PET-1 IS REQUIRED ACROSS DIFFERENT STAGES OF LIFE TO REGULATE SEROTONERGIC FUNCTION

by

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Pet-1 Is Required Across Different Stages of Life to Regulate Serotonergic Function

ABSTRACT

By

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An intrinsic transcriptional cascade directs the generation of 5-HT synthesizing neurons in the embryonic brain and germ line targeting of individual factors in the cascade leads to profound behavioral alterations in adulthood including elevated anxiety, aggression and maternal neglect of infant care. However, the mechanisms through which factors in the cascade program adult 5-HT-modulated behaviors are not well understood. Expression of several cascade transcription factors persists into adulthood raises the possibility that they are required for proper maturation of the 5-HT system after 5-HT neuron birth and perhaps in adulthood to maintain 5-HT-modulated behavior. Here, we developed temporally controlled targeting approaches to investigate regulatory roles for one such cascade factor, Pet-1, after it has fulfilled its initial role in 5-HT neuron differentiation. Our findings show that Pet-1 continues to control key developmental events after 5-HT neuron differentiation including serotonergic target innervation patterns and 5HT neuron firing properties. In addition, specific ablation of Pet-1 in adult ascending 5-HT system leads to increased anxiety-like
behaviors in multiple behavioral paradigms, which demonstrates for the first time a requirement for adult Pet-1-directed serotonergic transcription in the preservation of normal anxiety responses.
CHAPTER I: INTRODUCTION

A. Discovery of serotonin and its functions in the periphery.

The chemical 5-Hydroxytryptamine was discovered by independent work from two research groups in the early twentieth century. In 1935, Italian scientist Dr. Vittorio Erspamer discovered a previously unknown amine from enterochromaffin cells of the gastroinetrinal mucosa. He then named the substance “enteramine” and found it was able to cause contraction of smooth muscles. The same substance was later isolated from blood serum by Rapport and colleagues at the Cleveland Clinic and named serotonin for its vasoconstrictor activity (Rapport et al., 1948). Further analysis determined its chemical structure as 5-hydroxytryptamine, abbreviated as 5-HT (Rapport, 1949).

A majority (95%) of the body’s 5-HT is synthesized in intestinal enterochromaffin cells (EC), where it regulates intestinal motility and secretion (Gershon and Tack, 2007). 5-HT made from the gastrointestinal (GI) tract outflows into the blood through which it reaches other major organs and regulates a wide variety of physiological processes including vascular tone, hemostasis, cardiac functions, the development of mammary gland, and reproductive activities (Berger et al., 2009).

B. Vertebrate central 5-HT system: anatomy and projections.
Shortly after its discovery in the periphery, 5-HT was found to exist in the brain (Twarog and Page, 1953). Since 5-HT synthesized outside the CNS cannot cross the brain-blood barrier, it is almost exclusively synthesized and secreted in the brain by 5-HT neurons located in the ventral hindbrain.

The vertebrate central 5-HT neurotransmitter system consists of a relatively small number of neurons clustered into nine phylogenetically conserved nuclei (B1-B9) in the pons and ventral medulla (Dahlstrom and Fuxe, 1964; Steinbusch, 1981). 5-HT neurons are born into two main clusters along the anterior and posterior axis in the developing hindbrain. The anterior cluster gives rise to 5-HT neuron groups in the pons, including dorsal (B6 and B7), median (B5 and B8) raphe nuclei, and the supralemniscal nucleus (B9), whereas the posterior cluster becomes medullary 5-HT neurons (B1-B3) whose cell bodies are located in raphe obscurus (B2), raphe pallidus (B1), and raphe magnus (B3). The “B” nomenclature was originally introduced by Dahlstrom and Fuxe mainly by positions of cell bodies in the adult brain. However, significant heterogeneity exists within each anatomically defined nucleus as 5-HT neurons from the same B nuclei often have distinct sizes (Jacobs and Azmitia, 1992; Tork, 1990), projection patterns (Vertes, 1991; Vertes et al., 1999), rhombomeric origins (Jensen et al., 2008), other co-transmitters (Belin et al., 1991; Glazer et al., 1981; Holtman et al., 1984; Varga et al., 2009), gene expression profile (Wylie et al., 2010), as well as electrophysiological properties (Beck et al., 2004).
Despite their small numbers, brain 5-HT neurons comprise the most expansive neurotransmitter system in the mammalian CNS (Jacobs and Azmitia, 1992). This complex system can be divided into two major groups based on their projection patterns: the ascending system (B4-B9) including 5-HT neurons in the dorsal, median raphe, and the supramniscal nuclei that mainly project to forebrain and midbrain targets; the descending system which contains medullary 5-HT neurons (B1-B3) that mostly extend their axons into the spinal cord (Tork, 1990). Ascending and descending 5-HT pathways innervate nearly all levels of the brain and the spinal cord through extensive axonal collateralization and modulate diverse behavioral and physiological processes including mood, cognition and appetite, to name just a few (Jacobs and Azmitia 1992).

C. 5-HT metabolism in the central nervous system.

1. Synthesis.

The biosynthesis of 5-HT consists of two enzymatic reactions. The first and the rate-limiting step involves the hydroxylation of amino acid L-tryptophan into 5-hydroxytryptophan (5-HP) by tryptophan hydroxylase (TPH). Aromatic amino acid decarboxylase (AADC) then catalyzes the decarboxylation of 5-HP and converts it into 5-HT (Jaeger et al., 1984). In addition to its presence in brain 5-HT neurons, AADC is also expressed in other monoamine neurons and involved in the biosynthesis of dopamine and norepinephrine.
Two forms of TPH encoded by distinct genes (Tph1 and Tph2) have been discovered in both rodents and primates (Walther and Bader, 2003; Zill et al., 2004). In the periphery, Tph1 is expressed in the EC cells whereas Tph2 is located in enteric neurons; both enzymes contribute to 5-HT synthesis outside the CNS. In the brain, Tph2 is selectively expressed within 5-HT neurons, while Tph1 is mostly found in the pineal gland where it participates in the biosynthesis of melatonin (Patel et al., 2004; Sakowski et al., 2006).

Germline ablation of Tph1 gene has no significant effect on the levels of 5-HT in the brain even though 5-HT in the duodenum and whole blood is almost completely abolished (Walther et al., 2003a). In contrast, Tph2 deficient mice lack most brain 5-HT, while its concentration in the periphery remains unchanged (Alenina et al., 2009; Gutknecht et al., 2008; Savelieva et al., 2008). These data suggest that Tph1 is responsible for most of the 5-HT production in the periphery, whereas 5-HT in the CNS is synthesized primarily through the activity of Tph2.

While its levels are very low and often undetectable in the adult raphe (Patel et al., 2004; Sakowski et al., 2006; Zhang et al., 2004), Tph1 expression has been detected in brain 5-HT neurons during the late developmental period (~P21) (Nakamura et al., 2006b). Its expression during this critical developmental period is required for the maturation of sensorimotor gating (Nakamura et al., 2006a). Furthermore, Tph1 expression is sensitive to environmental stressors and can be up-regulated after repeated immobilization stress (Abumaria et al., 2008; Chamas et al., 1999; Chamas et al., 2004). These findings suggest that
although Tph1 function may be dispensable for the synthesis and maintenance of 5-HT in the brain, it still plays important roles in the development and may serve as a homeostatic regulator for brain 5-HT tone in response to environmental challenges in adulthood.

2. Vesicular packing.

5-HT synthesized within 5-HT neurons and EC cells is actively packed into secretory vesicles before release through excytosis. This process is mediated by two mammalian vesicular monoamine transporters (Vmat1 and Vmat2) (Schuldiner et al., 1995). While Vmat1 resides in peripheral tissues, Vmat2 is predominantly expressed in neuronal cells and responsible for the package of 5-HT into synaptic vesicles in the brain (Adam et al., 2008; Gonzalez et al., 1994; Peter et al., 1995; Weihe et al., 1994).

Unlike Tph2, Vmat2 is present in other brain monoaminergic neurons. Mice lacking the Vmat2 gene have a severe loss of 5-HT, dopamine (DA), and norepinephrine (NE), as monoamines cannot be transported into synaptic vesicles resulting in rapid degradation in the cytoplasm (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997b). As a result, Vmat2 homozygous null mice (Vmat2 -/-) die within a few days after birth with enhanced cell death in the cerebral cortex (Stankovski et al., 2007). Mice heterozygous for the Vmat2 gene (Vmat2 +/-), which have a mild loss of brain monoamines, are viable and fertile, but display depressive-like behaviors in adulthood (Fukui et al., 2007).
3. Reuptake.

5-HT neurotransmission begins with its regulated release from presynaptic terminals and terminates following its re-uptake from the synaptic cleft by the 5-HT transporter (Sert or 5-HTT) (Blakely et al., 1991; Chang et al., 1996; Hoffman et al., 1991). Within the CNS, the Sert protein is primarily located on the neurites of presynaptic 5-HT neurons, except that it is also transiently expressed during development in other non-serotonergic cells including those in the thalamus, hypothalamus, limbic cortex, and retina (Cases et al., 1998; Hansson et al., 1998; Lebrand et al., 1996; Lebrand et al., 1998). The transient capture of 5-HT by other neurons may serve important developmental functions (Gaspar et al., 2003). First, 5-HT may be used as a “borrowed transmitter” as Vmat2 is often co-expressed with Sert in those non-serotonergic neurons, in which 5-HT is found in small dense-core vesicles and synaptic boutons (Dori et al., 1998). Second, the transient uptake may provide an extra mechanism for the clearance of extracellular 5-HT during development, when serotonergic innervation is not fully established. Finally, it is proposed that the uptake of 5-HT in distinct brain areas may help create a morphogenetic gradient for 5-HT signaling in the developing brain (Gaspar et al., 2003).

Sert activity determines the levels of extracellular 5-HT available for signaling and therefore impacts a wide variety of physiological processes and behaviors. Sert protein is a common target for many psychiatric drugs. Inhibiting its function by selective serotonin reuptake inhibitors (SSRIs) leads to enhanced
serotonergic neurotransmission and has been widely used to treat several neuropsychiatric disorders, although mechanisms underlying the therapeutic effects remain to be elucidated.

4. Degradation.

5-HT recycled from the extracellular spaces is finally degraded into 5-hydroxyindoleacetic acid (5-HIAA) within cells that express monoamine oxidases (Maos) (Weissbach et al., 1957). Two forms of Mao mRNAs (MaoA and MaoB) encoded by different genes have been discovered in the mammalian brain. MaoA and MaoB share high sequences identities, but differ in their expression pattern, inhibitor sensitivity, and substrate specificities (Edwards, 1980). MaoA is present in a variety of brain structures including major monoamine groups, cerebral cortex, hippocampus, thalamus, and hypothalamus, whereas the distribution of MaoB is limited to a few regions, such as the area postrema, the subfornical organ, and the dorsal raphe (Jahng et al., 1997). While both MaoA and MaoB are present in 5-HT neurons, MaoA has a higher affinity for 5-HT than MaoB, and is therefore believed to play a major role in 5-HT catabolism. A substantial increase in brain 5-HT levels (up to 9 fold in early postnatal stages) is only observed in MaoA, but not in MaoB knockout mice (Cases et al., 1995; Grimsby et al., 1997).

D. 5-HT signaling.

1. 5-HT receptors.
The broad physiological functions of 5-HT are mediated by a range of different 5-HT receptors. Fifteen genes encoding functional 5-HT receptors have been identified and are classified into seven main receptor subtypes based on their structural and sequence similarities (Hoyer et al., 2002). With the exception for 5-HT$_3$ receptor subtype, which encodes ligand-gated cation channels, all other 5-HT receptors (5-HT$_1$, 2, 4, 5, 6, & 7) are metabotropic G-protein-coupled receptors (GPCRs), which when activated, elicit diverse intracellular signaling events in virtually all major organ systems (Barnes and Sharp, 1999).

Members of the 5-HT$_1$ receptor subtype (5H$_{1A}$, 5H$_{1B}$, 5H$_{1D}$, 5H$_{1E}$ and 5H$_{1F}$) are coupled to the inhibition of adenylyl cyclase (AC) via pertussis-toxin-sensitive G$_{ai}$ and/or G$_{ao}$ proteins (De Vivo and Maayani, 1986). In addition to their expression in other cell types, 5-HT$_1$ receptors are also present in brain 5-HT neurons as autoreceptors. Among them, the 5-HT$_{1A}$ receptor is primarily located on the soma and dendrites of raphe 5-HT neurons and is functionally coupled to a G-protein inward rectifying potassium channel (GIRK) (Aghajanian and Lakoski, 1984). Activation of somatodendritic 5-HT$_{1A}$ receptor in 5-HT neurons does not preferentially inhibit cAMP pathways, but rather opens GIRK channels which leads to an outflow of positively charged potassium currents and inhibits the firing of 5-HT neurons (Bayliss et al., 1997). 5-HT neurons deficient in 5-HT$_{1A}$ receptors demonstrate increased spontaneous neuronal activities, suggesting that 5-HT$_{1A}$ controls normal serotonergic tone through negative feedback inhibition (Richardson-Jones et al., 2010). Another 5-HT$_1$ autoreceptor, 5-HT$_{1B}$, is located on axons and axon terminals (Sari, 2004). Unlike 5-HT$_{1A}$, 5-HT$_{1B}$ is not required
for the control of spontaneous firing of 5-HT neurons as DRN 5-HT neurons in 5-HT$_{1B}^{-/-}$ mice demonstrated normal spontaneous activities compared to the wildtype controls (Evrard et al., 1999). However, increased 5-HT release was observed in the midbrain and forebrain in 5-HT$_{1B}^{-/-}$ mice (Pineyro et al., 1995), suggesting 5-HT$_{1B}$ is involved in local inhibitory control of 5-HT release in target areas (Bockaert et al., 2006). The developmental onset of 5-HT$_{1A}$ and 5-HT$_{1B}$ autoreceptor expression is critical for the maturation of 5-HT neuron functional properties, including 5-HT neuron firing and transmitter release. Their mRNAs within 5-HT neurons are not detectable until several days after 5-HT neuron generation (Liu and Deneris unpublished data); however, the genetic mechanism that coordinates their induction in 5-HT neurons remains poorly understood. The 5-HT$_{1D}$ receptor shares high structural homology with 5-HT$_{1B}$ receptor and has been detected in both central and peripheral nervous systems (Bonaventure et al., 1998). Activation of 5-HT$_{1D}$ in the trigeminal ganglia may be involved in the suppression of meningeal neurogenic inflammation and trigeminal nociception, suggesting that the 5-HT$_{1D}$ receptor could be a potential drug target for treating migraines (May and Goadsby, 2001). The 5-HT$_{1E}$ receptor has been detected in both rodent and human brains (Miller and Teitler, 1992) and is negatively coupled to adenylate cyclase (Amlaiky et al., 1992). Its function however, remains poorly understood due to a lack of specific pharmacological blockers or mutant animal models (Lanfumey and Hamon, 2004). 5-HT$_{1F}$ mRNA has been detected in the dorsal raphe, hippocampus, cortex, striatum, thalamus, and hypothalamus (Lanfumey and Hamon, 2004). Activation of 5-HT$_{1F}$ also produces antimigraine
effect and its selective agonist LY334370 is able to inhibit c-fos expression in brain stem nociceptive neurons (Johnson et al., 1997).

The 5-HT2 receptor subtype consists of three GPCRs including 5-HT2A, 5-HT2B, and 5-HT2C receptors. Activation of the 5-HT2 receptors stimulates the hydrolysis of inositol phosphates through Gαq/11 pathway to elevate the levels of cytosolic Ca2+. The 5-HT2A receptor is expressed in a wide range of central and peripheral tissues. Activation of 5-HT2A receptor in the hypothalamic neurons promotes the secretion of several hormones including ACTH, corticosterone, oxytocin, and prolactin (Van de Kar et al., 2001). Furthermore, it has been proposed that common hallucinogens, such as lysergic acid diethylamine (LSD), produce behavioral syndromes via the actions of 5-HT2A receptors (Aghajanian and Marek, 1999). As a result, 5-HT2A receptor antagonists have been developed for the treatment of psychotic disorders such as schizophrenia. The 5-HT2B receptor mRNA has been found in several organs including the gut, kidney, heart, lung and the brain (Kursar et al., 1994). It plays a critical role in regulating cardiac structure and functions (Nebigil et al., 2000a). In the brain, 5-HT2B is expressed in a few regions that includes the cerebellum, lateral septum, hypothalamus, and amygdala (Duxon et al., 1997), where it regulates 5-HT release and mediates 3,4-methylenedioxymethamphetamine (MDMA) induced hyperlocomotion (Doly et al., 2008). 5-HT2C was originally identified in the choroid plexus and later found in many other regions in the brain (Pasqualetti et al., 1999). At least 14 different functional 5-HT2C receptor isoforms exist through adenine deaminase editing of the receptor pre-mRNA (Burns et al., 1997;
Fitzgerald et al., 1999). The adenosine-to-inosine editing is associated with reduced constitutive and agonist-stimulated activity and the amount of editing changes in response to acute stresses or chronic fluoxetine treatments (Englander et al., 2005). 5-HT$_{2C}$ receptor is involved in the regulation of several CNS functions. For example, 5-HT$_{2C}$ knockout mice demonstrate increased seizure susceptibility, cognitive defects, and sleep disturbances (Frank et al., 2002; Tecott et al., 1995). Moreover, these mice also exhibited increased food intake and obesity starting at six months of age. Remarkably, the rescue of 5-HT$_{2C}$ expression in hypothalamic pro-opiomelanocortin (POMC) neurons alone is sufficient to correct the hyperphagia and obesity phenotypes observed in 5-HT$_{2C}$-/- mice (Xu et al., 2008).

The 5-HT$_3$ receptor is a heteromer of five subunits (pentamer), forming non-selective cation channels that are permeable to sodium, potassium, and calcium ions. To date, five 5-HT$_3$ receptor subunit genes (5-HT$_{3A-E}$) have been identified. These subunits form receptors that mediate fast excitatory responses via transient inward depolarizing currents (Niesler et al., 2007). Fully functional 5-HT$_3$ receptors appear to require 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits in the assembly (Davies et al., 1999; Niesler et al., 2007), and receptors with different subunit compositions exhibit quantitatively different functional properties (Niesler et al., 2007). 5-HT$_3$ receptor subunits are detected in both peripheral and central nervous system (Costall and Naylor, 2004). In the periphery, these receptors are found in the sensory nerve endings, heart, and gastrointestinal tract, where they play important roles in modulating sensory processing, cardiovascular function,
as well as gastrointestinal motility and secretion (Thor et al., 2007). $5-HT_3$ receptors are also abundant in several CNS structures, including the nucleus tractus solitarius (NTS), the superficial layers of the dorsal horn, as well as several limbic regions, such as the amygdala and hippocampus (Laporte et al., 1992). Their CNS functions have been implicated in central regulation of nociception, emesis, and anxiety (Costall and Naylor, 2004; Kelley et al., 2003; Zeitz et al., 2002).

$5-HT_4$ receptors positively couple to adenylate cyclase to promote cAMP formation and are found in the gut, smooth muscle, cardiac atria, and the brain. These receptors are products of a large complex gene (at least 38 exons spanning over 700kb in humans) that gives rise to various receptor isoforms due to alternative splicing (Bockaert et al., 2004). In the CNS, these receptors are located in limbic structures, olfactory tubercle, septum, hippocampus, basal ganglia, and substantia nigra (Bockaert et al., 2004), where they modulate neuronal excitability by inhibiting potassium channels through PKA pathways (Ansanay et al., 1995). Moreover, many pharmacological studies demonstrate that activation of $5-HT_4$ receptors increases the release of neurotransmitters (acetylcholine, dopamine, and 5-HT) and enhances synaptic transmission (Consolo et al., 1994; Ge and Barnes, 1996; Porras et al., 2002).

Two $5-HT_5$ receptor genes ($5-HT_{5A}$ and $5-HT_{5B}$) have been identified in rodents (Matthes et al., 1993). $5-HT_{5A}$ is found in humans, whereas the putative human $5-HT_{5B}$ gene does not produce a functional protein due to the presence of stop codons in the coding sequences (Grailhe et al., 2001). Human $5-HT_{5A}$
The 5-HT

receptor may functionally couple to the Gαi/o pathway and has been shown to inhibit adenylate cyclase activity when transfected into HEK293 cells (Francken et al., 1998). Other studies have demonstrated that it couples to the inward rectifying potassium channel (GIRK) when expressed in Xenopus oocytes (Grailhe et al., 2001). The 5-HT

5A receptor demonstrates a broad expression domain in the CNS, whereas the expression of 5-HT

5B in the rodent brain is limited (Duncan et al., 2000; Geurts et al., 2002; Wang et al., 2000). While studies have demonstrated that 5-HT

5A knockout mice showed increased locomotion in a novel environment and blunted responses to the LSD-induced increase in locomotion, the underlying mechanisms as well as other physiological functions of 5-HT

5 receptors await further analysis (Grailhe et al., 1999).

The 5-HT

6 receptor is positively coupled to adenylyl cyclase via Gαs to promote intracellular levels of cAMP (Ruat et al., 1993). Its expression is almost exclusively limited to the CNS and has been found in the amygdala, nucleus accumbens, striatum, hippocampus, cortex, and olfactory tubercle (Ward et al., 1995). Both pharmacological and genetic association studies have indicated that the 5-HT

6 receptor may be involved in the regulation of learning and memory, affective, and feeding behaviors (Woolley et al., 2004). Nevertheless, 5-HT

6 receptor knockout mice do not demonstrate behavioral deficits in a wide range of tests except for reduced responses to the ataxic and sedative effects of ethanol (Bonasera et al., 2006).

The 5-HT

7 receptor is found both in the brain and peripheral tissues and positively modulates cAMP formation through Gαs pathways (Bard et al., 1993;
Alternative spicing of the 5-HT$_7$ receptor gene generates multiple isoforms (5-HT$_7$A-7D) that differ in their C-termini, among which the 5-HT$_7$A splice variant is the most abundant in both rat and humans (Heidmann et al., 1997). Nevertheless, all known 5-HT$_7$ receptor isoforms in the rat are functionally competent with no obvious differences in their respective pharmacology, adenylate cyclase coupling, or tissue distribution, although variations in receptor phosphorylation and intracellular localization may still exist (Heidmann et al., 1998). 5-HT$_7$ receptor mRNAs are found in the thalamus, hypothalamus, cerebral cortex, hippocampus, and amygdala (Neumaier et al., 2001). Expression of postsynaptic 5-HT$_7$ receptors in the suprachiasmatic nucleus (SCN) regulates the firing of local GABAergic neurons and may mediate the non-photic phase shifts of circadian rhythms by 5-HT receptor agonists (Kawahara et al., 1994; Lovenberg et al., 1993). Furthermore, 5-HT$_7$ receptor function in discrete nuclei of the hypothalamus may be needed for the regulation of body temperature, as 5-HT induced hypothermia is absent in 5-HT$_7$ receptor null mice (Gustafson et al., 1996; Hedlund et al., 2003). In addition, 5-HT$_7^{-/-}$ mice demonstrate antidepressant-like behaviors in the forced swim test suggesting that this receptor may also be involved in the modulation of emotional behaviors (Guscott et al., 2005).

2. Serotonylation.

Evidence supporting a receptor-independent mechanism for 5-HT signaling emerged recently from several studies in the periphery. This process,
coined serotonylation, was first described by Walther who found 5-HT covalently binds to small GTPases such as Rab4 and RhoA during platelet activation and aggregation, rendering these GTPases constitutively active (Walther et al., 2003b). The serotonylation process is catalyzed by platelet transglutaminase (TG) FXIIIa which cross-links 5-HT and the γ-carboximide group of glutamine residues on the GTPases (Dale et al., 2002). The physiological function of serotonylation was illustrated by the impaired hemostasis in Tph1−/− mice, in which the adhesion of 5-HT depleted platelets was reduced, resulting in prolonged bleeding times (Walther et al., 2003b). Furthermore, recent studies implicate a critical role for serotonylation in the pathogenesis and progression of pulmonary hypertension (PH). Increased serotonylation of RhoA and its elevated GTPase activity was detected in patients with idiopathic PH as well as in transgenic mice that developed spontaneous PH (Guilluy et al., 2009). Finally, serotonylation was also observed in pancreatic beta-cells where 5-HT regulates insulin secretion by serotonylation of small GTPases such as Rab3a and Rab27a (Paulmann et al., 2009). It remains to be determined whether serotonylation also occurs in brain 5-HT neurons. If it should exist, it would be important to study how such a process impacts normal function and physiology of 5-HT neurons.

E. Physiological functions of central 5-HT signaling.

1. Mood.
An increasing body of literature emphasizes a critical role of central 5-HT signaling in the establishment and maintenance of normal emotional behaviors. Dysfunction of 5-HT neurotransmission has been implicated in the pathophysiology of a variety of neuropsychiatric disorders, such as major depression (Arango et al., 2002), anxiety (Akimova et al., 2009), bipolar disorders (Mahmood and Silverstone, 2001), and schizophrenia (Abi-Dargham, 2007). Evidence that supports the neuromodulatory function of central 5-HT neurotransmission on emotional states is briefly discussed below.

a. Pharmacological studies.

A serendipitous finding in the 1950s that drugs (Tricyclics, TCAs and Mao inhibitors) enhancing 5-HT signaling have clinical effects in ameliorating depressive symptoms gave rise to the serotonin hypothesis of depression which suggests a causative role of 5-HT deficiency in the etiology of human depressive disorders (Coppen, 1967). This hypothesis gained further support following the discovery of several 5-HT abnormalities in certain depressed patients, including lower levels of plasma tryptophan (Cowen et al., 1989), reduced uptake of 5-HT by blood platelets (Coppen et al., 1978), and lower levels of CSF 5-HIAA (Mann et al., 1996), all of which are indicative of 5-HT deficits in the CNS. Together, these findings led to the development of SSRIs, such as fluoxetine (Prozac™) and sertraline (Zoloft™), which are now frequently prescribed for not only depression, but also for other psychiatric disorders including obsessive-
compulsive disorder (OCD), posttraumatic stress disorder (PTSD), and premenstrual dysphoric disorder (PMDD) (DSM IV).

However, mechanisms underlying the clinical effects of SSRI treatments remain to be elucidated. Although SSRI promptly increases extracellular levels of 5-HT in the brain, weeks of continuous treatment is necessary for the antidepressant effect to become fully manifested. Furthermore, while boosting the extracellular levels of 5-HT by inhibiting Sert function relieves neuropsychiatric symptoms, consistent evidence that supports a direct causal relationship between lower levels of 5-HT and depression or any other neuropsychiatric disease is still lacking (Cowen, 2008; Lacasse and Leo, 2005). Dietary depletion of tryptophan, the precursor of 5-HT, produces relapses in recovered depressed patients, but has no significant effects on healthy volunteers (Ruhe et al., 2007; Smith et al., 1997). Similar depletion studies using 5-HT neurotoxins (pCPA and 5, 7-DHT) were carried out in rodents, but generated mixed results (Harro et al., 2001; Lieben et al., 2006).

Much of the inconsistency arises not only from the methodological variability throughout a multitude of studies, but also from the inherent limitations of these pharmacological manipulations. For example, dietary tryptophan depletion in humans only produces a transient reduction of 5-HT (usually less than 1 day), while long-term effects of 5-HT deficiency on emotional states have not been evaluated (Bell et al., 2001). Additionally, non-specific activity and other side effects of 5-HT neurotoxins, such as pCPA and 5, 7-DHT, remain
problematic and can potentially confound the interpretation of the data (Breese and Cooper, 1975; Breese et al., 1978; Kronick et al., 1987). Finally, most of these studies are performed in adulthood, and therefore, fail to address the effects of embryonic 5-HT deficiency on adult behaviors.

b. Mutant mice models.

The requirement of 5-HT neurotransmission for the development of normal emotional behaviors is better demonstrated using transgenic mice models. Mice with targeted deletions in 5-HT synthesis enzymes (Tph1 or Tph2), transporter (Sert), and 5-HT receptors have recently been generated, which provide valuable animal models to study the impact of 5-HT neurotransmission on adult emotional behaviors.

Tph2 knockout mice lack the enzyme for 5-HT synthesis in the CNS and lose almost all 5-HT in the brain (Alenina et al., 2009; Gutknecht et al., 2008; Savelieva et al., 2008). Most of the 5-HT neurons are still present in the Tph2 knockout brain, even in the absence of 5-HT, suggesting that 5-HT synthesis is not required for the survival of 5-HT neurons (Gutknecht et al., 2008). Although growth retardation was observed in early postnatal life (growth returns to normal levels by 4 months of age), fertility and life span were both normal when compared to controls (Alenina et al., 2009). Nevertheless, pronounced aggressive behavior was found in both Tph2−/− mice and mutant mice with significantly reduced Tph2 activity (Alenina et al., 2009; Beaulieu et al., 2008). The increase in aggression in these mice may be associated with the aberrant
activation of GSK3 beta signaling in the brain and can be attenuated by genetically inactivating GSK3 beta activity (Beaulieu et al., 2008).

Mice lacking the Sert gene have a diminished ability to clear 5-HT from extracellular spaces, resulting in a 4-6 fold increase in forebrain extracellular 5-HT (Mathews et al., 2004). These mice demonstrated a reduction in the number of 5-HT neurons (Bengel et al., 1998; Lira et al., 2003), decreased neuronal firing in the dorsal raphe (Mannoury la Cour et al., 2001), and altered binding densities of several 5-HT receptors (Fabre et al., 2000; Li et al., 2000; Li et al., 2003). Nevertheless, Sert knockout mice were viable, fertile, and produced normal number of progeny when they were bred with each other (Bengel et al., 1998; Lira et al., 2003). Behavioral phenotyping of these mice revealed a “depression-like” response to inescapable stress compared to their littermate controls in both forced swim and tail suspension tests on a congenic 129S6 background (Holmes et al., 2002). Increased anxiety-like behaviors in the elevated plus maze, dark↔light exploration, and open field tests on the C57BL/6J background were also observed in Sert knockout mice (Holmes et al., 2003a; Holmes et al., 2003b). Conversely, mice that over-expressed a human Sert transgene demonstrated a decreased anxiety-like phenotype (Jennings et al., 2006).

Perturbations of adult emotional behaviors are often observed in mice with targeted disruptions in 5-HT receptors. For example, 5-HT$	extsubscript{1A}$ receptor knockout mice had been generated by several research groups and have consistently
exhibited elevated anxiety-like phenotype in the open-field, elevated zero/plus maze, and novel-object assays (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). These mice also demonstrated reduced immobility in forced-swim and tail-suspension tests, which resembles an “anti-depressive effect” in wildtype mice treated with antidepressants. Mice lacking the $5-HT_{1B}$ receptor did not show increased anxiety-like behaviors, but demonstrated augmented aggression in the resident-intruder assay (Ramboz et al., 1996; Saudou et al., 1994). Germline deletion of $5-HT_{2A}$ receptor in mice produced anxiolytic effect (less anxiety) on their performance in several anxiety-related tests and the restoration of function in the forebrain alone was sufficient to normalize the behavioral alterations to wildtype levels (Weisstaub et al., 2006). A similar anxiolytic phenotype was also observed in mice lacking the $5-HT_{2C}$ receptor, and this behavioral alteration may be associated with the blunted activation of corticotrophin-releasing hormone (CRH) neurons in the amygdala under stress (Heisler et al., 2007).

Each of the 5-HT receptors has its own spatiotemporal expression pattern throughout a variety of CNS structures. Activation of individual 5-HT receptors in different brain areas may subserve discrete functions and therefore, may have a varying impact on adult behaviors. To further understand 5-HT receptor functions underlying circuit modulation of emotional behaviors, it is necessary to develop conditional targeting strategies to study their spatiotemporal roles within distinct neuronal populations as well as contributions of individual brain circuits to the development and maintenance of normal emotional behaviors.
Disruption of other 5-HT metabolic genes also leads to alterations in animal emotional behaviors. Mice with reduced levels of \textit{Vmat2} (\textit{Vmat2}^{+/−}) exhibited psychomotor retardation, anhedonia, and enhanced behavioral despair to inescapable stress, all of which resembled core symptoms of human depressive disorder (Fukui et al., 2007). Germline deletion of \textit{MaoA} led to elevated aggression in adulthood (Cases et al., 1995), whereas knocking-out both \textit{MaoA} and \textit{MaoB} genes (with greater elevated monoamines than single knockouts) resulted in increased anxiety-like behaviors (Chen et al., 2004). Since these genes are also present in other monoaminergic neurons, the global deletion approach, while targeting serotonergic neurons, also affects the homeostasis of other monoamines in the brain. Therefore, the observed behavioral alterations may derive from not only a 5-HT deficit, but also deficiencies in other transmitter systems.

c. Functional genetic variance in humans.

With the rapid development of novel sequencing and genotyping techniques, much of the efforts have been spent in searching for genetic variants in 5-HT genes that are overrepresented in various neuropsychiatric disorders.

To date, dozens to hundreds of single-nucleotide polymorphisms (SNPs) have been identified within individual human genes involved in 5-HT metabolism and signaling (dbSNP Build 130 for human). Many of them have been associated with mood disorders, including depression, OCD, bipolar disorders (Cichon et al., 2008), panic disorders, and attention-deficit/hyperactivity disorder (ADHD)
(Baehne et al., 2009). While it remains largely unknown how individual SNPs or haplotypes increase an individual’s vulnerability to certain neuropsychiatric conditions, several polymorphisms have been shown to alter either serotonergic gene expression or their functions. For example, Zhang et al. identified a SNP in the coding region of human Tph2 gene (G1463A) that resulted in a 80% loss of its enzymatic activity and found that this SNP was enriched in a group of patients with unipolar major depression (Zhang et al., 2005). The SNP, however, is extremely rare and cannot be found within other larger groups of patients or controls (Glatt et al., 2005; Van Den Bogaert et al., 2005; Zhou et al., 2005).

Lemonde et al. described a C>G SNP (rs6295) in the upstream regulatory region of 5-HT1A gene which is associated with major depression and suicide. This G (-1019) allele fails to bind to the identified repressors Deaf-1 and Hes5, which is thought to cause the aberrant up-regulation of 5-HT1A receptor expression (Albert and Lemonde, 2004; Lemonde et al., 2003). Genetic variants altering Sert functions have been identified in both coding and non-coding regions of the human Sert gene and are associated with several neuropsychiatric disorders (Caspi et al., 2003; Collier et al., 1996; Kendler et al., 2005; Mann et al., 2000; Murphy and Lesch, 2008). Among them, Lesch et al. reported a functional repeat length variation in the serotonin-transporter-gene-linked polymorphic region (5-HTTLPR) (Lesch et al., 1996). The short (s) allele which contains fewer repeats than the long (l) allele has lower transcriptional efficiency of Sert gene resulting in less serotonin uptake activity and may be responsible for individual’s susceptibility to depression and suicide after adverse life events (Caspi et al.,
Moreover, Wendland et al. demonstrated a common C>T SNP (rs25532) located within 5-HTTLPR that was responsible for reduced expression of Sert and was associated with OCD in a large case-control group (Wendland et al., 2008). Altogether, these findings provide further evidence that dysfunction of 5-HT signaling is involved in the pathophysiology of several mood disorders.

2. Breathing.

The establishment and maintenance of normal respiratory pattern and stability during the perinatal period is essential for neonatal survival. The Pre-Bötzinger Complex (preBötC) serves as a central respiratory pattern generator in mammals, whose activity can be modulated by several neurotransmitters including 5-HT (Hilaire et al., 1997; Hilaire and Duron, 1999; Richerson, 2004; Thoby-Brisson et al., 2005). Recent studies using 5-HT deficient mice demonstrate that 5-HT signaling plays a critical role in the maturation of normal breathing behavior. For example, depressed breathing frequency and prolonged respiratory pauses were observed during early postnatal period (P0-P4.5) in Pet-1−/− neonates whose brain had only 10-15% of 5-HT compared to the wildtype levels (Erickson et al., 2007). More severe respiratory defects were observed in mice that had conditional deletion of Lmx1b in brain 5-HT neurons, resulting in an almost complete loss of brain 5-HT (Hodges et al., 2008) and in the Tph2−/− mice that was deprived of brain serotonin (Alenina et al., 2009). Altogether these findings support the concept that although 5-HT is not required for the generation of respiratory rhythm, it modulates the maturation and stability of respiratory
patterns by providing excitatory input to the central respiratory network (Pena
and Ramirez, 2002).

More importantly, increased early postnatal mortality was observed in all
three 5-HT deficient mice mentioned above (Alenina et al., 2009; Erickson et al.,
2007; Hodges et al., 2009). These findings suggest that the breathing deficits
caused by the central 5-HT deficiency may contribute to the increased risk for
early mortality and raise the possibility that similar defects may be involved in the
pathophysiology of Sudden Infant Death Syndrome (SIDS). Indeed, recent
studies have identified multiple brainstem serotonergic abnormalities, including
lower levels of medullary Tph, brain 5-HT, and 5-HT_{1A} receptor binding in infants
dying from SIDS (Duncan et al.; Paterson et al., 2006). These findings thus
support the hypothesis (Erickson et al., 2007) that altered development of 5-HT
system is responsible for SIDS vulnerability (Kinney et al., 2009).


5-HT neurons located in the ventral medulla project to superficial laminae
of the dorsal horn and have long been implicated in the descending modulation
of pain processing in the spinal cord (Suzuki et al., 2004). A large body of
evidence supports an anti-nociceptive role for 5-HT signaling in reducing the
response capacity of spinal nociceptive neurons (Millan, 2002). However, recent
findings suggest that descending 5-HT pathways, acting through distinct 5-HT
receptors (e.g. 5-HT_{3}), can also mediate pro-nociceptive effects (Lima and
Almeida, 2002; Oatway et al., 2004).
Traditional studies rely heavily on pharmacological manipulations to either block 5-HT receptor function in the spinal cord or deplete 5-HT in descending 5-HT pathways. These methods are often limited by nonspecific drug actions and therefore, there is difficulty in evaluating the specific impact of descending 5-HT input on pain processing, especially on different pain modalities. Using transgenic mice that have no brain serotonin neurons, Zhao et al. examined the behavioral responses to different types of nociceptive pains and found normal thermal pain thresholds and visceral pain responses in these mice, suggesting that central 5-HT signaling has minimal effect on thermal and visceral pain. However, these mice demonstrated less sensitivity to mechanical stimuli, but increased responses to inflammatory pain, which provided the evidence that descending 5-HT input facilitates mechanical sensitivity while inhibiting inflammatory pain \textit{in vivo} (Zhao et al., 2007a). Furthermore, a strong attenuation of morphine-induced analgesia was also observed in these mice, while no changes were found in morphine tolerance or morphine reward, suggesting that central 5-HT signaling mediates opioid induced analgesia (Zhao et al., 2007b).


The proper expression of maternal behavior is essential for reproductive fitness and offspring survival. However, the genetic mechanisms underlying the development and maturation of this behavior remain poorly understood. Human post partum depression (PPD) leads to poor quality of maternal care and can be alleviated by SSRI treatment, suggesting that 5-HT signaling may be involved in
the modulation of maternal behavior. Indeed, brain areas involved in the maternal care, including the medial preoptic area (MPA), are densely innervated by 5-HT axons (Simerly et al., 1984; van de Kar and Lorens, 1979). Using the Pet-1\(^{-/-}\) dams, Lerch-Haner et al. provided the first *in vivo* evidence that demonstrated an essential role for 5-HT signaling in the expression of normal maternal behavior (Lerch-Haner et al., 2008). Although normal numbers of pups were born to Pet-1\(^{-/-}\) dams with normal body weight upon birth, none of the pups survived in the following days due to a profound loss of maternal care from Pet-1\(^{-/-}\) dams, including failures of nest building, pup retrieval, and huddling (Lerch-Haner et al., 2008). In comparison, pups born to Pet-1\(^{-/-}\) dams survived when nurtured by wildtype dams in a cross-fostering experiment. Subsequent studies in Tph2\(^{-/-}\) dams revealed similar (but less severe) maternal defects and corroborated the findings in the Pet-1\(^{-/-}\) mice that central 5-HT signaling is needed for normal maternal behaviors (Alenina et al., 2009). It remains to be determined whether 5-HT signaling is only needed during development for the establishment of neural circuitry that give rise to normal maternal behaviors or whether a proper 5-HT tone is constantly needed in adulthood for this behavior.

**F. Developmental requirement of 5-HT signaling in shaping adult behaviors.**

The profound behavioral deficits observed in various 5-HT or 5-HT receptor-deficient mice suggest that 5-HT signaling plays a critical role in shaping
adult behaviors, especially emotion related behaviors. Nevertheless, how exactly perturbations in 5-HT signaling lead to alterations in adult emotional behaviors remain to be elucidated. Recent studies demonstrated a requirement of 5-HT signaling during development for the establishment of normal adult emotional behaviors (Leonardo and Hen, 2008). For example, 5-HT1A receptor knockout mice had been generated in several laboratories and consistently demonstrated increased anxiety-like phenotypes in several anxiety-related behavioral paradigms (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Using a spatiotemporal rescue experiment, Gross et al. showed that the correction of the behavioral deficit depended on the expression of this receptor during a critical developmental period (P5-P21), while its adult function was dispensable (Gross et al., 2002). Similarly, Ansorge et al. reported that early life (P4-P21) blockade of 5-HT transporter (Sert) function by SSRI (fluoxetine) treatment was sufficient to cause impaired responses to novelty and stress that were similar to those observed in the Sert−/− mice (Ansorge et al., 2004). Others have demonstrated that treatment with another SSRI (escitalopram) at the same developmental stage caused lasting depressive-like syndromes in adulthood (Popa et al., 2008). Altogether, these experiments highlight an essential role for normal 5-HT signaling during development in the maturation of neural circuits that give rise to adult emotional behaviors.

An increasing number of findings suggest that many human neuropsychiatric disorders, including aggression, anxiety, and schizophrenia have a developmental origin; that is; impaired brain development contributes to
the increased susceptibility and vulnerability to these diseases in adulthood. Numerous studies stress the importance of proper 5-HT signaling as a major regulator of brain development (Gaspar et al., 2003; Sodhi and Sanders-Bush, 2004). The regulatory effect of 5-HT signaling on embryonic development occurs even before the appearance of brain 5-HT neurons, when maternal derived 5-HT plays a crucial role in the early embryogenesis. Mice deficient in the Tph1 gene have no 5-HT in the periphery (Savelieva et al., 2008; Walther et al., 2003a), and serve as a good rodent model to evaluate the impact of maternal 5-HT on embryonic development. Despite their genotypes, embryos born to Tph1\(^{-/-}\) dams had significantly reduced sizes as compared to those born to Tph1\(^{+/+}\) or wildtype dams with abnormalities in the entire brain region, suggesting maternal source of 5-HT is necessary for normal CNS morphogenesis (Cote et al., 2007).

While the impact of maternal 5-HT on CNS development fades as the brain blood barrier develops, 5-HT in the CNS is then supplied by brain 5-HT neurons which are among the earliest neurons to be generated during development. 5-HT is released by growing serotonergic axons and diffuses through volume transmission before conventional synapses are established (Lidov and Molliver, 1982). Highest levels of 5-HT immunoreactivity are detected during neonatal stages (Whitaker-Azmitia, 2001), when several critical developmental events including axonal outgrowth, dendritic aborization, and synaptogenesis are rapidly developing. The impact of 5-HT signaling on brain development is probably best demonstrated using transgenic mice that have disturbed brain 5-HT homeostasis (Gaspar et al., 2003). Genetic targeting of
genes encoding 5-HT degradation (MaoA) or reuptake (Sert) leads to significantly elevated levels of extracellular 5-HT (Bengel et al., 1998; Cases et al., 1995). The excessive build-up of 5-HT acting through the 5-HT\(_{1B}\) receptor (Salichon et al., 2001) disrupts the clustering and segregation of thalamocortical axons, resulting in the loss of barrels that are normally formed in the somatosensory cortex (Cases et al., 1996; Persico et al., 2001). Similar defects were also observed in the visual system, where retinal axons failed to segregate into eye specific domains in both MaoA\(^{-/-}\) and Sert\(^{-/-}\) mice (Salichon et al., 2001; Upton et al., 1999). A critical role for 5-HT on cortical development was revealed by pharmacological manipulations at late embryonic stages (E12-17), when inhibition of 5-HT synthesis with chlorophenylalanine (pCPA) caused significant reduction in dendritic length and arborization in S1 layer III and layer V pyramidal neurons and delayed migration and incorporation of interneurons into cortical plate (CP) (Vitalis et al., 2007).

The broad regulatory functions of 5-HT on brain development are mediated by a repertoire of 5-HT receptors. For example, the 5-HT\(_{1A}\) receptor exhibits an early and dynamic expression pattern in the developing brain (Bonnin et al., 2006) and is highly expressed in the dentate gyrus. Stimulation of 5TH\(_{1A}\) receptors with its specific agonist 8-OH-DPAT promotes the generation of new granule neurons in fetal hippocampal cultures (Gould, 1999). Such pro-neurogenic effect persists into adulthood and underlies the actions of antidepressants on neurogenesis in adult dentate gyrus (Santarelli et al., 2003). Furthermore, activation of 5-HT\(_{1A}\) receptors facilitates dendritic differentiation, as
5-HT_{1A} agonist treatments increase the total dendritic length and branching points in fetal rat septal neurons (Riad et al., 1994) and prevent the decrease of dendritic spines in hippocampal neurons induced by neonatal 5-HT depletion (Yan et al., 1997). 5-HT_{1B} is present in a wide variety of brain structures during development (Bonnin et al., 2006). They are transiently expressed on developing thalamocortical axons (TCAs) (Bennett-Clarke et al., 1993). Activation of 5-HT_{1B/1D} receptors mediates the switches of TCAs' responsiveness to netrin-1 from attraction to repulsion in posterior dorsal thalamus neurons (Bonnin et al., 2007). Moreover, 5-HT_{1B} modulates the refinement of the TCAs' arbors in the somatosensory cortex. Excessive stimulation of the 5-HT_{1B} receptor in MaoA^{-/-} mice (Salichon et al., 2001) perturbs both the formation of collateral axon branches and the retraction of misplaced axon collaterals so that TCAs couldn't properly segregate into clusters, resulting in the altered formation of barrels (Rebsam et al., 2002). A similar role for 5-HT_{1B} in afferent patterning is also observed in the superior colliculus (SC), where it is required for the normal segregation of retinal ganglion cell (RGC) fibers (Upton et al., 2002).

Members of 5-HT_{2} receptors are expressed early during embryogenesis and display overlapping spatiotemporal patterns in a wide variety of embryonic tissues (Lauder et al., 2000). The early appearance of these receptors mediates the morphoregulatory function of 5-HT during early development, as exposure of mouse embryos to selective 5-HT_{2} receptors antagonist, ritanserin, induced morphological defects in the cephalic region, heart, and the neural tube (Choi et al., 1998; Choi et al., 1997). Among 5-HT_{2} receptors, 5-HT_{2A} receptors have
been demonstrated to promote the survival and differentiation of cortical glutamatergic neurons in several in vitro studies (Dooley et al., 1997; Lavdas et al., 1997). Furthermore, overstimulation of 5-HT$_{2A}$ receptors by either excessive 5-HT or 5-HT$_{2A}$ specific agonist treatment alters the dendritic morphology of phrenic motor neurons, suggesting that 5-HT$_{2A}$ may also be required for dendritic maturation (Bou-Flores et al., 2000). Nevertheless, loss of function studies do not support an essential requirement for 5-HT$_{2A}$ signaling during brain development, as no obvious CNS abnormalities have been found in the 5-HT$_{2A}$ null mice (Weisstaub et al., 2006). In contrast, genetic targeting of 5-HT$_{2B}$ receptors led to early lethality with severe developmental deficits, including cardiac malformation, ventricular hypoplasia, and defects in neural tube closure (Nebigil et al., 2000a). These findings are consistent with several in vitro observations that activation of 5-HT$_{2B}$ receptors promoted proliferation (Nebigil et al., 2000b), differentiation (Fiorica-Howells et al., 2000), and inhibited apoptosis (Nebigil et al., 2003). Another 5-HT$_2$ receptor, 5-HT$_{2C}$ has been implicated in the regulation of synaptic plasticity during late developmental stages. It demonstrated a transient columnar pattern within layer IV of the developing visual cortex, which coincided with the critical period for ocular dominance plasticity (Dyck and Cynader, 1993). Local infusion of 5-HT$_{2C}$ receptor blocker altered the long-term potentiation (LTP) (Edagawa et al., 2001) and reduced ocular dominance plasticity in kitten visual cortex (Gu and Singer, 1995; Wang et al., 1997a).
G. Molecular mechanisms governing 5-HT neuron generation.

The likelihood that altered serotonergic function during development contributes to the pathogenesis of mental illnesses has stimulated interest in understanding how 5-HT neurons are generated in the brain. Thanks to the rapid advancement in mouse molecular genetics and gene targeting approaches, our understanding about the molecular mechanisms that control the early differentiation and specification of brain 5-HT phenotype was significantly improved over the last ten years (Cordes, 2005; Gaspar et al., 2003; Scott and Deneris, 2005).

Prior to the generation of brain 5-HT neurons, early patterning of the neural tube is achieved by gradients of a combination of diffusing signaling molecules that define three-dimensional territories along the anterior-posterior and dorsal-ventral axes in the developing hindbrain. Each of these territories contains spatiotemporal cues so that progenitors at different positions obtain distinct molecular identities to generate neural diversity.

5-HT neurons in the mouse brain arise in two broad domains along the anterior and posterior axes of the developing hindbrain between E9.5 and E11.5 (Jacob et al., 2007; Pattyn et al., 2003a). The most anterior group of 5-HT neurons (derived from rhombomere 1, r1) is the first to be specified (around E9.5) and later gives rise to all 5HT neurons in the adult dorsal raphe (Jacob et al., 2007; Jensen et al., 2008). The positioning of this earliest born 5-HT group along the anterior-posterior axis is established by local signals (Fgf8) from the isthmic
organizer (IsO) that defines the boundary between the midbrain and the anterior hindbrain (Ye et al., 1998). All 5-HT neurons are specified caudally to the IsO in the hindbrain, whereas midbrain DA neurons arise rostral to the IsO (Ye et al., 1998). The position of IsO is determined by the expression of two homeodomain transcription factors Otx2 and Gbx2 (Broccoli et al., 1999; Millet et al., 1999). Otx2 is expressed directly rostral to the IsO, whereas Gbx2 protein is located immediately caudal to the IsO. Interestingly, in transgenic mice (En1+/Otx2), in which the Otx2 expression and IsO are shifted caudally, midbrain DA neuron population expands and appears in more caudal regions, whereas the number of hindbrain 5-HT neurons is reduced. In contrast, in Otx mutant mice (Otx1−/−, Otx2+/−), the population of 5-HT neurons enlarges and extends ectopically to more rostral regions (Brodski et al., 2003). Altogether, these findings demonstrate that the location and number of 5-HT neurons are determined by the position of the IsO.

The dorsal-ventral positioning of 5-HT neuron groups is established by gradients of sonic hedgehog (Shh) derived from the floor plate and notochord (Briscoe and Ericson, 1999). In transgenic mice that express a constitutively active form of Shh receptor Smoothened (Smo-M2), 5-HT precursors are dorsalized and 5-HT immunoreactive cells appear ectopically in the developing cerebellum (Hynes et al., 2000). Moreover, Shh signaling acting via two transcription factors Gli2 and Gli3 is required in the ventral hindbrain to induce 5-HT cell fate, as a 50% reduction in the number of 5-HT neurons is found in the Gli2 mutants (Matise et al., 1998). Graded Shh signaling further establishes
distinct progenitor domains in the ventral neural tube which later give rise to distinct neural identities. One such progenitor domain, marked by the expression of homeodomain factor *Nkx2.2*, contains a common pool of progenitors that sequentially generate visceral motor neurons (vMNs) and 5-HT neurons (Briscoe et al., 1999). *Nkx2.2*, together with *Nkx6.1*, acting downstream of *Shh* signaling, is essential for the induction of vMNs and 5-HT cell fates (Craven et al., 2004; Pattyn et al., 2003b). In the *Nkx2.2* mutant mice (*Nkx2.2<sup>-/-</sup>*), all 5-HT neurons are missing with the exception for those derived from r1 where *Nkx2.2* function may be compensated by a closely related gene *Nkx2.9* (Craven et al., 2004).

The neurogenesis in the *Nkx2.2* progenitor domain occurs in two waves. VMNs in r2-r7 are born first from E9.5 to E10.5, which is then followed by the generation of 5-HT neurons between E10.5 to E11.5 (Pattyn et al., 2003a). The forkhead transcription factor *Foa2* plays a critical role during the transition from vMNs to 5-HT neurogenesis (Jacob et al., 2007). During the period of vMNs generation, the expression of *Foa2* is suppressed by a vMNs fate determinant *Phox2b*, but later increases from E10.5, while 5-HT neurogenesis is ongoing. Expression of *Foa2* is sufficient to repress *Phox2b* induced vMNs generation in r2-r7, but not r4, where *Foa2* is not normally expressed in *Nkx2.2<sup>+</sup>* progenitors and vMNs neurogenesis persists into later stages (Jacob et al., 2007; Pattyn et al., 2003a).

In addition to *Nkx2.2* and *Foa2*, a bHLH transcription factor, *Mash1*, is also required for the generation of both central and periphery 5-HT neurons.
Mash1, acting as a proneural gene, is expressed within Nkx2.2+ progenitors. It is not required for the vMNs neurogenesis, but is essential for the generation of postmitotic 5HT precursors and the induction of several postmitotic 5-HT determinant factors, including Gata3, Lmx1b, and Pet-1. In the Mash1−/− mice, all 5HT neurons are lost in the developing hindbrain, whereas the expression of Nkx2.2, Phox2b, and vMNs differentiation are not perturbed (Pattyn et al., 2004).

Two Zinc finger transcription factors Gata2 and Gata3 demonstrate broad and overlapping expression domains during early development (Nardelli et al., 1999) and both factors play critical roles in the specification of the 5-HT phenotype. Gata2 acts downstream of Shh signaling and is sufficient to activate the expression of other 5-HT- fate determinants, including Gata3, Lmx1b, and Pet-1. Furthermore, mis-expression of Gata2 in chick neural tube induces ectopic 5-HT neurons along the dorsoventral axis in r1 at the expense of other neuronal cell fates (Craven et al., 2004). Gata2−/− mice die during early development (between E9.5-E10.5) from pan-hematopoietic failure, thus preventing the examination of 5-HT neuron generation in these mice (Tsai et al., 1994). Nevertheless, midbrain and hindbrain explants from E8 Gata2−/− mice failed to generate any 5-HT neurons suggesting an essential role for Gata2 in 5-HT fate specification (Craven et al., 2004). Expression of Gata3 immediately follows that of Gata2 and is present in postmitotic 5-HT precursors (van Doorninck et al., 1999). Loss of Gata3 function appears to have a bigger impact on the differentiation of caudal 5-HT neurons, as majority of 5-HT neurons in r5-
r7 are lost in Gata3 mutant mice, whereas a milder decrease (~30%) in 5-HT neuron numbers is observed in rostral 5-HT neuron groups (Pattyn et al., 2004).

In addition to the Gata factors, several other transcription factors are also present in postmitotic 5-HT neurons. The LIM homeodomain transcription factor Lmx1b is first detected within Nkx2.2+ precursors in regions lateral to the floor plate around E10.75. Lmx1b acts downstream of Shh/Nkx2.2 pathway and is absent in Nkx2.2-/- mice. Lmx1b plays an essential role in the generation of 5-HT neurons. Expression of several 5-HT neuron markers including Tph2, Sert and Vmat2 is completely lost in Lmx1b-/- mice. As a result, no 5-HT positive cells can be detected in the Lmx1b-/- brain (Cheng et al., 2003; Ding et al., 2003). Another zinc-finger gene, Insm1, is widely expressed (Farkas et al., 2008) in the CNS, including postmitotic 5-HT precursors. Mash1 directly transactivates the expression of Insm1 in the hindbrain and is necessary for its expression in 5-HT precursors. In Insm1-/- mice, the expression of several 5-HT fate determinants as well as 5-HT synthesis enzyme Tph2 is severely reduced at all axial levels, suggesting that Insm1 is a key component of the regulatory network that specifies 5-HT neuron cell fate (Jacob et al., 2009).

A novel ETS domain transcription factor, Pet-1 (PC12 ets factor 1), was first identified in a screen for ets gene expression in adrenal-medullary-tumor derived PC12 cells by Deneris and colleagues in 1998 (Fyodorov et al., 1998). RNase protection analyses revealed that Pet-1 had a restricted expression pattern, and its mRNA was primarily detected in neural tissues including the brain.
(Fyodorov et al., 1998). To understand its function in the central nervous system, Hendricks et al. used in situ hybridization to investigate the expression profile of Pet-1 in both developing and adult rat brain and found that Pet-1 mRNA in the brain was exclusively limited to 5-HT neurons throughout adulthood (Hendricks et al., 1999). Subsequent to its discovery in rat, homologues of Pet-1 were found in zebrafish, mouse, and human with similar restricted expression patterns in brain 5-HT neurons (Lillesaar et al., 2007; Peter et al., 1997; Pfaar et al., 2002). The human Pet-1 gene (also known as Fev) shares 96% protein sequence identity with mouse Pet-1 and is selectively expressed within raphe 5-HT neurons (Iyo et al., 2005; Maurer et al., 2004; Pfaar et al., 2002). Notably, expression of Fev in Pet-1−/− mice was sufficient to restore normal numbers of Tph+ cells, brain 5-HT levels, and behavioral deficits, suggesting that Fev is an orthologue of mouse Pet-1 and may perform similar functions in human 5-HT neurons.

The induction of Pet-1 expression in the developing hindbrain requires activities of several early cascade members, including Nkx2.2, Mash1, Foxa2, Gata2, and Insm1. Pet-1 expression is decreased in Nkx2.2−/− and Insm1−/− mice and is completely absent in Mash1−/−, Gata2−/−, and Foxa2 targeted mice (Cheng et al., 2003; Craven et al., 2004; Jacob et al., 2007; Jacob et al., 2009; Pattyn et al., 2004). More recently, Krueger et al. investigated the cis-regulatory control of Fev and identified phylogenetically-conserved consensus GATA factor binding sites upstream of the gene (Krueger and Deneris, 2008). Gata2 protein directly binds to these sites both in vitro and in vivo, and this interaction is required for serotonergic Fev transgene expression in the mouse embryonic hindbrain.
The earliest onset of Pet-1 expression occurred in the developing rat hindbrain around E12.5 in a small number of cells, just caudal to the mesencephalic flexure. The number of Pet-1-positive cells then gradually increases, and, by E13.0, Pet-1 mRNA appears on a sagittal section as a longitudinal band extending from rhombomere 1 (r1) to r3. A second Pet-1 expression domain, caudal to the pontine flexure (r5-r7), begins to appear around E14.0 (Hendricks et al., 1999). The two Pet-1 domains correspond precisely to the location of the developing rostral and caudal 5-HT neuron clusters and are separated by r4, where 5-HT neurons are never generated (Lidov and Molliver, 1982; Pattyn et al., 2003a; Wallace and Lauder, 1983).

The observation that Pet-1 expression precedes the first appearance of 5-HT immunoreactivity in the developing hindbrain raised the possibility that Pet-1 is induced at the terminal stage of 5-HT neuron differentiation for the transcription of key serotonergic genes. In support of this hypothesis, conserved consensus Pet-1 binding sites were identified near or within several 5-HT genes, including Tph, Sert, AADC, and 5-HT1A. Furthermore, these sites interact with Pet-1 protein in vitro and are capable of supporting transcriptional activation in luciferase reporter assays (Hendricks et al., 1999). To investigate the in vivo function of Pet-1, Hendricks et al. generated a Pet-1 knock-out mouse. Although normal numbers of 5-HT neuron precursors were present in the Pet-1−/− mice, over 70% of them failed to become 5-HT neurons, resulting in an 80% loss of 5-HT in the CNS (Hendricks et al., 2003). The expression levels of several 5-HT genes, including Tph2, AADC, Sert, and Vmat2, were also greatly reduced in Pet-1−/−.
brain at both embryonic and adult stages, indicating that Pet-1 plays a critical role in coordinating the induction of these genes (Hendricks et al., 2003). Moreover, using a BAC transgenic approach, Lerch-Haner et al. delivered human Fev transgene into Pet-1-/− mice resulting in various levels of Fev expression in multiple founder lines (Lerch-Haner et al., 2008). Interestingly, the levels of Tph2 and Sert mRNA in distinct founder lines were positively correlated with levels of Fev, suggesting that the in vivo expression of Tph2 and Sert was sensitive to the level of Fev-directed transcription (Lerch-Haner et al., 2008).

Despite a severe loss of brain 5-HT, Pet-1 null mice are still viable and fertile. Nevertheless, adulthood behavioral alterations were evident in these mice. Pet-1−/− males showed elevated inter-male aggression in the resident-intruder assay and demonstrated increased anxiety-like behaviors in the elevated plus maze and open field tests (Hendricks et al., 2003). Pet-1−/− females were not obviously anxious, but had a profound defect in specific maternal behaviors. For example, Pet-1−/− dams spent significantly less time crouching over their pups and never organized them into huddles. As a result, all pups born to Pet-1−/− dams died within days after birth. Altogether, these findings reveal, for the first time, that Pet-1-dependent serotonergic transcription is important for the normal expression of maternal nurturing behavior.

In addition to the observed behavioral deficits, alterations in other physiological processes were also found in Pet-1 null mice. For example, newborn Pet-1−/− neonates manifested significantly depressed breathing frequency with higher incidence of prolonged respiratory pauses relative to the
wildtype littermates (Erickson et al., 2007). While the breathing frequency normally stabilizes in wildtype animals by postnatal (P) day 4.5, irregular breathing patterns in Pet-1−/− pups persisted until P9.5, suggesting a developmental delay in the maturation of central respiratory rhythms. Importantly, environmental challenges such as pathogen exposure and hypoxia increased the magnitude of the breathing deficits in Pet-1−/− neonates, a phenomenon that was not observed in wildtype littermates, indicating that arrested 5-HT neuron development resulting from the loss of Pet-1 function contributed to the increased vulnerability to environment-induced respiratory failures (Erickson et al., 2007).

Cell bodies of 5-HT neurons have a scattered distribution in the adult hindbrain. Prior to the discovery of Pet-1, studies of the 5-HT system function were hindered by a lack of genetic approaches to selectively manipulate gene function in the 5-HT system. Traditional dietary or pharmacological manipulations have inherent limitations such as incomplete depletion, peripheral effects, and non-specific actions (Chang et al., 1979; Choi et al., 2004; Knuth and Etgen, 2004; Stokes et al., 2000). Moreover, these approaches are not able to alter gene expression specifically within 5-HT neurons.

To develop novel genetic tools to study gene function in the 5-HT system in vivo, Scott et al. investigated the genetic mechanism underlying Pet-1’s restricted expression pattern in the mouse brain and identified a 40 kb upstream enhancer sequence (ePet) that was sufficient to limit reporter transgene expression in brain 5-HT neurons (Scott et al., 2005a). Based on this observation, researchers used the 40 kb Pet-1 enhancer sequences to drive Cre recombinase
for 5-HT neuron specific gene ablations; Yfp for marking and purification of 5-HT neurons; and many other transgenes for numerous applications (Richardson-Jones et al., 2010; Scott et al., 2005b; Wylie et al., 2010). Notably, the cis-regulatory control region of human Fev was conserved with that of mouse Pet-1 and was sufficient to direct transgene expression specifically in mouse embryonic and adult 5-HT neurons (Krueger and Deneris, 2008). Furthermore, a shorter conserved DNA fragment immediately upstream of Pet-1 (1.8 kb) or Fev (2.2 kb) was also able to direct transgene expression in brain 5-HT neurons (Krueger and Deneris, 2008; Scott et al., 2005a), which can be useful for in vivo viral delivery of gene expression in mammalian 5-HT neurons. Altogether, the ePet transgenic approach revolutionized the way 5-HT neurons were studied and made significant contributions to the studies of serotonin neuron biology.

H. Aims of thesis.

Embryonic targeting of individual factors in the transcriptional cascade that governs 5-HT neuron differentiation causes alterations in adult emotional behaviors (Hendricks et al., 2003; Lerch-Haner et al., 2008). However, the mechanisms through which these factors regulate adult behavior remain poorly understood. While genetic targeting of individual factors normally causes a severe loss of brain 5-HT (Hendricks et al., 2003; Zhao et al., 2006), 5-HT depletion studies in both adult animals and humans suggest that many of the behavioral deficits cannot be explained by a simple loss of 5-HT (Booij et al.,
2003; Ruhe et al., 2007), suggesting other development defects are likely involved.

Our current understanding of the transcriptional mechanisms during neural development is largely limited to its function during early differentiation and cell fate specification (Lee and Pfaff, 2001), while its role in subsequent maturation events remains essentially unknown. The maturation of 5HT system spans a protracted period of time and is not fully complete until 3 weeks after birth. Many new factors need to be synthesized at distinct stages in order to support specialized serotonergic development so that complex transcriptional mechanisms are likely involved. Expression of several members of the transcription cascade persists following 5-HT neuron differentiation (Hendricks et al., 1999; Zhao et al., 2008; Zhao et al., 2006), suggesting that in addition to their early role in 5-HT fate specification, these transcription factors might still be required for other developmental events during 5-HT system maturation.

Immediately following the differentiation of 5-HT neurons, further maturation of the system depends on the successful achievements of a series of developmental milestones including, for example, proper routes of cell body migration, axonal innervations in the target areas, as well as acquisitions of 5-HT neuron specific firing properties. Therefore, it would be interesting to study whether continued functions of different transcription factors would be required for these later developmental events and if they would have common or distinct roles during 5-HT system maturation.
On the other hand, while it is well known that transcriptional programs are required to establish the serotonergic circuitry needed for adult emotional behaviors, it has yet to be determined whether such programs still operate in the adult brain to preserve the normal functions of 5-HT system and 5-HT modulated behaviors. The concept of the maintenance mechanism gains support from drug, toxin, and dietary perturbation studies in both adult animals and humans that highlight the importance of ongoing presynaptic serotonergic function in maintaining the behavioral integrity. To test this hypothesis, one would need to develop a novel genetic strategy that allows for gene inactivation only in brain 5-HT neurons in a temporally controlled manner so that the adult function of a transcription regulator can be studied after the normal development of 5-HT system is complete.

The major goal of this thesis project is to identify and characterize a persistent transcription program that is constantly needed to support 5-HT system maturation and adult maintenance and to illustrate the potential physiological impact of serotonergic transcription at different stages of life on adult emotional behaviors.
CHAPTER: II Pet-1/Fev-dependent transcription is required across different stages of life to regulate serotonergic function.

A. Summary

Transcriptional cascades are required for specification of 5-HT neurons and 5-HT modulated behaviors. Expression of several cascade factors extends across lifespan suggesting their control of behavior may not be temporally restricted to programming normal numbers of 5-HT neurons. We applied new mouse conditional targeting approaches to investigate ongoing requirements for Pet-1, a cascade factor required for the initiation of 5-HT synthesis but whose expression persists into adulthood. We found that Pet-1 was required after 5-HT neuron generation, for multiple steps in 5-HT neuron maturation including axonal innervation to the somatosensory cortex, firing properties, and 5-HT$_{1A}$ and 5-HT$_{1B}$ autoreceptor expression. Targeting Pet-1 in adult 5-HT neurons showed that it was still needed to preserve normal anxiety-related behaviors through direct autoregulated control of serotonergic gene expression. These findings show that Pet-1 is required across lifespan and therefore behavioral pathogenesis can result from both developmental and adult-onset alterations in serotonergic transcription.
B. Introduction

The brain serotonin (5-HT) transmitter system is a critical homeostatic modulator of neural circuits that shape emotional behaviors in response to stressors in the environment (Holmes, 2008). A widely discussed theory supported by a rich literature emphasizes the importance of 5-HT function for the maturation of neural circuits and the development of normal adult emotional behaviors (Ansorge et al., 2007). Altered serotonergic signaling and gene expression during embryonic development disrupts cortical dendritic arborization (Vitalis et al., 2007), differentiation, and patterning of forebrain afferents (Bonnin et al., 2007; Salichon et al., 2001). Other studies show that postnatal perturbation of the serotonergic system can cause emotional disorders in adult animals (Ansorge et al., 2004; Gross et al., 2002; Maciag et al., 2006). These findings together with correlative studies of serotonergic indices and gene variants in monkeys and humans support the idea that alterations in serotonergic function are involved in establishing vulnerability for several mood and neurological disorders (Holmes, 2008; Jans et al., 2007).

The likelihood that altered serotonergic function during development contributes to behavioral pathogenesis has stimulated interest in the genetic mechanisms that direct the formation of the 5-HT system (Scott and Deneris, 2005). A cascade (Figure 8) of transcriptional regulators has been identified that progressively restricts multi-potent neuronal progenitors to a 5-HT neuron fate in the embryonic ventral hindbrain (Cordes, 2005). Gene targeting of factors in the
cascade causes alterations in adult 5-HT-modulated emotional responses (Hendricks et al., 2003), thus providing a link between transcriptional regulation of 5-HT neuron birth and adult behavior. Nevertheless, the mechanisms through which transcription factors in the cascade regulate behavior are poorly understood and may not be, simply, the result of programming normal 5-HT neuron numbers and 5-HT levels. For example, although all of the factors known to compose the cascade have been shown to be necessary for the initiation of 5-HT synthesis at the cell fate specification stage, transcriptional control of subsequent steps in 5-HT system maturation may also be crucial for programming normal 5-HT-modulated behaviors. However, whether or not factors in the cascade are responsible for additional transcriptional events in the maturation of the system has not been investigated. Furthermore, it is not known whether the critical period for transcription directed by these developmental determinants extends into adulthood to regulate maintenance of 5-HT signaling and preserve behavioral integrity. The concept of a transcriptional maintenance mechanism is potentially of critical importance in understanding the regulation of behavioral and psychiatric pathogenesis as drug, toxin, and dietary perturbation studies in adults including humans demonstrate the importance of ongoing presynaptic serotonergic function in emotional and behavioral processing (Jans et al., 2007).

Expression of the rodent Pet-1 ETS domain transcription factor (human orthologue, Fev) is restricted in the CNS to 5-HT neurons and is induced in postmitotic precursors just prior to the initiation of 5-HT synthesis in the ventral
hindbrain (Hendricks et al., 1999). *Pet-1* plays a pivotal role in the cascade through its coordinate induction of the enzymatic pathway responsible for 5-HT synthesis in immature postmitotic precursors (Hendricks et al., 2003). Interestingly, *Pet-1* expression is never extinguished and appears to continue undiminished in all adult 5-HT neurons (Hendricks et al., 1999). This persistent expression suggests that *Pet-1* may be required for events in 5-HT neuron maturation that occur subsequent to their specification and possibly in adulthood for transcriptional maintenance of the 5-HT system. Here, we applied new 5-HT neuron-specific and temporally-restricted conditional targeting approaches to investigate requirements for continued *Pet-1*-dependent transcription in the 5-HT system.
C. Results.

**Conditional deletion of Pet-1 after 5-HT neuron generation**

To investigate Pet-1 function after its initial role in 5-HT neuron generation, we inserted two loxP sites in introns on each side of exon 3, which encodes most of the Pet-1 protein coding sequences including the ETS DNA binding domain (Fig. 1a). In situ hybridization (Fig. 1b, c) and quantitative reverse transcriptase PCR (RT-qPCR) (Figure 9) indicated that Pet-1 expression was indistinguishable in mice carrying either one copy of the wildtype (+) or floxed (fl) Pet-1 allele together with a constitutive null allele. Excision of exon 3 generated a deleted Pet-1 allele (∆) in which all protein coding sequences except those encoding 42 amino acids at the N terminus were eliminated (Fig. 1a). In situ hybridization showed that Pet-1 expression was absent in mice heterozygous for the deleted and null Pet-1 alleles (Fig. 1d). Furthermore, normal numbers of TPH+ neurons were present in Pet-1fl/fl mice but not in Pet-1fl/fl mice (Figure 9). Thus, the unrecombined floxed allele is functionally equivalent to the wildtype allele and the conditionally deleted Pet-1 allele is functionally equivalent to the Pet-1 constitutive null allele.

We crossed floxed Pet-1 mice with ePet::Cre transgenic mice (Guscott et al., 2005), which express Cre recombinase only in postmitotic 5-HT neurons, to generate Pet-1 early conditional knockout mice (Pet-1fl/fl, ePet::Cre, designated Pet-1εCKO). Nearly all 5-HT neurons derived from progenitors in rhombomere 1 and 2 are born by embryonic days 10 and 11 respectively (Jacob et al., 2007;
Pattyn et al., 2003a). However, we showed previously (Zhao et al., 2006) that reduced serotonergic gene expression is not evident with this ePet::Cre transgene until about E12.5. Thus, Pet-1 expression should be maintained in Pet-1eCKO mice for about 2 days after fulfilling its early role in 5-HT neuron generation. Based on this reasoning, we predicted that normal numbers of 5-HT neurons would be generated in Pet-1eCKO mice.

Consistent with our expectation, we found that expression of Pet-1 and other 5-HT neuron markers including tryptophan hydroxylase 2 (Tph2), serotonin transporter (Sert or Slc6a4) and another transcription factor Gata3 was indistinguishable between Pet-1eCKO and the wild-type controls in the anterior hindbrain at E11.5 (Fig. 1f, h, and Figure 10). Furthermore, immunohistochemistry, using anti-sera against 5-HT showed that normal numbers of 5-HT+ neurons were generated in Pet-1eCKO mice as compared with the controls (Fig. 1j, k). In comparison, very few 5-HT+ cells could be detected in Pet-1−/− mice at this stage (Fig. 1l). Reduced Pet-1 transcripts were not observed until E12.5 (Fig. 1g), while the expression of Gata3 was not altered (Fig. 1i).

Concomitant with the conditional deletion of Pet-1 at E12.5 was diminished expression of Tph2, Sert and 5-HT in Pet-1eCKO mice (Figure 10). Importantly, Pet-1 is not required for 5-HT neuron survival (Krueger and Deneris, 2008), and all Pet-1 deficient cells were present in the Pet-1eCKO brain through adulthood (Figure 11). These findings indicate that Pet-1eCKO mice provide a means to investigate Pet-1 function in 5-HT system maturation after it has fulfilled its initial role in 5-HT neuron generation.
Continued *Pet-1* function controls serotonergic innervation

Immediately following the birth of 5-HT neurons, maturation of the 5-HT system depends on proper routes of cell body migration, axon pathfinding, and innervation in terminal fields (Lidov and Molliver, 1982). To facilitate an investigation of *Pet-1* function in these maturation events, we used Cre-mediated activation of the $R26R^{Yfp}$ (Srinivas et al., 2001) reporter allele to permanently mark *Pet-1* deficient 5-HT neurons in $Pet-1^{eCKO}$ mice. *Pet-1* deletion was spared in a small subset (~15%) of 5-HT neurons in $Pet-1^{eCKO}$ mice (Figure 11) and therefore *Pet-1* deficient Tph$^-$ cells were situated side by side with untargeted Tph$^+$ 5-HT neurons (Fig. 2a, arrows). Examination of R26R-Yfp$^+$ cells in $Pet-1^{eCKO}$ mice showed these *Pet-1* deficient 5-HT neurons extended axons from their cell bodies, similar to intermingled wildtype Tph$^+$ cells, and therefore *Pet-1* was not essential for proximal axonal outgrowth (Fig. 2a, arrowheads). Indeed, Yfp immunostaining in $Pet-1^{eCKO}$ mice revealed that axon bundles from *Pet-1* deficient 5-HT neurons crossed the midbrain-hindbrain boundary and entered the midbrain at E14.5 (Fig. 2b).

To determine whether these axons could properly reach their forebrain targets, we performed a retrograde tracing experiment by injecting tracer (Texas Red-conjugated dextran, with a molecular mass of 3 kDa) into the somatosensory cortex, which receives serotonergic afferents mainly from 5-HT neurons located in the dorsal raphe nucleus (DRN) (Fig. 2c) (Vertes et al., 1999). Stereotaxic injection into 3 week-old wildtype and $Pet-1^{eCKO}$ mice resulted in the
retrograde labeling of both 5-HT and non-5-HT neurons in the DRN (Fig. 2d, e, f). Tracer intensity observed in Pet-1 deficient 5-HT neurons was comparable to that in other retrogradely labeled cells, suggesting Pet-1-deficient cells were able to retrogradely transport tracer (Fig. 2f). Interestingly, we found a significant decrease in the total number of tracer labeled cells across the entire DRN in Pet-1eCKO mice (Fig. 2g, h). In wildtype mice, the majority of retrogradely labeled cells were 5-HT neurons (Fig. 2i, k). In contrast, far fewer Pet-1 deficient Yfp+ 5-HT neurons were labeled with similarly performed tracer injections (Fig. 2j, l), indicating significantly disrupted serotonergic innervation of the somatosensory cortex from DRN 5-HT neurons in the Pet-1eCKO brain.

Continued Pet-1 function controls autoreceptor pathways

Another critical event in the maturation of brain 5-HT neuron function is the acquisition of 5-HT neuron-specific firing properties (Le Francois et al., 2008). To study whether continued Pet-1 function is required for normal firing of 5-HT neurons, we analyzed Pet-1 deficient R26R-Yfp+ cells in postnatal brain slices from Pet-1eCKO mice with whole cell recordings under current clamp conditions. Compared with the aged-matched controls, many Pet-1 deficient cells demonstrated increased spontaneous firing of action potentials (Fig. 3a, b, c). The increased excitability could be due to alterations in 5-HT1A autoreceptor signaling that normally inhibits the firing of 5-HT neurons through negative feedback inhibition (Fornal et al., 1994). As previously described (Bayliss et al.,
1997), activation of 5-HT$_{1A}$ receptor with its specific agonist 8-OH-DPAT elicited strong inwardly rectifying potassium currents in control Yfp$^+$ 5-HT neurons under voltage clamp (Fig. 3d, f). In striking contrast, 8-OH-DPAT at either low (1 µM) or high (10 µM) concentrations did not elicit a change in baseline currents in Pet-1 deficient Yfp$^+$ 5-HT neurons (Fig. 3e, f). To investigate the mechanism that accounts for the loss of 8-OH-DPAT responses, we used in situ hybridization to examine the expression of the 5-HT$_{1A}$ receptor and found greatly reduced levels of 5-HT$_{1A}$ receptor mRNA in the Pet-1$^e$CKO DRN (Fig. 3g, h).

A second prominent serotonergic autoreceptor that functions in serotonergic presynaptic terminals for regulation of 5-HT release is the 5-HT$_{1B}$ receptor (Sari, 2004). In situ hybridization in Pet-1$^e$CKO mice revealed that Pet-1 was also required for expression of the 5-HT$_{1B}$ gene (Fig. 3i, j). The residual expression of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors mRNAs is most likely from the remaining untargeted 5-HT neurons in Pet-1$^e$CKO mice since the expression of the two autoreceptors in the DRN was almost completely abolished in the Pet-1$^{-/-}$ mice (Figure 12). Our in situ hybridization studies (data not shown) indicated that the onset of 5-HT$_{1A}$ and 5-HT$_{1B}$ autoreceptor gene expression occurs after embryonic day 14 in nearly all 5-HT neurons, which is consistent with their onset in the forebrain at E14.5 (Bonnin et al., 2006). Thus, our findings show that ongoing Pet-1 expression is required, well after it has completed its role in the initiation of 5-HT synthesis in immature precursors, for maturation of essential serotonergic autoreceptor characteristics that controls firing patterns and transmitter release.
**Gata3 is not required for 5-HT_{1A} autoreceptor responses**

We investigated whether the establishment of normal 5-HT neuron firing properties requires parallel ongoing activity of other serotonergic developmental control genes or *Pet-1* plays a special role in this event. Germ line targeting of the zinc finger transcription factors, *Gata2* and *Gata3*, has demonstrated requirements for both factors in 5-HT neuron differentiation (Craven et al., 2004). We found that Gata2 protein expression began to dramatically decline in differentiated 5-HT neurons at E12.5 and was not detectable at E14.5. In contrast, Gata3 expression persisted in all 5-HT neurons through adulthood (**Figure 13**).

To compare the role of *Gata3* in differentiated 5-HT neurons with that of *Pet-1*, we crossed floxed *Gata3* mice (Zhu et al., 2004) with ePet::Cre mice to generate *Gata3* conditional knockout mice (*Gata3^{fl/fl}, ePet::Cre*, designated as *Gata3^{eCKO}*). Like *Pet-1*, *Gata3* was not required for cell survival as normal numbers of *Gata3* deficient 5-HT neurons survived in the adult brain (**Fig. 4a**). However, we did observe a substantial reduction in the number of Tph immunoreactive cells (**Fig. 4b, d**), 5-HT levels (**Fig. 4f, g**) and expression of several other 5-HT genes (**Fig. 4e**) in the *Gata3^{eCKO}* DRN. Persistent *Gata3* and *Pet-1* expression appears to be maintained by independent regulatory pathways, as neither of them was required for each other’s expression (**Fig. 4c and Fig.1i**). These findings suggest that, *Gata3* and *Pet-1* function in parallel pathways to coordinate the expression of normal levels of serotonergic gene expression and 5-HT in the brain.
Although *Gata3* and *Pet-1* share several common transcriptional targets, *Gata3*-deficient 5-HT neurons demonstrated normal 5-HT$_{1A}$ expression (Fig. 4e) indicating distinct requirements for *Pet-1* and *Gata3* in the regulation of gene expression in 5-HT neurons. These findings further suggest that *Gata3* may not be required for serotonergic firing characteristics. Indeed, whole cell recordings of slices from *Gata3$^{eCKO}$* mice revealed firing properties and 5-HT$_{1A}$ agonist responses typical of wildtype 5-HT neurons (Fig. 4h, i).

**Targeting of *Pet-1* in the adult ascending 5-HT system**

Having demonstrated that ongoing *Pet-1* function is needed for multiple steps in 5-HT system maturation, we sought to determine whether a *Pet-1*-dependent transcriptional program still operates in adulthood to support serotonergic function and 5-HT modulated behaviors. Thus, we used *Pet-1* regulatory elements to generate a transgene that directed tamoxifen (TM) inducible CreERT$^{T2}$ (Feil et al., 1997) expression specifically in brain 5-HT neurons. We identified several different founder lines with inducible Cre activity by injecting pregnant females with a single dose of TM (150 µg/g, intraperitoneal, i.p.) at E11.5 and scoring at E16.5 for Cre-activated βgal expression from the R26R$^{βgal}$ allele (Figure 14). Eight week-old Cre reporter mice (R26R$^{βgal+}$, ePet::CreERT$^{T2}$) were then given a single daily dose of TM or vehicle for five consecutive days. Expression of βgal, 5 or 30 days after the last TM treatment, was detected in the majority of 5-HT neurons in the DRN and median raphe nucleus (MRN).
Recombination was strictly dependent upon TM treatment as no Cre activity could be detected in the absence of TM. Importantly, Cre activity was not detected in other regions of the CNS after TM injections (Figure 14). In one of the transgenic lines, designated ePet::CreERT2^T2ascend, we found differential targeting efficacies following adult TM treatments between 5-HT neuron raphe nuclei that give rise to ascending and descending 5-HT systems (Fig. 5b, c). Double-labeling to detect β-galactosidase and Tph revealed TM-activated Cre activity in up to 80% of 5-HT neurons in the DRN (B6, B7), MRN (B5, B8), and B9 nucleus. In contrast, far fewer 5-HT neurons were β-galactosidase+ in the medullary nuclei (B1-B3) in response to TM treatments (Fig. 5d, e).

To determine the efficacy of the ePet::CreERT2^T2ascend line for excision of Pet-1 in Pet-1\textsuperscript{fl/fl} mice, we crossed ePet::CreERT2^T2ascend, Pet-1\textsuperscript{−/−} and Pet-1\textsuperscript{fl/fl} mice to generate Pet-1\textsuperscript{fl/fl}; ePet::CreERT2^T2ascend mice, designated as Pet-1\textsuperscript{aCKO}. Six to eight week-old Pet-1\textsuperscript{aCKO} mice were given single daily TM treatments for 5 days and sacrificed either 5 or 30 days after TM treatments for evaluation of Pet-1 expression in the adult brain. Using in situ hybridization, we observed that TM treatment abolished the majority of Pet-1 expression in adult DRN (B6, B7 nuclei), MRN (B5, B8 nuclei) and the B9 group of 5-HT neurons (Fig. 5f, g). In contrast, Pet-1 mRNA was not decreased in the B1-B3 groups of 5-HT neurons in the ventral medulla (Fig. 5h, i). The reduction of Pet-1 in Pet-1\textsuperscript{aCKO(+TM)} mice was further quantified with RT-qPCR, which revealed a more than 70% loss of Pet-1 mRNA in pontine tissue containing B5-B9 serotonergic nuclei, but no significant change in tissue containing the medullary B1-B3 nuclei (Fig. 5j). The loss of Pet-
mRNA in B5-B9 nuclei demonstrated that ePet::CreER°T²ascend could be used for highly reproducible and stage-specific disruption of Pet-1 expression in adult ascending 5-HT neurons.

Adult Pet-1 is required for normal anxiety-like behaviors

Germ line targeting of Pet-1 results in increased anxiety-like behaviors in the adult. It remains unknown, however, whether Pet-1-dependent transcription is only needed during development or is also required in adulthood to modulate normal anxiety responses. To address this question, we treated 6-8 week old Pet-1\textsuperscript{aCKO} mice with TM to delete Pet-1 in the ascending 5-HT system. The impact of adult Pet-1 deletion on anxiety-related behaviors was then investigated 4 weeks after the last TM treatment.

Using similar dosages, it was recently demonstrated in mice that multiple tamoxifen treatments have no effect in several anxiety-related behavioral tests (Vogt et al., 2008). We verified this finding on a separate cohort of wild type mice treated with either vehicle or TM (Figure 15). We then examined TM-treated control and Pet-1\textsuperscript{aCKO} mice with the elevated plus maze test and found that Pet-1\textsuperscript{aCKO+TM} mice spent significantly less time and initiated fewer entries into the open unprotected arms of the maze (Fig. 6a, e). In addition, Pet-1\textsuperscript{aCKO} mice spent less time in the hub area but significantly more time in the closed arm (Fig. 6b, c). No differences between genotypes were found in overall explorative
activities determined as number of total open/closed arm entrances (Fig. 6d). The increased avoidance of the aversive properties of height and openness suggests an augmented anxiety-like behavior in Pet-1aCKO(+TM) mice. To further study this behavior, we tested the same mice in the light↔dark exploration paradigm, which presents the mice with a similar conflict between the curiosity to explore a novel environment versus the aversive features of a brightly illuminated open field. As compared to TM-treated littermate controls, Pet-1aCKO(+TM) mice spent significantly more time in the dark chamber (Fig. 6f, g) with a trend of reduced latency to enter the dark area from the beginning of the test (Fig. 6h). The increased time in the dark area and thus avoidance of the bright open areas further supported an increased anxiety-like behavior in Pet-1aCKO(+TM) mice. Finally, in further support of increased anxiety-like behavior, Pet-1aCKO(+TM) mice spent significantly less time (Fig. 6i) than controls in the center of an open field. Importantly, a second independent cohort of control and Pet-1aCKO mice showed similar significant increases in all three tests of anxiety-like behaviors in Pet-1aCKO(+TM) mice (Figure 16). Furthermore, the increased anxiety-like behavior seen in all three tests depended on reduced Pet-1 levels as no differences were observed in a separate cohort of Pet-1aCKO mice treated with vehicle (Figure 17). Overall growth measured as body weight was similar in control and Pet-1aCKO mice following TM treatments (data not shown).

Adult Pet-1 is required for serotonergic gene expression
The altered anxiety-like behaviors in Pet-1aCKO(+TM) mice demonstrate that Pet-1 is required in adulthood to maintain serotonergic function. To explore the mechanisms underlying the alterations in serotonergic function in Pet-1aCKO(+TM) mice, we first measured levels of brain 5-HT and 5-HIAA in Pet-1aCKO and control mice sacrificed 5 days after TM treatments. HPLC analysis demonstrated that both 5-HT and 5-HIAA were significantly reduced in the forebrain of Pet-1aCKO(+TM) mice (Fig. 7a, b), but as predicted, 5-HT levels were not altered in the spinal cord (data not shown), which is innervated by the descending 5-HT system. Western blotting using a monoclonal antibody against both Tph1 and Tph2 indicated that Tph protein levels were reduced by about 50% in Pet-1aCKO(+TM) mice relative to controls (Fig. 7c, d). Consistent with these findings, we found a comparable decrease in Tph2 mRNA levels in Pet-1aCKO(+TM) mice as early as five days after the last TM treatment (Fig. 7e, f). Further, Tph2 mRNA was still decreased 30 days after the last treatment and we did not find a compensatory increase in Tph1 expression with the loss of Tph2 (Fig. 7e). Together these findings indicate that Pet-1 is required in adult 5-HT neurons to regulate 5-HT synthesis through maintenance of Tph2 expression.

To determine whether disruption of the 5-HT1A autoreceptor pathway might have contributed to the abnormal anxiety-like behavior, we performed whole cell recordings in Pet-1aCKO(+TM) slices but found that Pet-1 was no longer required in the adult brain for spontaneous firing, autoreceptor agonist responses (Fig. 7j, k), or 5-HT1A gene expression (Fig. 7e). To explore other potential deficits beyond the loss of 5-HT, we analyzed the set of additional genes that are
known to depend on Pet-1 in the embryonic hindbrain as well as other genes that are important for 5-HT synthesis and metabolism (Fig. 7e, f–i). Sert and the vesicular glutamate transporter 3 (Vglut3 or Slc17a8) were also significantly reduced (Fig. 7e, h), but for Vglut3, decreased expression was not observed until 30 days after TM treatments (Fig. 7e). In contrast to the dramatic decreases in Tph2, Sert, and Vglut3 gene expression, Pet-1 was no longer required in adulthood to maintain mRNA levels for Aadc and Vmat2 whose embryonic expression is dependent on Pet-1 function (Fig. 7e).

**Direct autoregulation of serotonergic gene expression**

The studies presented so far do not distinguish direct vs. indirect transcriptional control of target genes by Pet-1. We previously identified a consensus Pet-1 binding sequence GGAART upstream of Sert and showed that Pet-1 protein interacted with these sites in vitro (Hendricks et al., 1999). Further analyses identified conserved putative Pet-1 ETS binding sites in highly conserved upstream regulatory regions of Tph2 and Sert genes (Figure 18). To probe the mechanism through which Pet-1 regulates Tph2 and Sert in 5-HT neurons, we investigated the possibility that Pet-1 directly regulates their transcription through interactions with conserved upstream regulatory elements.

Because we have been unsuccessful in preparing a suitable Pet-1 antibody for chromatin immunoprecipitation (ChIP), we generated a new
transgenic mouse line that expresses a myc-epitope tagged Pet-1 protein in the
Pet-1−/− brain with Pet-1 promoter/enhancer sequences (Guscott et al., 2005).
We found that expression of the ePet::mycPet-1 transgene recapitulated
endogenous Pet-1 expression in both developing and adult hindbrain resulting in
the rescue of normal numbers of 5-HT neurons in the Pet-1−/− brain (Figure 19).
We then used this rescue line for ChIP to determine whether Pet-1 directly
interacted with Tph2 and Sert promoter sequences, in vivo. Chromatin was
harvested from E12.5 mouse hindbrain before extensive 5-HT neuron dispersion
began to scatter these cells, although the dissected tissue was still largely
composed of non-serotonergic cells. Sheared chromatin was immunoprecipitated
with an anti-myc antibody and analyzed by RT-qPCR for anti-myc enrichment of
genomic fragments that included predicted Pet-1 binding sites upstream of Tph2
and Sert as well as in the intron of Sert (+11390). Compared to the control, no
enrichment was detected near the Sert intron sequence. In contrast, several fold
enrichment of upstream Tph2 and Sert sequences was observed compared to
both negative control region untr17 and to the Sert intron sequence in two
independent immunoprecipitation assays (Fig. 7l and data not shown).

Finally, we used the ePet::CreERT2ascend transgene as a reporter for Pet-1-
dependent regulation of its own enhancer in Pet-1aCKO mice. We found CreERT2
expression in the adult DRN was significantly reduced in Pet-1aCKO mice treated
with TM, but not in Pet-1aCKO mice treated with vehicle, thus demonstrating that
adult expression of Pet-1 depends on positive autoregulation (Fig. 7m, n).
Inspection of the upstream Pet-1 promoter/enhancer sequences (Guscott et al.,
revealed conserved Pet-1 consensus binding sites at -465 and -621 relative to predicted transcription start site (Figure 18). ChIP for genomic fragments with these binding sites revealed a ten-fold enrichment relative control immunoprecipitations (Fig. 7I and data not shown). These findings suggest that transcriptional regulation of 5-HT synthesis and serotonergic gene expression in adulthood depends on direct positive autoregulatory maintenance of Pet-1 expression.
D. Discussion

In this study, we tested the idea that Pet-1, a critical component of an embryonic transcriptional cascade that generates 5-HT neurons in the ventral hindbrain, continues to regulate subsequent milestones in 5-HT system maturation and 5-HT function in adulthood. We find that Pet-1 function is not restricted to the induction of serotonergic characteristics in embryonic 5-HT neuron precursors. Instead, our findings demonstrate that ongoing Pet-1 directed transcription is required across life span for multiple regulatory events that shape and maintain the serotonergic neurotransmitter system. A further conclusion supported by our findings is that the etiology of behavioral pathogenesis is not limited to dysfunction of the serotonergic system during development but may also result from adult onset alterations in serotonergic transcription.

The present findings together with our previously published studies (Hendricks et al., 2003) define three general but distinct stages of Pet-1 function. The initial stage occurs during serotonergic neurogenesis in which Pet-1 regulates a late phase of 5-HT neuron generation by coordinating the induction of key serotonergic genes required for transmitter synthesis, reuptake and vesicular transport in immature postmitotic precursors (Hendricks et al., 2003). Here, we uncovered a second stage of Pet-1 function using a conditional targeting approach that did not interfere with Pet-1 expression until about 2 days after the completion of serotonergic neurogenesis in the anterior hindbrain. This transcriptional stage coincides with the prolonged period of 5-HT neuron
maturation when these cells must negotiate complex axonal growth and pathfinding decisions and are acquiring their characteristic firing properties. We identified multiple requirements for Pet-1 at this second stage, which showed that Pet-1 is essential for proper 5-HT system maturation. For example, retrograde tracing of R26R-Yfp-marked Pet-1 deficient 5-HT neurons revealed a substantial deficit in the number of serotonergic projections to the somatosensory cortex. However, initial serotonergic axon-like outgrowth did not appear compromised in Pet-1eCKO mice suggesting that Pet-1 dependent transcription regulates subsequent pathfinding decisions that help to build the ascending serotonergic system. The innervation defects in Pet-1eCKO mice were not likely contributed by the reduction of brain 5-HT. Although pharmacological disruption of embryonic 5-HT signaling alters neuronal organizations in the presubicular cortex (Janusonis et al., 2004) and 5-HT regulates thalamocortical axon pathfinding by modulating axonal responsiveness to guidance cues (Bonnin et al., 2007), recent studies of Tph2 targeted mice, which are devoid of brain 5-HT synthesis, do not detect defects in serotonergic innervation patterns (Baehne et al., 2009).

We also identified a special role for continued Pet-1 directed transcription, relative to Gata3, in regulating the maturation of 5-HT neuron firing frequency through the control of 5-HT autoreceptor mediated inhibitory responses. Similar to the innervation defects, the defects in firing frequencies and autoreceptor mediated inhibitory responses were not likely caused simply by reduced 5-HT as 5-HT is also reduced in the Gata3eCKO brain. Instead, our findings showed that Pet-1 transcriptionally controls spontaneous firing frequency and inhibitory
responses through regulation of $5-HT_{1A}$ autoreceptor gene expression. We further found that Pet-1 was required for expression of the presynaptic $5-HT_{1B}$ autoreceptor. Because expression of $5-HT_{1A}$ and $5-HT_{1B}$ do not normally occur until several days after 5-HT neuron generation, our findings strongly support the hypothesis that persistent Pet-1 directed transcription is essential for maturational steps during which 5-HT neurons acquire key functional characteristics.

We identified a third stage of Pet-1 function using a tamoxifen-inducible targeting approach that resulted in a severe and selective reduction of Pet-1 in the adult ascending 5-HT system. Significantly, the targeting of Pet-1 in adult 5-HT neurons revealed that this late stage of Pet-1 function is required in the adult ascending 5-HT system to maintain emotional behaviors. Our conclusion of altered emotional behavior in Pet-1\textsuperscript{aCKO(+TM)} mice was supported with three different tests of rodent anxiety-related behavior performed on two independent cohorts of mice that were tested several months apart from one another. The simplest interpretation of our findings is that the accompanying reduction of 5-HT levels in the ascending serotonergic system accounted for the elevated anxiety in Pet-1\textsuperscript{aCKO(+TM)} mice. However, the literature regarding the effect of adult neurotoxin-mediated depletion of 5-HT on anxiety in rats is conflicting with both anxiogenic and anxiolytic effects reported depending on experimental design (Ludwig and Schwarting, 2007; Pum et al., 2009; Sommer et al., 2001). Furthermore, as Pet-1 is likely to control a complex network of downstream transcriptional targets, the increased anxiety-like behavior in Pet-1\textsuperscript{aCKO(+TM)} mice
may have resulted from multiple alterations in adult serotonergic function. Indeed, we found a reduction in Sert and Vglut3 gene expression, which suggests complex changes in the 5-HT neuron genetic network. Interestingly, a recent study demonstrated that at least part of the ascending 5-HT system engages in dual serotonergic/glutamatergic fast synaptic transmission (Varga et al., 2009). Reduced Vglut3 expression in Pet-1aCKO(+TM) mice was not observed until 30 days after the TM treatments and thus, adult loss of Pet-1 expression may have elicited gradual plasticity changes in glutamatergic transmission that contributed to the behavioral phenotype of Pet-1aCKO(+TM) mice.

The normal expression of 5-HT1A autoreceptor and 5-HT1A mediated inhibitory responses in Pet-1aCKO(+TM) mice indicates that the observed increased anxiety-like behaviors following adult deletion of Pet-1 were not due to deficiencies in the 5-HT1A signaling pathway. This result is consistent with the findings that although germ line targeting of 5-HT1A leads to increased anxiety-related behaviors (Heisler et al., 1998; Ramboz et al., 1998) reduced 5-HT1A autoreceptor signaling in adulthood does not (Richardson-Jones et al., 2010). These findings, therefore suggest further that anxiety-like behavior in Pet-1aCKO(+TM) may be caused by a different process than that responsible for increased anxiety in Pet-1−/− mice. Nevertheless, our findings provide the first direct evidence in support of the concept that adult 5-HT-modulated behaviors are not hardwired during development but are transcriptionally regulated in the adult brain. Moreover, they highlight the potential importance of perturbations in serotonergic transcription at any stage of life in emotional pathogenesis.
Several characteristics of Pet-1 expression and function suggest it is a terminal selector gene analogous to the C. elegans ETS terminal selector gene, ast-1 that coordinates induction and maintenance of dopamine synthesis and transport in postmitotic neurons through a common conserved terminal selector motif (Flames and Hobert, 2009). Consistent with the fundamental properties of a terminal selector gene, Pet-1 is expressed throughout the life of postmitotic 5-HT neurons and is required not only to determine serotonergic-type identity but also to maintain it. However, like a terminal selector gene, it is not required for generic neuronal identity. Further key features of Pet-1 function that fits with the terminal selector gene classification is that it directly regulates and maintains expression of terminal differentiation genes that define serotonergic-type identity and positively autoregulates its own expression all through conserved ETS binding motifs (Hobert, 2008). It remains to be determined whether Pet-1 induces other transcription factors that then function cooperatively in a feed-forward loop for control serotonergic-type identity.

Our findings raise the question of why is Pet-1 still needed in adult 5HT neurons for regulation of a subset of its known embryonic targets but not for others such as Aadc, Vmat2 and 5-HT1A genes. Adult CNS expression of Tph2 and Sert are restricted to Pet-1 expressing 5-HT neurons and are rate-limiting for the essential serotonergic functions of 5-HT synthesis and reuptake. Interestingly, expression of Tph2 and Sert in the adult DRN are regulated by external stimuli such as selective serotonin reuptake inhibitors and different stress paradigms (Gardner et al., 2009; Shishkina et al., 2007). In addition, we showed earlier that
the levels of *Tph2* and *Sert* expression, *in vivo*, were sensitive to the levels of *Pet-1* expression (Lerch-Haner et al., 2008). We hypothesize (Figure 8) that environmentally induced alterations in *Tph2* and *Sert* expression may be mediated through direct transcriptional activation by *Pet-1*, which itself is subject to extrinsic regulation (Rivera et al., 2009), thus providing an efficient homeostatic transcriptional mechanism acting across life span to alter serotonergic function in response to environmental challenges.
E. Methods.

Mice

Animal procedures used in this study were approved by the CWRU School of Medicine Institutional Animal Care in compliance with the National Institutes of Health guide for the care and use of laboratory animals.

Floxed Pet-1 mice

An eleven kb genomic fragment that included Pet-1 was subcloned into a targeting construct designed to insert loxP sites around exon 3. Several rounds of electroporation and G418 selection were performed on genetically modified R1 ES cells containing a protamine Cre transgene (O’Gorman et al., 1997). A total of 176 colonies were isolated and screened by Southern blot analysis using an NcoI restriction digest and a 5’ external probe. Nine positive clones were identified and rescreened using a KpnI digestion and 3’ external probe. The 5’ probe hybridized to an 8.4 kb fragment in wildtype DNA and an 11.2 kb fragment in targeted DNA. The 3’ probe hybridized to an 8.0 kb fragment in wildtype DNA and a 10.6 kb fragment in targeted DNA. Two clones, i5h and i7c, were chosen for blastocyst injection. All resulting chimeras displayed germline transmission and were bred to mice of mixed 129Sv and C57BL/6 backgrounds. The F1 pups from male chimeras were screened for mice carrying either a floxed Pet-1 allele or a conditionally deleted Pet-1 allele using PCR genotyping with following primers p1:
5'-ACTCTGGCTTCCCTTTCTCC-3'; p2: 5'-ACTTGGAGGCCTTTTGCTCT-3'; p3: 5'-TAGGAGGGTCTGGTGTCTGG-3'; p4: 5'-GCGTCCTTGTGTGTAGCAGA-3'; p6: 5'-ATGCAAGAAGTTTCGGATGG-3' as shown in Supplementary Figure 2.

**ePet::CreER\textsuperscript{T2}**

DNA sequences encoding a fusion protein of Cre recombinase with a mutated estrogen receptor (**CreER\textsuperscript{T2}**, a gift from Dr. Pierre Chambon via Dr. Susan Dymecki) were first subcloned into the pSG5 vector (Stratagene, Cedar Creek, TX) between the β-globin intron and the simian virus 40 polyadenylation sequences. The \(\beta\)-globin-intron/C\(\text{CreER}\textsuperscript{T2}\) /poly (A) cassette was then released from the pSG5 vector and subcloned downstream of the \(\beta\)-globin minimal promoter in a modified BGZA vector in which LacZ was removed. The \(\beta\)-globin promoter/\(\beta\)-globin-intron/C\(\text{CreER}\textsuperscript{T2}\) /poly (A) region was subcloned downstream of the 40 kb ePet genomic fragment present in the modified pBACe3.6 vector (Guscott et al., 2005). The transgene was released from vector with an AscI digest and purified for pronuclear injections into hybrid c57B6/129 zygotes. Founders were identified by PCR with 5'-AAAATTTGCCTGCATTACCG-3' and 5'- ATTCTCCCACCGTCAGTACG-3' primers.

**ePet::mycPet-1**
DNA sequences encoding a myc tagged Pet-1 protein (gift from Dr. Qiufu Ma) as well as a simian virus 40 polyadenylation region were first subcloned downstream of the β-globin minimal promoter in a modified BGZA vector. The \( \beta \)-globin/Pet-1/poly (A) cassette was then released and subcloned downstream of \( ePet \) enhancer sequence present in pBACe3.6. The transgene was released from vector with an Ascl digest and purified for pronuclear injections into Pet-1\(^{-/-}\) fertilized eggs in a mixed C57BL/6 and 129 background. Founders were identified by PCR with 5'-GGGCCTATCCAAACTCAACTT-3' and 5'-GGGAGGTGTGGGAGGTTTT-3’primers.

**Histology**

Fluorescent and Diaminobenzidine (DAB) immunohistochemistry were performed as described (Lerch-Haner et al., 2008). The following primary antibodies were used: rabbit anit-5-HT (1:10,000, ImmunoStar, Hudson, WI), mouse anti-TPH (1:200, Sigma, St. Louis, MO), rabbit anti-GFP (1:1,000, Invitrogen, Carlsbad, CA), rabbit anti-Vmat2 (1:200, Millipore, Billerica, MA), goat anti-CHAT (1:200, Millipore, Billerica, MA), chicken anti-TH (1:100, Aves, Tigard, Oregon), mouse anti-NeuN (1:500, Millipore, Billerica, MA), mouse anti-GFAP (1:200, Imgenex, San Diego, CA), rabbit anti-β-galactosidase (1:5,000, MP Biomedicals, Solon, OH), rabbit anti-Cre (1:500, Covance, Princeton, NJ), Mouse anti-myc (9E10, Sigma). Secondary antibodies including FITC, TexRed, and Cyanine3 (1:200) were from Jackson ImmunoResearch (West Grove, PA).
Fluorescent and bright field images were collected using a SPOT RT color digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to an Olympus Optical BX51 microscope (Center Valley, PA). Confocal images were taken on a Zeiss LSM 510 confocal laser microscope.

**Retrograde Tracing**

Six wildtype (ePet::Yfp+) and six Pet-1<sup>−<sub>eCKO</sub> </sup>mice (~P22) were deeply anesthetized by 1.5% isoflurane in the air flow. Mice were placed into a stereotaxic frame and a small opening was made in the skull directly over the injection site (-0.5 mm, 3 mm, 0.5 mm from bregma). Coordinates for stereotaxic injections were obtained from the Paxinos mouse brain atlas. About 1 µl of Texas Red conjugated dextran (5% diluted in 0.5XPBS, 3000 Mw, Invitrogen, Carlsbad, CA) was pressure injected into the somatosensory barrel cortex using a Hamilton syringe. After injection, animals were allowed to survive for another 3 days before being sacrificed for histology.

**In Situ Hybridization**

Gene specific DNA oligonucleotide primers (Table 1) were designed to amplify ~600bp fragments using cDNA synthesized from adult raphe mRNA. Forward and reverse primers contained bacteriophage T7 or T3 promoter sequences at 5’ ends so that PCR products could be directly used as templates.
to synthesize digoxigenin (Roche, Burlington, NC) labeled sense and antisense riboprobes. In situ hybridization was performed using previously published lab protocols (Hendricks et al., 1999).

**Western Blot Analysis**

Mice were sacrificed thirty days after the last TM treatment. DRN tissue was dissected and homogenized in RIPA buffer containing 1x proteinase inhibitor (Sigma, St. Louis, MO). Proteins were quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL). Seven micrograms of each protein extract was separated by 10% SDS-PAGE (BioRad, Hercules, CA) and then transferred to a 0.45 µm nitrocellulose membrane (BioRad, Hercules, CA). Antibodies used were a monoclonal anti-TPH antibody (1:2000, Sigma), an HRP conjugated anti-mouse secondary antibody (1:2000, Cell Signaling Technology, Beverly, MA), and an anti-beta actin (1:3000, Millipore, Billerica, MA). The film was developed and then scanned on a HP Scanjet 8200. The mean band density was measured using ImageJ (http://rsb.info.nih.gov/ij).

**Tamoxifen Preparation and Treatment**

Tamoxifen (Sigma, St. Louis, MO) was dissolved in corn oil at 20 mg/ml according to Joyner Lab’s protocol http://www.mskcc.org/mskcc/html/77387.cfm. For TM treatment in the embryo, one single dose of TM (150 µg/g body weight)
either by i.p. injection or oral gavage was given to the mother at E11.5. For treatment in adulthood, 5 single daily doses of TM (150 µg/g body weight) were given to adult mice by i.p. injections.

Electrophysiology

Coronal slices including dorsal raphe (250 µm thick) were cut from brainstem of Pet-1eCKO, Pet-1aCKO(+TM), and control mice aged 3-5 weeks postnatally. Mice were anesthetized with isoflurane and decapitated. The brainstem was cooled and sliced in ice cold solution containing (in mM) 87, NaCl; 75, sucrose; 2.5, KCl; 0.5, CaCl₂; 7, MgCl₂; 1.25, NaH₂PO₄; 25, NaHCO₃; and 20, glucose bubbled with 95% O₂ and 5% CO₂ using a vibratome (VT1000S, Leica). Slices were stored for at least 1 hour at room temperature in recording artificial cerebrospinal fluid containing (in mM) 124, NaCl; 3, KCl; 2.5, CaCl₂; 1.2, MgSO₄; 1.23, NaH₂PO₄; 26, NaHCO₃, and 10, glucose bubbled with 95% O₂ and 5% CO₂. YFP+ cells were visually identified under an upright microscope (DMLFSA, Leica) equipped with a monochromator system (Polychrome IV, TILL Photonics). Whole-cell recordings were made from the cells in the dorsomedial subregion of the B7 DRN. During recordings, slices were continuously perfused with the external solution containing 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), 20 µM D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) and 20 µM picrotoxin at room temperature. Patch pipettes (2-4 MΩ) were filled with an internal solution with the following composition (in mM) 140, K-
methylsulfate; 4, NaCl; 10, HEPES; 0.2, EGTA; 4, Mg-ATP; 0.3, Na-GTP and 10, Tris-phosphocreatine (pH 7.3, adjusted with KOH). Membrane currents or voltages were recorded with an EPC10/2 amplifier (HEKA). The signals were filtered at 3 kHz and digitized at 50 kHz. The PatchMaster software (HEKA) was used for control of voltage and data acquisition. Off-line analysis was performed with Igor Pro software (Wavemetrics). 5-HT1A agonist, (±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT) was purchased from Tocris and bath-applied to slices.

**Quantitative Real-time PCR**

Mice were anesthetized with Avertin (0.5 g tribromoethanol/39.5 ml H2O, 0.02 ml/g body weight) and sacrificed by rapid decapitation. Brains were dissected and placed in RNase-free tissue culture plates. A sterile razor blade was used to cut a transverse section at Bregma area -2.92 mm and then again at Bregma area -5.46 mm to isolate the area containing the DRN and MRN. The tissue was placed immediately in Trizol (Invitrogen, Carlsbad, CA) and RNA was extracted according to the manufacturer’s manual. Genomic DNA was removed by DNase I treatment (Roche, Burlington, NC) and 1 µg RNA was used for first-strand cDNA synthesis (Invitrogen, Carlsbad, CA). For real-time RT-qPCR, a SYBR green detection system (Molecular Probes, Eugene, OR), fluorescein calibration dye (Bio-Rad, Hercules, CA), Platinum Taq (Invitrogen, Carlsbad, CA), specific primers (Table 2), and 2 µl of undiluted cDNA were used in 20 µl PCR
reactions. Each reaction was performed in triplicate. All real time RT-PCR reactions were performed in 40 cycles on the iCycler (Bio-Rad, Hercules, CA). Relative gene expression and statistics analysis were determined using Relative Expression Software Tool (http://www.gene-quantification.de/rest-paper.html).

**HPLC Analysis**

Tissues were collected as described (Lerch-Haner et al., 2008). HPLC analysis was performed by the Neurochemistry Core Lab at Vanderbilt University, Center for Molecular Neuroscience, Nashville, TN.

**Sequence Analysis**

Three kilobases upstream of predicted the human and mouse *Tph2*, *Sert*, and *Pet-1* transcription start sites were compared using ECR browser tool (http://ecrbrowser.dcode.org/) as previously described (Krueger and Deneris, 2008). The minimum criterion for significant sequence conservation was 70% identity over 100 bp. Gene annotation information was derived from NCBI (*Pet-1*, GeneID, 260298; *Tph2*, GeneID, 216343; *Sert (Slc6a4)*, GeneID, 15567). Predicted conserved Pet-1 consensus binding sites (GGAAR(T)) were identified using rVista 2.0 (http://rvista.dcode.org/).
Chromatin Immunoprecipitation (ChIP) Assays

Hindbrain tissue from the mesencephalic flexure to the cervical flexure was removed from 56 E12.5 ePet::mycPet-1 transgenic embryos and quickly frozen on dry ice. MycPet-1 occupancy of genomic regions was tested by GenPathway, Inc. (San Diego), using goat anti-Myc antibody (Abcam ab9132) and quantitative PCR (qPCR) according to their protocols. Binding was tested in triplicate for the negative control region (untranscribed genomic region Untr17) and regions in or near predicted Pet-1 binding sites. Data are expressed as fold enrichment for each sample relative to binding at Untr17. Differences in binding among regions were calculated using one-way ANOVA with Bonferroni's Multiple Comparison Test (Prism 5.0, GraphPad Software, La Jolla, CA). Replication of the entire assay gave similar results. Sequences of primers used for qPCR and their positions relative to the predicted conserved Pet-1 binding sites for each of the test genes are shown below. Primers sequences are underlined. Pet-1 binding sites are shown in bold and italic characters.

Tph2:

TTTCCGTGGCCTTTCTAAAGTTGGAAAAAGTACAAATATAATCTTGTCTATGCC
TGTCAAATTGCTGGGTCTGATCAGGTCATAGATGGAGAGCAATAAAATTGTA
TCAGAAGAGATCATCAAAGGAATGATGGGCCTATGGGCATTTCATGGGCATTTC

ATTCC
Sert:

CCCCTTCTTTCCGCTCTATTTGATTAGCTAGGTCAGCCTCAGGTTGCT
GGGGAGATTCCAGGCCCTACTGTGGGTGGGACATCCGAAACAAGAGATTTCCCCTG
AGAGGGAGGAGATTGCTAGGTCAGCCTCAGGATTCCAGGCCTACTGTGGGTGGGACATCCGAAACAAGAGAGGGGTGTGGTAGCC
AGAGGGAGGAGGCTMCCTCTCTCTCTCTGTCACGCGGGTAAACAGAACAACAGGGCCAGAC
AGACAGATGGCAACCGGAGAGTTCC

Sert-intron:

CATCCTCAGTCAGAAGAGAGAGCCCCAGCTCTCTCCCTCTGTCACCGGGTGTGGTAGCC
GCAGTGAAGGTGAAGGTACAGCCT

Pet-1:

GGAAAACCGAGAAATCGAGGGAGGGATGGGGTCTCTCTAGGACCTAAAGAGAG
TAGGAAAAAGGAGGGAGAAGGACCGGGGGGTGGGGCAAGATGAGGAGAC
CACGGCAGCGCGGTAGCGCGGGCTGGGAGCGCGACAGGGGGGAGAGGAAAGGAGCGGAAAT

Behavioral Tests

All tests were carried out in the Case Western Reserve University Rodent Behavior Core. Six to eight week-old Pet-1aCKO (ePet::CreERT2; Pet-1fl/fl-) and
littermate controls (Pet-1fl/fl) were treated with TM for 5 consecutive days. After the last TM injection, mice were rested for another 4 weeks prior to testing with access to food and water ad libitum. All tests were performed during the light cycle between 10:30 am and 6:00 pm. Equipment was cleaned thoroughly with 70% ethanol between each test to remove odor cues. The elevated plus maze test was conducted first due to its sensitivity to prior experience. Individual tests were performed at least 48 hours apart from one another. The tester was blind to group identification. Cohort 1 was tested in autumn and Cohort 2 in spring.

Elevated Plus Maze

The elevated plus maze, equipped with infrared grid and video tracking system (Med Associates Inc, St. Albans, VA), was ~1 m high and consisted of two open and two closed arms forming the shape of a cross. Mice were placed in the center of the maze facing the open arm and their activity was recorded for five minutes. Total time spent in the open arm, closed arm, hub, and number of entries into each arm were measured. We did not observe differences in frequency of defecation, urination and head dips between control and Pet-1\(^{aCKO(+TM)}\) mice.

Light ↔ Dark exploration
The light/dark box consists of two square dark gray chambers. The lit open chamber (20X20 cm) was illuminated with a 100 W light 40 cm above the chamber floor and the “dark” chamber (15X15 cm) was entirely enclosed with a solid black plastic top. Mice were placed in the open chamber, facing away from the dark side, and their exploration pattern was tracked for 5 minutes. Latency to cross over into the dark chamber and total duration in light was scored. We did not detect differences in the number of re-entries into the illuminated chamber between control and Pet-1^{CKO(+TM)} mice.

*Open field*

The open field consisted of a 40 cm x 40 cm box located in a dimly lit room. Using EthoVision XT 5.0 (Noldus, Leesburg, VA), the area was digitally subdivided into a 20 cm x 20 cm center area and a peripheral area. The peripheral area was also divided into middle (inner 10 cm) and an outer area (outer 10 cm) to determine thigmotaxic behavior. Animals were placed in the open field and allowed to explore the enclosure freely for 15 minutes. During this period locomotor parameters such as total distance moved, velocity, angular velocity, and heading degrees were measured to determine basic locomotor activity and presence of stereotypies. Frequency and duration in the center, periphery and outer quadrants were collected to determine anxiety-like behavior. Additionally, data was nested into 5 minutes bins and distance moved during
each of these 3 periods was recorded to evaluate habituation differences across groups.

**Statistics**

All statistical measures on normally distributed data were done using either a two tailed t test between the control and mutant mice or one–way ANOVA with Bonferroni’s multiple comparison test to compare means between all combinations of groups. Statistical analysis in RT-qPCR experiment was carried out by using Pair Wise Fixed Reallocation Randomization Test (http://www.gene-quantification.de/rest-paper.html).

**F. Acknowledgements**

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Figure 1
Figure 1 Conditional deletion of Pet-1 after 5-HT neuron fate specification. (a) Targeting strategy. From the top, schematic of the Pet-1 mRNA; wildtype Pet-1 allele (+); floxed Pet-1 allele (fl); and the conditionally deleted Pet-1 allele (Δ, bottom). (b–d) In situ hybridization to detect Pet-1 transcripts in the dorsal raphe (DRN) of mice heterozygous for the Pet-1 null allele and either the wild-type (b), floxed (c) or conditionally deleted Pet-1 alleles (d). (e) Time frame of Pet-1 expression in Pet-1<sup>−/−</sup>, Pet-1<sup>eCKO</sup> (Pet-1<sup>fl/fl</sup>, ePet::Cre), and wildtype mice. (f–i) In situ hybridization to detect Pet-1 and Gata3 mRNAs in control (Pet-1<sup>fl/fl</sup>, ePet::Cre) and Pet-1<sup>eCKO</sup> mice. (j–l) 5-HT immunostaining in control, Pet-1<sup>eCKO</sup>, and Pet-1<sup>−/−</sup> mice at E11.5. Scale bars are 100 µm in (i, l) and 200 µm in (d).
Figure 2
**Figure 2** Continued Pet-1 function is required for maturation of serotonergic axonal innervation patterns. (a) Co-immunostaining of Yfp and Tph in adult Pet-1<sup>eCKO</sup> mice; white arrows indicate untargeted 5-HT neurons; arrowheads indicate proximal axons extending from cell bodies of Pet-1 deficient 5-HT neurons. (b) Yfp immunostaining of Pet-1 deficient 5-HT neuron axon bundles at E14.5 in Pet-1<sup>eCKO</sup> mice. Arrowheads mark axons that have crossed the MHB and entered the midbrain (dashed lines, midbrain-hindbrain boundary, MHB). (c) Schematic of the retrograde tracing experiment. Tracer injected into the somatosensory cortex labeled (d) Yfp<sup>+</sup> Pet-1 deficient 5-HT neurons (e) in the dorsal raphe. (f), merge of (d) and (e). (g–l) Significantly fewer retrogradely labeled cells were found in the DRN of the Pet-1<sup>eCKO</sup> brain (g, h; 49.9±3.1%, mean ± s.e.m, relative to control, n=6 for each genotype). Overlay of tracer signal with Yfp immunostaining (i, j) showed that 83.0±1.8%, mean ± s.e.m, of the retrogradely labeled DRN cells in control mice were ePet::Yfp<sup>+</sup> 5-HT neurons (k), whereas only 19.3±1.7% Yfp<sup>+</sup> Pet-1 deficient 5-HT neurons (l) were labeled by the same tracer injection in Pet-1<sup>eCKO</sup> brain. *** p<0.001, two tailed t test. Scale bars are 20 µm in (a, f) and 200 µm in (l).
Figure 3
Figure 3 Continued Pet-1 function is required for 5-HT neuron firing properties and inhibitory autoreceptor function. (a, b) Whole cell current clamp recordings measuring spontaneous firing of Yfp* neurons with indicated genotypes (c) Quantification of firing frequencies in (a, b), +/+; Pet-1^eCKO, n=19; * p<0.05, two tailed t-test. (d, e) Whole cell voltage clamp recordings measuring current changes of Yfp* neurons induced with either 1 or 10 µM of 5-HT_{1A} agonist 8-OH-DPAT. A ramp voltage was applied at 200 mV/s. The intersection voltage, -87 mV, of the control and 8-OH-DPAT trace in (d) was close to the estimated K^+ equilibrium potential (-99 mV), considering that recordings were not corrected for the liquid junction potential of around 10 mV. (f) Quantification of current changes at -110 mV in (d, e); * p<0.05, *** P<0.001; two tailed t-test (g–j) In situ hybridization of 5-HT_{1A} (g, h) and 5-HT_{1B} (i, j) in control and Pet-1^eCKO mice. Scale bar is 200 µm. Error bar is mean ± s.e.m.
Figure 4
Figure 4 Continued Gata3 expression is needed to maintain 5-HT gene expression but not autoreceptor function. (a) Yfp Immunostaining in adult DRN. (b) Tph immunostaining. (c) In situ hybridization of Pet-1 mRNA. (d) Counts of Tph⁺ cell bodies in control versus Gata3eCKO mice in individual adult B nuclei, n=3 for each genotype. (e) RT-qPCR of Aadc, Sert, Tph2, Vmat2, and 5-HT₁A mRNAs in control versus Gata3eCKO mice, control n=7, normalized to 100%; Gata3eCKO n=11, * p<0.05, ** p<0.01, two tailed t test. (f, g) HPLC analysis of 5-HT (f) and 5-HIAA (g) levels in forebrain and spinal cord of control (n=7) and Gata3eCKO (n=5) mice. ** p<0.01, *** p<0.001, two tailed t test. (h) Whole cell current clamp recordings of spontaneous firing of R26R-Yfp⁺ Gata3 deficient cells. (i) Whole cell voltage clamp recordings of current changes in response to 5-HT₁A agonist, 8-OH-DPAT, in R26R-Yfp⁺ Gata3 deficient cells. Scale bars are 200 µm. Error bars represent s.e.m except for s.d in (d).
Figure 5
Figure 5 Stage-specific disruption of *Pet-1* in the adult ascending 5-HT system. (a) Adult stage specific deletion of *Pet-1* in *Pet-1<sup>aCKO</sup>* mice. (b, c) Co-immunostaining of βgal and Tph in adult dorsal (b) and medullary (c) raphe. (d, e) Percentage of TPH<sup>+</sup> cells expressing CreER activated βgal in individual adult B nuclei (d) and in 5-HT neurons of ascending versus descending pathways (e, 68.5±9.5% in the pons (B4-B9) and 12.1±6.8% in medullary nuclei (B1-B3). n=7, mean ± s.d, *** p<0.001, two tailed t test). (f–i) In situ hybridization of *Pet-1* mRNA in coronal sections of adult *Pet-1<sup>aCKO</sup>* mice treated with either TM or vehicle (Veh). (j) RT-qPCR of *Pet-1* mRNA in TM-treated adult *Pet-1<sup>aCKO</sup>* mice (pons, n=30, 28.0±2.5% relative to control, n=35; medulla, n=12, 105.9±5.8% relative to the control, n=15). Each dot represents a sample from the indicated group, mean ± s.e.m, two tailed t test. Scale bars are 200 µm.
Elevated plus maze

(a) Open time %
(b) Close time %
(c) Hub time %

(d) Total entrances
(e) Open entrances %

Dark ↔ light exploration

(f) Duration in dark
(g) Dark/light ratio
(h) Latency

Open field

(i) Time in center
(j) Distance moved

Figure 6
Figure 6 Disruption of Pet-1-dependent transcription in the adult ascending 5-HT system causes elevated anxiety-like behavior. Six to eight-week-old male Pet-1<sup>aCKO</sup> mice (n=12) and their littermate controls (Pet-1<sup>fl/fl</sup>, n=12) were treated with TM for 5 consecutive days and then acclimated for another 4 weeks before behavioral testing. (a–e) Elevated plus maze, * p<0.05, ** p<0.01, *** p<0.001; two tailed t test. (f–h) Dark ↔ light exploration, * p<0.05, ** p<0.01; two tailed t test. (i, j) Open field, * p<0.05; two tailed t test. Error bars represent s.e.m.
Figure 7
**Figure 7** 5-HT synthesis and Sert expression is maintained in the adult ascending 5-HT system through positively autoregulated direct Pet-1 transactivation. (a,b) HPLC analysis of 5-HT and 5-HIAA levels in the forebrain of tamoxifen-treated control ($n=7$) and Pet-1<sup>aCKO</sup> ($n=7$) mice (**<i>P</i> < 0.001, two-tailed t-test). (c,d) Western blotting analysis of Tph protein in DRN of tamoxifen-treated control ($n=8$) and Pet-1<sup>aCKO</sup> mice ($n=7$; 50.3 ± 4.0% relative to the control; **<i>P</i> < 0.01, two-tailed t-test). (e) RT-qPCR analysis of serotonergic gene expression in Pet-1<sup>aCKO</sup> or control mice either 5 days (control, $n=11$; Pet-1<sup>aCKO</sup>, $n=11$) or 30 days (control $n=14$; Pet-1<sup>aCKO</sup>, $n=16$) after treatment with tamoxifen or vehicle (*<i>P</i> < 0.05, **<i>P</i> < 0.01, ***<i>P</i> < 0.001). (f–i) *In situ* hybridization to detect Tph2, Slc6a4, Maob, and Lmx1b mRNAs in coronal sections from Pet-1<sup>aCKO</sup> mice treated with tamoxifen or vehicle. (j) Whole-cell current-clamp recordings of spontaneous firing in YFP<sup>+</sup> Pet-1-deficient cells in tamoxifen-treated Pet-1<sup>aCKO</sup> mice. (k) Whole-cell voltage-clamp recordings of current changes in response to 8-OH-DPAT in YFP<sup>+</sup> Pet-1-deficient cells. (l) RT-qPCR analysis of chromatin immunoprecipitations. Values represent fold enrichment in binding to the indicated regions as compared to negative control region (Untr17). Untr, untranscribed genomic region; #, <i>P</i> < 0.0001 for Tph2, Slc6a4, Pet-1 versus Untr17 or Slc6a4 intron, one-way ANOVA with Bonferroni's Multiple Comparison Test. (m,n) *In situ* hybridization to detect CreER<sup>T2</sup> mRNA in adult Pet-1<sup>aCKO</sup> mice treated with tamoxifen or vehicle. Scale bars, 200 μm. Error bars represent s.e.m except for s.d. in (l).
Figure 8
**Figure 8** Transcriptional control of 5-HT neurons across life span. Top, transcriptional cascade that directs 5-HT neuron development. Middle, Distinct requirements for continued *Pet-1, Gata3* and *Lmx1b* expression during 5-HT neuron maturation. ?, not determined. Bottom, Proposed adult homeostatic transcriptional mechanism for regulation for 5-HT modulated behaviors.
Figure 9
**Figure 9** Analysis of the floxed and conditional deleted *Pet-1* alleles. (a) PCR genotyping using primer sets identified predicted wild-type (+), floxed (fl) and conditionally deleted (Δ) *Pet-1* alleles. (b) RT-qPCR to measure relative *Pet-1* expression levels in the indicated *Pet-1* genotypes (*Pet-1*+/−, n=6, normalized to 1; *Pet-1*fl/−, n=6; mean + s.e.m; p>0.05, two tailed t test). (c–f) Tph immunoreactivity in adult dorsal raphe nucleus (DRN) demonstrated that the number of DRN Tph+ neurons in *Pet-1*+/− (c) and *Pet-1*fl/− (d) mice was similar and the number in *Pet-1*Δ/− (e) and *Pet-1*−/− (f) mice was similar. Scale bar is 200 µm.
Figure 10
Figure 10 Down-regulation of Tph2, Sert and 5-HT levels at E12.5 following conditional deletion of Pet-1 in Pet-1\textsuperscript{eCKO} embryos. (a–d') In situ hybridization to detect Tph2 and Sert mRNAs in control (Pet-1\textsuperscript{fl/+}, ePet::Cre) and Pet-1\textsuperscript{eCKO} embryos. (e, e') 5-HT immunostaining in control (e) and Pet-1\textsuperscript{eCKO} (e') embryos at E12.5. Scale bars are 100 µm.
Figure 11 Neurochemical fate mapping of Pet-1 deficient 5-HT neurons in Pet-1<sup>eCKO</sup> mice. (a, b) Xgal staining showing disrupted organization of 5-HT neurons in DRN (black arrows in b) in Pet-1<sup>eCKO</sup> mice. (c–f) Confocal microscopy showing co-immunostaining of R26R-Yfp with NeuN (c), GFAP (d), ChAT (e) or TH (f) in the DRN of Pet-1<sup>eCKO</sup> mice. Pet-1 deficient cells maintained their generic neuronal–type identity as they expressed the neuronal marker NeuN, but not markers for other transmitter identities such as those for motor neurons, dopamine or noradrenergic neurons. (g, h) Pet-1 in situ hybridization showing Pet-1 deletion was spared in a small number of 5-HT neurons in Pet-1<sup>eCKO</sup> mice. Scale bar is 50 µm in (f), 200 µm in (b) and (h).
Figure 12
Figure 12 Loss of 5-HT$_{1A}$ and 5-HT$_{1B}$ autoreceptor expression in the DRN of Pet-1$^{-/-}$ mice. (a-b') 5-HT$_{1A}$ analysis in DRN (arrow heads, B6 nucleus) and dorsal tegmental nucleus (arrows) in wild type or Pet-1$^{-/-}$ mice. (c-d') 5-HT$_{1B}$ analysis in DRN (c, c') and Purkinje cells (d, d') in wild type or Pet-1$^{-/-}$ mice. Loss of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor expression was observed only in raphe 5-HT neurons as their expression in neighboring structures were not affected (b, b', d, d'). Scale bar is 200 µm.
Figure 13
**Figure 13** Gata3 expression persists, while Gata2 expression decreases following 5-HT neuron differentiation. (a–c’’) Co-immunostaining for Gata2 (a, b, and c) and ePet::Yfp (a’, b’ and c’) in coronal sections of the rostral hindbrain at E11.5, E12.5, and E14.5. (a’’), (b’’) and (c’’), Merge of (a), (a’); (b), (b’); and (c), (c’’) respectively. (d–e’) Coronal views of embryonic rostral (r2, d, d’) and caudal (r7, e, e’) hindbrain sections showing ePet::Yfp immunostaining (brown) after Gata3 in situ hybridization (blue) at E12.5. (d’’) and (e’’), Higher magnification of boxed areas in (d) and (e). (f–k’’) Co-immunostaining of adult coronal sections for Gata3 (f, i) and Vmat2 (g, j) in DRN (f–h’) and ventral medulla (i–k’’). (h), (k), Merge of (f), (g); and (i), (j) respectively. (h’), (k’), and (k’’), Higher magnification of boxed areas in (h) and (k). Scale bars in (c’’), and (k) are 200 µm. Scale bars in (e’), (h’), and (k’’) are 50 µm.
Figure 14
Figure 14 Generation and analysis of 5-HT neuron specific tamoxifen inducible $\text{CreER}^{T2}$ mice. (a) Schematic of the ePet::$\text{CreER}^{T2}$ transgene. One line, \textit{ePet::$\text{CreER}^{T2}_{\text{ascend}}$}, was selected for subsequent experiments as it showed selective targeting efficacies in ascending versus descending 5-HT neurons. (b) Xgal staining of an E16.5 sagittal section. r4, rhombomere 4. (c, d) Xgal staining of adult coronal sections. (e–h) X-gal staining of adult coronal sections from TM treated ePet::$\text{CreER}^{T2}$, \textit{R26R}$^{\beta\text{gal}+}$ mice. (e) ctx, cortex; (f) hp, hippocampus; (g) cb, cerebellum; (h) sc, spinal cord. Scale bars are 200 µm.
Elevated plus maze

(a) Open time %
(b) Close time %
(c) Hub time %

Open field

(i) Time in center
(j) Distance moved

Dark ↔ light exploration

(d) Total entrances
(e) Open entrances %

(f) Duration in dark
(g) Dark/light ratio
(h) Latency

Figure 15
Figure 15 Adult TM treatments do not alter behavioral performance in anxiety related tests. Six to eight-week-old male wildtype mice were treated with either vehicle (n=7) or TM (n=7) for 5 consecutive days and then acclimated for another 4 weeks before behavioral testing. (a–e) Elevated plus maze. (f–h) Dark ↔ light exploration. (i, j) Open field. Error bars represent s.e.m.
Figure 16

Elevated plus maze

a. Open time %

b. Close time %

c. Hub time %

CON aCKO

Open field

i. Time in center

j. Distance Moved

CON aCKO

Total Entrances

d. Total Entrances

CON aCKO

Open entrances %
e. Open entrances %

CON aCKO

Dark ↔ light exploration

f. Duration in dark

CON aCKO

CON aCKO

g. Dark/light ratio

h. Latency
Figure 16 Adult deletion of Pet-1 causes elevated anxiety-like behavior in a second independent cohort. Six to eight-week-old male Pet-1^{aCKO} mice (ePet::CreER^{T2}; Pet-1^{fl/–}, n=10) and their littermate controls (Pet-1^{fl/–}, n=10) were treated with TM for 5 consecutive days and then acclimated for another 4 weeks before behavioral testing. (a–e) Elevated plus maze. * p<0.05, two tailed t test. (f–h) Dark ↔ light exploration. * p<0.05, p=0.056 for (g), two tailed t test. (i, j) Open field. * p<0.05, two tailed t test. Error bars represent s.e.m.
Figure 17
Figure 17 Anxiety-like behavior is not observed in vehicle treated Pet-1aCKO mice. Six to eight-week-old male Pet-1aCKO mice (ePet::CreER\textsuperscript{T2}; Pet-1\textsuperscript{fl/fl}, n=7) and their littermate controls (Pet-1\textsuperscript{fl/fl}, n=7) were treated with vehicle for 5 consecutive days and then acclimated for another 4 weeks before behavioral testing. (a–e) Elevated plus maze. (f–h) Dark ↔ light exploration. (i, j) Open field. Error bars represent s.e.m.
Figure 18
Figure 18 Phylogenetically conserved consensus Pet-1 binding sites. Blue dots identify positions of predicted Pet-1 binding sites (GGAART) upstream of the Tph2, Sert and Pet-1 genes. Colored peaks indicate significant sequence conservation. Red, intergenic sequences; yellow, 5' untranslated exons; green, repetitive sequences; blue, protein coding sequences; orange, introns. Right axis shows percent identity between human and mouse sequences.
Figure 19
**Figure 19** *Pet::mycPet-1* expression recapitulates endogenous *Pet-1* expression and rescues 5-HT neuron number in *Pet-1<sup>−/−</sup>* mice. (a) Schematic of the *ePet::mycPet-1* transgene injected into pronuclei derived from *Pet-1<sup>−/−</sup>* female fertilized eggs. (b) Anti-myc immunohistochemistry in E11.5 coronal hindbrain sections. (c) In situ hybridization of *Pet-1* mRNA in E11.5 wildtype embryos. (d–f) Co-staining of adult coronal sections for myc (d) and Vmat2 (e) in *Pet-1<sup>−/−</sup>; ePet::mycPet-1<sup>+</sup>* mice demonstrating that expression of *ePet::mycPet-1* transgene (hemizygous) rescued the number of Vmat2<sup>+</sup> 5-HT neurons in the *Pet-1<sup>−/−</sup>* background (g). (f), Merge of (d) and (e). Inset: higher magnification showing co-localization of mycPet-1 and Vmat2 proteins within 5-HT neurons. Scale bar in (c) is 50 µm. Scale bar in (g) is 200 µm.
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**Table 1** Primer pairs for synthesis of antisense riboprobes.
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<th>Reverse Primer (5'→3')</th>
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**Table 2** Primer Pairs for RT-qPCR.
CHAPATER: III Discussion

A. Summary

Transcriptional programs play an essential role in the generation of the CNS serotonergic cell fate, but it has yet to be determined whether these programs continue to function in the maturation and maintenance of the 5-HT system. The persistent expression of several early transcriptional regulators that govern the differentiation of the central 5-HT neuron phenotype into adulthood suggests developmental determinants of serotonergic cell fate may perform additional roles in developing and adult 5-HT neurons. In this study, we have developed spatiotemporal conditional targeting approaches to investigate the requirements for continued Pet-1 function in embryonic and adult 5-HT neurons. Our findings identify several new regulatory roles for Pet-1 once it has fulfilled an early role in 5-HT neuron differentiation, including control of 5-HT cell body distribution in the DRN, axonal innervation patterns in the somatosensory cortex, 5-HT autoreceptor expression, and 5-HT neuron firing properties. Furthermore, by conditionally targeting Pet-1 expression in the adult ascending 5-HT system, we find that Pet-1 is still needed in adult 5-HT neurons to maintain Tph2 and Sert gene expression, but not other genes whose earlier induction depends on Pet-1. In addition, the loss of Pet-1 expression in the adult ascending 5-HT system leads to alterations in several tests of anxiety-like behavior. Together, our findings provide new insights into the transcriptional mechanisms that direct 5-HT neuron development and reveal a previously unrecognized transcriptional
maintenance mechanism that is continuously required to support adult 5-HT-modulated behaviors.

B. Mechanisms underlying Pet-1 regulation of adult emotional behaviors.

Germline deletion of the Pet-1 gene results in behavioral perturbations in adulthood (Hendricks et al., 2003; Lerch-Haner et al., 2008); however, the mechanisms through which Pet-1 regulates adult behaviors are not fully understood. Our previous studies demonstrated that Pet-1 expression was induced at the terminal stage of 5-HT neuron differentiation for the transcription of key serotonergic genes encoding 5-HT synthesis, re-uptake, and vesicular transport. As a result, loss of Pet-1 function in 5-HT neuron precursors caused arrested 5-HT neuron development and a severe loss of brain 5-HT (Hendricks et al., 2003).

However, does Pet-1 regulate adult emotional behavior only at the stage of 5-HT neuron differentiation by programming normal numbers of 5-HT neurons and 5-HT levels? Our present studies revealed that Pet-1 function was still required, after it fulfilled its initial role in 5-HT neuron generation, for multiple developmental milestones during 5-HT system maturation. Without Pet-1, 5-HT neurons in Pet-1\textsuperscript{eCKO} mice failed to properly form the dorsal raphe nucleus, innervate the somatosensory cortex, and have normal spontaneous firing properties. These developmental defects should also exist in the Pet-1\textsuperscript{−/−} mice
and are likely to contribute to the observed adulthood behavioral alterations. Moreover, the loss of 5-HT1A autoreceptor expression in Pet-1+/− mice provides a potential mechanism underlying emotional perturbations in Pet-1+/− mice. Somatodendritic 5-HT1A autoreceptors play an essential role in regulating 5-HT neuron firing (Richardson-Jones et al., 2010). Several postmortem and positron emission tomography studies of 5-HT1A binding reported altered levels of binding in subregions of the raphe in subjects with emotion-related psychiatric illness (Savitz et al., 2009) and SIDS (Paterson et al., 2006). These studies suggest that the transcriptional regulation of 5-HT1A receptor expression may be relevant to the pathogenesis of psychiatric diseases. Indeed, Albert and colleagues have identified a genetic variation in the 5-HT1A receptor upstream promoter region that can influence transcription of the autoreceptor and are associated with a risk for depression and panic disorder (Czesak et al., 2006; Lemonde et al., 2003). In rodents, 5-HT1A null mice consistently demonstrated increased anxiety-like phenotypes that were similar to those observed in the Pet-1 null mice. Altogether, our findings suggests that adult behavioral deficits in Pet-1+/− mice are not simply caused by a loss of 5-HT, but rather are at least in part, due to a combination of developmental defects during 5-HT system maturation.

How does Pet-1 regulate multiple developmental processes during 5-HT system maturation? The observed displacement of cell bodies in the dorsal raphe may indicate a migration defect, as Pet-1 deficient 5-HT neurons move away from the midline to more lateralized locations. Alternatively, it could also reflect failures of cell adhesion and clustering necessary to form the dorsal raphe
nucleus. Notably, the defect of cell body positioning is not caused by the loss of brain 5-HT, as formation of the dorsal raphe nucleus is normal in Tph2 targeted mice that have no brain 5-HT. It is possible that Pet-1 regulates the expression of guidance or cell-adhesion proteins during 5-HT neuron maturation. Interestingly, expression of the cell adhesion molecule Cadherin 8 (Cdh8) is highly enriched in 5-HT neurons (Allen brain atlas). It is thus intriguing to study whether the positioning deficit in Pet-1\textsuperscript{eCKO} mice could result from possible defects of Cdh8 function and whether Cdh8 expression is transcriptionally controlled by Pet-1.

The observed innervation defects in the somatosensory cortex could result from aborted pathway extension. Alternatively, mis-routing in termination zones, possibly secondary to mis-positioning of serotonergic cell bodies in the DRN, may be responsible for the pathway defects in the Pet-1\textsuperscript{eCKO} cortex. Loss of function studies have implicated the axonal guidance factors growth associated protein 43 (GAP43) (Donovan et al., 2002), Slits 1/2 (Bagri et al., 2002), and protocadherin-α (Pcdhα) (Katori et al., 2009) in the guidance and arborization of ascending serotonergic axons. The GAP43 gene is strongly expressed in adult 5-HT neurons (Bendotti et al., 1991), and our recent gene profiling of purified embryonic 5-HT neurons detected moderate expression of GAP43, Slit 1/2, and Pcdha at E12.5 (Wylie et al.). These findings raise the possibility that the pathway defects detected in Pet-1\textsuperscript{eCKO} brain result from deficiencies in the Pet-1-dependent transcriptional regulation of GAP43, Slits, or Pcdha.
Our findings provide insight into the mechanism through which Pet-1 determines spontaneous firing frequency and inhibitory responses by showing that ongoing Pet-1 function is essential for the developmental onset of 5-HT\textsubscript{1A} gene expression in 5-HT neurons. Expression of genes required for 5-HT synthesis and re-uptake occurs before the first appearance of brain 5-HT and can be first detected by in situ hybridization in the rostral hindbrain around E11 (Zhao et al., 2006). In contrast, the developmental onset of 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B} autoreceptor expression is “late” in 5-HT neurons, as Bonnin et al. found “no detectable expression of 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B} in the mouse brain” at E12.5 (Bonnin et al., 2006). Furthermore, in transgenic mice expressing Cre recombinase in 5-HT\textsubscript{1A} expressing cells, Sahly et al. found 5-HT\textsubscript{1A} driven Cre activity in several structures in the E13 embryo, while no Cre activity was detected in the developing hindbrain (Sahly et al., 2007). To probe the developmental onset of 5-HT autoreceptor expression in 5-HT neurons, we carried out in situ hybridization on developing brain slices at E12.5, E14.5, and E17.5. We verified that there was no detectable 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B} expression at E12.5; however, their mRNA began to appear in a subset of 5-HT neurons at E14.5 (Figure 20 and data not shown). Altogether, these findings suggest that continued Pet-1 function is needed at a later developmental stage for the induction of 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B} expression. This process is essential for the maturation of 5-HT neuron functional properties.

Continued expression of the zinc-finger factor Gata3 in differentiated 5-HT neurons was required for the normal expression of several 5-HT genes and might
also program adult maternal behaviors that are required for infant survival
(Figure 21). However, our findings revealed requirement for Pet-1, but not Gata3,
for induction of 5-HT$_{1A}$ and 5-HT$_{1B}$ autoreceptor expression. In addition, the early
postnatal loss of all 5-HT neuron cell bodies in conditionally targeted Lmx1b mice
(Zhao et al., 2006) but not in conditionally targeted Pet-1 or Gata-3 mice
suggests a selective requirement for Lmx1b in the regulation of genes involved in
5-HT neuron survival. Taken together, our findings support a model in which
different transcriptional regulators in differentiated 5-HT neurons modulate both
common and distinct 5-HT neuron properties. Their coordinated functions are
required to maintain proper levels of 5-HT gene expression and support different
aspects of 5-HT neuron development.

To fully understand how Pet-1 regulates the maturation of the 5-HT
system, it is necessary to examine the full complement of Pet-1 downstream
targets at different developmental stages by profiling and comparing the
transcriptomes of wildtype and Pet-1 deficient 5-HT neurons. Since all Pet-1
deficient 5-HT neurons are permanently labeled by R26R::Yfp in Pet-1$^{eCKO}$ mice,
these cells can be purified from Pet-1$^{eCKO}$ brain using Fluorescence Activated
Cell Sorting (FACS) (Wylie et al., 2010). The gene expression profile of Pet-1
deficient 5-HT neurons can be determined by both cDNA microarray and RNA
sequencing analyses and compared to that of the wildtype controls. We will
examine whether expression of certain families of axon guidance or cell adhesion
molecules are altered in Pet-1 deficient 5-HT neurons, which may help explain
the positioning and innervation defects observed in Pet-1$^{eCKO}$ mice. In addition,
we may find alterations in the expression of a new list of genes that are important for the development of 5-HT neurons, which may further indicate new functions for Pet-1 during 5-HT system maturation. To determine whether Pet-1 directly transactivates or repress the expression of these genes, genome alignment and conserved binding site prediction programs will be used to identify putative Pet-1 binding sites near or within these identified genes. Chromatin immunoprecipitation analysis using chromatin extracted from hindbrain tissues in the ePet::mycPet-1 mice will help determine whether Pet-1 protein directly interacts with those phylogenetically conserved binding sequences in vivo.

The observation that Pet-1-/- mice had increased anxiety-like behavior in adulthood does not address whether the critical period for the regulation of adult anxiety behavior by Pet-1 is limited to developmental stages or if Pet-1 is constantly needed in adulthood to maintain normal anxiety responses. While 5-HT signaling plays a critical role during development in establishing neural circuits that give rise to normal adult anxiety traits (Leonardo and Hen, 2008), the idea that ongoing serotonergic transcription is needed in the adult CNS to maintain emotional behaviors has not been previously addressed. Our development of a tamoxifen-inducible targeting approach for perturbation of Pet-1 in the adult DRN and MRN demonstrated a further requirement for Pet-1 dependent transcription in the maintenance of normal anxiety responses in the open field, elevated plus maze, and light-dark box. These findings demonstrated, for the first time, that emotional pathogenesis can arise from defects in
serotonergic transcription not only during early developmental but also adult stages.

We have shown in previous studies that embryonic targeting of Pet-1 led to additional behavioral alterations in adulthood, including increased inter-male aggression, altered fear conditioning responses (Schaefer et al., 2009), and a profound loss of maternal nurturing behavior (Hendricks et al., 2003; Lerch-Haner et al., 2008). It is not known, however, whether these adult behavioral alterations are consequences of Pet-1 deficiency during critical developmental periods, or if ongoing Pet-1-dependent transcription is constantly needed throughout life to modulate these behaviors. A reasonable starting point would be to investigate whether TM-induced adult deletion of Pet-1 would still affect aggression, fear conditioning, and maternal behaviors. These behaviors could be altered in Pet-1aCKO+TM mice, therefore establishing a requirement for ongoing serotonergic transcription in adulthood for the maintenance of these behaviors. Alternatively, Pet-1 may no longer be needed in adulthood for the modulation of aggression, fear conditioning, or maternal behaviors, suggesting that Pet-1-dependent transcription is required only during developmental stages for the proper establishment of circuitry responsible for these adult behaviors. If this is the case, it is important to define the temporal requirement of Pet-1-dependent transcription for these behaviors by conditional targeting of Pet-1 with TM treatments at various developmental stages. For example, the deletion of Pet-1 expression can be induced at a late embryonic period (around E14.5-E18.5) when ascending 5-HT axons are extending and innervating their midbrain and
forebrain targets. Alternatively, TM can be given during an early postnatal period (P7-P21) when synaptogenesis is actively ongoing between 5-HT neurites and other brain circuits. In either case, the results will help elucidate how perturbations in 5-HT system development might affect the establishment of normal adult behaviors and provide insights about potential mechanisms underlying behavioral pathogenesis.

The mechanism through which Pet-1 regulates anxiety responses in the adult ascending 5-HT system needs to be further explored. Our present studies demonstrate that Pet-1 regulates the function of the adult ascending 5-HT system through direct trans-activation of Tph2, Sert, and its own expression through interactions with phylogenetic conserved ETS binding sites. However, the increased anxiety-like behavior is not likely to be caused by the reduction of Sert expression, as blockade of Sert function in the adult by SSRI treatment produces anxiolytic effects (Katzman, 2009), an effect which was not seen in our TM-treated Pet-1aCKO mice. Lower levels of 5-HT and 5-HIAA in the forebrain resulting from a decrease in the Tph2 protein may have contributed to the elevated anxiety. Indeed, similar anxiety-like behaviors were observed in the elevated plus maze and open field tests when rats received lesions of serotonergic innervations in specific forebrain structures, such as the striatum (Ludwig and Schwarting, 2007) and median prefrontal cortex (Pum et al., 2009) by 5-HT neurotoxin 5,7-dihydroxytryptamine (5,7-DHT). Interestingly, a similar lesion in the amygdala had no effects on the plus maze test, but an anxiolytic effect in another behavioral conflict test (Sommer et al., 2001). These studies
suggest that 5-HT signaling in different brain structures may mediate both anxiogenic and anxiolytic effects (Deakin and Graeff, 1991). It remains to be determined whether adult Pet-1 deletion results in differential decreases of 5-HT neurotransmission in forebrain structures, such as the amygdala and hippocampus that have been implicated in the regulation of anxiety (Oler et al., 2010).

Our current studies have only examined the expression of a short list of genes that are required for 5-HT metabolism and signaling in Pet-1aCKO+TM mice. However, Pet-1 may regulate the expression of other genes in adult 5-HT neurons, whose deficiency may have also contributed to the increased anxiety-like phenotype. In order to reveal the full complement of Pet-1 transcriptional targets in adult 5-HT neurons, we can purify the Yfp+ 5-HT neurons using Laser Capture Microdissection (LCM) and compare the transcriptomes of Pet-1 deficient 5-HT neurons to that of controls. Identification of Pet-1 transcriptional targets in adult 5-HT neurons may help better elucidate the molecular and cellular mechanisms underlying the behavioral alterations.

Furthermore, our present study demonstrated that Pet-1 was no longer needed in adult 5-HT neurons to maintain mRNA levels of Aadc, Vmat2, and 5HT1A, whose embryonic expression depends on Pet-1 function. A similar phenomenon was recently reported outside the CNS, where Pax7 was required for the expression of markers for myogenic satellite cells in development, but not in adulthood (Lepper et al., 2009). These findings indicated that Pet-1 regulates
the expression of distinct sets of genes at different life stages. Therefore, by comparing the transcriptional targets of Pet-1 between embryonic and adult stages will not only test our hypothesis that the function of Pet-1 changes as the animal matures, but will also help us better understand how Pet-1 transcriptional regulation controls serotonergic function and influences emotional behaviors at different life stages.

C. Spatiotemporal gene manipulation in brain 5-HT neurons.

Recent studies showed that genetic targeting of certain 5-HT genes at distinct life stages often led to different, if not paradoxical, behavioral consequences. For example, embryonic or early postnatal deletion of 5-HT$_{1A}$ resulted in increased anxiety-like behaviors in rodents, whereas anxiety-like deficits were not observed when this receptor was deleted in the adult brain (Gross et al., 2002; Ramboz et al., 1998; Richardson-Jones et al., 2010). Similarly, germline deletion of Sert led to depressive-like phenotypes in adulthood (Holmes et al., 2003a), while adult blockade of Sert function by SSRI treatments had a well-known anti-depressive effect. These findings indicate that certain aspects of the adult behavior are determined by gene functions at distinct “critical periods”; therefore 5-HT genes, functioning at distinct life stages, may have a differential impact on adult behaviors. To fully understand the impact of gene functions at different developmental and adult stages, we developed a novel genetic tool that allows for temporally controlled gene
activation/inactivation specifically in brain 5-HT neurons (Figure 22) so that the adult function of a gene can be evaluated independently from its developmental role.

While a single dose of TM in the embryo was sufficient to induce Cre activity in majority of 5-HT neurons, multiple treatments (i.p.) were necessary to activate Cre function in adult 5-HT neurons. This discrepancy may result from the reduced permeability of TM into the adult brain. Furthermore, TM may not penetrate uniformly in all brain areas. As a result, levels of TM can be higher in certain brain regions than others, which might explain the differential responsiveness to TM treatments between 5-HT neurons in the pons versus those in the medulla. Since the CreER\textsuperscript{T2} protein was still present in majority of the medullary 5-HT neurons in the adult (Liu, C. unpublished observation), increasing the dosage of TM may help activate CreER\textsuperscript{T2} in more of these cells. Nevertheless, higher dosages of TM had detrimental effects on the overall health of the animals. In fact, nearly 50% of mice died after 5 days of TM treatment with a higher dosage (225 µg/g body weight, Liu, C. unpublished observation). Alternatively, one might construct a CreER\textsuperscript{T2} transgene that is driven by the regulatory sequences upstream of the Tph2 or Sert genes. Since expression of either gene is more abundant in 5-HT neurons than that of Pet-1, higher levels of transgene expression are expected. Therefore, a smaller dose of TM may be sufficient to activate CreER\textsuperscript{T2} function in more medullary 5-HT neurons. Indeed, a recent study reported a transgenic mice line, in which CreER\textsuperscript{T2} was expressed in 5-HT neurons under the regulatory control of Tph2 promoter/enhancer
sequences (Weber et al., 2009). Adult TM treatment in this transgenic line resulted in the induction of \textit{Cre} function in up to 90\% of 5-HT neurons in both pontine and medullary 5-HT nuclei, although leaky \textit{Cre} activity was also detected in the absence of TM. A third strategy that can be useful for adulthood gene manipulation in 5-HT neurons is to construct viral-based vectors that contain an expression cassette (RNAi or toxin) preceded by a floxed stop-codon and delivery of them into adult \textit{ePet-Cre} mice using stereotaxic injections. The specificity of the targeting is achieved by the restricted serotonergic \textit{Cre} expression so that exogenous construct is only expressed within \textit{Cre} expressing 5-HT neurons. Furthermore, RNAi or toxin construct can be locally injected into individual adult 5-HT nuclei, which may help further dissect gene function in individual 5-HT groups and its contribution to adult physiological processes and behaviors.

With the newly generated \textit{ePet::CreER}^{T2} mice, we are now able to ablate gene function within 5-HT neurons at virtually any stage of life. One important application of this tool is to study gene functions specifically in adult 5-HT neurons. For example, we have demonstrated that expression of two other transcriptional regulators \textit{Gata3} and \textit{Lmx1b} persists in all 5-HT neurons throughout adulthood. It would be interesting to study whether these factors are also required in the adulthood for the maintenance of normal 5-HT traits and 5-HT modulated behaviors.

Moreover, \textit{ePet::CreER}^{T2} mice can be used as a novel genetic model to
study the impact of adult 5-HT deficiency on physiological processes and behaviors. Reduced levels of TPH protein in Pet-1aCKO+TM mice led to a significant decrease of 5-HT in the adult forebrain and thus raised the possibility that increased anxiety-like behavior in Pet-1aCKO+TM mice could result from lower levels of forebrain 5-HT signaling. However, as Pet-1 is likely to regulate the expression of many genes other than Tph2 in adult 5-HT neurons, it is difficult to conclude that the observed anxiety-like behaviors in Pet-1aCKO+TM mice are simply caused by an adult 5-HT deficiency.

On the other hand, studies of the impact of adult 5-HT deficiency on emotional-related behaviors have been hindered by a lack of genetic approaches that allows for selective and irreversible depletion of 5-HT in the adult brain. While increased impulsivity and mood reduction have been reported in humans after acute tryptophan depletion (Walderhaug et al., 2007), traditional dietary and pharmacological manipulations fail to generate consistent results that support a direct neuromodulatory role for 5-HT on mood regulation.

When introduced into floxed Tph2 mice, ePet::CreERT2 can selectively ablate the expression of Tph2 in a majority of the adult 5-HT neurons that comprise the ascending 5-HT pathways and thus, greatly reduce 5-HT levels in the adult forebrain. Our study demonstrated that the expression of another 5-HT synthesis enzyme Tph1 was not up-regulated following the loss of Tph2. Therefore, the loss of 5-HT in ePet::CreERT2; Tph2fl/fl forebrain is likely to be irreversible. As a result, adult Tph2 conditional knockout mice (Tph2aCKO) would be a valuable rodent model to study both short- and long-term effects of adult-
specific 5-HT deficiency on anxiety and many other physiological processes and behaviors. Furthermore, varying levels of Tph2 knockdown and 5-HT depletion are expected after treating mice with different dosages of TM, which would help determine whether the onset and severity of certain behavioral alterations could be correlated to a specific range of 5-HT reduction in the adult forebrain. Finally, depletion of 5-HT can be induced by TM treatments not only in the adult but also throughout development, which will help distinguish whether behavioral pathogeneses such as aggression, depression, and circadian and sleep disturbances stem from developmental deficiency of 5-HT signaling or disrupted maintenance of normal serotonergic tone in the adult brain.

In addition to deficiencies of 5-HT synthesis and reuptake, physiological and behavioral alterations may arise from altered 5-HT neuron physiology. Since many 5-HT neurons also produce and release other neurotransmitters such as glutamate (Varga et al., 2009), GABA (Fu et al., 2010), galanin (Arvidsson et al., 1991; Melander et al., 1986), TRH (Arvidsson et al., 1992; Johansson et al., 1981), and substance P (Johansson et al., 1981), altered 5-HT neuron physiology is likely to perturb the neurotransmission of not only 5-HT, but also other transmitters, and possibly cause additional defects in 5-HT system function.

To evaluate the impact of deficiencies in 5-HT neuron activity on animal physiology and behaviors, ePet::CreERT² transgene can be introduced into floxed tetanus toxin light chain mice (R26R::TeNTfl/fl) to generate double transgenic mice (ePet::CreERT²; R26R::TeNTfl/fl) in which the expression of TeNT can be induced within adult 5-HT neurons following TM treatments. TeNT
cleaves the synaptic vesicle associated membrane protein (VAMP2)/synaptobrevin2, a key component of the SNARE complex, and therefore, blocks the fusion of synaptic vesicles to the membrane (Kim et al., 2009; Schiavo et al., 2000). As a result, the release of 5-HT and other neurotransmitters is likely to be minimized following the induction of TeNT. Behavioral tests that are related to aggression, depression, and anxiety will be carried out using both TM-treated control and ePet::CreER\textsuperscript{T2}; R26R::TeNT\textsuperscript{fl/fl} mice. These findings may corroborate those from the 5-HT-depleted mice (ePet::CreER\textsuperscript{T2}; Tph2\textsuperscript{-/-}), and both mouse models will help reveal critical adult modulatory roles of the ascending 5-HT system in animal physiology and behaviors.

D. Implication of adult Pet-1-dependent transcription in human anxiety disorders.

Numerous studies have emphasized the importance of a critical developmental periods during which brain circuits are “wired” for the normal expression of normal adult anxiety responses and suggested that anxiety disorders are mainly caused by neurodevelopmental defects (Leonardo and Hen, 2008). Nevertheless, our findings suggest that in addition to developmental processes, transcriptional mechanisms acting in adulthood play a critical role in preserving anxiety-related behavior.
Furthermore, our observation that the loss of *Pet-1* function in the adult ascending 5-HT system caused increased anxiety-like behavior provided the first genetic evidence that anxiety-related pathogenesis can result from adult-onset deficiencies in serotonergic transcription in mice that have completely normal brain development. The identification of an ongoing *Pet-1*-dependent transcription program for the maintenance of normal anxiety responses in adulthood is relevant to human anxiety related disorders, as this program could be altered in adulthood in response to exposure to adverse life events, such as physical or psychological trauma, and the alteration may contribute to the pathogenesis of human anxiety disorders e.g. the posttraumatic stress disorder (PTSD).

Interestingly, the expression of both *Tph2* and *Sert* are often modulated by environmental stressors as a number of studies have demonstrated that different paradigms of stresses alter both *Tph2* and *Sert* mRNA in subregions of the dorsal raphe (Filipenko et al., 2002; Gardner et al., 2009; McEuen et al., 2008; Shishkina et al., 2007; Vollmayr et al., 2000). Our earlier and current studies demonstrated that the levels of *Tph2* and *Sert* positively correlates with the amount of *Pet-1* in vivo and with the idea that *Pet-1* controls the expression of both genes through direct interactions with conserved ETS binding sequences upstream of these genes (Lerch-Haner et al., 2008). Our findings thus raise the possibility that adulthood stress-induced perturbations of 5-HT gene expression and adaptive behaviors may result from alterations in adult *Pet-1*-dependent transcription.
Two critical questions remain to be explored in future studies. First, it is important to study whether the expression of Pet-1 or function in adult 5-HT neurons alters in response to adverse stresses (e.g. restrain stress and social defeat) that are known to influence the expression of Tph2 or Sert in rodents (Filipenko et al., 2002; Gardner et al., 2009; McEuen et al., 2008; Shishkina et al., 2007; Vollmayr et al., 2000). These studies can be carried out using adult ePet::mycPet-1, Pet-1−/− mice so that the protein levels of Pet-1 can be measured by western blot analysis with anti-myc antibodies. In addition to Pet-1, expression of other transcription regulators in adult 5-HT neurons including Lmx1b and Gata3 will also be measured, as these factors may also regulate the expression of Tph2 and Sert in adult 5HT neurons and their levels may also change after exposures to stresses. We may find that the expression of these factors is not altered after stresses, which suggests that the observed alterations in Tph2 and Sert expression are caused by other unknown transcriptional regulators or factors that are important for mRNA stability. On the other hand, we may find that the expression of Pet-1, Lmx1b or Gata3 alters and correlates to the changes of Tph2 and Sert in above stress paradigms, suggesting that external stressors can alter adult serotonergic transcription. Finally, we will test whether stress induced alterations in serotonergic transcription is responsible for changes in subsequent adaptive behaviors by either increasing or decreasing the levels of these transcription factors in adult 5-HT neurons (for example, viral injection of RNAi or over-expression constructs) and determining whether these genetic manipulations would be able to attenuate or exacerbate stress induced
behavioral alterations. If so, these findings will establish a novel mechanism about how environmental factors interact with the function of brain 5-HT circuitry and alters 5-HT modulated emotional behaviors.

E. Conclusion

Our present findings expand our current understanding about transcriptional mechanisms required during 5-HT neuron development by revealing continued and temporally complex roles for Pet-1-mediated transcription at multiple steps in 5HT system development.

Our study demonstrated that Pet-1 function extends beyond an early role in 5-HT neuron differentiation and continues to be required for other developmental milestones, including cell body migration, axon target innervation patterns, induction of autoreceptors expression, and spontaneous firing properties. These findings suggest that the behavioral and physiological phenotypes of mice deficient in Pet-1 function during development (Hendricks et al., 2003; Lerch-Haner et al., 2008) are likely to result from a combination of defects at many critical steps in 5HT neuron development.

By generating a TM inducible CreERT2 line that allows for gene inactivation in adult 5-HT neurons, our study further demonstrated that ongoing Pet-1 function is still needed in adult 5-HT neurons to maintain proper levels of 5-HT synthesis and re-uptake. The identification of an adult maintenance program supports the novel concept that, in addition to developmental processes,
transcriptional mechanisms play a critical role in preserving emotion-related behavior and highlight the potential importance of perturbations in serotonergic transcription across the life span in emotional pathogenesis.
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Figure 20.
**Figure 20.** In situ hybridization detecting $5HT_{1A}$ (a) and $5HT_{1B}$ mRNA (b) in developing hindbrain at E14.5. Arrow head, $5HT_{1B}$ mRNA was only detected in a few cells in the posterior part of the hindbrain. Scale bar is 200 µm.
Figure 21.
Figure 21. Left, number of pups born to wildtype and $Gata3^{eCKO}$ ($ePet::Cre$, $Gata3^{fl/fl}$) dams. Right, survival of litters born to wildtype and $Gata3^{eCKO}$ dams. $n=8$ for each indicated genotype.
Figure 22.
Figure 22 Spatiotemporal gene manipulation in brain 5-HT neurons. Top, schematic of the ePet::CreER\textsuperscript{T2} transgene. Bottom, CreER is normally sequestered in the cytoplasm by heat shock protein (HSP) 90. However, upon binding with tamoxifen, the CreER chimeric protein undergoes a conformational change that releases it from HSP90. CreER then enters the nucleus and induces recombination between two loxP sites to excise the target DNA sequence.
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