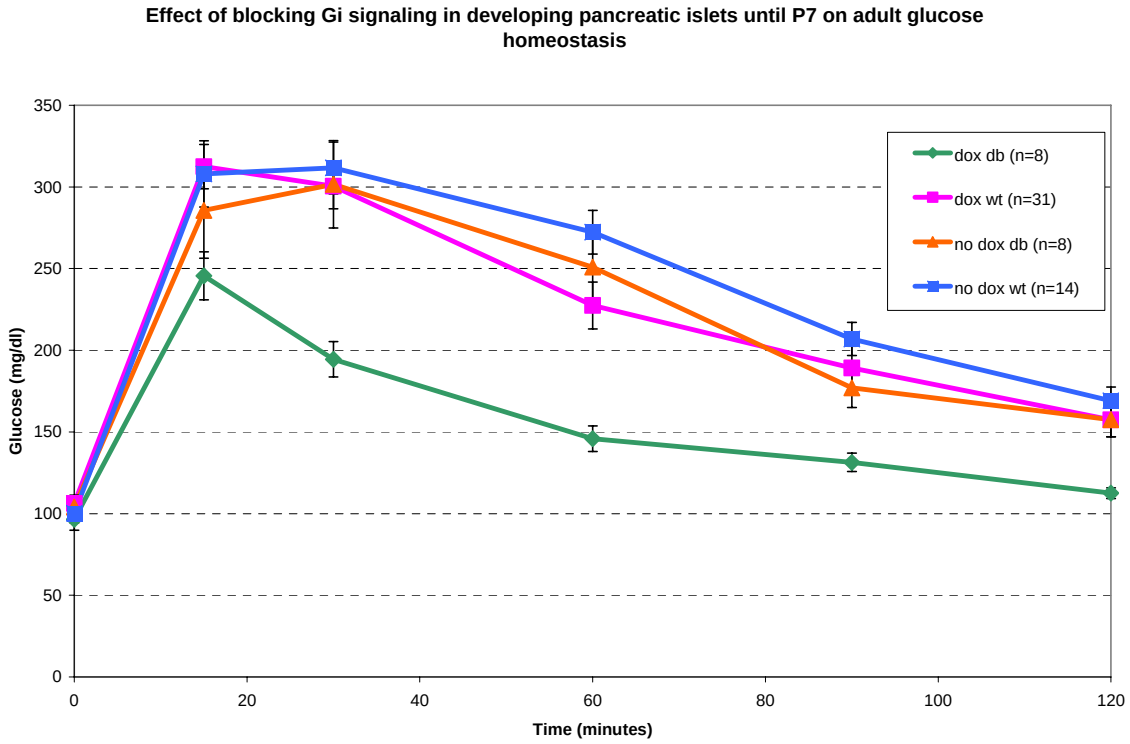
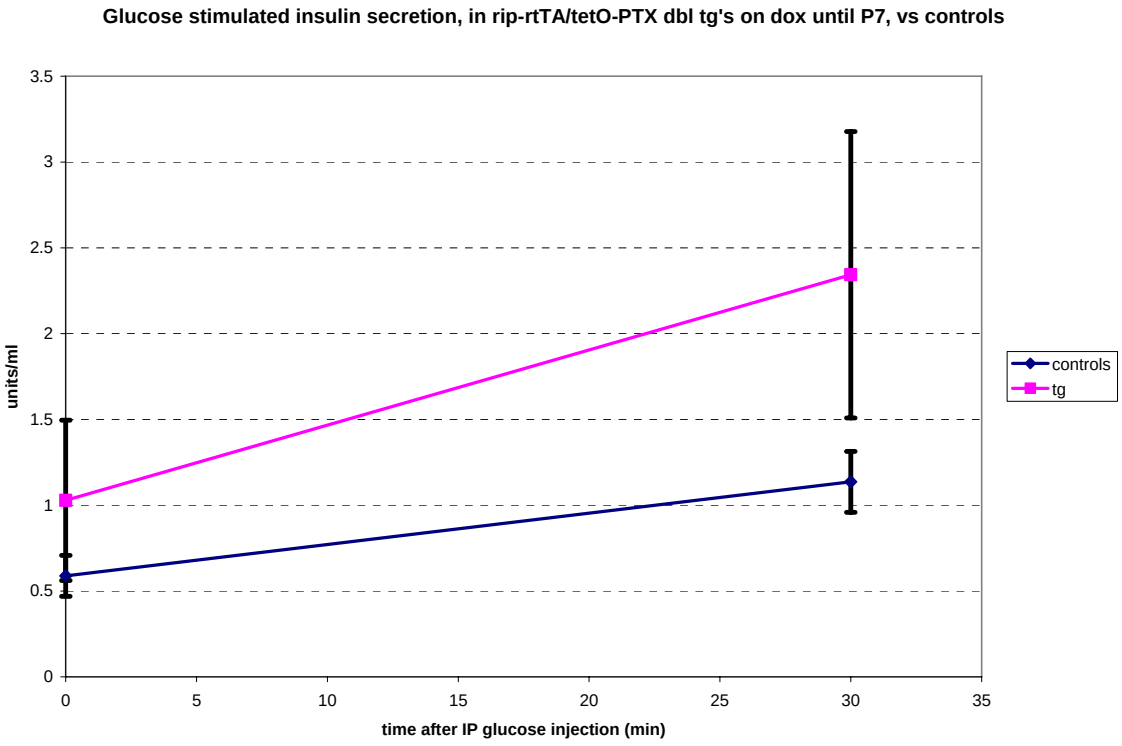


Fig 3D



Note: For the figure above, p=.1 for the two sets of mice at time=0, while p<.02 for the two groups at t=30 minutes.

Table 1

Genbank Name	common name
ADMR	adrenomedullin receptor
ADORA1	adenosine A1 receptor
ADORA2A	adenosine A2A receptor
ADORA2B	adenosine A2B receptor
ADORA3	adenosine 3 receptor
ADRA2A	alpha 2A adrenergic receptor
ADRB2	Beta 2 adrenergic receptor
CASR	Calcium sensing receptor
CCKAR	cholecystekinin type A receptor
CCRL1	
CD97	leukocyte adhesion receptor
CELSR1	Protocadherin Flamingo 2
CELSR2	FLAMINGO 1
CELSR3	Protocadherin Flamingo 1
CHRM3	Muscarinic acetylcholine receptor M3
CHRM4	Muscarinic acetylcholine receptor M4
CRHR2	Corticotropin Releasing Factor Receptor 2 precursor
CXCR6	Chemokine receptor 6

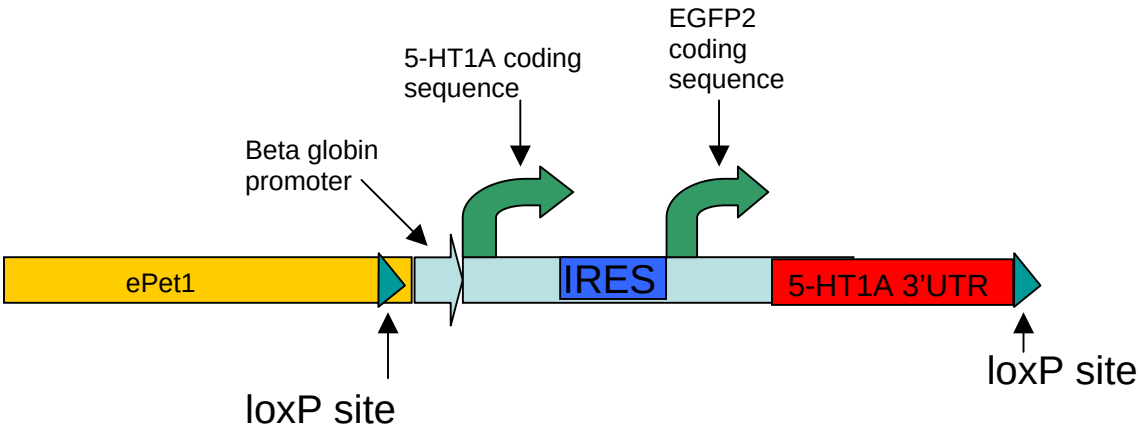
DJ287G14	orphan receptor, no common name
EBI2	EBV-induced G protein-coupled receptor 2 (EBI2)
EDG2	Lysophosphatidic acid receptor (EDG-2)
EDG4	orphan receptor, no common name
EDG5	LYSOSPHINGOLIPID RECEPTOR EDG5
EDNRA	Endothelin-1 receptor precursor (ET-A)
F2RL1	Proteinase activated receptor 2 precursor (PAR-2)
FZD2	Frizzled 2
FZD3	Frizzled homolog 3 (Frizzled-3) (WNT receptor frizzled-3)
FZD5	Frizzled 5
FZD6	Frizzled 6
FZD7	Frizzled 7
GABBR1	Gamma-aminobutyric acid type B receptor, subunit 1 precursor (GABA-Breceptor 1) (GABA-B-R1) (Gb1)
GALR1	Galanin receptor 1
GALR2	Galanin receptor 2
GALR3	Galanin receptor 3
GCGR	glucagon receptor
GHSR	Ghrelin receptor
GIPR	Gastric inhibitory polypeptide (GIP)
GLP1R	glucagon like peptide 1 receptor
GPR10	Prolactin releasing hormone receptor
GPR105	P2Y-like receptor
GPR107	orphan receptor, no common name
GPR108	orphan receptor, no common name
GPR18	orphan receptor, no common name
GPR19	GPR-NGA
GPR21	orphan receptor, no common name
GPR22	orphan receptor, no common name
GPR26	orphan receptor, no common name

GPR27	Super conserved receptorexpressed in brain 1
GPR3	Probable G protein-coupled receptor GPR3 (ACCA orphan receptor)
GPR30	estrogen receptor
GPR31	orphan receptor, no common name
GPR39	Ly6/Plaur domain containing 1 receptor
GPR40	free fatty acid receptor
GPR41	orphan receptor, no common name
GPR43	free fatty acid receptor
GPR44	Putative G protein-coupled receptor GPR44 (Chemoattractant receptor-homologous molecule expressed on Th2 cells)
GPR48	LGR4
GPR51	Gamma-aminobutyric acid type B receptor, subunit 2 precursor (GABA-Breceptor 2) (GABA-B-R2) (Gb2) (GABABR2) (G protein-coupled receptor51) (GPR 51) (HG20)
GPR54	G protein-coupled receptor AXOR12
GPR56	TM7XN1 protein precursor
GPR63	PSP24-2
GPR75	orphan receptor, no common name
GPR82	orphan receptor, no common name
GPR85	Super conserved receptorexpressed in brain 2, (PKrCx1, SREB2)
GPR86	FKSG77, 2010001L06RIK, GPR68, P2RY13
GPR87	orphan receptor, no common name
GPRC5B	A-69G12.1, HYPOTHETICAL 44.8 KDA PROTEIN
GPRC5C	orphan receptor, no common name
GPRC6A	GPCR Class C group 6 member A, similar to calcium sensing receptor, VIRC8
GRCA	Gpr162, protein A-2
H963	Gpr171

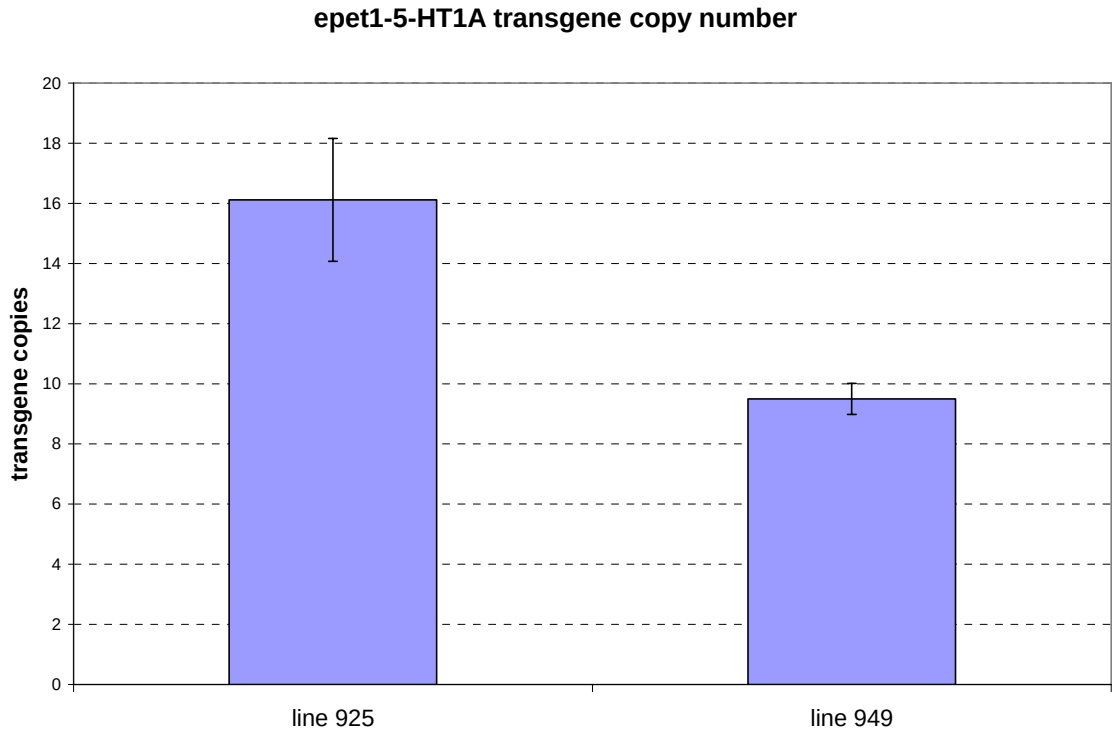
HGPCR2	Gpr119, similar to beta-2 AR
HM74	PUMA-G
HTR1B	Serotonin 1B receptor
HTR2B	Serotonin 2B receptor
IL8RB	interleukin 8 receptor
KIAA0758	DJ365O12.1, GPR116
LEC2	Lectomedin-2
LEC3	Lectomedin-3
MAS1	MAS proto-oncogene
MC5R	Melanocortin-5 receptor
MrgA3	MAS-related receptor A3
MRGE	orphan receptor, no common name
OPN3	Opsin 3 (Encephalopsin) (Panopsin
OPRL1	Nociceptin receptor (Orphanin FQ receptor) (Kappa-type 3 opioidreceptor)
OXTR	Oxytocin receptor
P2RY1	P2Y purinoceptor 1 (ATP receptor) (P2Y1) (Purinergic receptor)
P2RY12	P2Y12 platelet ADP receptor (G-protein coupled receptor SP1999)
P2RY2	P2U purinoceptor 1 (ATP receptor) (P2U1) (Purinergic receptor)
P2RY6	P2Y purinoceptor 6
P2Y5	P2Y purinoceptor 5 (P2Y5) (Purinergic receptor 5) (RB intron encodedG-protein coupled receptor)
PGR2	Gpr142
PGR21	V1RA7, Similar to TEM5
PGR22	GPR155, similar to frizzled
PGR4	Similar to NPY2R
PGR8	Similar to GPR30
PTGDR	Prostaglandin D2 receptor (Prostanoid DP receptor) (PGD receptor)
PTGER1	Prostaglandin E2 receptor, EP1 subtype (Prostanoid EP1 receptor) (PGEreceptor, EP1 subtype)
PTGER3	Prostaglandin E2 receptor, EP3 subtype (Prostanoid EP3 receptor) (PGEreceptor, EP3 subtype)

PTGER4	Prostaglandin E2 receptor, EP4 subtype (Prostanoid EP4 receptor) (PGEreceptor, EP4 subtype)
RAI3	PUTATIVE G PROTEIN-COUPLED RECEPTOR (CDNA FLJ10899 FIS, CLONENT2RP5003506) (RETINOIC ACID INDUCED 3)
SMOH	Smoothened homolog precursor (SMO)
SREB3	Super conserved receptor expressed in brain 3
SSTR1	somatostatin receptor 1
SSTR3	somatostatin receptor 3
TA11	Trace amine receptor 11
TA7	Trace amine receptor 7
TAR1	trace amine receptor 1
TM7SF1	transmembrane 7 superfamily member 1 (upregulated in kidney); transmembrane 7 superfamily member 1 (upregulated in [Homo sapiens]
TM7SF1L1	orphan receptor, no common name
TM7SF3	orphan receptor, no common name
TPRA40	seven transmembrane domain orphan receptor; transmembrane domain protein regulated in adipocytes 40 kDa
TSHR	Thyrotropin receptor precursor (TSH-R) (Thyroid stimulating hormonereceptor)
VIPR1	Vasoactive intestinal polypeptide receptor 1 precursor (VIP-R-1)(Pituitary adenylate cyclase activating polypeptide type II receptor)(PACAP type II receptor) (PACAP-R-2)
VLGR1	Very large G-protein coupled receptor-1

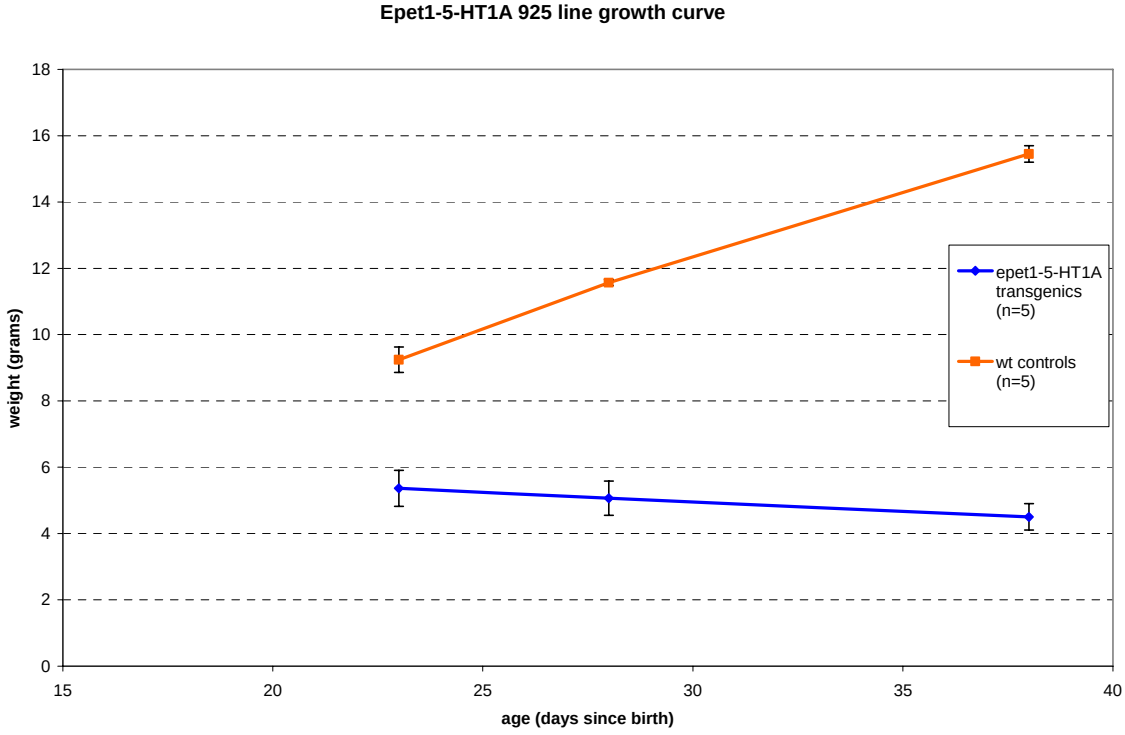
Supplemental Fig 1A



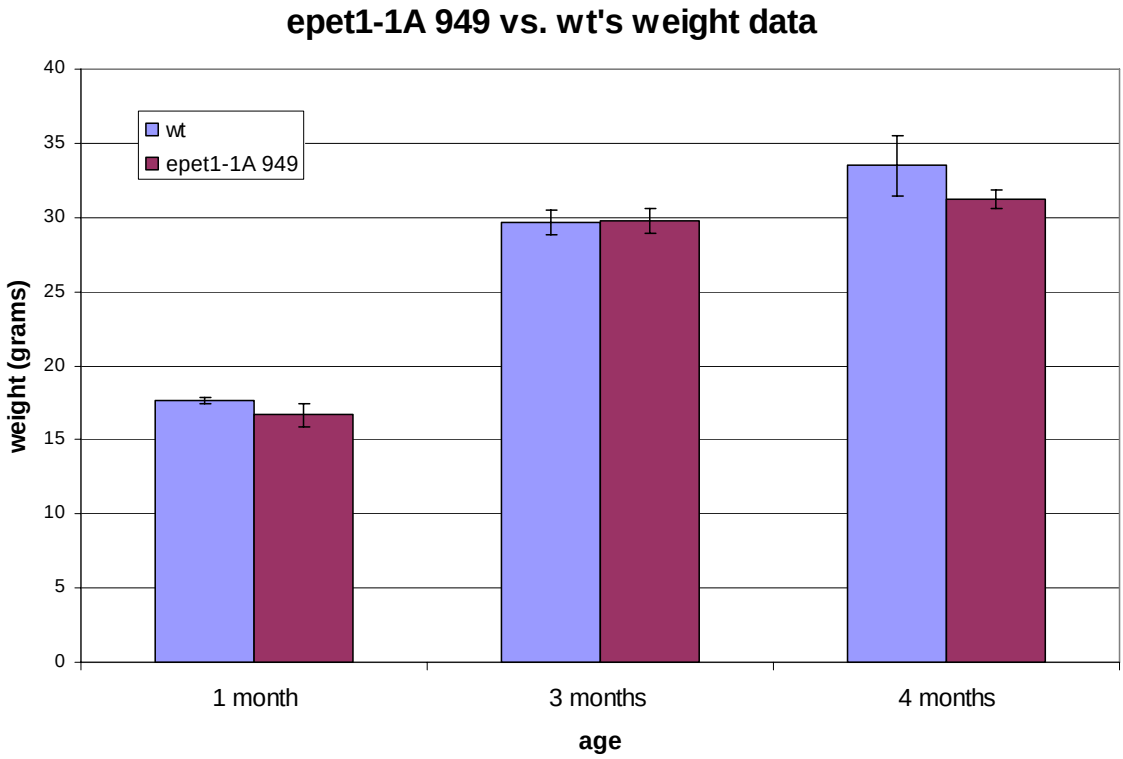
Supplemental Fig 1B



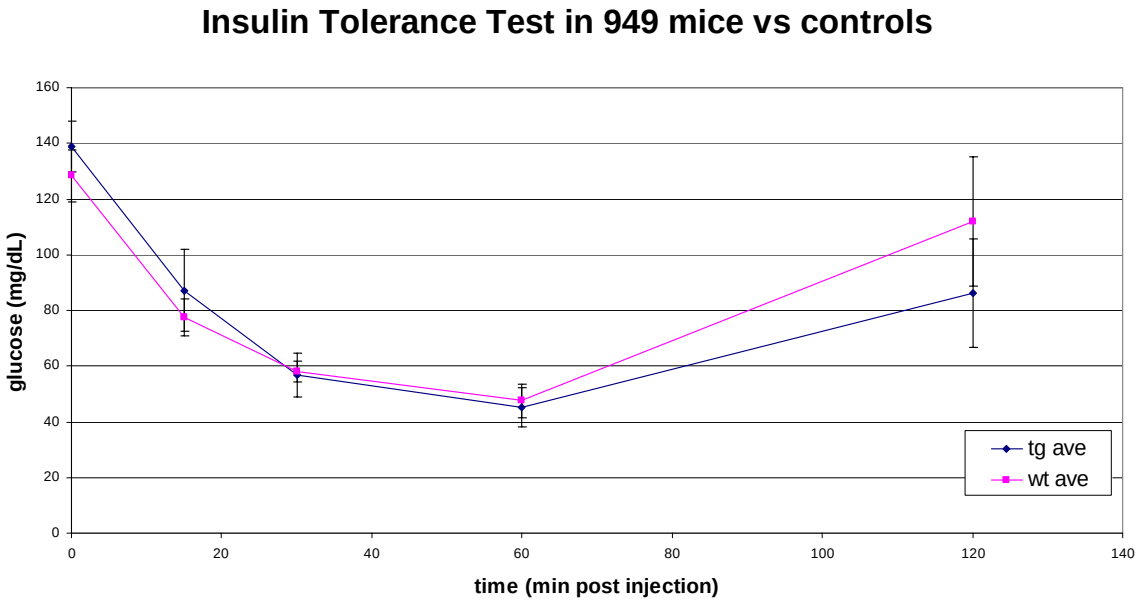
Supplemental Figure 1C



Supplemental Fig 2A



Supplemental Fig 2B



Supplemental Fig 3

Chapter IV. Serotonin System Gene Knockouts: a Story of Mice with Implications for Man

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Abstract: Serotonin modulates a dizzying array of mammalian physiological and behavioral processes thru some 15 specific receptor subtypes in the CNS and periphery, from cardiac development to aggressive behavior to energy balance. To better understand how serotonin uses specific receptor subtypes to modulate these disparate biological phenomena, a number of groups have generated 'knockout' mice lacking individual receptor subtypes over the last decade. Overall, 10 of the 15 serotonin receptor subtypes have been knocked out in mice, as well as several genes that regulate the development and activity of serotonin neurons (e.g. Pet-1, the serotonin transporter, and tryptophan hydroxylase). These genetic studies and complementary pharmacologic approaches paint a rich picture of how serotonin orchestrates such disparate biological processes. Here I review these studies, discuss their relevance to the pathophysiology of clinical disorders (with a primary focus on neuropsychiatric disorders), and I close with a perspective on where the field is likely to head in the future.

Key Words: Transgenic and Knockout Mice, Neuropsychiatric Disorders, Anxiety, the Neural Circuitry of Complex Behavior, SERT, Pet-1, Tryptophan Hydroxylase 2.

Selective Serotonin Reuptake Inhibitors (SSRI's) like Prozac™ are used to treat many psychiatric disorders ranging from intermittent explosive disorder to obsessive-compulsive disorder to major depression and panic disorder (1), even though these disorders bear little resemblance to one another behaviorally. How does one drug class treat these disparate disorders?

One possibility is that the various serotonin receptor subtypes (15 have been cloned to date) selectively regulate neural circuits that underlie specific behaviors. According to this view, alterations in the activity of different serotonin receptors (or alterations in the activity of a subpopulation of a given receptor) may perturb distinct neural circuits and therefore contribute to the characteristic behavioral dysregulation of a specific psychiatric illness.

To examine the role of individual serotonin receptor subtypes in the neural circuitry of these behavioral abnormalities and in normal behavior, many laboratories have generated "knockout" mice lacking individual serotonin receptor subtypes. Though it is wise to note that mice are not just miniature humans (2), mice and humans do express the same serotonin receptor subtypes (with the sole exception of the 5-HT5B receptor) with a roughly similar anatomic distribution (3). Moreover, mice and humans share many neurobehavioral processes such as explicit and implicit memory, ingestion of drugs of abuse, aggression, and anxiety-like behavior.

Thus, studies of serotonin receptor knockout mice are likely to enhance our understanding of the roles these receptors play in the neural circuitry of complex behavior in both mouse and Man.

Indeed, over the last decade, 10 out of the 15 serotonin receptor subtypes have been knocked-out, and studies of these knockout animals have confirmed existing hypotheses and revealed new insights into the roles of these receptors. Perhaps the most surprising finding is that none of these 'knockouts' causes a lethal CNS defect. While many of the knockout lines have intriguing behavioral abnormalities, that none have lethal CNS defects suggests that central serotonergic activity subtly modulates the neural circuitry underlying these behaviors without being absolutely necessary for brain functions or behaviors necessary to support life.

Many of the behavioral abnormalities in serotonin receptor knockout mice enrich our understanding of the specific serotonergic pathways that may be dysregulated in psychiatric disorders. For example, 5-HT1A knockout mice display anxiety-like behavior in numerous behavioral tests (4), and more recent work has begun to elucidate the dysregulated neural pathways that may underlie their anxiety phenotype (5; 6; 7). These data suggest possible mechanisms for the pathogenesis of anxiety and depressive disorders, and they imply future directions for therapeutic strategies.

While the behavioral phenotypes seen in 5-HT-R knockout mice are intriguing, it can be challenging to discern the full range of behavioral processes disrupted by the knockout of a 5-HT-R. For example, a behavioral abnormality in a single test paradigm could arise from many different underlying causes, just as a single symptom like anxiety appear in the context of many different medical and/or psychiatric illnesses. A mouse that performs poorly in a test of spatial memory may indeed have impaired spatial memory, or it may have a vision defect or simply a change in overall motor activity. Thus, the more different behavioral phenotypes investigated in

a given knockout mouse, the more confident we can be about our interpretation of a given abnormality.

Yet no matter how confident we can be of the specificity of a behavioral phenotype, another issue with knockout studies is that they often do not provide a complete understanding of how the loss of a receptor leads to a given phenotype. Standard knockout models ablate the expression of the knocked-out gene throughout development in all tissues where it would normally be expressed. It may thus be unclear whether a behavioral phenotype is due to the absence of the receptor in the adult animal, the absence of the receptor during development, or other compensatory alterations due to the chronic loss of the receptor. In addition, identifying which subpopulations of the knocked-out receptor are most responsible for the altered phenotype may be difficult. Newer techniques such as the Cre/loxP and Tet inducible systems (8), RASSLs (9) and chemical genetic approaches (10) allow investigators to study these subpopulations of receptors with spatial and/or temporal control, and will clearly play an increasingly important role in the future.

The application of mouse genetics to the study of serotonin receptor biology over the last ten years has revolutionized our understanding of the roles that these receptors play in the neural circuitry of behavior. While these studies have confirmed existing ideas about links between serotonergic tone and affective behavior, they have also provided surprising new insights into classic questions (7). Recent studies have also begun to clarify the roles of other genes like Pet-1, tryptophan hydroxylase (TPH2), and the serotonin transporter (SERT) that regulate the development and activity of serotonin neurons; future work is likely to focus on how mutations in these genes may perturb serotonergic function and on developing drugs to modulate their

activity. In this chapter we review these studies, discuss their relevance to the pathophysiology of human psychiatric disorders, and close with a perspective of future directions in this field.

5-HT1A receptor

Role of CNS 5-HT1A receptors in anxiety- and depression-related behavior

The 5-HT1A receptor is perhaps the most extensively studied of all the serotonin receptors: a recent medline search for "5-HT1A" found 4,505 papers. In accord with this intense interest, three independent groups (including our own) simultaneously generated 5-HT1A receptor knockout mice (11; 12; 13). All three groups reported increased anxiety-like behavior in these mice, although each group maintained the knockout allele on a different genetic background, highlighting the robust influence of *5-htr1a* in regulating anxiety-like behavior.

Specifically, these studies found that 5-HT1A-R null animals showed increased anxiety-like behavior in the open field, elevated plus maze, elevated zero maze, and novel object tests. 5-HT1A-R knockouts were also less immobile in the forced swim and tail suspension tests, the same response induced in these tests by antidepressant drugs. However, it is unclear whether this decreased immobility truly reflects an antidepressant-like coping reaction mediated by increased 5-HT transmission, or whether it simply reflects an increased stress response in these anxiety-prone mutant mice (12).

The striking anxiety-like phenotype of 5-HT1A-R KO mice raises an important question: does this phenotype reflect the absence of normal receptor activity in the adult animal, or indirect compensatory responses its absence? Initially all three groups proposed that the KO phenotype was due to the absence of the receptor in adult animals, in part because administration of a 5-

HT1A antagonist induced an anxiety-like response in wild type mice in the open field test similar to the baseline response seen in 5-HT1A-R knockouts (13).

5-HT1A receptors are expressed widely throughout the cerebral cortex and are densely present in the hippocampus and the brainstem raphe nuclei, which also raises the question of the relative contribution of these 5-HT1A-R subpopulations to the anxiety-like phenotype. In the raphe nuclei, 5-HT1A receptors are expressed on 5-HT neurons and act as inhibitory autoreceptors (5-HT1A-AR) that restrain the firing of these neurons (14). Since increased serotonergic tone has been linked to anxiety (15, and references therein), the loss of raphe 5-HT1A-AR could enhance anxiety by disinhibiting 5-HT neurons. In line with this hypothesis, the serotonin neurons of 5-HT1A KO mice fire at a higher rate on average (16) although increased basal 5-HT release has been found in 5-HT1A KO mice in only one study (17; 18; 19; 20).

Tissue 5-HT content is not increased in the brains of 5-HT KO mice (21), and the majority of microdialysis studies have found no baseline differences in extracellular 5-HT between 5-HT1A KO animals and controls. Furthermore, lowering 5-HT synthesis with the tryptophan hydroxylase inhibitor PCPA was not sufficient to normalize the decreased immobility of 5-HT1A knockouts in the forced swim test (22), indicating that this phenotype may not simply result from the acute loss of 5-HT1A-AR and disinhibited 5-HT release. This finding, along with the fact that 5-HT1A-R antagonists induce some but not all of the anxiogenic responses seen in 5-HT1A KO mice (13; 23, and supplementary data therein) suggest that much of the 5-HT1A-R knockout behavioral phenotype may not simply reflect disinhibited serotonin release due to the loss of 5-HT1A-AR. Instead, much of the behavioral phenotype of 5-HT1A-R KO mice may reflect compensatory events distal to the actual loss of the 5-HT1A receptor protein.

Indeed, the loss of hippocampal 5-HT1A receptors disrupts the dendritic maturation of CA1 pyramidal neurons during development (6), and 5-HT1A-R KO mice have impaired hippocampal synaptic transmission and impaired hippocampal-dependent learning and memory (24). Furthermore, forebrain expression of transgenic 5-HT1A receptors in a 5-HT1A-R KO background during early-post natal development, but not in adulthood (23), prevented the appearance of an anxiety-like phenotype in adult animals. This genetic strategy resulted in significant receptor expression in regions of the hippocampus that normally do express the 5-HT1A-R, but it also led to receptor expression in forebrain regions that do not normally express this receptor but which have been implicated in the control of fear and anxiety-like behavior (25). Nonetheless, when taken together these findings suggest that compensatory events due to the loss of forebrain receptors during early post-natal development may contribute to the anxiety phenotype of 5-HT1A-R KO mice.

What might these compensatory events be? Recent work shows that 5-HT1A-R KO mice, at least on the Swiss-Webster background, have decreased expression of the $GABA_A$ receptor subunits $\alpha 1$ and $\alpha 2$ and do not display normal anxiolytic responses to benzodiazepine $GABA_A$ agonists (5). However, these findings were not seen in 5-HT1A-R KO mice on the C57Bl/6J background, indicating that these alterations in the GABA system observed on the Swiss-Webster background are due to a specific genetic modifier in the Swiss-Webster genome. These data fit nicely with human studies showing that SSRI administration increases cortical GABA release (26), also suggesting that serotonergic pathways enhance GABAergic signaling. Additional studies indicate that the hippocampi of 5-HT1A KO mice display large alterations in the expression of many different genes (27), suggesting that these widespread alterations may perturb hippocampal circuitry and could increase anxiety-related behavior in these mutant mice.

These findings in 5-HT1A-R KO mice are paralleled by human data showing lowered forebrain 5-HT1A-R binding in patients with panic disorder (28) and depression (29), suggesting that decreased forebrain 5-HT1A-R signaling may play a role in causing the anxiety and affective symptoms of these patients. The decreases in 5-HT1A-R forebrain binding in patients with panic disorder are also intriguing because these patients are frequently insensitive to benzodiazepines and have decreased $GABA_A$ binding (30; 31), similar to 5-HT1A-R KO mice on the Swiss-Webster background (5). This similarity suggests that the decreased 5-HT1A-R binding in these patients may lead to lower $GABA_A$ subunit expression and benzodiazepine resistance, changes that may increase anxiety symptoms.

Since some patients with anxiety disorders and depression display decreased forebrain 5-HT1A-R binding, it is possible that these patients may have altered CA3-CA1 hippocampal circuitry similar to that seen in 5-HT1A-R KO mice (6). Since partial 5-HT1A agonists like buspirone exert anxiolytic and antidepressant effects only after chronic administration (32), it is intriguing to speculate that these drugs ameliorate anxiety symptoms by chronically increasing hippocampal 5-HT1A-R signaling to 're-wire' and normalize hippocampal circuitry that might become dysregulated in the absence of normal 5-HT1A-R signaling.

Consistent with this hypothesis, a recent study suggests that the anxiolytic and antidepressant behavioral effects of SSRIs and drugs like buspirone in mice depend on 5-HT1A-dependent increases in hippocampal neurogenesis (7). Whether these drugs work in humans by a similar mechanism is unknown, but indirect evidence suggests they may: depressed patients show hippocampal volume loss and antidepressants may protect against this loss (33). In any case, how newly born hippocampal neurons may modulate the neural circuitry that underlies

anxiety and affect regulation is obscure, and much future work is likely to focus on this important question.

Meanwhile, another set of studies in mice and humans has focused on the role of 5-HT1A-AR. A line of mice that show a wide variety of depressive-like traits exhibit 5-HT1A-AR overexpression (34; 35), and conversely, a line of rats specifically bred for increased 5-HT1A responsivity also show depressive-like behavior (36). Similarly, 5-HT1A-AR overexpression and/or hyperactivity have been found in imaging studies in depressed patients (37) and in post-mortem studies of depressed suicide victims (38). A variant allele of *5-htr1a* that increases autoreceptor expression has also been found, and is tied to depression and suicide (39; although this result was not replicated by 40), anxiety disorders (41), neuroticism (42), and poor SSRI treatment response (43), and schizophrenia, substance abuse disorder and panic attacks (44). High 5-HT1A-AR expression is also associated with longer latency to treatment response in depressed patients (45), which suggests that desensitizing or downregulating 5-HT1A-AR's may be a rate-limiting step in SSRI treatment response. Given the well-described role of 5-HT1A-AR in restraining the activity of 5-HT neurons (14), these studies suggest that increased 5-HT1A-AR expression may excessively inhibit 5-HT neurons and lead to a hyposerotonergic state associated with depression (as proposed by the monoamine hypothesis), and perhaps with resistance to SSRI treatment response. To model this phenomenon, we have generated transgenic mice that overexpress 5-HT1A-AR, and we are currently analyzing affective behavior and serotonergic neurotransmission in these animals (M. Berger and L. Tecott, unpublished data).

Taken together, these studies in mouse and Man implicate decreased forebrain 5-HT1A-R expression and increased midbrain 5-HT1A-AR expression in anxiety and affective disorders, and they suggest that drugs specifically modulating the activity or expression of these distinct

receptor pools could help treat these disorders. Further studies with conditional 5-HT1A-R mouse mutants will further our understanding of the role this receptor plays in regulating the neural circuitry of affective behavior.

5-HT1B-R KO

Role of CNS 5-HT1B receptors in aggression and drug abuse

The 5-HT1B-R was the first 5-HT receptor to be knocked out, and mice lacking this receptor were initially found to have increased aggression (46; 47). Conversely 5-HT1B-R agonists (termed 'serenics') reduce aggression in wild type mice and this effect can be reversed by 5-HT1B-R antagonists (48; 49), suggesting that the aggression phenotype of 5-HT1B KO's may arise directly from the absence of 5-HT1B-R rather than from secondary compensations to its loss.

Other studies found increased pre-pulse inhibition and decreased startle responses in 5-HT1B-R KO's (50, 51). Later work showed that these mice display decreased anxiety-related behavior (52), although another study showed increased autonomic response to novelty in these mice (53). Meanwhile, viral overexpression of 5-HT1B autoreceptors in the raphe nucleus led to increased stress-induced anxiety behavior (54). This finding, together with decreased anxiety-like behavior in the 5-HT1B KO's, suggests that 5-HT1B-R-mediated suppression of serotonergic tone may increase anxiety responses, and that inhibiting this 5-HT1B-R activity may have an anxiolytic effect. This hypothesis is consistent with the anxiolytic activity of serenics (48).

Further work showed that 5-HT1B-R KO mice have enhanced spatial memory and are resistant to age-related declines in spatial learning and memory (55). These cognitive phenotypes

may be caused by increased activity at glutamatergic CA1-subiculum terminals lacking 5-HT1B receptors or by increased cholinergic transmission to the hippocampus due to the loss of inhibitory 5-HT1B receptors on medial septal cholinergic terminals (55, and references therein). 5-HT1B receptors also regulate serotonergic projections from the median raphe that innervate the forebrain: 5-HT1B KO mice display elevated hippocampal 5-HT release in response to SSRI's (19) and have a compensatory increase in 5-HT1A-R activity on these projections (56). 5-HT1B-R KO mice also display sexually dimorphic differences in serotonergic activity: 5-HT1B-R KO females, but not males, display lower tail suspension and forced swim test immobility (57), and this anti-depressant-like phenotype can be reversed by depleting serotonin with p-chloro-phenylalanine. 5-HT1B-R KO females also display an enhanced decrease in immobility time in response to fluoxetine when compared to males (57), further suggesting that the loss of the 5-HT1B-R alters serotonergic activity to a much larger extent in female than male mice. These findings are of considerable interest given the widely reported increased prevalence of affective disorders in women; might this increase relate to sexually dimorphic serotonergic activity (perhaps via similar 5-HT1B-related mechanisms)?

5-HT1B-R KO mice also displayed increased alcohol consumption and decreased alcohol sensitivity: they consumed more alcohol than control animals, displayed less alcohol-induced ataxia, and developed less tolerance to alcohol (58). However, later studies failed to replicate these findings and suggested that increased ethanol consumption in 5-HT1B-R KO's may be due to increased size of the KO's and a general increase in fluid consumption (59; 60). Alternatively, the increased ethanol consumption seen originally in 5-HT1B-R KO's may reflect genetic drift from wild type controls (61, and references therein); careful breeding schemes can minimize this possibility (61).

Although 5-HT1B-R's have been implicated in the serotonergic suppression of feeding, an obesity phenotype (increased adiposity) has not been observed in these KO's. One report did find that 5-HT1B-R KO mice weigh, eat and drink more than littermate controls (60), although this finding has not been consistently replicated. Another recent study suggested that 5-HT1B receptors mediate a tonic satiety signal in brain regions that control food intake (62). 5-HT1B-R KO's also display a reduced hypophagic response to d-fenfluramine, though this effect may arise from secondary adaptive changes in 5-HT2C receptor signaling or other pathways rather than the acute absence of the 5-HT1B-R (63).

5-HT1B-R KO mice are also resistant to the locomoter effects of 3,4-methylene dioxy methamphetamine (MDMA, also known as ecstasy), although they still exhibit MDMA-induced hypophagia and MDMA-induced suppression of exploratory behavior (64; 65). Other studies have demonstrated increased cocaine self-administration in these mice (66), and suggested that loss of the 5-HT1B-R sensitizes mice to the behavioral effects of cocaine (67). However, 5-HT1B-R antagonists do not increase cocaine self-administration in wild type mice (68, 65), which implies that this phenotype in 5-HT1B-R KO's is due to compensatory changes rather than the absence of the 5-HT1B-R itself. Indeed, several studies have demonstrated other alterations in the reward circuitry of 5-HT1B KO mice. These mice have enhanced dopamine release in the nucleus accumbens (NAcc; 65; 69), which may result from the loss of the 5-HT1B receptor on GABAergic terminals projecting to the substantia nigra and ventral tegmental area (70). 5-HT1B-R KO mice also have higher striatal expression of deltaFosB and AP-1 (67), two transcription factors whose expression is increased by cocaine. Taken together, these findings suggest that adaptations secondary to the loss of the 5-HT1B-R place animals in a behavioral and neurochemical state at baseline similar to that induced in wild type mice by cocaine (67).

Additional evidence also suggests that the 5-HT1B-R KO cocaine phenotype is not due to the actual loss of the 5-HT1B-R itself: overexpression of 5-HT1B-R on serotonergic efferents to the NAcc sensitizes animals to cocaine (71), a phenotype similar to that seen in KO mice lacking the 5-HT1B-R! The 5-HT1B-R KO cocaine phenotype may therefore result from other adaptive changes, just as the 5-HT1A-R KO anxiety-like phenotype may result from adaptive changes rather than the acute loss of the receptor protein. However, the discrepancy between the KO and overexpression phenotypes can also be explained in another way. Since the 5-HT1B-R is expressed so widely, both as an autoreceptor on serotonin neurons and as a heteroreceptor on cholinergic, GABAergic and glutametergic neurons, different 5-HT1B-R populations may play opposing roles in the regulation of cocaine intake and its behavioral effects. As such, the specific overexpression of 5-HT1B receptors on NAcc efferents could produce the same phenotype as the loss of the receptor on other neuronal populations in the full KO. Conditional genetic manipulations will be necessary to remove the 5-HT1B-R from distinct neurochemical subpopulations of neurons in specific anatomic regions to fully understand how specific receptor populations regulate the neural circuitry underlying this and other phenotypes seen in the full KO's.

Despite the observation of behavioral phenotypes in 5-HT1B-R KO's, there is little evidence that *5-htr1b* alterations contribute to behavioral dysregulation or mental illness in humans. Numerous studies have failed to show any association between the C129T or C861G *5-htr1b* polymorphisms and suicide or alcoholism, although a recent report did find an association between the 861G allele and major depression and substance abuse disorder (72). Neither of these mutations changes the amino acid sequence of the 5-HT1B-R, but both variants are in linkage disequilibrium with several other mutations in the 5-HT1B promoter that lead to

decreased receptor expression. Indeed, a ~20% decrease in 5-HT1B-R binding has been found in the brains of individuals with the 861G allele (73), suggesting that clinical disorders associated with the 861G allele may actually be due to these other closely linked promoter mutations. Future studies directly examining these promoter mutations in patients may lead to a clearer understanding of any possible relationship between 5-HT1B-R expression/activity and psychopathology.

5-HT2A-R

Role in hallucinogenic drug response and psychosis

The 5-HT2A-R is a primary molecular target of the atypical antipsychotic drugs (74). As expected, 5-HT2A-R KO mice have reduced cellular responses to hallucinogenic drugs with high affinity to the 5-HT2A-R like lysergic acid diethylamide (LSD; 75), and may display a decrease in anxiety-like behavior (76).

Another set of studies have examined 5-HT2A-R function by using antisense oligonucleotides (ASO) to block expression of this receptor. Intra-cerebro-ventricular (ICV) anti-5-HT2A-R ASO injection of mice caused a significant decrease in immobility in the forced swim test similar to that seen with antidepressant administration (77), leading the authors to suggest that 5-HT2A-R downregulation may be an important part of antidepressant response. Another report using the same technique in rats found that anti-5-HT2A-R ASO injection into different brain regions had opposing effects on ethanol (ETOH) intake (78) and anxiety measures; intra-PFC injection increased ETOH intake, while ASO injection into the central nucleus of the amygdala decreased ETOH intake. These studies suggest that different 5-HT2A-R populations may play opposing roles in modulating the circuitry underlying these behaviors. Future studies

utilizing careful ASO injections and both traditional and conditional 5-HT2A-R KO's are likely to yield new insights into the role of this important receptor in regulating anxiety, antidepressant responses, ETOH intake and various other behaviors.

Several studies have examined the potential role of human 5-HT2A-R variants in psychotic behavior and memory. There is a T102C silent polymorphism in the first exon of *5-ht2a*, which may lower 5-HT2A-R expression through its linkage disequilibrium with a –A1438G promoter polymorphism (79), although other reports have not supported this claim (80; 81). Nonetheless, the 102C allele has been associated with delusions and agitation/aggression (82; 83), visual and auditory hallucinations (84), and psychosis (85) in Alzheimer's disease patients, and with schizophrenia and other phenotypes as well (86 and references therein; 82 and references therein). These findings are surprising given that the 102C allele may lower 5-HT2A-R expression, since 5-HT2A-R agonists like LSD produce psychosis in humans while many antipsychotic drugs work by blocking the 5-HT2A-R (74).

A rare polymorphism (H452Y) in the C-terminal tail of the 5-HT2A-R has also been found that may alter agonist-induced receptor activity (87). Carriers of this rare 452Y allele were found to have decreased verbal memory performance in a delayed free recall test (88), and it will thus be of interest to examine what effect this polymorphism has on other cognitive and emotional processes.

5-HT2B-R

Role in Cardiac Development

5-HT2B-R KO mice have been generated, but ~50% of homozygous KO's die by the first week of life due to defects in cardiac development (89). Some mutant mice do survive to six

weeks and beyond, although they too have a variety of cardiac defects and display decreased fertility (89), while mice overexpressing cardiac 5-HT2B-R's exhibit cardiac abnormalities of the opposite type (90). These findings are of interest in particular due to the increased incidence of cardiac disease in patients with depression who also demonstrate alterations in central and peripheral serotonergic activity (91): might some of this increased cardiac disease incidence be due to altered 5-HT2B-R activity in the heart?

The 5-HT2B-R is also expressed on the cardiac valve fibroblasts, and the cardiac valvulopathy induced by drugs such as d-fenfluramine has been attributed to excessive activation of this population of 5-HT2B receptors (92; 93). A similar phenomenon has been observed in Parkinson's disease patients treated with the ergot derived dopamine agonists pergolide and cabergoline (94), in patients treated with the anti-migraine ergot alkaloid ergotamine (95), and in patients with carcinoid tumors that release 5-HT (96, and references therein). Pergolide, ergotamine and MDMA have mitogenic effects on human valvular interstitial cells via the 5-HT2B-R (97), providing a mechanistic explanation for these clinical findings and suggesting that chronic MDMA users may also be at risk for cardiac valvulopathy.

5-HT2B receptors are also expressed at low levels in the CNS (98) and 5-HT2B-R antagonists have effects on theta activity and sleep/wake states (99). However, to our knowledge, no reports to date have examined the behavior or neurochemistry of 5-HT2B KO mice. This may be due to the confounding effects of the cardiac deficits in these mice, in which case conditional genetic approaches to delete only neural 5-HT2B receptors may be necessary. To our knowledge, there are no known associations between 5-HT2B-R polymorphisms and any clinical disorder.

5-HT2C-R

Role in obesity and energy balance

Role in epilepsy

Role in regulation of Dopamine system activity and drug abuse

A major contribution of 5-HT2C receptors to the appetite suppressant actions of central serotonin systems was indicated by a feeding and obesity phenotype in 5-HT2C-R KO mice (100), which were the first line of knockout mouse model of obesity. Accordingly, these animals displayed reduced sensitivity to the anorectic effects of the nonselective serotonergic releasing agent dexfenfluramine (101), which fits well with recent work showing that 5-HT2C-R antagonists block the hypophagic and hyperlocomotor responses to MDMA (64). 5-HT2C-R KO mice exhibit a 25-30% increase in food intake, beginning in young adulthood. Interestingly, the obesity phenotype does not develop until the "middle-age" period of the mouse life span (beginning at 5-6 months of age; 102). Young adult mutants are able to compensate for their increased energy consumption and maintain normal body composition. However, compensatory processes appear to falter later in life, leading to body weight gain and enhanced adiposity. The exact nature of these compensatory processes that fail later in life is unclear, but they may include changes in the expression of beta 3 adrenergic receptors or uncoupling proteins in adipose tissue (103). Subsequent studies also revealed that progressive increases in the energy efficiency of physical activity may contribute to the late onset of obesity in these animals (104). In addition, a role for 5-HT2C receptors in the central regulation of glucose homeostasis was indicated by the enhanced susceptibility of the mutants to type 2 diabetes mellitus (102). These and other features of this obesity syndrome mimic common forms of human obesity. Partly on the basis of this work, a number of pharmaceutical companies are developing 5-HT2C receptor agonists as potential appetite-suppressants. Although 5-HT2C-R's are expressed in many CNS

sites implicated in energy balance, recent attention has focused on the arcuate nucleus of the hypothalamus, where 5-HT2C-R's activate neurons expressing melanocortins (105).

Initial studies of 5-HT2C receptor mutants also revealed evidence of enhanced neuronal network excitability. Videotape monitoring of mutant mice revealed infrequent and sporadic tonic-clonic seizures, and increased susceptibility to seizures induced by the $GABA_A$ receptor antagonist pentamethylenetetrazole (100). In addition, the mutants were found to be highly sensitivity to fatal audiogenic seizures— the first instance in which a gene underlying such a phenotype had been identified (106). Subsequent studies showed that 5-HT2C-R KO's have a globally enhanced sensitivity to a variety of convulsant stimuli, such as electroshock, electrical kindling of the olfactory bulb, and the chemoconvulsant flurothyl (107). These results implicate 5-HT2C receptors in the serotonergic inhibition of neuronal network excitability, and indicate a potential direction for anticonvulsant drug development.

Additional studies highlighted a role for 5-HT2C receptors in the actions of cocaine. Mutant mice displayed enhanced locomotor responses to cocaine and elevated cocaine self-administration in an operant progressive ratio paradigm (108). In vivo microdialysis studies revealed enhanced cocaine-induced elevations of extracellular dopamine levels in the nucleus accumbens. These findings were in accord with pharmacological studies indicating that 5-HT2C receptors mediate tonic inhibition of the mesoaccumbens dopamine projection. In accord with this, mutants also displayed enhanced novelty-induced locomotion, a behavior associated with mesolimbic dopamine system activation and the susceptibility to self-administer drugs of abuse.

Recent work has also suggested a role for the 5-HT2C-R in modulating responses to antidepressants. 5-HT2C-R KO mice display enhanced antidepressant-like behavioral responses to fluoxetine, as well as enhanced fluoxetine-induced elevations of extracellular serotonin levels

(109). No differences in either of these parameters were observed in the absence of fluoxetine. Very similar findings were independently observed in rats treated with SSRIs and 5-HT2C receptor antagonist compounds (109). Taken together these data suggest that 5-HT2C antagonists may augment the efficacy of SSRI's.

Several other behavioral abnormalities have also been described in 5-HT2C-R KO's. These mice display several types of repetitive behaviors that have been likened to compulsions (110). 5-HT2C-R KO mice also display altered stress responses (111), a deficit in maternal behavior (E.E. Storm, unpublished data), decreased spatial memory performance in the Morris Water maze and decreased long term potentiation (LTP) in medial perforant path-dentate gyrus synapses (112).

Indeed, several of the phenotypes seen in 5-HT2C KO mice are related to clinical disorders associated with polymorphic variants of *5-htr2c*. *5-htr2c* promoter variants have been associated with diabetes and obesity (113), while 5-HT2C-R agonists cause weight loss and decrease subjective feelings of hunger in people (114). Antipsychotic drugs like clozapine and olanzapine are 5-HT2C-R antagonists (115), which raises the possibility that the significant weight gain caused as a side effect of these drugs may be due to their action at this receptor. Indeed, the -759T variant allele of the 5-HT2C promoter was reported to protect schizophrenic patients from clozapine-induced weight gain (116; 117), although other studies have failed to replicate this association (118; 119; 120).

A structural polymorphism has also been found in *5-htr2c*, which results in a cys23serine change in the N terminus of the receptor. This polymorphism has been associated with major depression and bipolar disorder (121; 122), and with increased risk of bipolar disorder among women with a family history of mental illness (123; 124). This polymorphism has also been

associated with increased risk for depression and psychopathology among patients with Alzheimer's disease (125), a more severe clinical course in schizophrenic patients (126), and with weight loss and anorexia nervosa among teenage girls (127). Individuals carrying this allele show significantly higher CSF levels of the norepinephrine metabolite 3-methoxy-4-hydroxyphenyl-ethyleneglycol (MHPG; 128). Elevated CSF MHPG levels have also been found in alcoholic violent offenders, and although the *5-htr2c* 23ser allele was not found at an increased rate in this group, this allele has been associated with impulsivity in another study (129). This 23ser variant has also been shown to increase constitutive activity of the 5-HT2C-R while also making it less sensitive to 5-HT and other agonists (130).

The finding that the 23ser allele alters the constitutive activity of the 5-HT2C-R is of interest because the constitutive activity of this receptor is tightly regulated. Five adenosines in the 5-HT2C mRNA are converted to inosines by the enzyme adenosine deaminase, a process known as RNA editing. This mRNA editing process alters the amino acid coding sequence of the receptor protein and the basal activity of the receptor, and the editing pattern shows significant alterations in depressed suicide victims that result in decreased receptor activity in the prefrontal cortex (131; 132). Mouse studies show that this editing process is controlled by serotonergic tone (133), suggesting that the alterations in 5-HT2C-R editing in depressed suicide victims reflect defective editing regulation by serotonin (134).

Taken together, these data from mouse and Man suggest that the 5-HT2C-R plays an important role in the regulation of neural circuitry that underlie many behaviors, from drug abuse and satiety to energy balance and affective state. Future studies with conditional knockouts and viral rescue strategies are under development to explore the involvement of distinct 5-HT2C-R subpopulations in these processes, and to understand how their dysregulation perturbs behavior.

5-HT3A-R KO:

Receptor structure and activity

Role in regulating nociception,

Role in regulation of anxiety, alcohol intake

The 5-HT3A-R and 5-HT3B-R are unique among the known 5-HT-R's because they encode a serotonin gated ion channel rather than a G-protein-coupled-receptor. The 5-HT3A-R is sufficient to produce excitatory channel activity on its own as a homopentamer (135), although it also forms heteromeric complexes with and is modulated by the 5-HT3B-R (136). Nonetheless, since the 5-HT3B-R is incapable of reconstituting channel activity on its own, the 5-HT3A-R is probably a core component of all serotonin-gated channels.

5-HT3A-R KO mice were recently generated and found to have a variety of phenotypes related to the role of this receptor in both the peripheral and central nervous systems. These KO's have no change in acute pain responses to a variety of stimuli, but do display a significant reduction in tissue injury-induced persistent nociception even though they display normal post-injury edema (137). Furthermore, this phenotype was replicated in control mice treated with a 5-HT3-R antagonist by the intrathecal or intraplantar route, suggesting that the reduction in persistent nociception in the KO's is due to the acute loss of the receptor rather than secondary adaptations in its absence.

These findings may be explained by the observation that 5-HT3A-R are expressed mostly on myelinated Aδ afferents and a unique population of C fibers, few of which co-express the vanilloid/capsaicin receptor VR1 or the pro-edema factor Substance P (137). Consistent with this

expression pattern, deep dorsal horn neurons in the KO's fire at a lower rate than those of control animals during exposure to nociceptive stimuli

5-HT3A-R KO mice also display decreased nociceptive behavioral responses to the intrathecal admistration of 5-HT, although the injection of a selective 5-HT3-R agonist was insufficient to produce nociceptive behavioral effects itself (137). Taken together, these findings suggest that 5-HT3A-R activation is necessary but not sufficient for the pro-nociceptive behaviors elicited by 5-HT in the spinal cord, and suggest that chronic tissue injury pain responses are partly mediated by both central and peripheral 5-HT3-R populations.

5-HT injection into the spinal cord can also have anti-nociceptive or analgesic behavioral effects at high doses, although this effect did not differ between KO's and control animals (137). However, antisense oligos against the 5-HT3-R were reported to decrease the analgesic effects of intrathecal 5-HT administration (138). The discrepancy between these findings may reflect a non-specific effect of the antisense oligos, compensatory changes in the 5-HT3-R KO's, or other differences in experimental paradigms.

The 5-HT3A-R may also play a role in modulating anxiety-like behavior, a possibility previously suggested by pharmacologic studies and now supported by the finding of decreased anxiety-like behavior in 5-HT3A KO mice. Specifically, these mice spent more time in the illuminated zone of a light/dark box, and made more entries into the open arms of an elevated plus maze (139). The KO animals also investigated more closely a novel object placed in an open field enclosure (139). A second study also found that the KO's display a significant anxiolytic-like phenotype in the elevated plus maze, and found an anxiolytic-like trend in their behavior in the open field and light-dark tests (140). Furthermore, wild-type mice treated with a specific 5-HT3A-R antagonist also displayed decreased anxiety-like behavior in the elevated

plus maze (141), suggesting that the anxiolytic-like phenotype of the KO animals is due to the loss of 5-HT3A-R activity rather than secondary compensations in its chronic absence.

However, 5-HT3A-R KO mice also displayed enhanced freezing behavior in both the conditioning and later phases of a fear conditioning test (140). This finding suggests that 5-HT3A-R loss enhances fear memory formation, which may be due to the loss of 5-HT3A-R-driven GABA-ergic inhibition of the lateral amygdala (142; 143), and/or an upregulation of CRH mRNA in the central nucleus of the amygdala (140), two brain regions thought to play a role in the neural circuitry of fear behavior (25).

Despite this increase in fear-related behavior, the 5-HT3A-R KO mice actually display decreased ACTH release in response to restraint stress or LPS injection, although the KO's did not differ from controls in their CRH expression or sensitivity to CRH (140). This decrease in stress-induced ACTH release may thus be due to lower arginine vasopressin (AVP) mRNA expression in the paraventricular nucleus (PVN) of 5-HT3A-R KO mice (140), since AVP release in the PVN potentiates CRF-induced ACTH release (144).

5-HT3A-R KO females also displayed significantly increased immobility in the forced swim test, and males showed a similar trend (145). Increased immobility in this test is commonly thought to reflect a depressive-like state since antidepressant medications decrease immobility (146). However, another interpretation is that increased behavioral activity (measured as decreased immobility) in the forced swim and tail suspension tests actually reflects increased somatic anxiety (4); according to this view, the increased immobility seen in 5-HT3A-R KO mice may be simply another reflection of decreased anxiety-like behavior in these animals. The 5-HT3A-R KO mice also displayed sex differences in the defensive withdrawal test (145). The finding of sex differences in this test and the forced swim test suggest a sexually dimorphic role

for this receptor in the serotonergic modulation of affective behavior, an intriguing finding that merits further study.

A long literature suggests that 5-HT3A-R activation contributes to the reinforcing properties of alcohol, since 5-HT3A-R antagonists decrease alcohol consumption (147, and references therein; 148, and references therein). It was surprising to find, then, that 5-HT3A-R KO mice do not display any difference from controls in alcohol intake or preference, or in the locomotor response to alcohol (149). This study also replicated previous reports that 5-HT3A-R antagonists decrease alcohol consumption in wild type mice, but not in 5-HT3A-R KO animals, suggesting that this effect is due to a specific interaction of these drugs with the 5-HT3A-R (149). Furthermore, the fact that these drugs decrease alcohol intake by specifically blocking the 5-HT3A-R while the corresponding KO mice do not drink less alcohol than controls provides behavioral evidence for secondary compensations in the KO's that obfuscate the normal role of the 5-HT3A-R in alcohol consumption. Nonetheless, this study is consistent with the larger literature in suggesting that 5-HT3-R antagonists may be useful clinically in treating alcoholism and related disorders.

Aside from this role of presumably central receptor populations in modulating alcohol intake and affective behavior, peripheral 5-HT3A-R's may play a role in urinary system function. A line of mice was recently described that contain a val13ser point mutation in the M2 channel lining domain of the 5-HT3A-R, which makes the channel more sensitive to serotonin and increases its constitutive activity when combined with 5-HT3B-R subunits (150). These 5-HT3A 'hypermorph' mice died prematurely from an obstructive uropathy, and displayed significant urinary system dysfunction. Heterozygous male mice and homozygous female mice

with this mutation live for ~4-6 months, though, and future studies could also examine behavioral changes related to changes in central 5-HT3A-R function in these animals.

In addition to this line of 5-HT3-R 'hypermorph' mice, a line of mice has been created that overexpresses the 5-HT3A-R in forebrain regions. These mice display an interesting set of behavioral phenotypes such as altered ethanol responses (151). However, the relevance of these findings to the normal role of the endogenous 5-HT3A-R is limited by the extreme nature of this genetic manipulation: the overexpressing mice express the 5-HT3A-R in the forebrain at a level two orders of magnitude higher than normal (151). These animals display significant ectopic expression of the 5-HT3A-R in brain regions such as amygdala and in cellular populations such as pyramidal neurons that do not normally express this receptor (151; 152).

Nonetheless, phenotypic abnormalities observed in 5-HT3A-R KO mice, together with pharmacologic studies and clinical trials using the 5-HT3-R antagonist ondansetron (153), suggest that 5-HT3A-R antagonists could be used to treat chronic pain, anxiety disorders, and alcoholism in addition their established role as anti-emetics. Further support for a role for this receptor in regulating affective behavior comes from recent studies on a polymorphism (C178T) in the 5' untranslated region of the 5-HT3A-R mRNA, which may increase 5-HT3A-R expression by improving translational efficiency *in vitro* (154). This polymorphism was associated with decreased harm avoidance in women in a recent study (155), and replicated in a second group of women. This finding was also corroborated by the finding of higher social desirability and lower indirect aggression scores in the non-conformity section of the Karolinska Scales of Personality test (155). The 178T allele was more common among bipolar patients than controls in another study (154). Future studies will be necessary to replicate these intriguing findings and to study what effect this polymorphism may have on neural 5-HT3A-R expression

in vivo; these findings also raise the possibility that similar phenotypes may be seen in 5-HT3A-R KO mice.

5-HT4-R

Role in Novelty and Stress responses:

5-HT4-R KO mice were recently generated and characterized (156). These mice have decreased viability (a decreased percentage of homozygous null animals were born from a heterozygote cross than expected), which may be due to a lower seizure threshold in these mice. 5-HT4-R are present on GABA-ergic interneurons and increase the firing of these cells (157; 158), which raises the possibility that the seizure phenotype of the KO mice is due to a loss of 5-HT4-R-driven GABA release. The 5-HT4-R is also expressed on rhythm-generating respiratory neurons in the lower brain stem Pre-Boetzinger complex (159), raising the possibility that 5-HT4-R KO animals may have impaired ventilatory regulation.

5-HT4-R KO gained weight and ate normally, but they displayed a lack of restraint-induced anorexia and weight loss compared to controls (156). Despite this apparent difference in stress sensitivity, the KO's did not display a stress-induced change in corticosterone levels. The 5-HT4-R KO mice also displayed lower activity in an open field during a first exposure but no difference in thigmotaxis or other validated measures of anxiety. These KO's also displayed normal home cage activity. Taken together, these findings suggest that the 5-HT4-R may play a role in neural circuits that underlie stress responses and reactivity to novelty. However, it is unclear whether these phenotypes seen in the KO mice are due to the acute loss of the receptor or secondary compensations in its absence.

Few studies have examined any potential relationship between the human 5-HT4-R and psychiatric disorders, although *5-htr4* polymorphisms have been linked to both bipolar disorder and schizophrenia (160; 161). These findings are of particular interest because *5-htr4* maps to 5q32, a genomic region linked to bipolar disorder in a Costa Rican kindred study (162; 163) and in another ethnic sample as well (164). Future work will be necessary to corroborate these findings and clarify the potential role of 5-HT4-R polymorphisms in bipolar disorder, but these findings suggest it would be of interest to examine a wide variety of other affective behaviors in the 5-HT4-R KO mice.

5-HT5A-R

Regulation of novelty related behavior/exploratory activity

The 5-HT5A-R is among the least explored of the serotonin receptors (a medline search for 5-HT5A yields only 39 papers), yet 5-HT5A-R KO mice display increased exploration in the open field, elevated plus maze and novel object tests (165). This phenotype appears specific to increased exploratory activity, because the KO's displayed no significant alterations in anxiety-related measures in these tests. The KO's also display a blunted increase in locomotor activity induced by LSD, suggesting that some of the behavioral effects of LSD may be due to its interaction with the 5-HT5A-R. It is difficult to know whether these phenotypes reflect the absence of 5-HT5A-R signaling in the adult brain, or indirect compensatory responses in its absence; this question is difficult to answer in part because of the absence of specific 5-HT5A antagonists. Several studies have examined polymorphisms of the human *5-htr5a* gene in schizophrenia or affective disorders, but consistent findings have yet to emerge (166; 167; 168). Mice also express a 5-HT5B-R, but humans lack this receptor because its gene has been

interrupted by stop codons (169); to our knowledge this is the only serotonin receptor expressed in either mouse or Man without a functional homologue in the other species.

5-HT6

Receptor Structure and activity

Role in regulating alcohol intake

Pharmacological and antisense oligonucleotide studies support a role for the 5-HT6-R in fear conditioning, and cognition (170, and references therein), though similar phenotypes were not found in 5-HT6-R KO's (S. Bonasera and L. Tecott, unpublished data). This discrepancy may be due to species differences, compensatory processes in the KO mice, or limitations of the tools used in the pharmacologic and antisense studies. Although 5-HT6-R KO mice display normal behavior in a wide array of tests, they do display reduced sensitivity to the ataxic and sedative effects of alcohol (S. Bonasera and L. Tecott, unpublished data). Several studies have looked for an association between 5-HT6 polymorphisms and various neuropsychiatric disorders, but consistent findings have yet to emerge.

5-HT-7-R KO

Role in regulating thermal homeostasis

Role in regulating depressive/anxiety-like behavior

5-HT7-R KO mice have recently been generated and shown to have decreased contextual fear conditioning, which may be due to decreased LTP in the CA1 region of their hippocampus (171). Studies with these mice also suggest that the 5-HT7-R may regulate body temperature changes in response to serotonergic drugs (172). These animals also display decreased

immobility in both the forced swim and tail suspension test, and they spend less time in REM sleep (173). Taken together these results suggest that 5-HT7-R antagonists warrant consideration as potential antidepressant agents (174). Several studies have looked for an association between *5-htr7* polymorphisms and various neuropsychiatric disorders, but consistent findings have yet to emerge.

Pet1

Role in CNS 5-HT neuron development, and affective behavior

Role in development of ENS, enterochromaffin cells.

Pet-1 is an ETS-domain transcription factor that was first discovered in adrenal chromaffin-derivd PC12 cells, where it was found to induce transcription of the nicotinic acetylcholine β4 receptor (175). Pet-1 is expressed in the adrenal medulla as well as the eye and the brain (175), and within the brain it is expressed exclusively in serotonin neurons (176; 177). Pet-1 expression appears within these neurons approximately one half day before they express 5-HT itself, and functional Pet-1 binding sites are found in the promoter regions of the human and mouse 5-HT1A receptor, the serotonin transporter, tryptophan hydroxylase, and aromatic L-amino acid decarboxylase (176), suggesting that Pet-1 might coordinately regulate many of the genes necessary for the final differentiation of serotonin neurons.

To evaluate this possibility, Hendricks and colleagues then generated Pet-1 KO mice. These mice were subsequently found to lack ~70% of the normal number of serotonin neurons, and the remaining 30% of serotonin neurons displayed defective expression of tryptophan hydroxylase and the serotonin transporter (178). Furthermore, the Pet-1 KO mice display an 85-90% decrease in both 5-HT and the serotonin metabolite 5-hydroxyindole-acetic acid (5-HIAA)

in their cortex, hippocampus and caudate nucleus, though these animals did not display any noticeable differences in overall cortical or cytoarchitectural structure. Other studies have given further support for this essential role for Pet-1 in the final differentiation of serotonin neurons, and suggest that Pet-1 acts in a molecular cascade containing several other transcription factors including Nkx2.2, Lmx1b, and Ascl1/Mash1 (179; 180; 181; 182).

Consistent with the neurochemical findings in Pet-1 KO mice and the widely reported inverse correlation between low serotonin levels and affective disorders, these mice also displayed significant anxiety-like behavior in the open field and elevated plus maze. These animals also displayed increased aggressive behavior (when compared to wild type controls) in the resident-intruder test (178), including an increased number of overall attacks on the intruder mouse. Furthermore, the Pet-1 KO mice frequently attacked the intruder within the first ten seconds of encountering it, a striking phenotype that was never observed with wild type control animals. Given the strong association between low serotonergic activity and depression as well as alcoholism, future studies are likely to evaluate these and other related behaviors in Pet-1 KO mice.

The human Pet-1 gene (also known as FEV) is also expressed exclusively within serotonin neurons in the CNS (183), as well as in megakaryocytes (184). To date, no association studies have examined whether mutations in the Pet-1/FEV gene are associated with neuropsychiatric disorders in humans; however, a linkage study in patients suffering from a severe, recurrent and early-onset form of major depression did show a significant peak at 2q36 (185), the genomic location of Pet-1/FEV. Future studies are thus likely to focus on the tantalizing possibility that this linkage peak is the result of mutations or polymorphisms in Pet-1/FEV. Indeed, the phenotype of Pet-1 KO mice suggests that deficient Pet-1 activity could

profoundly hinder the differentiation of serotonin neurons and result in a hyposerotonergic state that could lead to affective disorders, as proposed by the monoamine hypothesis.

SERT

Role in regulation of serotonergic tone

Role in affect regulation

Serotonin transporter (SERT) KO mice were initially generated by two groups (186; 187), and were found to display equivocal phenotypes in the tail suspension and forced swim tests (188), but they display a generally consistent phenotype of reduced novelty-induced exploratory activity and increased anxiety-like behavior in a variety of tests (189). SERT KO mice also display a decrease in 5-HT neuron firing rates (186; 190), and a 50% reduction in the number of 5-HT neurons (186). Despite this decrease in the firing rate and total number of 5-HT neurons, SERT KO mice have significantly higher baseline extracellular 5-HT levels as measured by microdialysis (191). Taken together, these data suggest a model in which the absence of SERT impairs either the embryonic differentiation or adult viability of 5-HT neurons and lowers the firing rate of the remaining 5-HT neurons; however, 5-HT re-uptake in these mice is so profoundly deficient that they still display almost 10-fold more extracellular serotonin than wild type controls.

One potential explanation for the decreased firing rate of 5-HT neurons in the SERT KO mice is that the additional extracellular serotonin present in these animals excessively activates 5-HT1A-AR, which then hyperpolarize the 5-HT neurons and decrease their firing rate. Consistent with a chronic enhancement of this process, SERT KO mice display alterations in 5-

HT1A-AR expression (192) and behavioral changes consistent with these 5-HT1A-AR alterations (189). In fact, overexpression of 5-HT1A receptors in specific brain regions of SERT KO mice can rescue some parts of the abnormal stress/anxiety phenotpe of these animals (193), further suggesting that this aspect of the SERT KO phenotype may actually result from altered 5-HT1A receptor expression/activity.

An alternative, though not necessarily mutually exclusive, hypothesis is that the SERT KO anxiety-like behavioral phenotype arises from developmental alterations in these mice. In line with this hypothesis, inhibiting SERT with SSRI's during a transient early post-natal period causes a variety of affective changes later in the resultant adult animals that are generally similar to those seen in SERT KO mice (194; 195). Furthermore, the effects of neonatal SSRI exposure are due specifically to SERT blockade because they are absent in mice genetically deficient in SERT (196).

Why does SERT blockade during development disrupt affective behavior later in life? One possibility is that this manipulation increases post-synaptic serotonin receptor signaling in the forebrain, which then exerts maladaptive compensatory changes that increase anxiety-like behavior later in life. Another possibility is that neonatal SSRI exposure increases serotonin concentrations in the raphe nucleus, which then excessively activates 5-HT1A-AR's. Excess 5-HT1A-AR activity during development could then impede the growth and differentiation of serotonin neurons, since 5-HT1A-AR may exert a negative control over 5-HT neuron development (197). Consistent with this hypothesis, rats treated neonatally with SSRI's show a persistent reduction in tryptophan hydroxylase expression as adults (198).

In either case, the anxiety-like phenotype of SERT KO mice fits well with data tying human SERT polymorphisms to affective disorders. The human SERT promoter contains a 44

base pair variable repeat sequence that commonly exists in either a short (s) or long (l) form, and the s allele is generally associated with lower SERT expression and activity. Furthermore, the s allele has been tied to increased amygdala reactivity to fearful faces (199), anxiety symptoms (200), and to an increased probability of depressive episodes after significant life stressors (201). These findings are similar to those seen in the SERT KO mice; in both cases, lower SERT activity is associated with negative affective behavior.

In the case of both the SERT KO mice and individuals carrying the s allele, the challenge is to understand how blocking SERT with antidepressant drugs generally improves affective symptoms while people or animals with lower SERT expression/activity are at risk for affective disorders. Since individuals with the s allele are likely to have lower SERT expression/activity throughout development and adulthood whereas SSRI's are usually prescribed only in adolescents or adults (who would have had higher SERT activity prior to taking the SSRI), it is possible that blocking SERT during development has opposite effects to blocking it in adulthood. This hypothesis fits well with the literature on the effects of neonatal antidepressant use in rodents, and future work is likely to focus on clarifying the molecular and cellular mechanisms by which this manipulation perturbs adult behavior and brain function. This work is likely to have an important clinical implication for pregnant women taking SSRI's, since many of them cross the placental barrier and may interact with the developing brain of the fetus.

TPH1 and TPH2

Enzyme structure and properties

Roles in CNS 5-HT synthesis

Roles in 5-HT synthesis outside the CNS

Until two years ago, it was thought that there was only a single tryptophan hydroxylase (TPH) gene. However, when this TPH gene was knocked-out in mice, the investigators were surprised to find normal 5-HT levels in the brain (202). This observation led to the cloning of a second TPH gene (TPH2), which is expressed predominantly in the brain (while the original TPH1 gene is expressed in the gut, pineal gland, spleen and thymus). A Pro447Arg mutation in TPH2 was then serendipitously found in two commonly used inbred mouse lines (BALB/cJ and DBA/2), while two other commonly used inbred lines (C57Bl/6J and 129/SvJ) have the normal proline residue at this position (203). This study also demonstrated that the 447Arg mutant enzyme is associated with a ~55% decrease in 5-HT synthesis rate, and the inbred lines carrying this mutant allele display a 40-45% decrease in tissue 5-HT content in several brain regions. These data suggest an interesting explanation for the fact that BALB/cJ and DBA/2 mice are sensitive to the effects of fluoxetine in the forced swim test, but C57Bl/6J and 129/SvJ mice are not (204): perhaps only animals with lower 5-HT neurotransmission will respond behaviorally to SSRI's.

The TPH2 Pro447Arg mutation has not been found in humans, but a nearby Arg441His mutation has been found that causes an ~80% reduction in 5-HT synthesis in *in vitro* assays (205). This 441His mutation was found in patients with unipolar major depression at ten times the rate at which it was seen in healthy controls (and it was not found at all in patients with bipolar disorder), suggesting that this functional polymorphism is a significant risk factor for unipolar major depression. Future studies will be necessary to confirm this finding and clarify the inheritance and penetrance pattern of this allele in affective disorders, but a variety of other studies already fit well with this finding. Several studies have found an association between the TPH2 gene and depression (206), and between TPH2 and suicidality and/or bipolar disorder

(207; 208; 209). Furthermore, another study showed significant linkage to a region of chromosome 12 just proximal to the TPH2 locus (at 12q21) among a large number of individuals suffering from depression (210).

Taken together, these findings suggest that TPH2 mutations can lead to affective disorders in humans, and a mouse model similar to these mutations already exists in the inbred mouse strains carrying the 447Arg allele. Further study of these mouse strains may help us understand how decreased serotonin synthesis leads to affective behavioral changes. These mouse strains can also serve as a model to help us evaluate new treatments and early interventions designed to ameliorate or even prevent the negative affective changes that result from chronically impaired 5-HT transmission.

Summary

The past ten years have been an intensely productive period in 5-HT receptor knockout studies- of the fifteen murine 5-HT-R's that have been cloned to date, ten have been 'knocked-out' and behavioral characteristics of these mice have been reported, while several other genes that regulate the development and activity of serotonin neurons (like Pet-1, SERT, and TPH) have been studied in mutant mice and human subjects. These studies are all the more remarkable when placed in historical context: serotonin was not identified as a neurotransmitter until just over 50 years ago (211), at which time it was not clear that receptors even existed as true physical entities. Pharmacologic and biochemical studies provided evidence for the existence of receptors over the following decades, and these studies were finally molecularly substantiated for the serotonin receptors as they were cloned over the last 15 years. Thus the field has undergone a

metamorphosis, from an initial finding of receptor-like activity in various tissue preparations, down to the focused molecular cloning of these receptors, and back up to the analysis of their roles in complex behavior with pharmacology studies and 5-HT-R KO mice.

These studies are illuminating for both what they have and what they haven't found: none of the KO's are lethal due to defects in CNS development or function, suggesting that while 5-HT receptors modulate the neural circuitry of diverse behaviors, they are not absolutely necessary for brain functions required to support life. These mutants have provided models to understand the neural circuitry of some complex behaviors with relevance to human diseases such as epilepsy and obesity, anxiety and affective disorders, substance abuse, and nociceptive processing. Careful study has provided evidence of alterations in synaptic connectivity (6) and the expression of other genes in some of these KO mice (5), and similar studies are likely to further enrich our understanding of these additional roles that 5-HT receptors play beyond the acute regulation of neuronal activity. Since KO mice lack the knocked-out receptor throughout development and adult life (a phenomena difficult to replicate with pharmacology), it provides a model to investigate other secondary processes that depend on the activity/expression of the missing receptor. Similarly, KO studies have greatly clarified our understanding of how relatively broad spectrum serotonergic drugs like SSRI's, MDMA, LSD and d-fenfluramine exert particular behavioral effects through their action at specific serotonin receptors.

Despite the benefits of KO studies, none of the 5-HT-R KO's precisely resembles a single human neuropsychiatric disease, just as human genetics studies suggest that no single human neuropsychiatric disease is perfectly correlated with mutations in a single 5-HT receptor gene. Instead, these studies suggest that 5-HT-R's modulate the neural circuits that underlie complex behavior, and that pharmacologic modulation of these receptors may be efficacious in

specific neuropsychiatric disorders even if these disorders are not 'caused' by a primary dysfunction in a 5-HT-R. Nonetheless, emerging data suggests that SERT, 5-HT1A-R and TPH2 mutations may influence susceptibility to affective disorders. Given the severe 5-HT deficit in Pet-1 KO mice, it will not be surprising if Pet-1 mutations are also associated with affective dysregulation. Taken together, these findings suggest the potential of a new horizon in clinical psychiatry, in which the careful diagnosis and specific treatment of mental health disorders may be based on genetic testing and/or neuro-imaging in combination with a clinical interview, and in which future treatments may be targeted to correct underlying genetic/neuro-anatomic lesions.

Our understanding of the roles 5-HT-R's play in complex behavior is likely to expand dramatically in future years with the increasing availability of conditional genetic mutants that rely on cell type- and tissue-specific promoters, microarrays and other tools to analyze compensatory changes in genetically modified mice at a genome and proteome wide level, and new behavioral testing equipment designed to allow high-resolution quantitative analysis of mouse behavior (212). Considering how far the study of these receptors has progressed in the last fifty or even just ten years, the next ten years and the decades beyond are likely to be a fascinating journey full of unexpected surprises, a story of small mice with big implications for Man.

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5-HT Receptors and the Neural circuitry of behavior*

Receptor knocked out:	**Behavioral phenotype:**	**Proposed neural circuit underlying phenotype:**
5-HT1A receptor	Increased anxiety-like behavior	-Loss of hippocampal 5-HT1A receptors in development[23, 6]? -Loss of 5-HT1A autoreceptors and disinhibited 5-HT release[17, 19]?
5-HT1B receptor	Decreased immobility in the tail suspension test, only in females	-Loss of 5-HT1B terminal autoreceptors and disinhibited 5-HT release[57]?
5-HT2C receptor	Increased food intake	-Altered hypothalamic feeding circuitry[105]? -Disinhibition of central dopamine pathways[150]?
5-HT3A receptor	Increased fear conditioning	-Loss of GABArgic inhibition of lateral amygdala[142, 143]?

*These examples are representative, not comprehensive. See chapter text for further discussion.

Chapter V- Dynamic modulation of affective behavior in Pet1 KO mice: potential roles for altered 5-HT2C receptor activity and dopaminergic transmission

Introduction and Results

Serotonin is known to regulate a wide array of behavioral processes in part through the 15 some serotonin receptors that I have discussed in chapter II, and low serotonergic transmission has been implicated in the pathophysiology of mood disorders (Bourne HR et al, 1967; Owens MJ, 2003). While each of the serotonin receptors modulates different brain circuits in a unique fashion, the loss of any single receptor does not phenocopy any specific clinical disorder (Berger M et al, 2006). Therefore, the finding of globally decreased serotonin transmission in clinical disorders implies that decreased ligand concentration (i.e. serotonin) and/or release must affect post-synaptic cell and brain circuit responses in a complex global fashion, rather than simply by reducing all signaling at any one specific serotonin receptor subtype.

Thus, the question of how deficient serotonergic activity/release perturbs brain circuits and animal (or human) behavior may be an irreducible phenomenon. Such a deficiency likely impacts brain function through simultaneous alterations in the activity of multiple receptors, which produce interactive effects through interlaced neural circuitry, which may be impossible to model simply by deleting one receptor at a time via knockout mice. Viewed this way, the best way to study how deficient serotonergic activity alters brain circuit dynamics and behavior may not be through single receptor KO mice, but rather by studying the circuit dynamics and whole animal behavior in animals with a generalized serotonergic deficiency.

To this end, I have examined the brain circuitry and affective behavior in a line of mice lacking pet1, a transcription factor that is essential for the formation and function of serotonergic neurons (Hendricks et al, 2003). Although these mice were originally

created to study the transcriptional cascade of serotonergic development, they provide a useful model of an extreme serotonergic deficiency similar to that seen in extreme psychiatric cases (Bourne HR et al, 1967); these mice were originally reported to have a 70% decrease in the total number of serotonin neurons, and an 80% decrease in brain serotonin tissue content levels. Thus, while these animals do have some brain serotonin, they clearly do have altered serotonergic release and/or transmission, and thus provide an excellent model for studying how global alterations in serotonergic transmission affect brain circuits and animal behavior.

We began our studies of these mice by reasoning that the deficient serotonergic transmission in these animals could affect each receptor subtype differently, especially depending on whether the expression of each specific receptor increased or decreased as a response to altered ligand availability. Thus, we used qPCR to measure the expression level of each serotonin receptor in the brainstem (Fig 1A), striatum (Fig 1B) and hypothalamus (Fig 1C) of the pet1 KO mice versus wild type controls. We observed slight changes in several of the receptors in each brain region, including a ~2 fold increase in 5-HT2C mRNA expression in the hypothalamus of pet1 KO mice, although we saw no change in 5-HT2C mRNA expression in the brainstem or striatum. However, given that the 5-HT2C receptor can upregulate by orders of magnitude at the protein level without any chance at the RNA level (A. Abbas and B. Roth, pers. communication), we then further examined 5-HT2C receptor expression at the protein level by radioactive ligand binding in the hippocampus and striatum (Fig 2A, 2B). Interestingly enough, we saw a ~5 fold increase in hippocampal and striatal 5-HT2C receptor binding in the pet1 KO mice, but no change in 5-HT2A binding in these regions (data not shown). Recent work has shown that 5-HT2C receptors in the ventral tegmental area (VTA) play a key role in restraining dopamine system activity and controlling related behaviors (Navailles S et al, 2006; Fletcher PJ et al, 2004), thus we examined 5-HT2C mRNA expression in

the VTA at cellular resolution by quantitative in situ hybridization. However, we saw no change in VTA 5-HT2C mRNA expression (Fig 1D) despite the increase in 5-HT2C protein expression seen in figure 2A and 2B, also potentially consistent with a disassociation between RNA and protein expression for the 5-HT2C receptor.

5-HT2C receptor activity can also be regulated by the enzyme adenosine deaminase, which converts a set of specific adenosine residues in the 5-HT2C mRNA into inosines, which are then read by the ribosome at cytosines (Schmauss C, 2005). This process, known as RNA editing, actually changes the 5-ht2C receptor coding sequence and thereby changes the relative amount of constitutive versus ligand induced 5-ht2C receptor activity. Recent studies have shown that altered serotonergic transmission can alter this editing process (Englander MT. et al, 2003), and that low serotonergic activity leads to increased 5-HT2C receptor activity and/or expression (Sharma et al, 1997; Heslop KE et al, 1999). Thus, we are currently examining 5-HT2C RNA editing in the striatum of wild type and pet1 KO mice, based on the hypothesis that there should be less 5-HT2C RNA editing in the KO mice, and a shift towards expression of the highly constitutively active 5-HT2C isoforms like the INI variant. Our preliminary data show a trend towards decreased A, B and C site editing in the brains of pet1 KO animals (Fig 2C), which would be consistent with the hypothesized shift towards higher expression of the less edited and thus more constitutively active isoforms of the 5-HT2C receptor.

These increases in 5-HT2C receptor expression (and potentially in 5-HT2C receptor activity) are especially interesting in light of prior data showing that 5-HT2C receptors constrain dopamine system activity (Navailles et al, 2006). Thus, we examined dopamine tissue content levels in the brainstem of pet1 KO mice, and found a roughly 40% decrease in brain stem dopamine content in these mice that remained stable from two months of age onwards (Fig 3A,B). However, no such deficiency was seen before

this time point (fig 3A). In performing these studies, we also found a consistent drop in tissue 5-HT and 5-HIAA levels (Fig 3C, D), thus corroborating the previously reported deficiency (Hendricks et al, 2003).

We next asked whether the decrease in gross tissue dopamine content also extends to a deficiency in extracellular dopamine levels in the pet1 mice. To this end, we measured extracellular monoamine levels in the dorsal striatum of pet1 KO versus wild type mice. Interestingly, we saw no drop in extracellular DA levels in the pet1 mice at two months of age (Fig 4A, but we did observe a 4-fold drop in extracellular DA in the pet1 animals at 4 months of age (Fig 4B).

In conjunction with these biochemical and neurochemical studies, we also examined the behavioral phenotype of the pet1 mice in several tests of affective behavior. In both the open field and zero maze assays, the pet1 KO mice consistently displayed decreased novelty-induced locomotion but no change in anxiety-related parameters (such as thigmotaxis, or an increase in time spent in the periphery of the open field, Fig 5A-D). Pet1 KO males and females also showed decreased novelty induced locomotion in the photobeam activity system (Fig 6A,C), yet they displayed increased locomotion during the dark cycle (Fig 6B,D), suggesting that they have a specific decrease in novelty induced locomotion rather than a generalized motor deficit.

We were surprised, however, by the absence of a direct anxiety phenotype in the pet1 KO animals, both because this phenotype was previously reported in pet1 animals (Hendricks et al, 2003) and because low serotonergic activity has been previously correlated with anxiety-related behaviors (discussed in Berger M et al, 2006). Thus, we examined whether the pet1 KO mice would have a serotonergic deficit-induced behavioral change in the tail suspension test, a test with predictive validity for monoamine antidepressant activity.

We saw no baseline difference in tail suspension immobility between pet1 KO, het or wild type animals (Fig 6E), a surprising finding given the pronounced serotonergic abnormalities we and others have found (Hendricks et al, 2003; Fig 3C-D) in these mice. Although the pet1 KO animals have a 70% decrease in the number of serotonergic neurons and a ~6 fold decrease in tissue 5-HT content (Fig 3C-D; Hendricks et al, 2003), we also found a ~20 fold reduction in serotonin transporter expression in these mice (Fig 1A). Thus, we reasoned that the pet1 KO animals may actually have a greater deficit in serotonin reuptake than in serotonin release, which could lead to roughly normal (or at least not grossly deficient) extracellular serotonin levels and could lead to normal behavioral phenotypes in some assays sensitive to serotonergic transmission (like the tail suspension test). Consistent with this hypothesis and the ~20 fold decrease in serotonin transporter expression in these animals, we also found no significant effect of the serotonin reuptake inhibitor citalopram in pet1 KO animals in the tail suspension test, though it did have a significant effect in wild type animals (Fig 6F). This finding fits nicely with data showing that polymorphisms in the human homolog of pet1, *fev*, are associated with antidepressant responsitivity in humans (J. Kraft and S. Hamilton, pers. communication).

Aside from this issue, the aforementioned finding of decreased novelty induced locomotion in the pet1 animals fits nicely with the known and well described role of dopamine release in regulating novelty responses in both experimental animals and humans (Knutson et al, 2006) and the dopamine system defects we observed in the pet1 mice (Figs 3A-B, 4A-B). Taken together, these findings suggest that the decreased novelty responses in the pet1 KO's may actually be due to this deficient dopamine system activity rather than deficient serotonergic activity. Since mesolimbic dopamine also plays an important role in regulating a variety of other behaviors, ranging from anxiety and fear responses to reward related behaviors (Schultz W, 2007), we examined

these behaviors in pet1 KO animals. In particular, we used fear sensitized startle paradigm to measure fear responses and the conditioned place preference paradigm to measure reward-related behavior. In the fear sensitized startle paradigm, we saw equal baseline startle in the pet1 KO mice versus controls, but deficient sound-based potentiation of the startle response in these mice at both 90 and 105 decibels (fig 7A). This phenotype is also consistent with lower dopamine system activity, given the prior described role of dopamine release in the amygdala in mediating sound induced startle response potentiation (Meloni EG et al, 2005). To rule out the possibility that this phenotype might be due to differential nociception between pet1 KO versus wild type animals, we also performed the hot plate test in these animals (fig 7B). We saw equal responses between genotypes, suggesting that pet1 KO animals do not have a deficit in peripheral nociception. This finding further suggests that the startle phenotype in the pet1 KO mice is due to a central defect in potentiation rather than a difference in peripheral nociception.

Given the well-established role of dopamine in mediating the rewarding effects of cocaine (Haile CN et al, 2007), we also asked whether the rewarding effects of cocaine would differ between the dopamine deficient pet1 KO animals and controls using the conditioned place preference test (CPP). Surprisingly, we found that pet1 KO mice show a larger preference for cocaine at 5 mg/kg than wild type controls (Fig 8A-C), but the same preference as wild type littermate for 10 mg/kg of cocaine (Fig 8D-E). Interestingly, pet1 KO animals showed less novelty-induced exploratory behavior than wild types when they were first introduced to the CPP chambers (Fig 8C), but the locomotor deficit in these animals disappeared after they were treated with cocaine (Fig 8C). This finding suggests that the pet1 KO mice may have increased sensitivity to the rewarding effects of cocaine, and/or that cocaine could be acting to remedy the underling dopaminergic deficits in these mice that may underlie their deficient novelty-induced locomotion

phenotype. We are currently determining whether the pet1 mice are intrinsically more sensitive to the behavioral effects of cocaine, by examining locomotor responses to cocaine in the pet1 KO mice versus wild types (E. Mangir and M Berger, studies in progress).

Taken together, these studies show that pet1 KO mice have a significant upregulation of the 5-HT2C receptor expression and activity, which could be the cause of the dopaminergic deficiencies and behavioral abnormalities seen in these animals. To examine whether this relationship is in fact causal, we are generating 5-htr2c$^{-/y}$, pet1 $^{-/-}$ double knockout animals and we will examine both the neurochemical and behavioral phenotype of these mice. We hypothesize that these mice (when compared to pet1-/- single knockouts) should have normal dopamine system function and normal reward and novelty-related behaviors, which would suggest that the dopaminergic deficiencies and behavioral abnormalities since in these KO mice are both due to increased 5-HT2C receptor activity.

Discussion

There are several novel implications of these findings. First, the finding of a significant dissociation between 5-HT2C mRNA and protein expression levels suggests a high degree of post-transcriptional control over 5-HT2C receptor expression. This is an interesting finding that raises questions about the use of gene expression methods that measure only RNA levels, and about the potential cellular and molecular mechanisms of this post-transcriptional control over 5-HT2C receptor expression. Second, these findings provide a potential link between the monoamine or low-serotonin hypothesis of mood disorders and the clinical finding that many of the symptoms of mood disorders reflect reward-related deficiencies that could be better explained by acute deficits in phasic dopamine signaling (Schultz, 2007) than by chronically diminished serotonergic

transmission per se. For example, phasic dopamine release in the striatum is thought to encode a reward prediction error function (Schultz W, 2007), and many depressive symptoms such as anhedonia reflect a deficit in this reward circuitry (Epstein J et al, 2006).

Thus, a central question in the basic science of affective disorders is how can deficient serotonergic activity lead to reward-related deficits, if serotonin itself does not encode reward? Our results suggest that chronically reduced serotonergic release can lead to a massive upregulation of 5-HT2C receptors, which can then perturb dopamine system function and reward-related behaviors, perhaps through the high basal activity of these receptors. These results also imply that blocking 5-HT2C receptor activity and/or expression could be useful in restoring reward-related abnormalities in patients with affective disorders and low serotonergic function.

Finally, these results also provide a generalizable model in general for how altered activity of a neurotransmitter system in a neuropsychiatric disorder can be studied, via a comprehensive analysis of receptor expression levels, neurochemical measurements, and behavioral phenotyping in a proper animal model. In principle, each neurotransmitter system can be viewed as a complex network consisting of a central node (the neurons that release the transmitter) and numerous interconnected secondary nodes (the post-synaptic cells expressing the various receptors for the given neurotransmitter). The studies we have performed here show how a complex network of this form can be dissected at a multi-nodal level, and how emergent behavioral phenotypes can be traced to specific altered nodes in the network. Similar approaches could be applied to further our understanding of similar neurochemical imbalance hypotheses in other psychiatric illnesses, such as the hyperdopaminergic hypothesis of schizophrenia (Miesenzahl EM et al, 2007), or the idea that acute increases in serotonin may underlie some cases of mania (Silverstone PH et al, 2004).

Fig 1A. qPCR of all 5-HT receptors in pet1 KO vs wt brainstem.

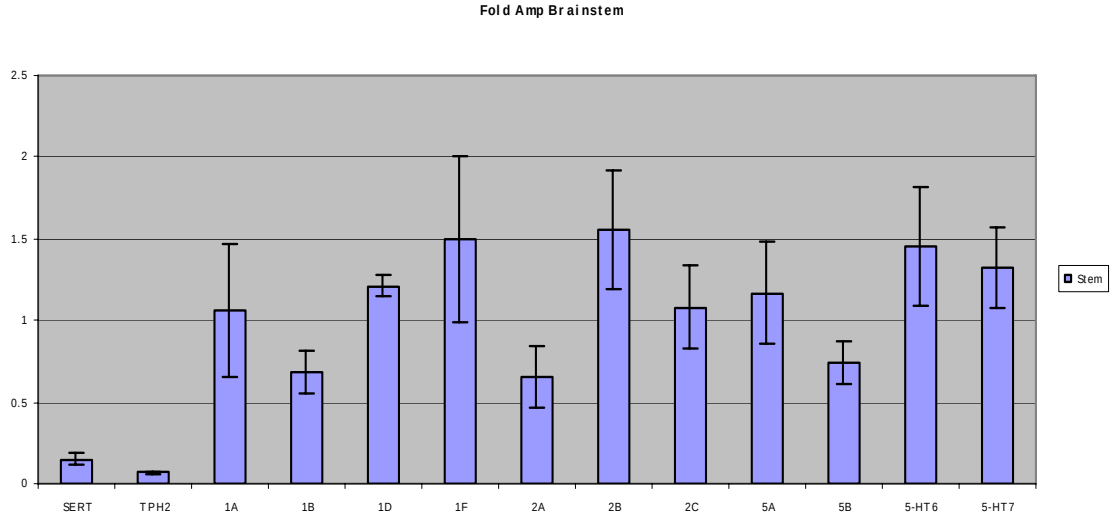


Fig 1B. qPCR of all 5-HT receptors in pet1 KO vs wt striatum.

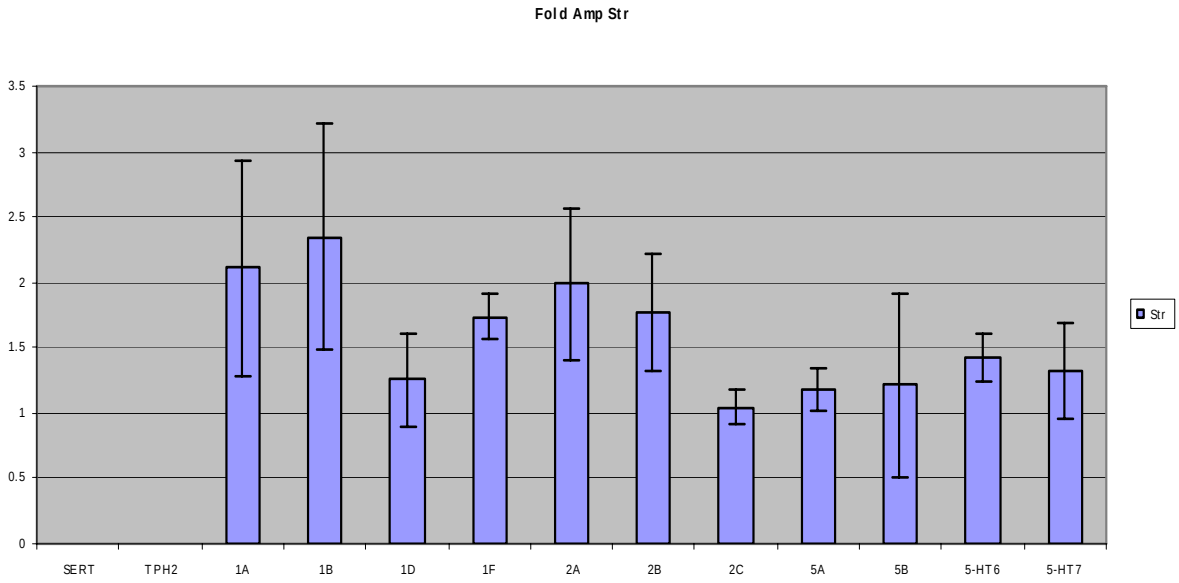


Fig 1C. qPCR of all 5-HT receptors in pet1 KO vs wt hypothalamus.

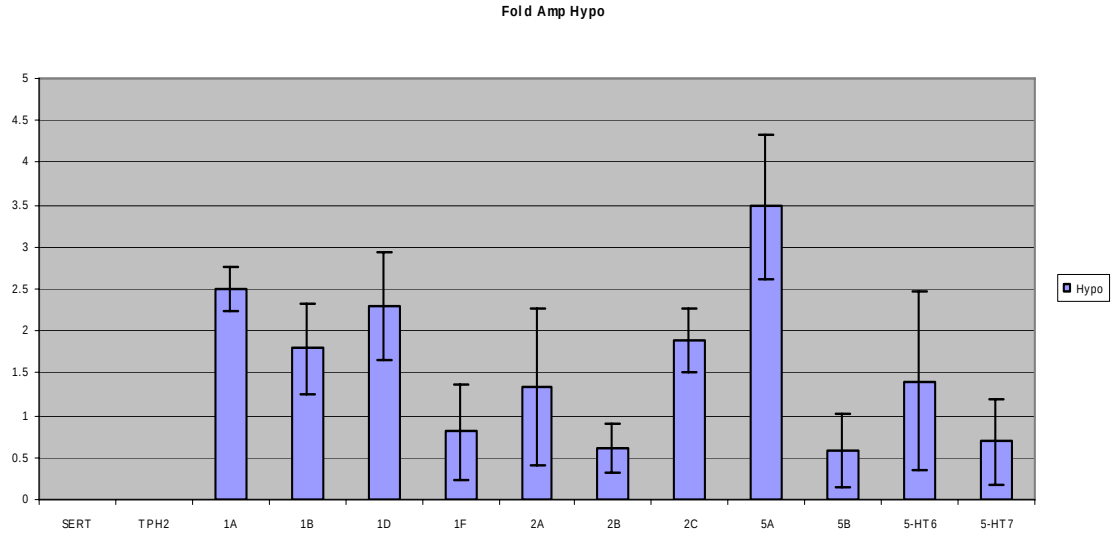


Fig 1D. *In situ* hybridization for 5-HT2C mRNA in pet1 KO vs wild type ventral tegmental area.

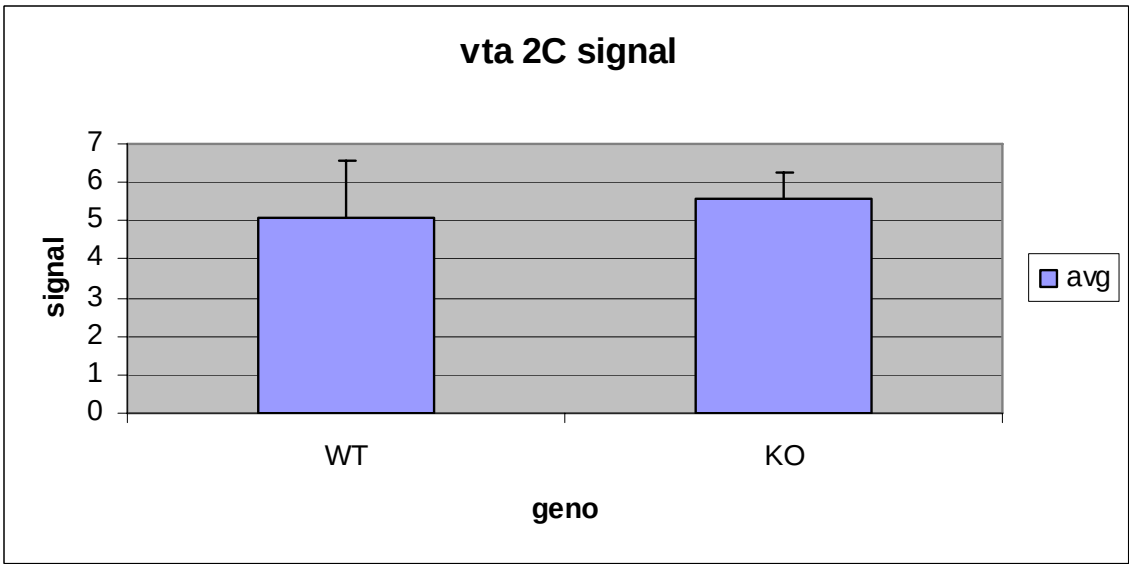


Fig 2A. radioactive ligand binding for 5-HT2C receptors in pet1 KO vs wt striatum.

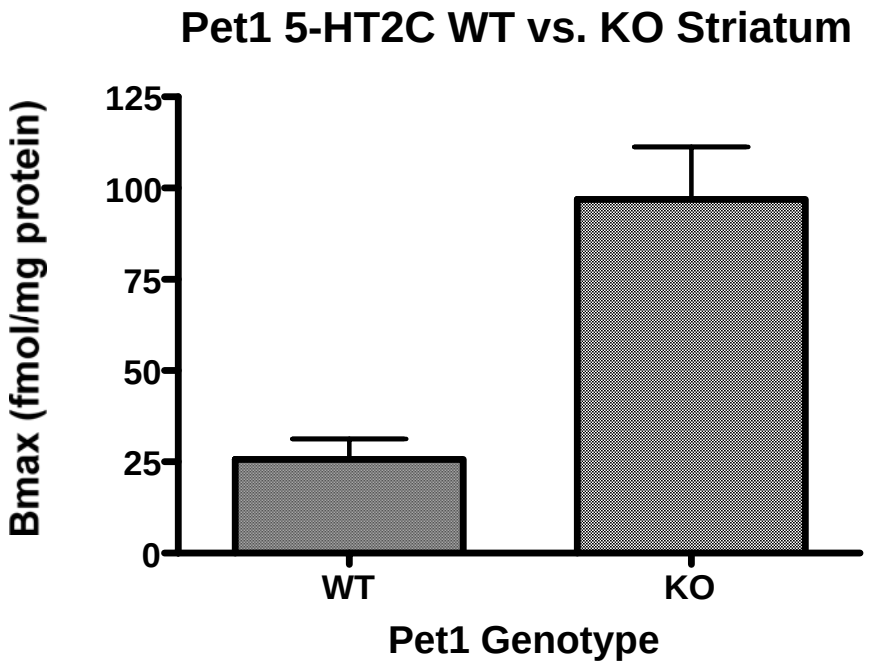


Fig 2B. radioactive ligand binding for 5-HT2C receptors in pet1 KO vs wt hippocampus.

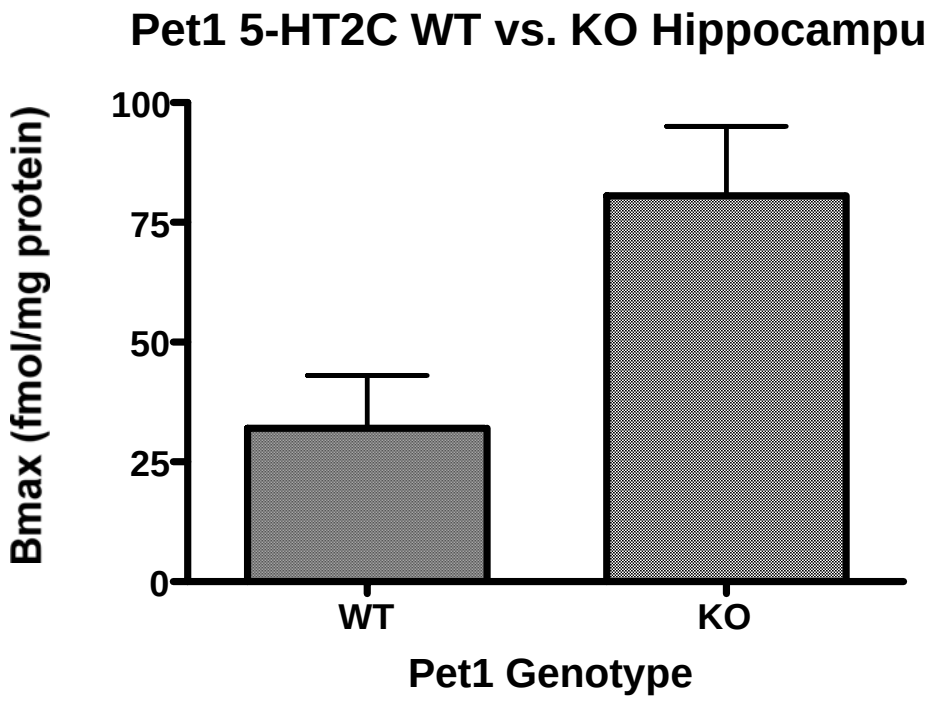


Fig 2C. RNA editing at A through E sites in pet1 KO versus wild type mouse brains.

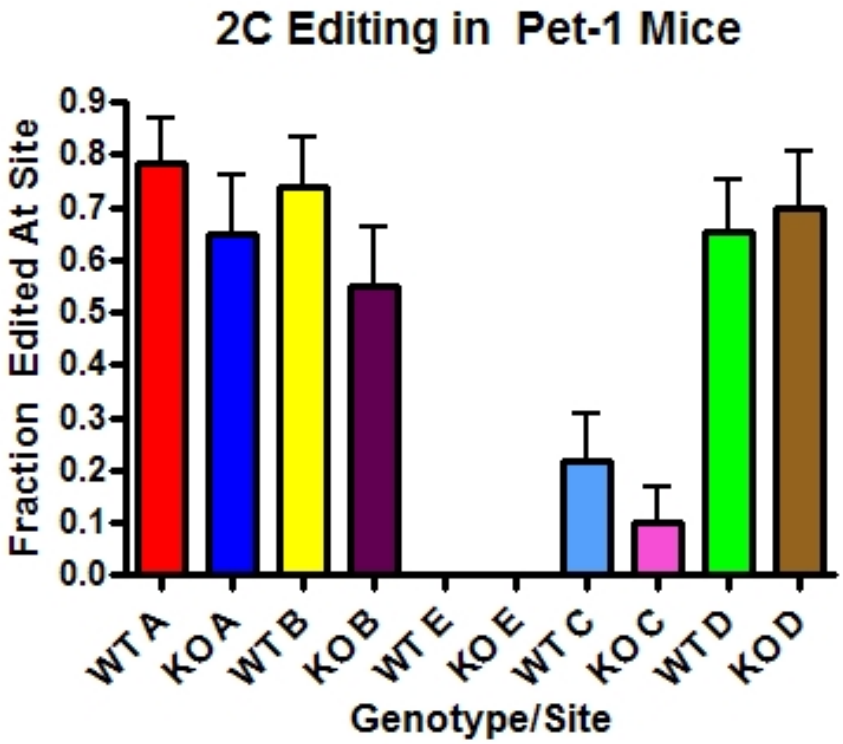


Fig 3A. Brainstem tissue DA content time course in pet1 KO vs wt mice.

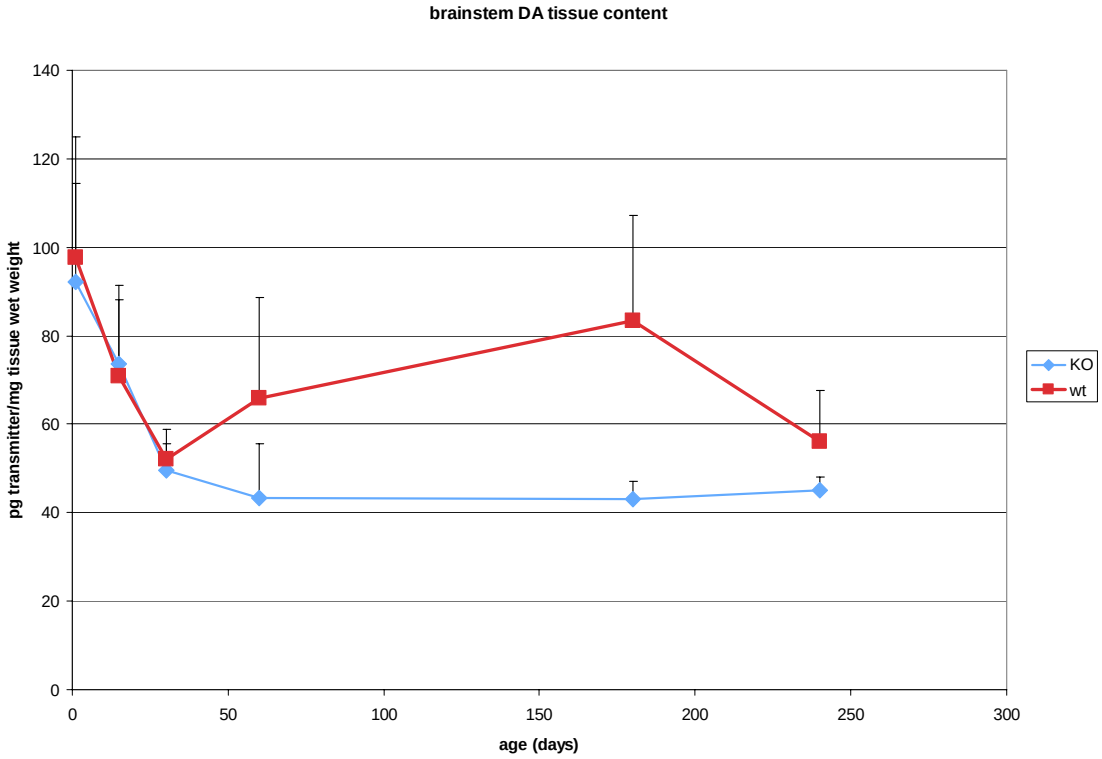
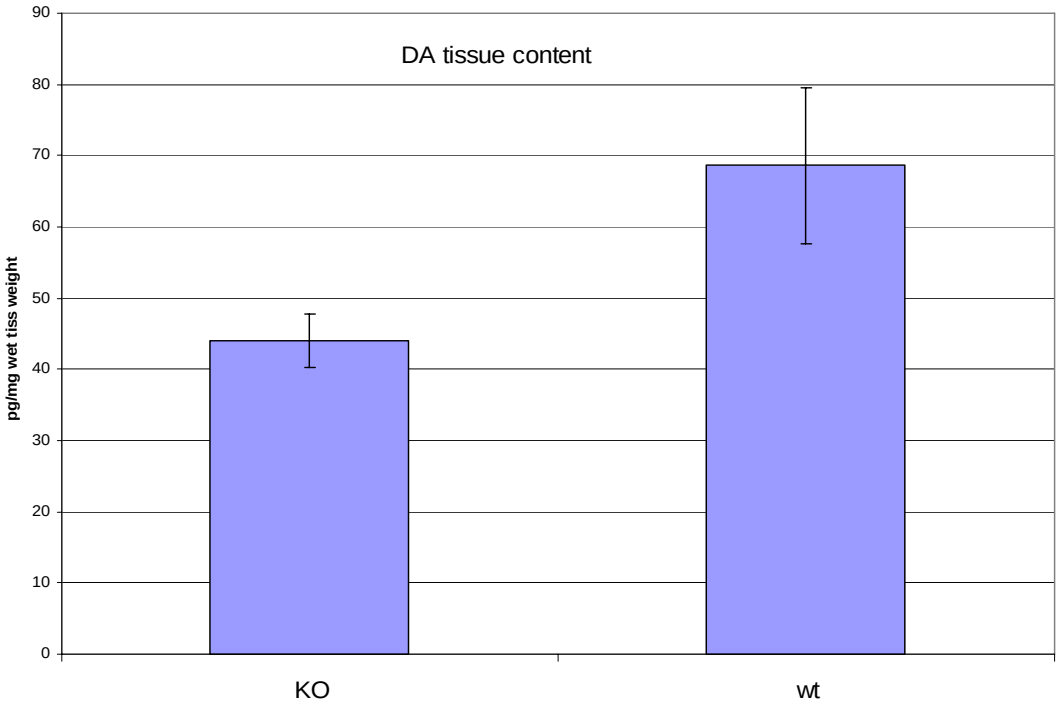


Fig 3B. Average brainstem tissue dopamine content in adult pet1 KO vs wt mice.



P<.05 between groups.

Fig 3C. Time course of 5-HT tissue content in pet1 KO vs wt brainstem.

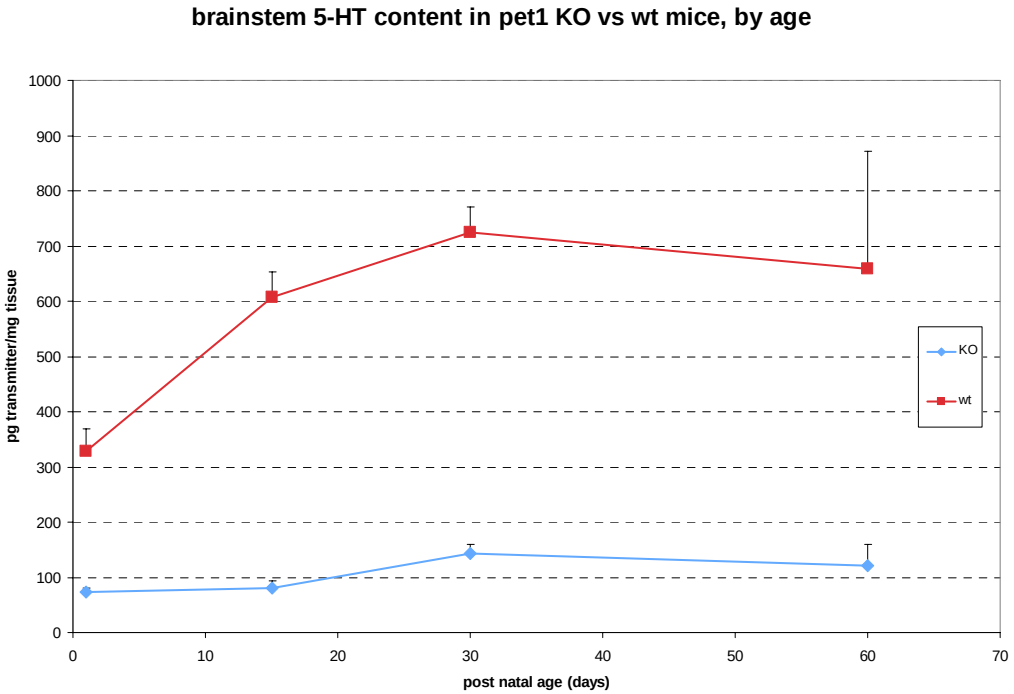


Fig. 3D: Brainstem tissue 5-HIAA content in pet1 KO vs wt mice.

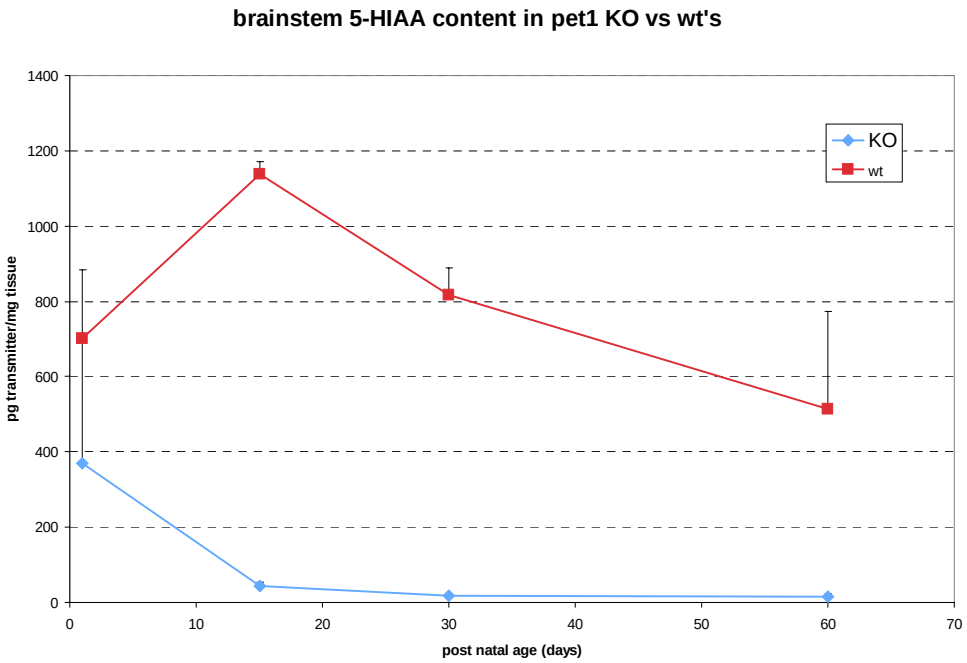


Fig 4A: striatal extracellular DA in pet1 mice at 2 months old

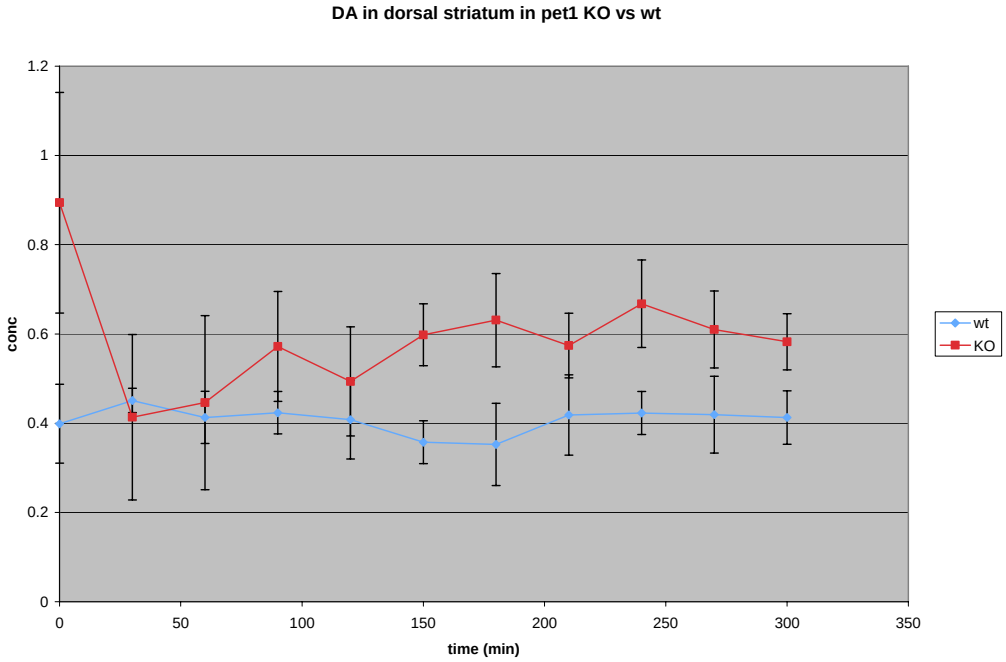


Fig 4B: striatal extracellular DA in pet1 mice at 4 months old

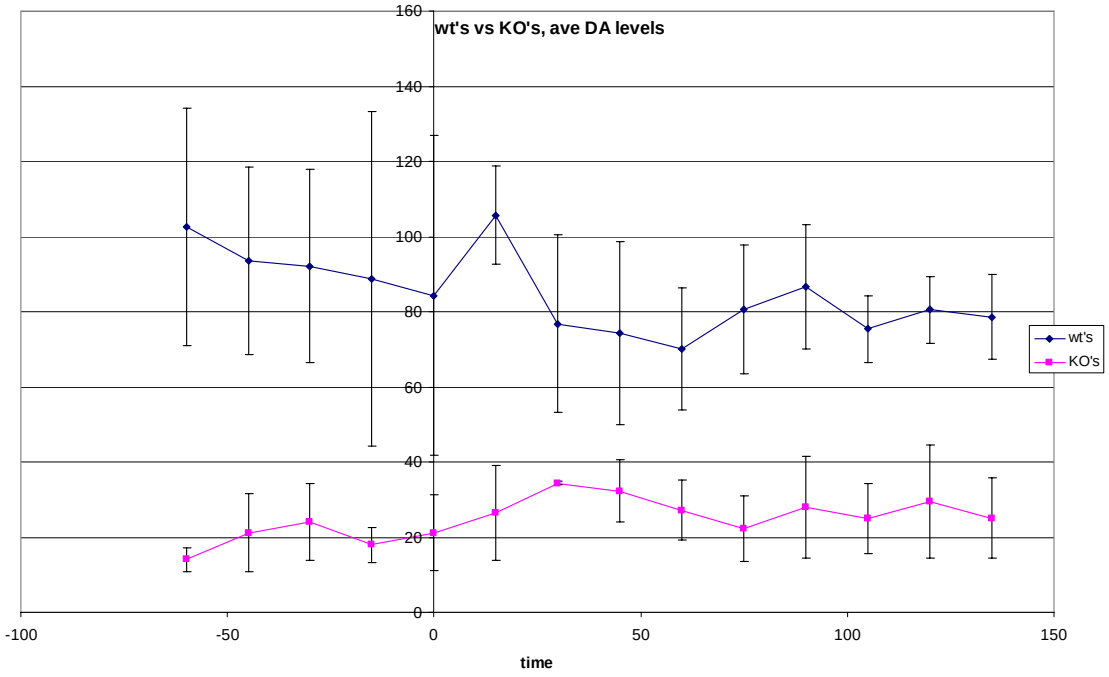


Fig 5A.

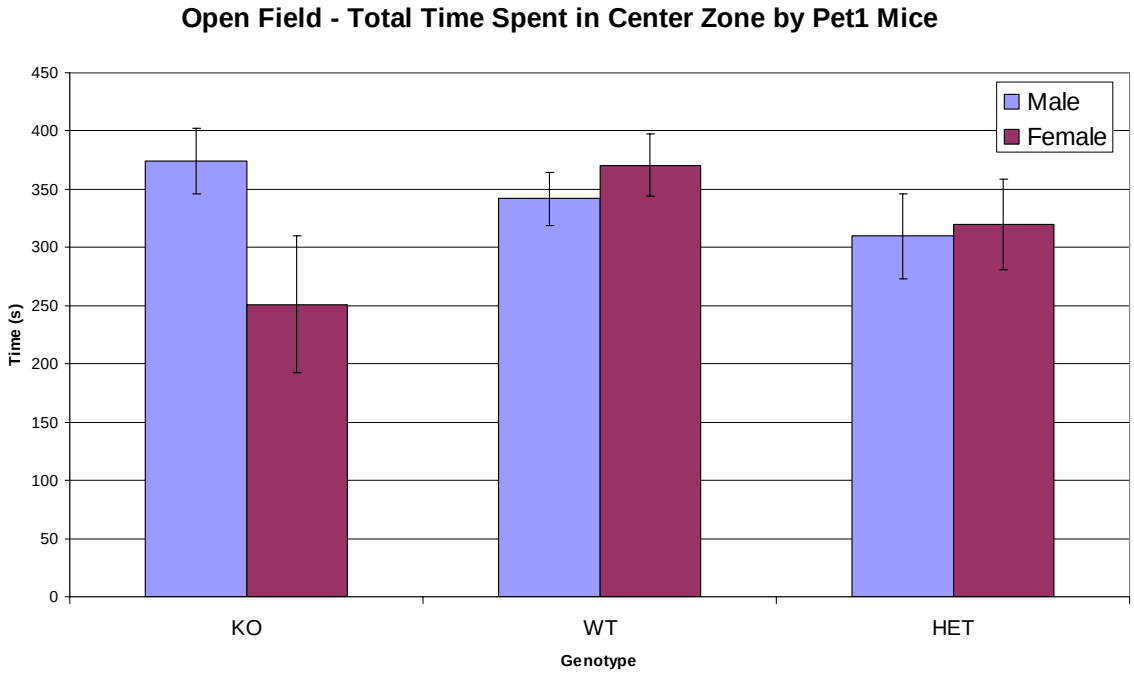


Fig 5B.

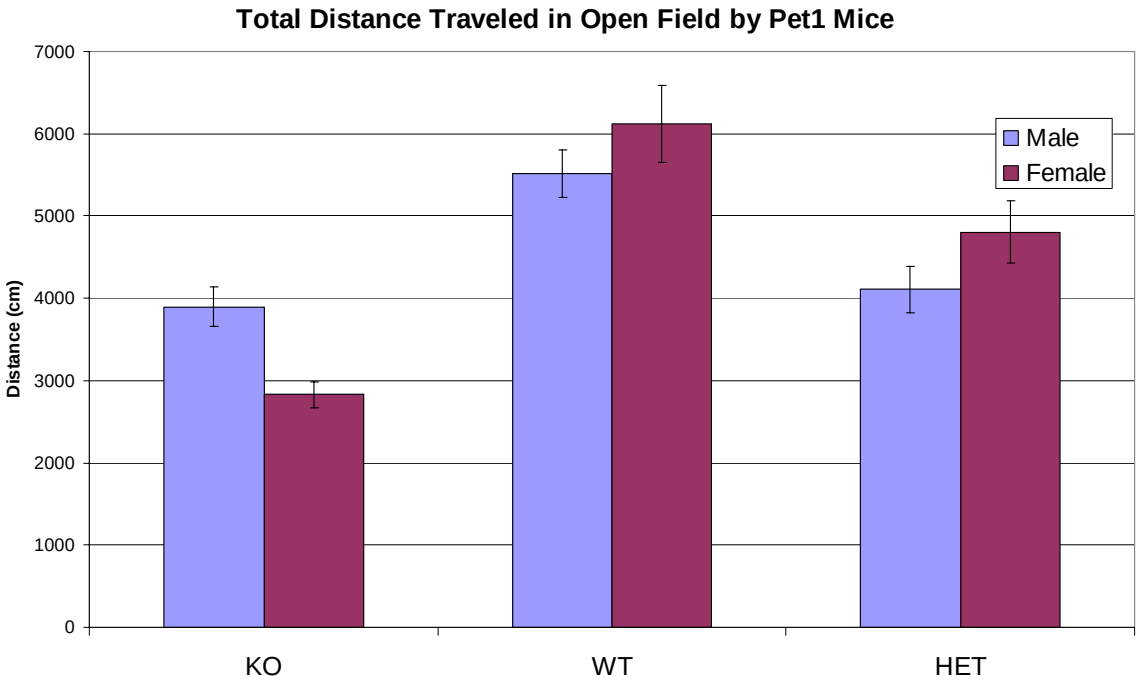

Total Distance Traveled in Open Field by Pet1 Mice
Distance (cm)
7000
6000
5000
4000
3000
2000
1000
0
Male
Female
KO
WT
HET

Fig 5C.

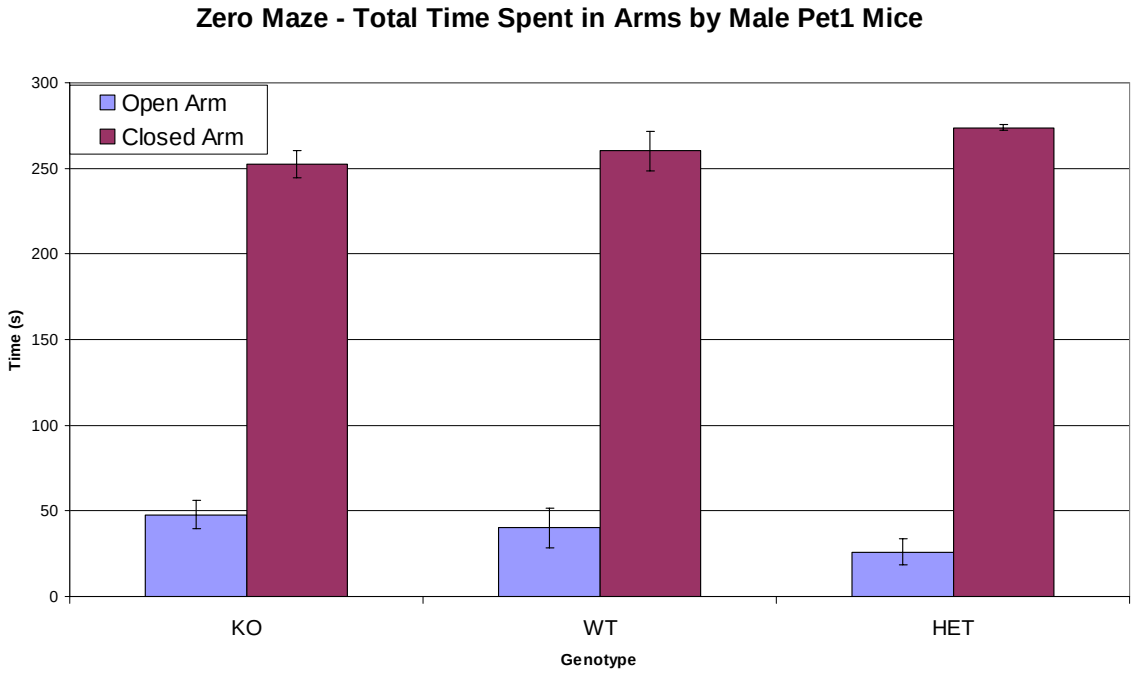

Zero Maze - Total Time Spent in Arms by Male Pet1 Mice
Time (s)
300
250
200
150
100
50
0
Open Arm
Closed Arm
KO
WT
HET
Genotype

Fig 5D.

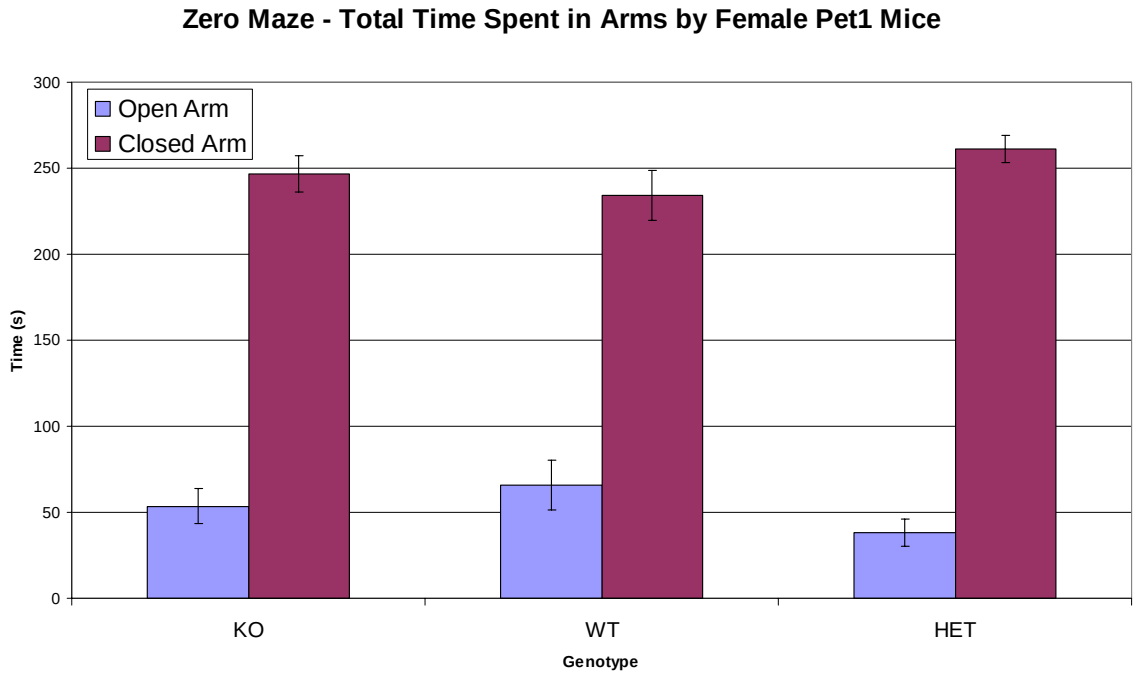


Fig 6A. Photobeam activity monitoring of Pet1 wt, het and KO.

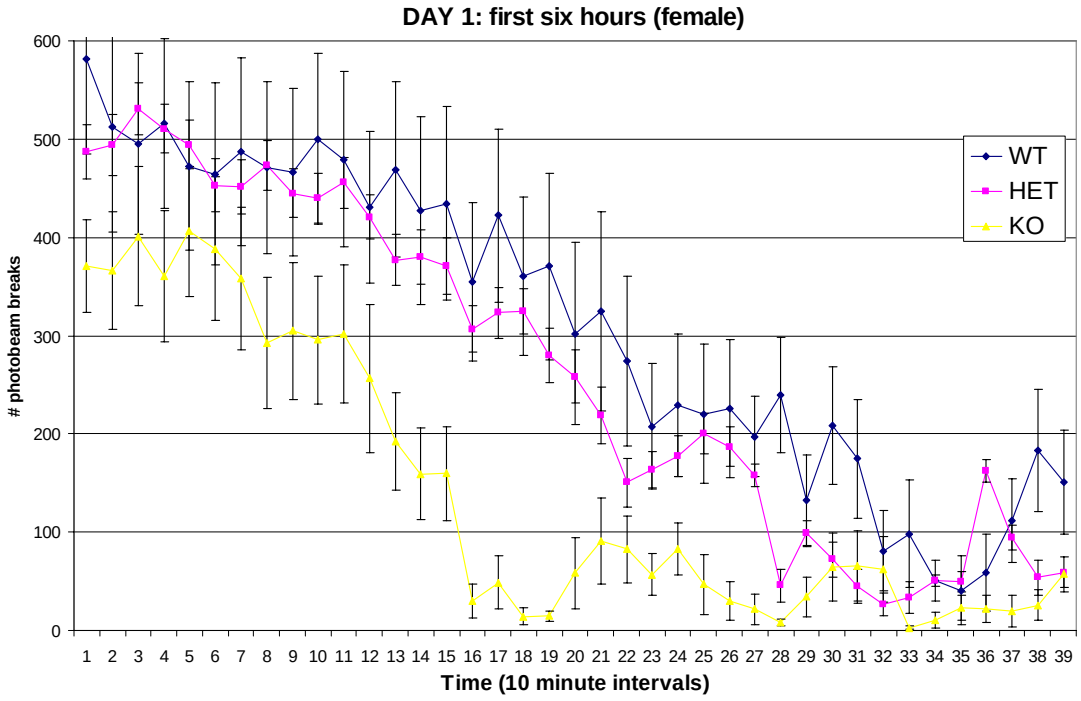


Fig 6B.

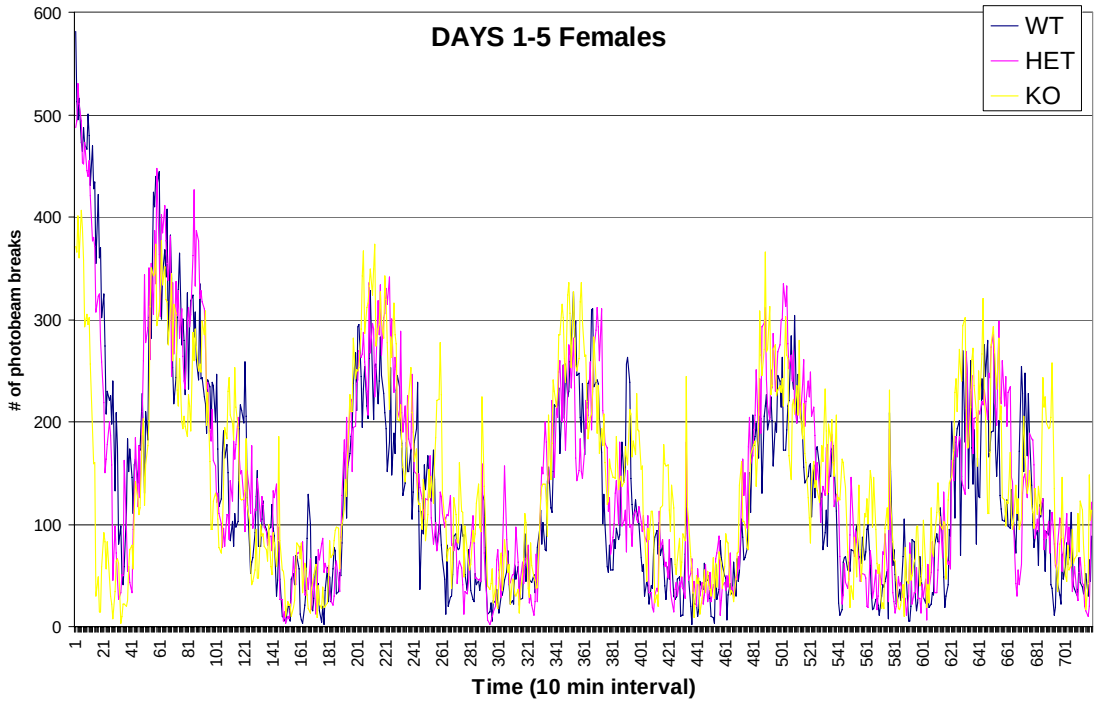

DAYS 1-5 Females
WT
HET
KO
# of photobeam breaks
Time (10 min interval)

Fig 6C.

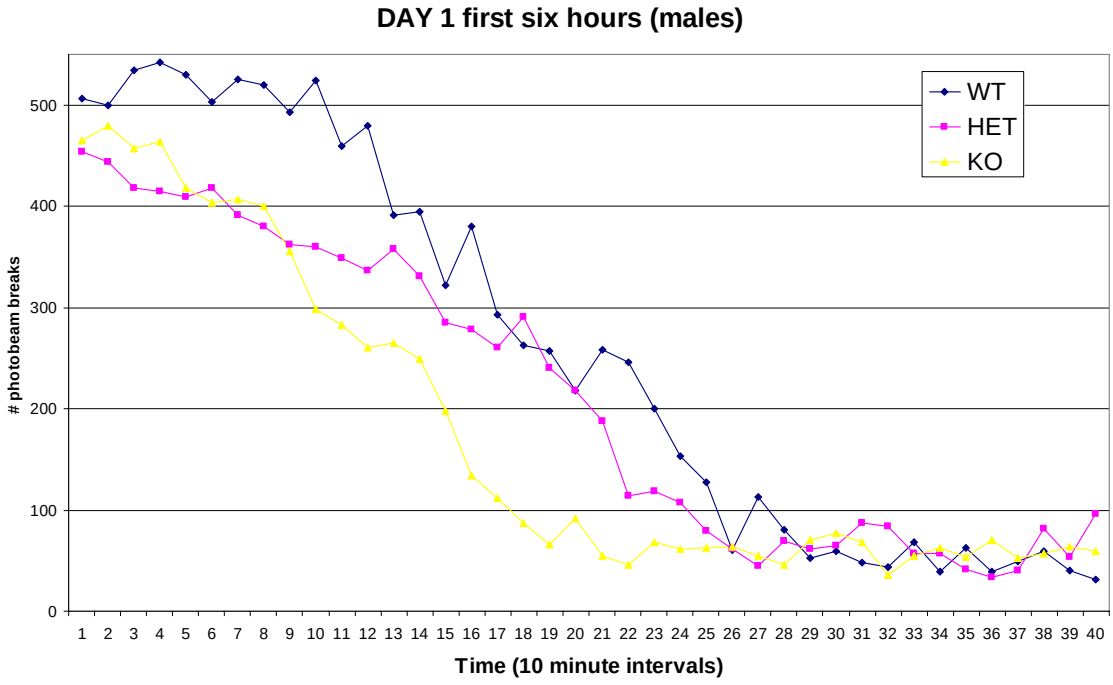

DAY 1 first six hours (males)
WT
HET
KO
photobeam breaks
Time (10 minute intervals)

Fig 6D.

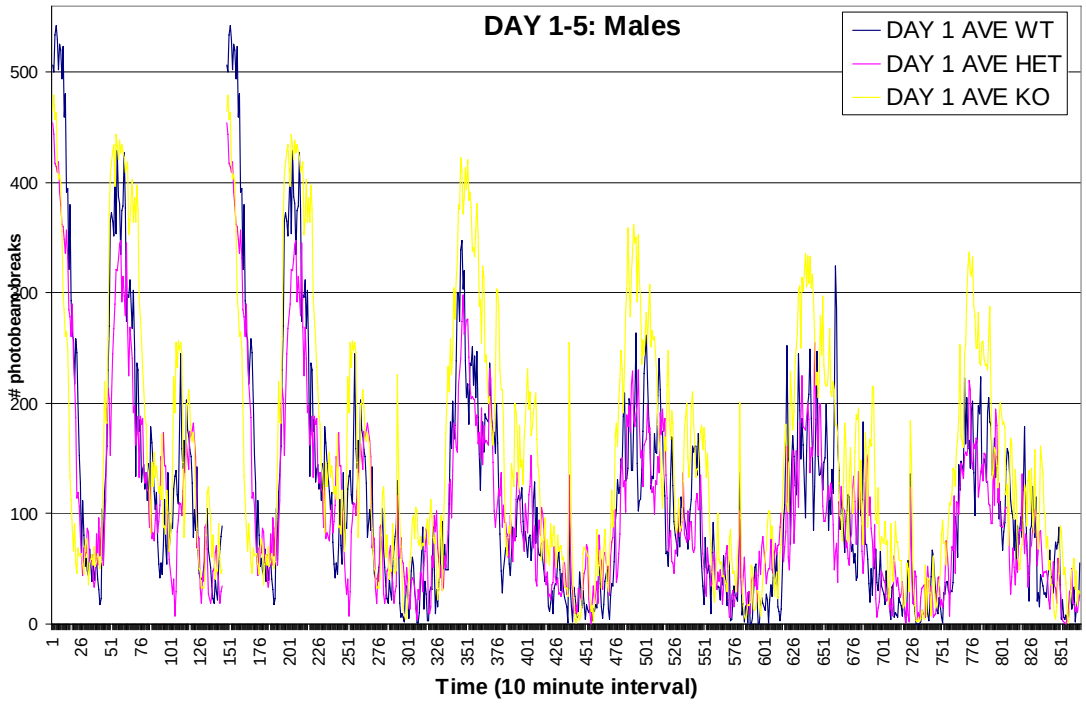

DAY 1-5: Males
DAY 1 AVE WT
DAY 1 AVE HET
DAY 1 AVE KO
photobeam breaks
0
100
200
300
400
500
1
26
51
76
101
126
151
176
201
226
251
276
301
326
351
376
401
426
451
476
501
526
551
576
601
626
651
676
701
726
751
776
801
826
851
Time (10 minute interval)

Fig. 6E

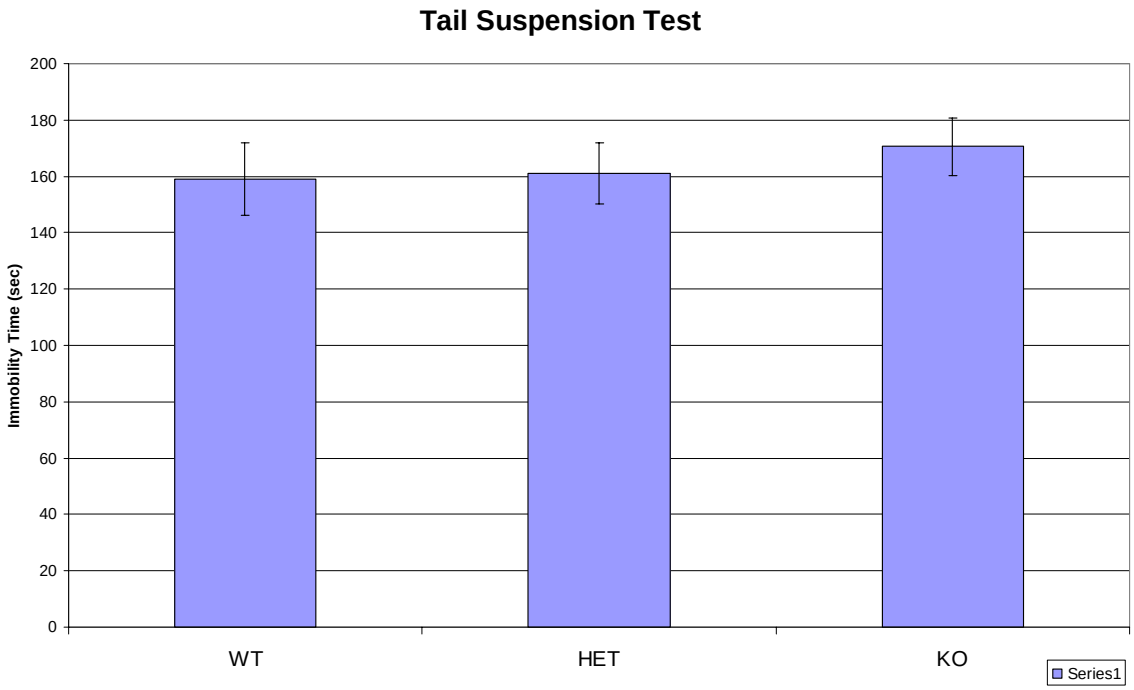

Tail Suspension Test
Immobility Time (sec)
0
20
40
60
80
100
120
140
160
180
200
WT
HET
KO
Series1

Fig. 6F. Effect of Citalopram in pet1 KO and wild type mice in the tail suspension test.

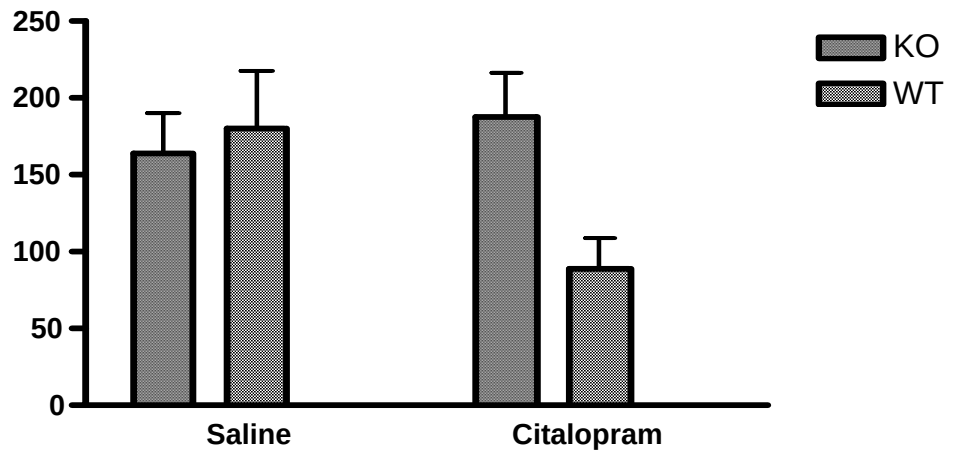


Fig 7A. Fear potentiated startle assay in pet1 vs wt;s

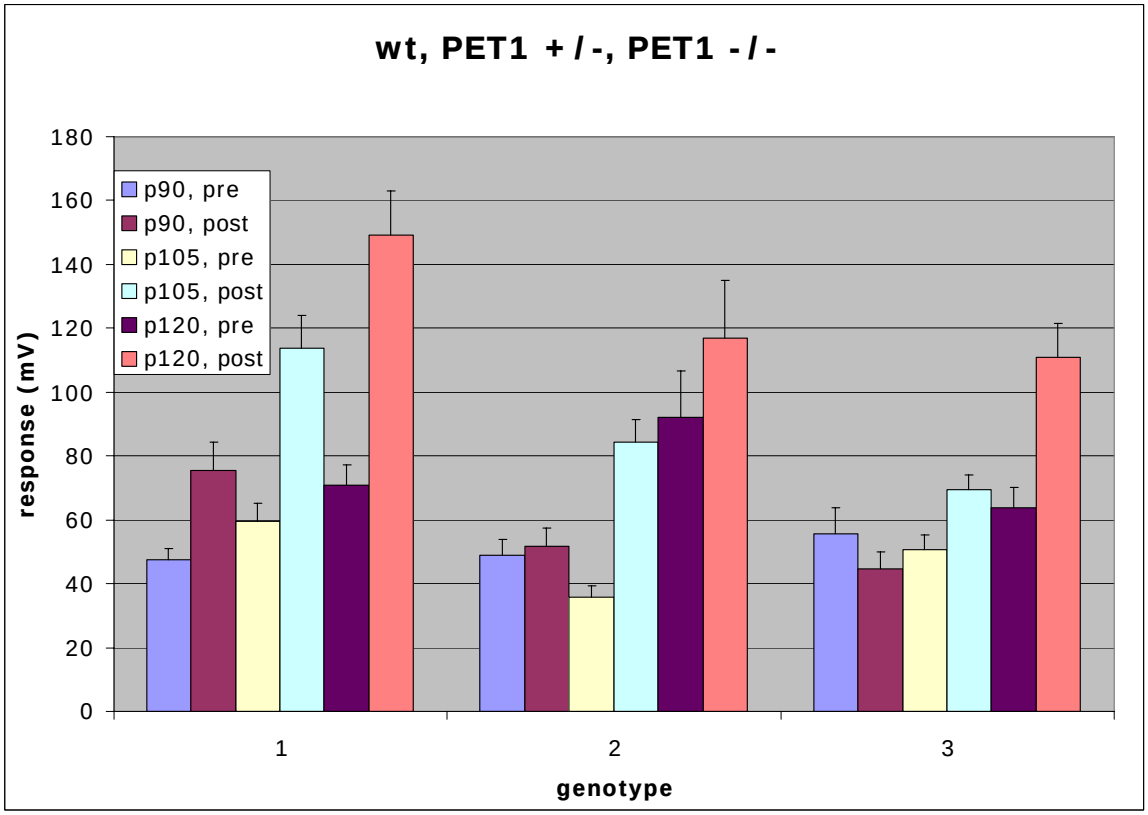


Fig 7B. hot plate test in pet1 mice vs wt's

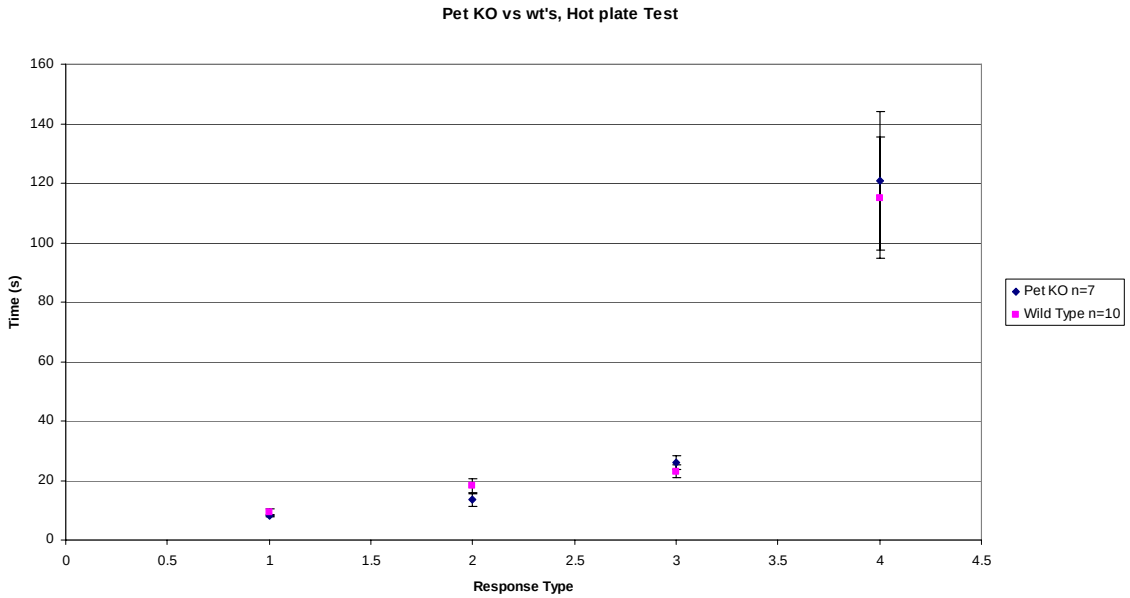


Fig 8A Conditioned Place Preference to Cocaine in Pet1 KO vs wild type mice.

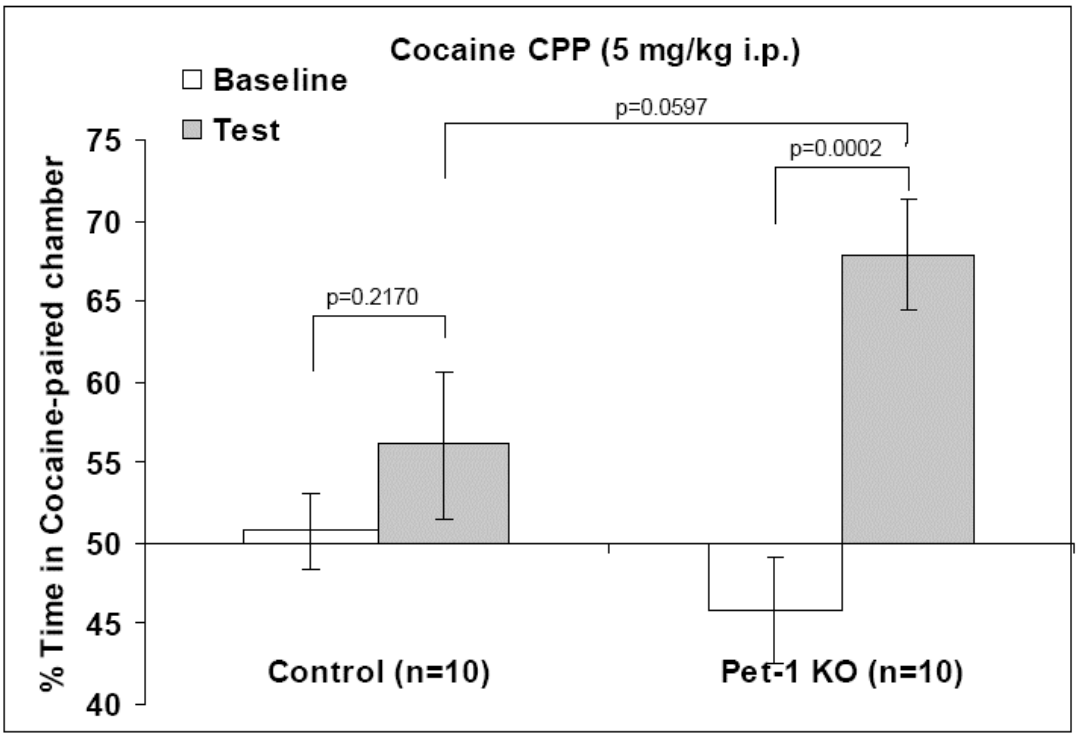


Fig 8B. CPP Diffference Score, in response to 5 mg/kg cocaine.

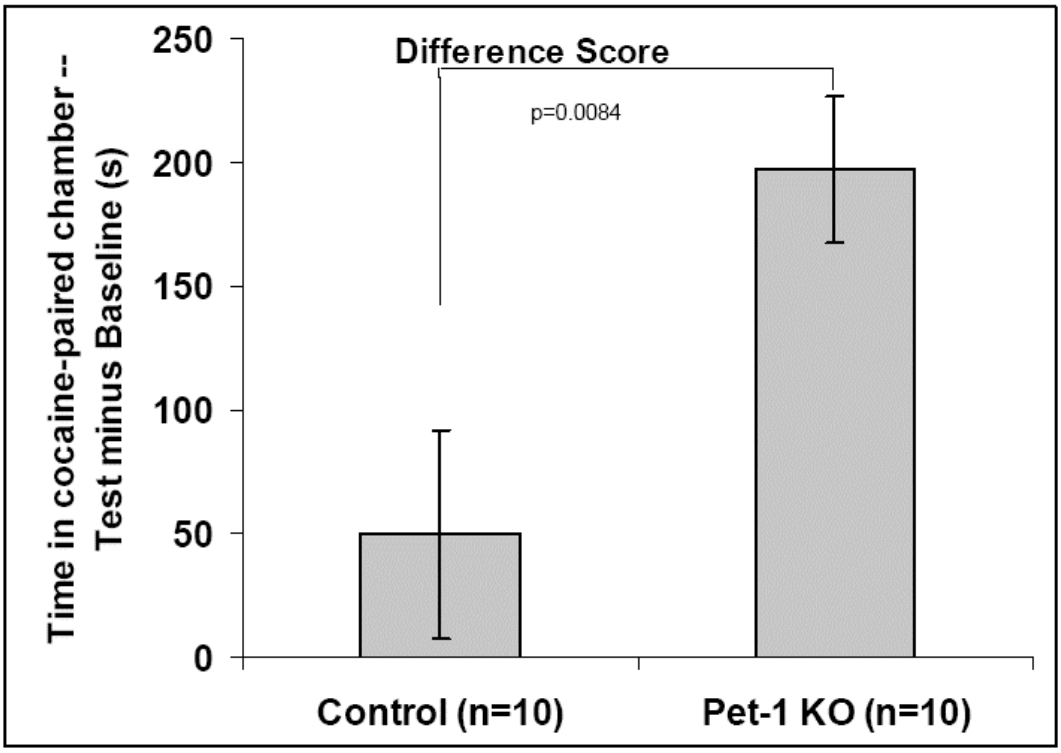
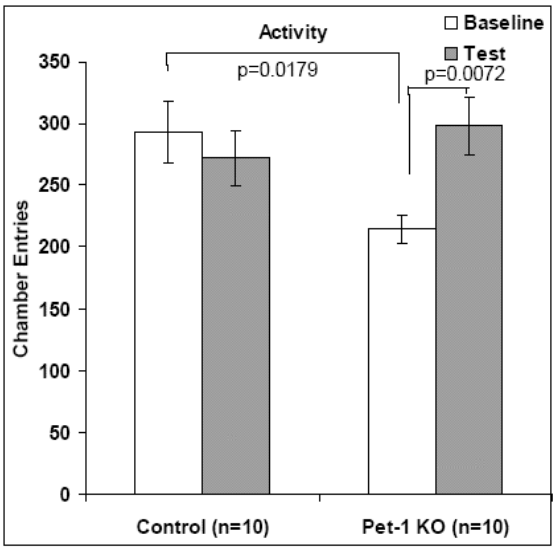


Fig 8C. Activity and Distance Traveled during 5 mg/kg cocaine CPP testing



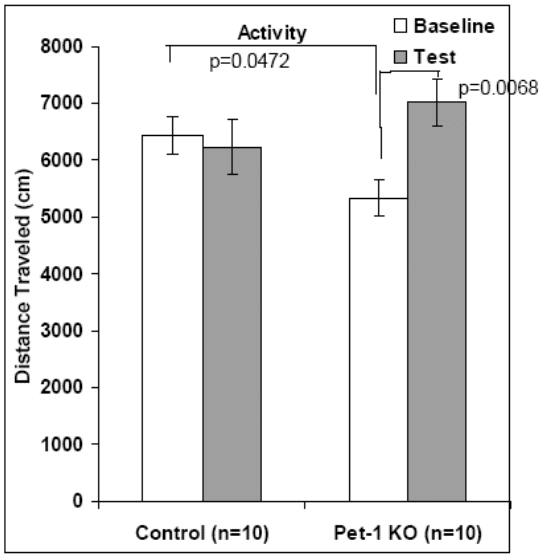


Fig 8D. Pet1 KO vs wt responses to 10 mg/kg cocaine in CPP paradigm.

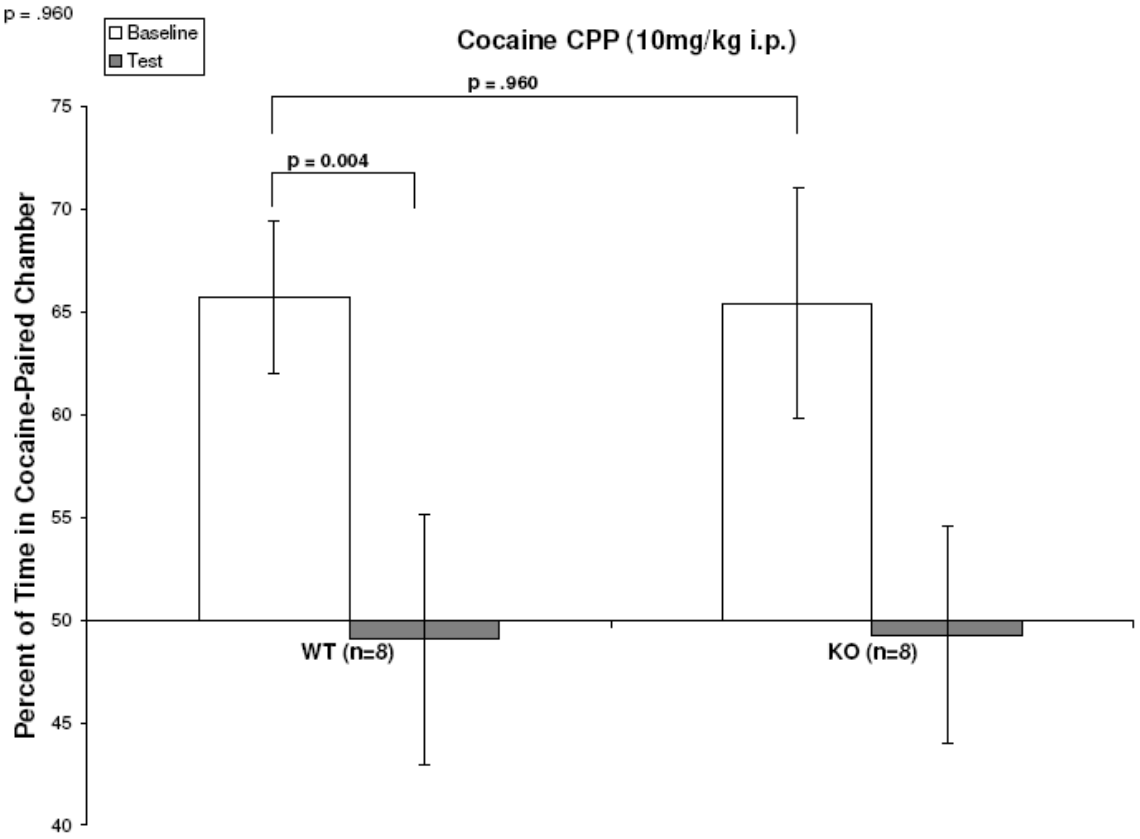
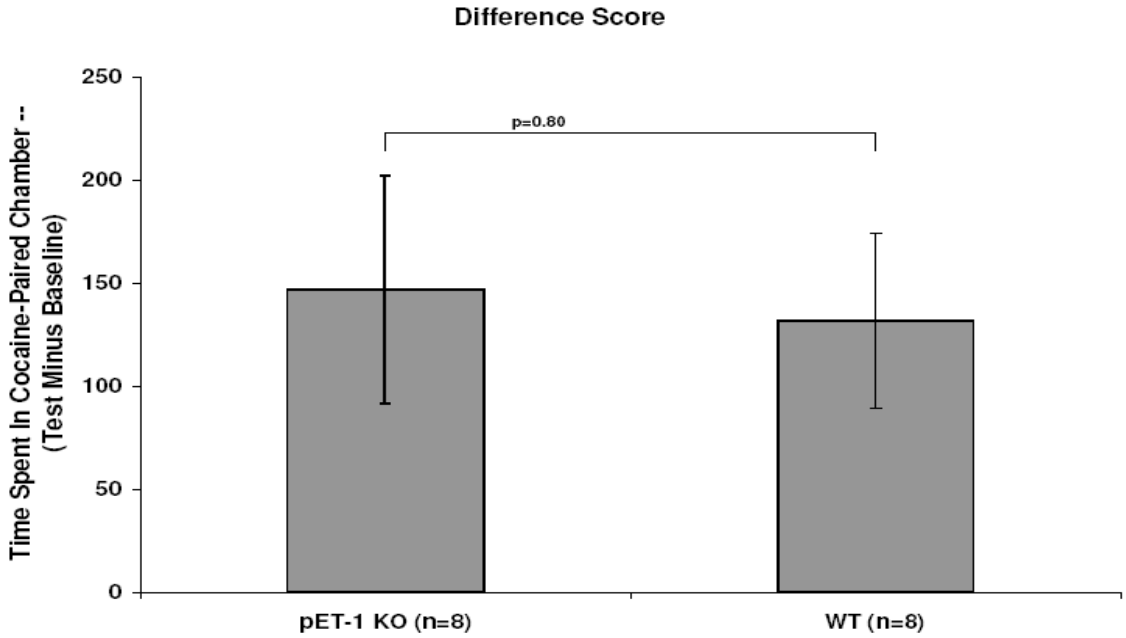


Fig 8E.



Contributions: M. Berger conceived of this project, analyzed all data, wrote this manuscript, mentored Y. Tang in performing qPCR studies, and oversaw L. Yu, P. Sharifnia, and E. Mangir in performing behavioral studies. T. Hnasko and E. Mangir performed the CPP studies, and J. Hsu performed the *in situ* study for 5-HT2C. M. Jongsma performed the in vivo microdialysis studies; A. Abbas performed the 5-HT2C binding and RNA editing studies in collaboration with M. Berger. L. Tecott oversaw all aspects of this project, analyzed all data and mentored M. Berger; B. Roth oversaw the 5-HT2C binding and RNA editing studies and mentored A. Abbas.

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Chapter VI. Future directions in serotonin and GPCR research

The last five decades have been witnessed a growth in biological knowledge unprecedented in human history. Watson and Crick's seminal discovery of the structure of DNA and the creation of the central dogma of molecular biology, that DNA makes RNA which makes protein, have laid the groundwork for an explosive rise in our understanding of how cells, organs and tissues, and whole organisms work. This knowledge has crossed the boundaries of academic disciplines, and ranges from the level of atomic structure to whole organism physiology and behavior. This knowledge has ranged from the detailed molecular structures of specific proteins and biological molecules made possible by X-ray diffraction methods, to the specific organization of the mammalian genome and the genes that make it up, to the increasingly detailed understanding of how proteins work together in networks to give rise to complex cellular functions (such as chemotaxis, secretion, and replication), and finally to the understanding of how large networks of cells work together to give rise to the emergent properties of entire organs, such as the heart pumping blood to the quadriceps of a champion cyclist, or the brain orchestrating the magnificent muscular contraction patterns that give rise to a ballerina's beautiful dance. Eventually, we can hope that these advances and this knowledge will lead to a better mechanistic understanding of human physiology and disease processes, new medical treatments, and ultimately to a detailed physical explanation of human mental processes, of the human mind.

But such discoveries are still a ways off: some lie in the near future, some further afield. In previous chapters, I have discussed general research trends in endocrinology and psychiatry, as well as my own research efforts in these areas. In particular, in chapters I and III, I discussed how the developmental and cell biology of pancreatic islet function may relate to understanding the pathophysiology of and devising new treatments for diabetes, while in chapter V I have discussed my efforts to better understand the neural circuitry of emotion in mice with a severe

serotonin deficiency. In the present chapter, I will discuss how new technological approaches can be used to explore these questions further, and how these novel methods could lead to the future discoveries mentioned above.

The future of beta cell biology research

Pancreatic beta cell biology is a competitive field of study, for obvious reason: understanding the biology of these cells will likely help us to understand the pathophysiology of and devise novel treatments for diabetes. Since type I diabetes is a disease of beta cell (and thus insulin) deficiency, simply replacing the missing beta cells should be sufficient to cure this disorder. Getting a large quantity of beta cells for transplantation, however, remains a hurdle. The supply of cadaveric beta cells is limited, which has led many to hope that human embryonic stem cells (hESCs) could be differentiated in insulin secreting beta cells for transplantation. In recent years, several groups have reported the differentiation of hESCs into beta cells, primarily by expressing the same lineage of transcription factors in these hESCs that specify beta cell fate during mammalian embryogenesis.

These results have excited many researchers and patients, and some centers are already attempting to do beta cell transplantations for diabetic patients, including the UCSF Diabetes center. However, these transplantations are not a standard treatment for the vast majority of diabetic patients, for two reasons. First, many of the hESC differentiation protocols lead to cells with low insulin content, and it is difficult to derive large numbers of these cells from hESCs. Second, the autoimmune attack that kills the original pancreatic beta cells in most type I diabetic patients still remains a fear: would the same auto-reactive B and T cell clones simply attack any newly grafted beta cells derived from hESCs?

Thus, an ability to derive large numbers of insulin-producing beta cells from hESCs is something of a holy grail in this field: transplanting larger numbers of cells would provide for

better control of blood glucose, and it could help ensure enough insulin-secreting capacity remains even if some cells are lost due to a recurrent autoimmune attack. There are thought to be homeostatic mechanisms that limit the number of terminally differentiated cells of any lineage (Lipsett et al, '06; Stanger et al, '07), but these normal physiological limit-setting mechanisms may be an obstacle to generating sufficient numbers of beta cells from hESCs *in vitro*. Thus, we may need to better understand these limits, and how to circumvent them, in order to generate sufficient numbers of beta cells for transplantation purposes.

But most scientific questions are not of this form. Most scientific investigations traditionally begin with a specific hypothesis about whether a given pathway or gene product is involved in a given cellular process (such as beta cell replication). These more typical investigations are tractable if time consuming; they involve either blocking or activating the pathway in question, and then measuring the effects of such manipulations at the cellular and/or whole animal level. I have performed a specific-hypothesis driven study of this form to examine the role of Gi-coupled receptor signaling in beta cells in chapter IV, and numerous other investigators are pursuing similar studies around the world. However, such studies depend on the ability to construct the given specific hypothesis, which usually depends on having prior biological knowledge about the gene(s) or pathways in question from other systems. But how can we proceed at a genomic level, knowing that there are over 30,000 genes in the human genome that could regulate any given cellular process, a large fraction of which have unknown functions?

This is a difficult question that biologists will likely struggle with in the years to come, as we begin to ask questions like "what are all the genes and signaling pathways that regulate a given cellular process in a cell autonomous fashion, whether that process is beta cell replication or hippocampal neurogenesis?" We currently do not have the methods to examine the potential causal role of multiple genes or signaling pathways in a cellular process in real time; rather, we have to infer such causation by either activating or blocking pathways one at a time.

However, there are two several potential shortcuts around this problem. First, expression analysis tools such as cDNA microarrays, qPCR techniques and proteomic methods are capable, in principle, of examining the expression of potentially thousands of RNA transcripts and/or protein molecules at once. These techniques can identify those genes expressed at the highest level within the cells of interest, and can thus point us in the direction of those genes that may be most likely to regulate the cellular function of interest. For example, I have used a qPCR array in this fashion to find all of the GPCRs expressed in developing pancreatic beta cells in chapter III.

Second, the genome is not random: many if not all genes fit into classes whose protein products share similar structures and functions. For example, approximately 3% of the human genome encodes GPCRs, as I have discussed in chapter I. Because these receptors signal through a small number of basic pathways, it is thus possible to ask whether a specific class of GPCRs is involved in a given cellular process by using wide spectrum pharmacologic agents that modulate signaling through all members of a given class. For example, all Gi coupled receptor signaling can be blocked by pertussis toxin, while all Gs-coupled receptor signaling can be activated by cholera toxin. Conversely, new tools like DREADS and RASSLs can be used to ask whether signaling through a given class of receptors could or would be sufficient to modulate a given cellular process (Armbruster et al, 2007; Coward et al, 1998), independent of which individual members of the gene class may normally modulate this cellular process under physiological conditions. These approaches can thus allow us to ask whether an entire gene class is involved in a cell process; if yes, then we can further investigate each gene in the class to find the relevant ones; while if the wide-spectrum studies show that the entire class is uninvolved in the process in question, then we can skip studying the entire class of genes.

Thus, by using genome-level expression analysis tools and wide spectrum inhibitors of entire gene classes, we can rapidly identify which specific genes are expressed in a given cell type and may be regulating our cell process of interest, and we can identify which gene classes

as a whole are important in regulating the given cellular processes. Then, detailed studies can be conducted on these target genes. In the future, perhaps protein specific live cell imaging or other methodologies could allow us to watch cellular processes happen at a single protein level resolution, and could potentially allow us to infer causality to specific proteins and gene products without the time consuming steps of detailed studies on a gene by gene basis. A fitting analogy would be the difference between using DNA testing to examine a blood specimen found at a crime scene, versus simply watching the crime happen in real time. It is entirely possible to find out whose blood is at the scene by DNA sequencing of the blood specimen, testing the DNA of all human beings, and looking for a match, but it would be much easier to watch the crime happen in real time and simply see who committed the crime. But until we have the ability to watch cellular processes at a single protein resolution-level, we will be forced to assign causality by the detailed and labor-intensive experimental methods I have used in chapter IV.

From Beta Cells to Serotonin Neurons, from endocrinology to psychiatry

Though my discussion of this issue thus far has focused on pancreatic beta cells and their role in glucose homeostasis, the same basic principles apply to the study of CNS serotonin neurons and their role in affect regulation. Just as beta cells are a key node in the complex physiological network of energy balance and blood glucose homeostasis (Figure 1), in principle CNS serotonin neurons can be viewed as a key node in the complex neural network that gives rise to emotional experience and behavior. I refer to these as key nodes, because lesioning either of these elements significantly disrupts the functioning of the entire circuit: a deficiency of beta cells produces hyperglycemia and diabetes (Gillespie KM, 2006), while a lack of serotonin severely perturbs emotional behavior in both humans and mice (Hendricks et al, 2003; Bourne et al, 1968).

And these cell types can be studied in similar ways as well. For example, just as the use of transgenic mice that express fluorescent reporters specifically in beta cells allowed us to

purify these cells and measure gene expression specifically within them in chapter III, similar reporter mice are currently being used to pursue similar studies of serotonin neurons (Scott MM et al, 2005). Thus, similar approaches to that we have taken here in chapter IV can be used to ask, what are all the receptors, ion channels and signaling proteins expressed in a specific neural network node, such as the central nucleus of the amygdala or the serotonergic neurons of the raphe nuclei. The relevant nodal cell populations can then be purified by FACS based cell sorting and/or laser capture microdissection techniques, using transgenic animals that use cell-specific promoters to drive expression fluorescent protein markers (such as GFP) in the specific cells and/or regions of interest. Though labor intensive and time consuming, these techniques can ultimately identify the specific receptors and molecules that could regulate each node in a circuit, and to dissect cellular pathways within these cells, just as we have done with pancreatic beta cells in chapter IV.

However, there is a major difference between serotonergic neurons and beta cells: while beta cells simply need to secrete insulin as a hormone to meet peripheral glycemic demands on a time frame of seconds and minutes (ref), serotonergic neurons likely encode information in their firing rate and pattern with millisecond temporal resolution (for example, a single action potential in these cells last 1 msec, while a burst may last X msec; ref). Although much research on the serotonin system has been guided by the monoamine hypothesis of psychiatry, the idea that deficient serotonin release/transmission underlies mood disorders, little work in the field has reflected the more complex underlying neural coding mechanisms of these cells. Thus, many studies of the serotonin system have simply examined whether drugs elevate extracellular serotonin levels in large brain regions on a time scale of minutes (reference), when much of the important synaptic signaling may be occurring on a time scale that is at least five orders of magnitude faster.

How do we study the role of serotonergic neuron activity *in vivo*, in behaving animals, on the time scale of single action potentials and stimulus trains? This is a major dilemma for the

entire field of behavioral neuroscience, of how to understand behavior in terms of the activity pattern of single cell types; it is a question facing behavioral neuroscientists studying virtually all CNS cell types. In general, this question demands three capabilities. First, we need to monitor cellular activity on a msec time scale *in vivo*, which is currently possible (though difficult) with *in vivo* electrophysiology studies (ref) or by watching genetically encoded optical activity markers (ref); this capability can allow us to correlate specific activity patterns with particular behaviors. Second, we need the ability to manipulate cellular activity on a similar time scale *in vivo*, and only in the cells of interest.

This second capability has been sorely lacking in behavioral neuroscience until recently. Chemical genetic methods such as RASSLs and DREADDs, and ligand gated ion channels that can silence cells (Coward et al, 1998; Armbruster et al, 2007; Lerchner WA et al, 2007) have been used in recent years to silence specific cell populations in awake animals, allowing investigators to ask questions about which cell types may be involved in a given behavioral process. Thus, these methods are primarily useful for silencing cells on timescales of seconds to minutes to hours; they simply lack the temporal resolution that is necessary to understand behavioral processes in terms of neural activity codes.

However, recent advances in the use of optical genetic methods may provide this long sought-after capability. In particular, Deisseroth and colleagues have succeeded in expressing the algal Ca^{++} channel channelrhodopsin in mouse CNS neurons (Boyden ES et al, 2005), and they have demonstrated that it can be activated by light pulses to control neuronal spiking on a msec time scale (Wang et al, 2007); more recently, they have also used an archaeal light-driven chloride pump (NpHR) from Natronomonas pharaonis to hyperpolarize and silence mammalian neurons on a similar time scale (Zhang et al, 2007). Future studies in transgenic mice expressing these genes on specific cell populations using tissue specific promoters could then be used to examine the causal relationship between specific neuronal firing patterns and whole animal behavior. Although delivering light pulses to the appropriate brain regions is currently a

challenge, this obstacle should be surmounted soon by advances in fiber optic technology and/or the potential ability to alter the activation spectrum of these proteins so that infrared light beams could be directly shined through the animal's head. Such studies are likely to represent a paradigm shift in neuroscience, because they will allow us to correlate the activity of specific neurons with animal behavior in an unprecedented fashion.

From cells to circuits

Although the optical genetic methods described above present a potential paradigm shift in our ability to understand how the activity of specific cells correlates with behavior, they may not resolve another problem in understanding the neural circuitry of behavior: How can one study the emergent or system-wide properties of a complicated multi-nodal network (such as the emergence of anxiety-related behavior from the activity of the limbic system), if these properties cannot be reduced or understood simply in terms of the activity of one node in the network?

For example, it may be that anxiety related behaviors such as freezing may depend on activity within an entire neural network, and such behaviors may not be reducible to the activity patterns of any one cell type. If this is the case, then it may be necessary to monitor and manipulate the activity of multiple neural cell types simultaneously to understand how properties of the entire circuit give rise to behavior. In principle, one could achieve this aim by expressing multiple different optical and/or chemical genetic sensors in different cell types and activating these sensors with the appropriate light wavelengths or chemical ligands in real time to simultaneously cause different populations of cells to spike or to go silent. One could also express fluorescent activity marker proteins in cell types of interest, such that cellular activity could be monitored optically without the need for cumbersome electrodes.

These are the types of studies which will be necessary to understand how the cellular activity of neurons gives rise to behavior and ultimately to subjective human mental states. They are technologically demanding and complex, which perhaps should not be surprising, given that

the brain is far and away the most complex human organ. While the control of energy balance also relies on an integrated network of organs and cell types, it is intrinsically simpler than the neural networks that give rise to mental experience, because the time scale of energy balance is at least three orders of magnitude slower, and the number of inter-cellular connections (or network nodes) is orders of magnitude smaller. Similarly, we can imagine that the pathophysiology of psychiatric disorders may be orders of magnitude more complex than the pathophysiology of metabolic disorders like diabetes.

Nonetheless, to young scientists at the turn of the twentieth century it must have been difficult to fathom that the dreaded disease of diabetes, which at that time was a guarantee of death within X months, would be well understood and treated in a mere two decades with Banting's isolation and discovery of insulin in 1922. A century ago this discovery would have been hard to imagine, and the entire endocrine system perhaps seemed like an enigma: the idea that hormones work through receptors, a basic tenet of modern endocrinology, had not even been formally stated at this point.

Similarly today, a century later, we stand on the precipice of finally understanding one of the questions that has plagued humankind for millennia- what is the physical nature of the human mind? In some ways we know a great deal about this question, via the vast body of cellular, molecular and systems neuroscience knowledge that we have attained over the last century or so since Ramon Y Cajal's fundamental neuroanatomical studies. Yet in other ways, this question seems further afield than ever, for we still know little about how the activity of specific populations of neurons gives rise to behavior and mental experience. Yet just as the biochemical methods already present in the early 1900's soon gave rise to the discovery of insulin and the treatment of diabetes, we may already have the tools today (in the combination of optically activated activity modulators and sensors, cell and tissue specific promoters, etc) to dissect this question in the days and years to come. Just as the first few decades of the twentieth century saw scientific advances that led to a rich understanding of and novel

treatments for diabetes and other endocrine disorders, we can expect that scientific advances in the decades to come will lead to rich new understandings and novel treatments for many neuropsychiatric disorders.

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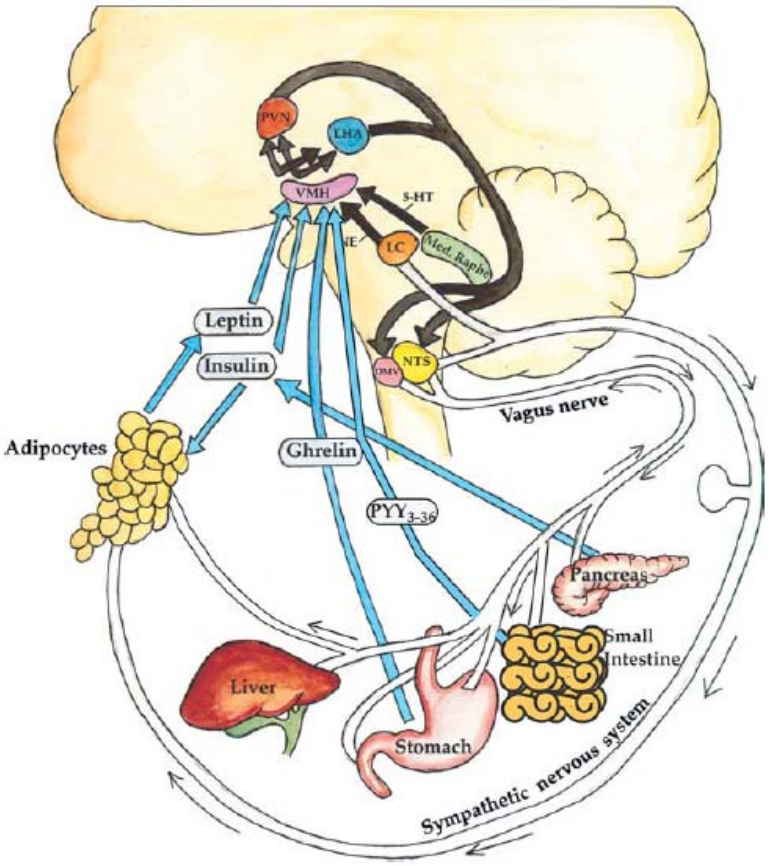

Figure 1. The physiologic regulation of energy balance, by the pancreas, liver, adipocytes, hypothalamic and brain stem nuclei. Reprinted here with permission from Dr. Robert Lustig.

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