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MOLECULAR GENETICS OF MOUSE SEROTONIN NEURONS ACROSS THE LIFESPAN

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Abstract—New molecular genetics approaches have been developed over the past several years to study brain serotonin (5-HT) neuron development and the roles of 5-HT neurons in behavior and physiology. These approaches were enabled by manipulation of the gene encoding the *Pet-1* ETS transcription factor whose expression in the brain is restricted to developing and adult 5-HT neurons. Targeting of the *Pet-1* gene led to the development of a mouse line with a severe and stable deficiency of embryonic 5-HT-synthesizing neurons. The *Pet-1* transcription regulatory region has been used to create several new 5-HT neuron-type transgenic tools that have greatly increased the experimental accessibility of the small number of brain 5-HT neurons. Permanent and specific marking of 5-HT neurons with *Pet-1*-based transgenic tools have now been used for flow cytometry, whole cell electrophysiological recordings, progenitor fate mapping, and live time lapse imaging of these neurons. Additional tools provide multiple strategies for conditional temporal targeting of gene expression in 5-HT neurons at different stages of life. *Pet-1*-based approaches have led to advances in understanding the role of 5-HT neurons in respiration, thermoregulation, emotional behaviors, maternal behavior, and the mechanism of antipsychotic drug actions. In addition, these approaches have begun to reveal the molecular basis of 5-HT neuron heterogeneity and the transcriptional mechanisms that direct 5-HT neuron-type identity, maturation, and maintenance. **© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: serotonin, Pet-1, raphe, transcription, transgenic.

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Abbreviations: DRN, dorsal raphe nucleus; Fev, Fifth Ewing Variant; ROb, raphe obscurus; Tph, tryptophan hydroxylase; VGlut3, vesicular glutamate transporter 3.

Brain serotonin (5-HT) neuron signaling is a well known form of neuromodulation that shapes many behaviors and physiological processes through interactions with at least 14 broadly distributed postsynaptic receptor subtypes (Berger et al., 2009; Filip and Bader, 2009). The seemingly ubiquitous neuromodulatory role of the 5-HT system is effected by about 26,000 neurons in the rodent brain (Ishimura et al., 1988). These small numbers of neurons are sparsely intermingled with many non-serotonergic neurons in most of the raphe nuclei. In addition, while many 5-HT neurons are clustered in the midline raphe about 35% of them are scattered off the midline in disparate regions of the midbrain, pons, and medulla (Steinbusch, 1981). Similar to most neuronal types, this experimentally unwieldy neuroanatomical organization has made it very difficult to access 5-HT neurons for molecular and cellular studies of their development and their impact on postnatal behavior and physiology. Although genetic loss and gain of function studies have implicated the broadly expressed serotonin transporter gene (*Sert*) in numerous behavioral and physiological processes (Murphy et al., 2008), genetic-based methods to selectively and stably alter 5-HT neuron gene expression at different stages of life have been lacking. The identification of *Pet-1* (Pheochromocytoma 12 ets) (Fyodorov et al., 1998), an ETS (E26-specific) transcription factor gene, provided a solution to this methodological shortcoming. This review describes how the unique expression pattern of *Pet-1* has been exploited to create 5-HT neuron-type genetic based experimental approaches. The power of these approaches is then illustrated by highlighting some of their recent applications to questions that have been impossible or difficult to address with previously established experimental approaches: (1) transcriptional mechanisms that control 5-HT neuron identity, (2) the impact, in the intact animal, of 5-HT neuron perturbation at different stages of life on behavior, physiology, and pharmacology, and (3) 5-HT neuron molecular heterogeneity.

Pet-1 encodes a class 1 ETS factor whose human ortholog is Fifth Ewing Variant (Fev), an ETS gene whose fusion to the Ewing's sarcoma gene locus in a chromosomal translocation was identified in a subset of Ewing's sarcoma family of tumors (Peter et al., 1997). A degener-

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ate RT-PCR screen, aimed at identifying ETS factors expressed in the nervous system, led to the discovery of *Pet-1* sequences in PC12 cell total RNA. RNase protections assays then revealed extremely weak expression of *Pet-1* in total RNA isolated from whole brain. Consistent with the weak protection signal, a follow-up *in situ* hybridization study indicated that *Pet-1* transcripts were restricted to the raphe nuclei. *In situ* hybridization combined with anti-tryptophan hydroxylase (Tph) immunostaining showed that *Pet-1* is expressed in what appeared to be all 5-HT-synthesizing neurons of the rodent brain (Hendricks et al., 1999). Expression of rat and mouse *Pet-1* in the brain is induced specifically in postmitotic 5-HT neuron precursors about 0.5 days before the initiation of brain 5-HT synthesis (Hendricks et al., 1999; Pfaar et al., 2002). Thus, *Pet-1* is induced and maintained specifically in this single neuronal type across the lifespan. *Pet-1* is unique among the known factors that constitute the transcriptional network that specifies serotonergic phenotype, as it is the only one expressed specifically in 5-HT neurons (Scott and Deneris, 2005). Significantly, the spatiotemporal pattern of expression of the zebrafish ortholog, *zPet-1*, in the zebrafish brain is similar as it is restricted to raphe 5-HT neurons and is induced about 5 h before the onset of *zTph2*, the gene encoding the isoform of the rate-limiting enzyme responsible for zebrafish hindbrain 5-HT synthesis. Interestingly, *zPet-1* is not expressed in other zebrafish 5-HT-synthesizing neurons present in the diencephalon of this species but not in that of rodents (Lillesaar et al., 2007, 2009). In support of its functional orthology, *Fev* is expressed in the human and primate raphe in a pattern that suggests it is restricted to 5-HT neurons (Maurer et al., 2004; Iyo et al., 2005; Lima et al., 2009). A recent study (Kriegebaum et al., 2010) reported detection of *Fev* transcripts by RT-PCR in various dissected human forebrain regions. However, the cellular source of the RNA template in these seemingly weak amplifications was not identified. *Pet-1* is also expressed in small number of peripheral cell types, including the 5-HT-synthesizing enterochromaffin cells of the intestine, adrenal medulla, and pancreatic islets (Fyodorov et al., 1998; Ota et al., 2005; Wang et al., 2010).

Pet-1-BASED APPROACHES

Manipulation of *Pet-1* has enabled the development of diverse mouse molecular genetic approaches for the study of 5-HT neurons (Table 1). The first approach, simple germline targeting of *Pet-1*, provided insight into the transcriptional induction of 5-HT neuron identity as acquisition of serotonergic phenotype failed to occur in most *Pet-1*$^{-/-}$ embryonic 5-HT neuron precursors (Fig. 1). Second, unlike 5-HT synthesis inhibitors and serotonergic neurotoxins whose effects may not be specific to 5-HT and whose physiological and behavioral effects may not depend on

Table 1. *Pet-1*-based transgenic and targeted mice

Name	Background	Description	Application	References
Pet-1$^{-/-}$	C57BL/6*129sv; SJL congenic, n=10; C57BL/6J congenic, n=10	Germline-targeted *Pet-1* null	5-HT neuron differentiation	Hendricks et al., 2003; Erickson et al., 2007; Bonnin et al., 2011
Pet-1 floxed allele	C57BL/6*129Sv	*Cre recombinase* conditional allele	Temporal conditional targeting of *Pet-1*	Liu et al., 2010
ePet-Cre	C57BL/6*SJL	*Cre recombinase* controlled by *Pet-1* enhancer sequences	Gene targeting and optogenetic viral gene expression in 5-HT neurons	Scott et al., 2005; Hodges et al., 2008; Samaco et al., 2009; Liu et al., 2010; Depuy et al., 2011
ePet1::Flpe	Unknown	*Flp recombinase* controlled by *Pet-1* enhancer sequences	Intersectional/subtractive fate mapping of 5-HT neurons	Jensen et al., 2008
ePet::CreER^{T2ascend}	C57BL/6*129	Tamoxifen-inducible *Cre recombinase* controlled by *Pet-1* enhancer sequences	Temporal control of gene expression in ascending 5-HT neurons	Liu et al., 2010
Pet1-CreER^{T2}	Unknown	Tamoxifen-inducible *Cre recombinase* controlled by *Pet-1* enhancer sequences	Temporal control of gene expression in adult 5-HT neurons	Song et al., 2011
eFev::LacZ (Fev60Z)	C57BL/6*129Sv	*LacZ* controlled by human *Fev* enhancer sequences	5-HT neuron marker	Krueger and Deneris, 2008
ePet-EYFP	C57BL/6*SJL	Enhanced yellow fluorescent protein controlled by *Pet-1* enhancer sequences	5-HT neuron flow cytometry, electophysiology, cell culture, live cell imaging	Scott et al., 2005; Wylie et al., 2010; Hawthorne et al., 2010; Hawthorne et al., 2011
ePet::mycPet-1	C57BL/6*129sv	Myc epitope-tagged Pet-1 cDNA controlled by *Pet-1* enhancer sequences	Chromatin immunoprecipitation	Liu et al., 2010
Pet1-tTS	129S6Sv*C57BL/6*CBA	*Tetracycline-dependent transcriptional suppressor* controlled by *Pet-1* enhancer sequences	Inducible suppression of Htr_{1A} autoreceptor	Richardson-Jones et al., 2010; Richardson-Jones et al., 2011

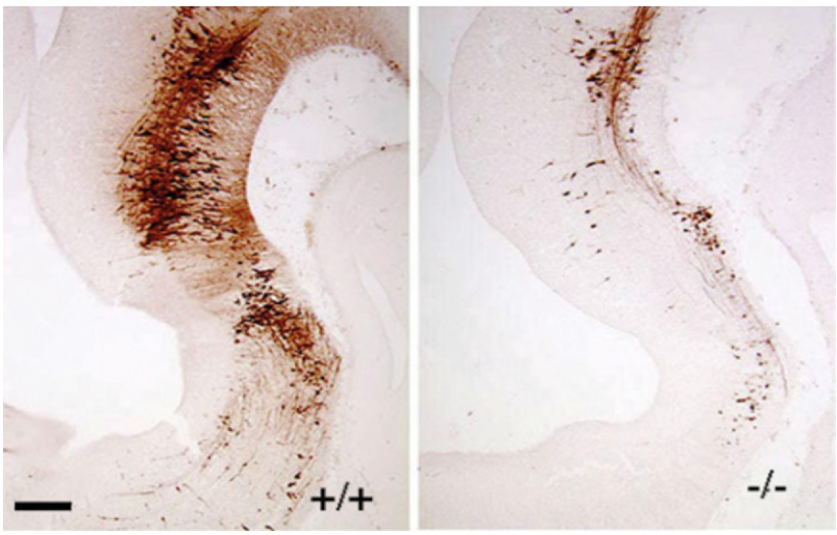

Fig. 1. Anti-5-HT immunoreactivity in the wild-type (left) and *Pet-1*$^{-/-}$ (right) anterior hindbrain at E11.5. Scale bar: 100 μm.

5-HT depletion (Choi et al., 2004; Knuth and Etgen, 2004; Dailly et al., 2006), *Pet-1* targeting resulted in the creation of a viable mouse strain in which the substantial deficiency of 5-HT-synthesizing neurons in *Pet-1* mutant embryos is invariant across the lifespan. The *Pet-1* mutant mouse, therefore, offered a new way with which to study the impact of an embryonic perturbation in serotonergic development on postnatal behavior and physiology. Third, the restricted expression of *Pet-1* to 5-HT neurons in the brain suggested that the *Pet-1* regulatory region could be used to create new genetic tools to specifically mark and manipulate the relatively small numbers of 5-HT neurons in the raphe as well as laterally scattered ones. This idea led to the isolation of the mouse *Pet-1* (Scott et al., 2005a) and human *Fev* (Krueger and Deneris, 2008) upstream enhancer regions (*ePet, eFev*). These enhancers were shown to direct highly reproducible transgene expression among independent transgenic lines in virtually all (>98%) 5-HT neurons in the developing and adult mouse brain with little or no ectopic expression, thus closely matching the spatiotemporal expression pattern of endogenous *Pet-1* (Fig. 2). A 40 kb *ePet* mouse BAC fragment and a 60 kb *eFev* human BAC fragment have been used to create several transgenes (Table 1) whose expression enabled permanent genetic marking of 5-HT neurons and constitutive and inducible control of gene expression specifically in these neurons (Scott et al., 2005b; Liu et al., 2010). In further support of the neuron-type specificity of *Pet-1*, a 5-HT neuron-specific enhancer region was isolated from the zebrafish *zPet-1* upstream region. The zebrafish enhancer directs eGFP expression in all 5-HT neurons of the zebrafish raphe with faint ectopic expression in scattered cells of the forebrain (Lillesaar et al., 2009).

TRANSCRIPTIONAL CONTROL OF 5-HT NEURONS ACROSS THE LIFESPAN

Because *Pet-1* expression in the brain is restricted to 5-HT neurons and precedes the onset of Tph2 expression and 5-HT synthesis, it is the earliest specific marker of the serotonergic lineage. This expression characteristic has aided studies of the transcriptional determinants that specify serotonergic progenitors (Pattyn et al., 2003; Jacob et al., 2007) and suggested that *Pet-1* regulatory elements could be used to mark early postmitotic serotonergic precursors to study their development. In fact, expression of an *ePet::EYFP* transgene specifically marks virtually all serotonergic precursors during the earliest periods of their migration (Hawthorne et al., 2010). These Yfp+ cells have a bipolar and radially shaped morphology; they express several markers of neuronal phenotype but not radial glia or markers of cellular proliferation. Consistent with their morphology and gene expression characteristics, time-lapse imaging of live Yfp+ precursors in slice cultures showed that 5-HT neurons migrate via somal translocation rather than by the more commonly studied radial glia-guided locomotion (Hawthorne et al., 2010).

Before the discovery of *Pet-1* expression in 5-HT neurons, very little was known about the intrinsic mechanisms that direct the development of these cells and how those mechanisms impact postnatal behavior and physiology (Rubenstein, 1998). Initial studies of *Pet-1* function focused on determining its role in the transcriptional network that specifies serotonergic identity (Hendricks et al., 2003). Germline targeting of the *Pet-1* locus created a null mutant in which no *Pet-1* RNA expression could be detected in the brain. Consistent with its induction at the postmitotic precursor stage, normal numbers of serotonergic precursors were detected in *Pet-1*$^{-/-}$ brain. However, about 70% of these precursors failed to induce the serotonergic-type gene battery, Tph2, AADC, *Sert*, Vmat2, MaoB encoding 5-HT synthesis, reuptake, vesicular storage, and enzymatic degradation. The aborted development of 5-HT neurons in *Pet-1*$^{-/-}$ mice caused an 80–90% deficiency of adult 5-HT levels in brain and spinal cord (5-HT levels are normal in adult *Pet-1*$^{+/-}$ brain). However, this gene defect did not trigger the apoptotic elimination of these mutant

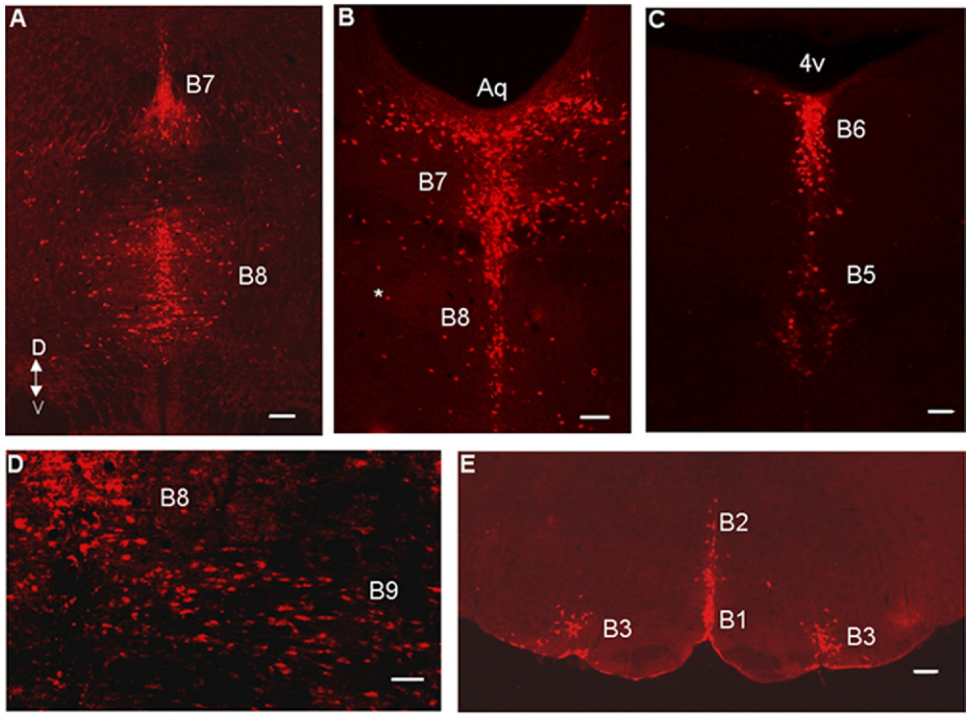

Fig. 2. A 40 kb mouse Pet-1 upstream genomic fragment directs LacZ transgene expression to virtually all 5HT neurons in the adult midbrain, pons, and medulla. Scale bars: (A, E) 200 μm; (B–D) 100 μm. B1, raphe pallidus; B2, raphe obscurus; B3, ventrolateral medulla; B5, pontine median raphe; B6, pontine dorsal raphe; B7, midbrain dorsal raphe; B8, midbrain median raphe.

cells at least in the dorsal raphe (Krueger and Deneris, 2008). Although the deficiency of 5-HT+ neurons in the *Pet-1*$^{-/-}$ brain is largely uniform across the nine raphe clusters in the midbrain, pons, and medulla, recent studies have shown that the 20–30% of *Pet-1* resistant 5-HT neurons that remain in the *Pet-1*$^{-/-}$ brain comprise a functionally and morphologically distinct subset of 5-HT neurons that continue to innervate circuitry involved in autonomic and stress responses (Kiyasova et al., 2011). Loss of *Pet-1* function does not affect the number of 5-HT producing enterochromaffin cells or the level of small intestinal mRNA encoding Tph1, the enzyme required for synthesis of peripheral 5-HT (Wang et al., 2010).

The ongoing expression of *Pet-1* into adulthood raised the possibility that it continues to function after fulfilling its initial role in serotonergic neurogenesis. Thus, two *Pet-1*-based conditional targeting approaches were developed to determine whether *Pet-1* regulates 5-HT system maturation during fetal life and maintenance of the serotonergic phenotype in adulthood. An *ePet-Cre* transgene that can direct recombination in 80–95% of 5-HT neurons, depending on the floxed target, was used to delete a floxed *Pet-1* allele. Because Cre recombinase activity was not evident with the *ePet-Cre* transgene until 1–2 days after the completion of serotonergic neurogenesis, this genetic strategy was used to investigate requirements for *Pet-1* during early 5-HT system maturation. Indeed, *Pet-1* expression levels in *Pet-1*$^{loxP/-;\ ePet\text{-}Cre}$ (*Pet-1*^{eCKO}) mice were normal at E11.5 when 5-HT neuron generation is largely complete but began to fade at E12.5 (Liu et al., 2010). A critical step in the maturation of 5-HT neuron function is the induction of the Htr1A and Htr1B autoreceptors that regulate 5-HT neuron firing frequency and transmitter release from the presynaptic terminal. Induction of these autoreceptor genes normally occurs at about E14 in the mouse (Liu et al., 2010). However, in *Pet-1*^{eCKO}, Htr_{1A} and Htr_{1B} induction failed in the majority of 5-HT neurons. Moreover, in whole cell recordings of *Pet-1* deficient 5-HT neurons that were genetically marked by *ePet-Cre*-directed activation of the *R26RYFP* locus, 8-OH-DPAT, a 5-HT_{1A} selective agonist, failed to elicit inwardly rectifying potassium currents that are characteristically elicited with the agonist in wild-type 5-HT neurons (Liu et al., 2010). These findings showed that induction of key autoreceptors during 5-HT system maturation depends on persistent *Pet-1* expression. In addition to regulating the induction of autoreceptors during 5-HT system maturation, ongoing *Pet-1* expression appears to be required to regulate innervation patterns of serotonergic axons (Liu et al., 2010). Injection of the retrograde tracer, Texas Red conjugated dextran, into the somatosensory cortex resulted in the marking of significantly fewer *Pet-1* deficient cell bodies in the *Pet-1*^{eCKO} dorsal raphe (DRN) compared to the number of tracer-positive 5-HT neurons in littermate control DRN.

A tamoxifen-inducible *Pet-1*-based targeting approach was then developed to investigate further functions of *Pet-1* specifically in adult 5-HT neurons (Liu et al., 2010). An *ePet-CreER*^{T2} transgenic line was established that expresses CreER^{T2} in nearly all 5-HT neurons of the midbrain, pons, and medulla. Tamoxifen-inducible targeting of LacZ expression in *ROSAR26*$^{ePet\text{-}CreERT2}$ mice was detected specifically in 5-HT neurons and not in other regions

of the brain. However, tamoxifen-inducible recombination directed by this line was largely restricted to the dorsal raphe, median raphe, and B9 cluster. Tamoxifen treatment of *Pet-1*$^{loxP/-;ePet\text{-}CreERT2}$ (*Pet-1*^{aCKO}) led to the targeting of *Pet-1* in about 75% of 5-HT neurons in the adult ascending 5-HT system comprising the DRN, median raphe, and B9 groups of 5-HT neurons; for reasons that are not yet clear *Pet-1* expression in the descending 5-HT subsystem was not significantly altered with this approach. The loss of *Pet-1* in the adult ascending system of *Pet-1*^{aCKO} mice caused substantial decreases in the expression of Tph2 and *Sert* demonstrating that *Pet-1* dependent transcription is required to regulate these two critical serotonergic genes across the lifespan. However, other genes such as AADC and Vmat2, whose embryonic induction depends on *Pet-1*, were not affected by loss of *Pet-1* in adult 5-HT neurons. In addition to Tph2 and *Sert*, loss of adult *Pet-1* resulted in a delayed loss of the vesicular glutamate transporter 3 (VGlut3) expression in the DRN. A subset of 5-HT neurons in the DRN normally express VGlut3, which may be required for glutamatergic/serotonergic co-transmission in these cells (Varga et al., 2009), and therefore, this finding raises the intriguing possibility that *Pet-1* may also be required for glutamatergic functions in 5-HT neurons. However, because VGlut3 is not restricted to 5-HT neurons, additional histological studies will be required to determine whether *Pet-1* is an intrinsic regulator of VGlut3 in 5-HT neurons.

Pet-1-based approaches have also been important for understanding the role of the LIM homeodomain protein, *Lmx1b*, in 5-HT neuron development. Nearly all ventral hindbrain precursors fail to acquire a serotonergic identity in germline-targeted *Lmx1b* mutant embryos demonstrating an essential role for *Lmx1b* in 5-HT neuron differentiation (Ding et al., 2003). However, *Lmx1b* null mutation is perinatal lethal (Chen et al., 1998), which precluded further studies of the *Lmx1b* deficiency in the postnatal period. To circumvent the lethality caused by global *Lmx1b* deficiency, an *Lmx1b* floxed allele was crossed to *ePet-Cre* mice in order to generate conditionally targeted *Lmx1b* mice (*Lmx1b*$^{f/f/p}$). *Lmx1b* expression was preserved during the period of serotonergic neurogenesis but began to decline at E12.5 in *Lmx1b*$^{f/f/p}$ mice. The conditional loss of Lmx1b resulted in a failure to maintain Tph2, *Sert*, and *Pet-1* expression. Interestingly, nearly all 5-HT neuron cell bodies were eliminated in the early postnatal mutant brain resulting in >99% loss of 5-HT, while peripheral 5-HT levels were unaltered. Thus, serotonergic conditional targeting of *Lmx1b* revealed a critical role for this factor in the maintenance and survival of 5-HT neurons (Zhao et al., 2006). Despite the profound deficit in brain 5-HT, the majority of *Lmx1b*$^{f/f/p}$ mice survive into adulthood and therefore this *Pet-1*-based conditionally targeted strain has been used in numerous studies aimed at determining the impact of a virtually complete serotonergic deficiency on behavior and physiology (see below).

Song et al. used a different *Pet-1* BAC to express CreER^{T2} for inducible targeting of *Lmx1b* in adult 5-HT neurons. This *Pet-1*-CreER^{T2} BAC transgene directed recombination in 85% of 5-HT neurons, but recombination was not detected elsewhere in the brain. Targeting of *Lmx1b* in adult 5-HT neurons revealed that similar to *Pet-1*, *Lmx1b* is also required to maintain Tph2 and *Sert* expression but not AADC expression in adult 5-HT neurons (Song et al., 2011). However, in contrast to *Pet-1*, *Lmx1b* was also required to maintain Vmat2 expression. Furthermore, unlike at embryonic stages *Lmx1b* was no longer required to maintain *Pet-1* expression in adult 5-HT neurons. Although *Lmx1b* is required for the maintenance of midbrain dopaminergic (DA) neurons, the 5-HT conditional targeting strategy had no effect on DA (and noradrenergic) phenotype, which further corroborates the 5-HT neuron-type specificity of the *Pet-1*-based targeting approaches.

PERTURBATION OF 5-HT SYSTEM DEVELOPMENT AND ITS IMPACT ON PHYSIOLOGY AND BEHAVIOR

Neonatal growth and survival. Because of their stable deficiencies of 5-HT synthesizing neurons, *Pet-1*$^{-/-}$ and *Lmx1b*$^{f/f/p}$ mice have been used extensively to determine the impact of embryonic disruption of serotonergic signaling on postnatal physiology and behavior. A highly anticipated experiment with *Pet-1*$^{-/-}$ mice was to determine whether embryonic disruption of 5-HT neurons would impair offspring viability. Although expected Mendelian proportions of viable *Pet-1*$^{-/-}$ births were found, 20–30% of the *Pet-1*$^{-/-}$ neonates did not survive to weaning, which was a significantly higher mortality compared to their +/+ and +/− littermates (Hendricks et al., 2003; Erickson et al., 2007). In addition, *Pet-1*$^{-/-}$ neonates had a significantly lower body weight at birth, which persisted into the second postnatal week but normalized in adulthood. Interestingly, *Lmx1b*$^{f/f/p}$ neonates were also found to have comparably impaired viability and growth trajectories with the later abnormality normalizing in young adulthood (Hodges et al., 2009). In support of a specific requirement for brain 5-HT synthesis, growth and viability was also found to be impaired, depending on the genetic background, in Tph2$^{-/-}$ neonates, which synthesize virtually no brain 5-HT but have normal peripheral (blood and gut) 5-HT levels (Alenina et al., 2009). Importantly, survival is normal in Tph1$^{-/-}$ mice that are diabetic and whose brain 5-HT levels are normal but peripheral 5-HT levels are <10% wild-type levels (Walther et al., 2003a,b). These findings support a model in which *Pet-1*/*Lmx1b*-transcriptional control of Tph2-dependent brain 5-HT synthesis but not peripheral 5-HT synthesis is required for normal neonatal growth trajectories and optimal viability.

Another experiment of particular interest was to test whether an embryonic deficiency of 5-HT neurons would lead to developmental abnormalities in brain cytoarchitecture. This question was stimulated by a large literature supporting a role for early 5-HT signaling in neural cell proliferation, differentiation, migration, and circuit formation in the brain (Gaspar et al., 2003). However, in contrast to the overt disruption of forebrain circuitry in mice with genetically engineered increases in the levels of 5-HT

(Cases et al., 1996), the substantial deficiency of 5-HT-synthesizing neurons in *Pet-1*$^{-/-}$ and the virtually complete absence of these cells in *Lmx1b*$^{f/f/p}$ mice did not cause gross structural alterations in CNS cellular organization (Hendricks et al., 2003; Zhao et al., 2006; Stankovski et al., 2007). Although subtle circuit alterations may yet be found, these findings raise questions about the impact of brain 5-HT on brain morphogenesis. It is important to keep in mind, however, that most 5-HT is synthesized outside the brain in multiple tissues and cell types. A widely discussed hypothesis posits that 5-HT produced in the periphery supplies the developing brain before blood–brain barrier formation, and it is this source of 5-HT that is critically important for the fetal brain. Significantly, Bonnin et al. has shown that 5-HT levels are normal in the *Pet-1*$^{-/-}$ fetal forebrain until E16.5 despite the dramatic deficiency in hindbrain 5-HT synthesis beginning at E10.5. Further studies showed that 5-HT is synthesized in the placenta by the Tph1/AADC pathway and transported via the fetal circulation to the fetal forebrain before the arrival of 5-HT raphe axons (Bonnin et al., 2011). This provocative finding has stimulated great interest in determining the impact of placental 5-HT synthesis on early fetal brain development and postnatal behavior.

Maternal nurturing. Although correlative evidence in non-human primates suggested serotonergic function might be required for maternal behavior, a clear link was not established until the idea was tested in *Pet-1*$^{-/-}$ mice. When *Pet-1*$^{-/-}$ females were crossed to +/+ or *Pet-1*$^{+/-}$ males, normal numbers of offspring were born but all of them failed to survive within 4 days of birth regardless of their *Pet-1* allelic genotype (Lerch-Haner et al., 2008). However, offspring mortality did not occur if the pups were cross-fostered to a wild-type mother at P0 or P1. Specific tests of maternal behavior showed significant deficits in pup retrieval and nest building and an absolute failure of the dams to huddle pups. These specific deficits were not accompanied by maternal olfactory deficits, changes in maternal anxiety-like behaviors, or failure to nurse (Lerch-Haner et al., 2008). Further support for serotonergic function and specifically brain 5-HT synthesis but not peripheral 5-HT synthesis in maternal behavior was the finding that although normal numbers of offspring were born to Tph2$^{-/-}$ dams most of them died within 2–3 days of birth unless cross-fostered to +/+ dams (Alenina et al., 2009). Similar to *Pet-1*$^{-/-}$ dams, Tph2$^{-/-}$ dams delivered milk to their pups but exhibited deficits in pup retrieval and eventually abandoned them. In contrast to *Pet-1*$^{-/-}$ dams, however, Tph2$^{-/-}$ dams often cannibalized their pups but did not show deficits in nest construction. The different genetic backgrounds of the *Pet-1*$^{-/-}$ and Tph2$^{-/-}$ mice might be responsible for specific differences in nurturing abnormalities in these two targeted strains. Alternatively, the different types of 5-HT system lesions in these strains (complete loss of 5-HT synthesis in Tph2$^{-/-}$ mice versus preservation of some 5-HT neurons in *Pet-1*$^{-/-}$ mice) are a potential explanation for the phenotypic differences.

Respiration and thermoregulation. 5-HT, substance P, and TRH released from medullary 5-HT neurons are well-established modulators of CNS respiratory circuitry, *in vitro*, and the preponderance of evidence supports a model in which the overall effect of 5-HT neuron firing is to stimulate respiratory drive (Hodges and Richerson, 2008). What has been more difficult to address is the role 5-HT neurons play in breathing within the intact animal. *Pet-1*-based strategies have been used to investigate this question and are now preferred over older pharmacological and neurotoxin lesion approaches that have produced inconsistent effects on serotonergic depletion and contradictory effects on breathing (Hodges and Richerson, 2010). Plethysmographic recordings from *Pet-1*$^{-/-}$ newborns (Erickson et al., 2007) raised in pathogen free conditions showed that the embryonic deficiency of 5-HT-synthesizing neurons caused a delay in respiratory maturation as evidenced by an initially depressed breathing frequency and increased incidence of prolonged apneas. If *Pet-1*$^{-/-}$ neonates were raised under pathogen-exposed conditions, the variability of respiratory parameters significantly increased and the incidence of hypoxia-induced apneas increased dramatically in comparison to *Pet-1*$^{-/-}$ neonates raised in pathogen-free conditions (Erickson et al., 2007). These abnormalities largely resolved by the second postnatal week. *In vitro* studies identified depressed and unstable hypoglossal nerve discharges in medullary slices taken from *Pet-1*$^{-/-}$ neonates, suggesting altered serotonergic modulation or development of the central respiratory rhythm generator is responsible for the abnormal neonatal breathing behavior (Erickson et al., 2007). Both *Pet-1*$^{-/-}$ neonates (Erickson and Sposato, 2009) and adults (St-John et al., 2009) can generate normal gasps, however, *Pet-1*$^{-/-}$ neonates elicited an abnormal gasping pattern in response to hypoxia-induced apnea that delayed restoration of eupenic breathing via autoresuscitation (Erickson and Sposato, 2009). In addition, *Pet-1*$^{-/-}$ neonates display lowered resting heart rate and an increased magnitude and incidence of spontaneous bradycardias compared to littermate controls (Cummings et al., 2010).

Consistent with their larger, nearly total absence, of brain 5-HT synthesizing neurons compared to *Pet-1*$^{-/-}$ mice, *Lmx1b*$^{f/f/p}$ neonates have more dramatic breathing abnormalities including severe hypoventilation and more frequent and longer apneas (Hodges et al., 2009). Similar to *Pet-1*$^{-/-}$ neonates these abnormalities largely resolved, albeit more slowly, as the animal matured. Furthermore, *Pet-1*$^{-/-}$ adult females but not males exhibit deficits in resting breathing, but these deficits were modest compared to those in female and male *Lmx1b*$^{f/f/p}$ mice (Hodges et al., 2011).

An optogenetic study of 5-HT neurons in the raphe obscurus (ROb) was aimed at determining what effect stimulation of these cells has on respiration, *in vivo* (Depuy et al., 2011). An adeno-associated viral construct was injected into the ROb for conditional expression of inverse oriented double-floxed channelrhodopsin-mCherry sequences in *ePet-Cre* mice. Co-immunostaining indicated that 97% of infected cells could be identified as Tph+ 5-HT

neurons and nearly all of these cells were within the limits of ROb. mCherry+ serotonergic axonal projections were identified in close association with all of the respiratory motor neuron groups in the medulla and the pre-Botzinger complex, the putative site of the respiratory rhythm generator. Photostimulation of the ChR2+ 5-HT neurons in the ROb caused increased breathing frequency and magnitude, which were attenuated by the serotonergic receptor antagonist, methysergide. These findings nicely demonstrate the power of 5-HT neuron specific optogenetic approaches, *in vivo*, for understanding the physiological roles of specific subsets of 5-HT neurons.

Pet-1 and *Lmx1b*$^{f/f/p}$ mice have also been used to clarify the role of the 5-HT system in thermoregulation, *in vivo*. Although adult *Lmx1b*$^{f/f/p}$ mice maintain normal circadian control of core body temperature under ambient conditions, they have a severe defect in maintaining core body temperature in response to moderate cold challenge. This defect is the result of abnormal shivering responses and sustained activation of brown adipose tissue and not from a defect in thermosensory perception (Hodges et al., 2008). Both female and male *Pet-1*$^{-/-}$ mice show a defect in defense to cold challenge, but it is less severe than that seen in *Lmx1b*$^{f/f/p}$ mice (Hodges et al., 2011).

Aggression, fear, and anxiety-related behaviors. A rich history of studies in humans, non-human primates, and rodents has demonstrated that alterations in 5-HT system function can have dramatic effects on stress and emotion-related behaviors (Holmes, 2008). For example, pharmacological blockade of *Sert* (Ansorge et al., 2008; Popa et al., 2008) or gene targeted loss of *Sert* function in mice causes highly reproducible alterations in anxiety-like and depressive-like behaviors (Murphy and Lesch, 2008). Non-conditional targeting of Htr1A (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998) and pharmacological blockade of 5-HT$_{1A}$ receptors in the early postnatal period also results in increased anxiety-like behavior (Lo Iacono and Gross, 2008). In addition, Tph2 loss of function has been reported to result in increased aggression (Beaulieu et al., 2008; Alenina et al., 2009) and anxiety-like behavior (Beaulieu et al., 2008), although other studies of Tph2 deficient mice have reported more mild behavioral alterations (Savelieva et al., 2008). Because of *Pet-1*'s role in the induction of Tph2, *Sert*, and Htr1A, a logical question was whether disruption of *Pet-1*-dependent transcription would also alter these behaviors. Behavioral analyses of *Pet-1*$^{-/-}$ mice reported significantly increased male aggression in the resident–intruder assay (Hendricks et al., 2003). Further evidence in support of serotonergic transcriptional control of aggression was the finding of increased male aggression in *ePet-Cre* direct loss of a floxed allele encoding the transcriptional repressor, methyl CpG binding protein (MeCP2), in 5-HT neurons (Samaco et al., 2009).

Pet-1$^{-/-}$ mice also showed striking increases in anxiety-like behavior in the elevated plus maze and open field versus +/+ littermate controls (Hendricks et al., 2003). A recent study used another ePet controlled transgene, *Pet1-tTS*, for 5-HT neuron-specific expression of a tetracycline-regulated transcriptional suppressor, tTS (Richardson-Jones et al., 2010). This transgene was used to specifically diminish Htr1A autoreceptor expression (but not Htr1A postsynaptic heteroreceptors) from a knock-in allele in which tetracycline transactivator operator sequences were introduced into the Htr1A promoter region. Interestingly, suppression of Hrt1A autoreceptors beginning in the embryo and continuing into adulthood led to increased anxiety-like behaviors in the open field and light/dark choice exploration tests (Richardson-Jones et al., 2011). In contrast, reducing the expression of Htr1A autoreceptors in adult 5-HT neurons by removing doxycycline from chow fed to mice aged 7 weeks led to increased stress responses but not increased anxiety-like behavior in the conflict-based assays (Richardson-Jones et al., 2010). These findings support a model in which *Pet-1*-dependent induction of Htr1A autoreceptors and/or *Sert* and Tph2 during development is required for development of normal anxiety-like behaviors. However, other laboratories failed to detect increased anxiety-like behaviors in *Pet-1*$^{-/-}$ mice in conflict-based aversive environments perhaps because of differing testing conditions (Schaefer et al., 2009; Kiyasova et al., 2011). Instead, *Pet-1*$^{-/-}$ mice showed significantly increased contextual and acoustic cue conditioned fear responses (Kiyasova et al., 2011). *Lmx1b*$^{f/f/p}$ mice also showed enhanced contextual fear memory but reduced anxiety-like behavior (Dai et al., 2008). Thus, notwithstanding inconsistent findings, studies of *Pet-1*$^{-/-}$ and *Lmx1b*$^{f/f/p}$ mice indicate that altered transcriptional induction of 5-HT neuron identity leads to susceptibility for altered emotional behaviors in adulthood.

To determine whether *Pet-1*-dependent transcription was required in adult 5-HT neurons to preserve normal anxiety-like behaviors, 6–8-week-old tamoxifen-treated *Pet-1*^{aCKO} mice were analyzed in the elevated plus maze, open field, and light–dark box tests. Two different cohorts of tamoxifen-treated but not vehicle-treated *Pet-1*^{aCKO} mice tested during different seasons showed significantly elevated anxiety-like behavior in all three tests compared to tamoxifen-treated littermate *Pet-1*$^{flox/-}$ controls (Liu et al., 2010); tamoxifen treatment did not alter these behaviors in wild-type mice. These findings revealed an ongoing requirement for *Pet-1* function in adult 5-HT neurons for transcriptional control of anxiety-like behavior.

Antipsychotic drug actions. *Pet-1*$^{-/-}$ mice have also been used in studies aimed at understanding the mechanisms of action of atypical antipsychotics. Atypical antipsychotics such as clozapine are highly efficacious in the treatment of schizophrenia but, unlike typical antipsychotics, do not trigger disabling extrapyramidal symptoms. Clozapine treatment is not without serious side effects, however, which underscores the need for development of newer atypical antipsychotics that are more effective but have fewer side effects. A critical gap in knowledge that has hindered efforts to develop better antipsychotics is the poor understanding of clozapine's mechanism of action. A clue, however, was the finding that clozapine and the

closely related atypical antipsychotic olanzapine but not the typical antipsychotic, haloperidol, display high affinity interactions with most 5-HT GPCRs and lower affinity interactions with D2, D3, and D4 dopamine receptors (Yadav et al., 2011). While these findings are consistent with the earlier postulated role of postsynaptic 5-HT receptors as substrates for clozapine action, genetic deletion of the 5-HT 2A receptor, a hypothesized principal target of clozapine, had little effect on clozapine's antipsychotic-like properties: normalization of disrupted pre-pulse inhibition (PPI) by the non-competitive NMDA antagonist and schizophrenia mimetic, phencyclidine. To determine whether 5-HT neurons were required for atypical antipsychotic-like actions, clozapine and olanzapine were tested in *Pet-1*$^{-/-}$ mice (Yadav et al., 2011). While pretreatment of +/+ mice with clozapine or olanzapine completely normalized PCP-disrupted PPI, normalization was abolished in *Pet-1*$^{-/-}$ mice. This unexpected requirement for 5-HT neurons suggests that development of more effective atypical antipsychotics may depend on drug actions that alter pre-synaptic serotonergic activity in addition to concomitant effects on various post-synaptic receptors (Yadav et al., 2011).

5-HT NEURON HETEROGENEITY

Classic immunohistochemical studies with anti-5-HT antisera (Lidov and Molliver, 1982; Wallace and Lauder, 1983) showed that rodent 5-HT neurons are born in two bilateral longitudinal domains on either side of the floor plate in the ventral hindbrain. The anterior or rostral domain positioned at the level of rhombomeres 1–3 was found to give rise to 5-HT neurons that populate the dorsal and median raphe, while the posterior or caudal domain at r5–r8 gave rise to 5-HT neurons that coalesce into the medullary raphe nuclei. Recently, a new molecular genetic approach, intersectional and subtractive genetic lineage tracing, has provided a more precise way to define the disparate embryonic hindbrain origins of different raphe nuclei (Jensen et al., 2008). This approach took advantage of the distinct serotonergic specification programs operating at discrete axial levels of the hindbrain to follow the anatomical fate of rhombomere-specific progenitor pools. A dual Flp and Cre recombinase conditional indicator allele was combined with an *ePet::FLPe* driver expressing flp recombinase specifically in 5-HT neuron precursors and different rhombomere-specific Cre drivers (Kimmel et al., 2000). Cells that had an intersecting history of expressing both Flp and Cre activated a LacZ indicator allele, thus identifying those mature 5-HT neurons that originated from specific rhombomeres. The findings of this study demonstrated the exclusive r1 origin of the entire dorsal raphe nucleus and revealed that the median raphe and the B9 cluster of 5-HT neurons are formed by a commingling of cells derived from progenitors that populate r1–r3. In addition, the intersectional gene expression approach offers a way to define and access subsets of hindbrain 5-HT neurons based on their distinct genetic lineages.

Different serotonergic lineages defined by intersectional fate mapping are consistent with the rich diversity of 5-HT neurons that is evident in their diverse axonal trajectories, anatomical locations, physiological properties, and molecular requirements. To directly identify the gene expression differences that might account for the diversity of serotonergic neurons, whole genome expression profiling was performed on FACS purified *ePet-EYFP* expressing rostral and caudal 5-HT neurons (Wylie et al., 2010). This study focused exclusively on E12.5 5-HT neurons and therefore was designed to identify expression of genes that were more likely to regulate serotonergic differentiation and maturation rather than progenitor specification. Enriched expression of hundreds of genes not previously associated with 5-HT neurons was identified. Furthermore, comparative analysis of rostral and caudal 5-HT neurons identified hundreds of genes, encoding transcription factors, axon guidance, intracellular signaling, and other kinds of proteins, whose expression was differentially enriched between these two groups of neurons. For example, expression of the closely linked Hmx2 and Hmx3 homeodomain genes was detected in rostral 5-HT neurons but not caudal ones while expression of several Hox genes was detected in caudal but not rostral 5-HT neurons. These findings revealed deep molecular and potential biological pathway differences between rostral and caudal 5-HT neurons, which may be responsible for generating 5-HT neuron heterogeneity. Functional studies will be required to determine the importance of rostrocaudal gene expression heterogeneity and whether distinct homeodomain codes specify subtypes of 5-HT neurons.

CONCLUSION

The experimental accessibility of 5-HT neurons has been greatly expanded in recent years with the development and application of 5-HT neuron-type transgenic tools. These tools have made possible studies that have revealed key features of the transcriptional mechanisms that direct differentiation, maturation, and maintenance of 5-HT neurons. Genetic disruption of 5-HT neuron development and function is beginning to provide a better understanding of the physiological roles of 5-HT neurons and the impact, *in vivo*, of serotonergic alterations at different stages of life. The demonstration that genetic disruption of 5-HT neuron development severely impairs maternal behavior and offspring survival has challenged prevailing wisdom about the dispensability of the 5-HT system for essential physiological processes. The wide range of behavioral and physiological phenotypes arising from disruption of the transcriptional programs that generate 5-HT neurons reinforces the idea that perturbation of serotonergic gene expression is a potential avenue to vulnerability for several disorders of the nervous system. Further mapping of diverse 5-HT neuron transcriptomes and the refinement of 5-HT neuron-type genetics approaches will help to define functional neuronal heterogeneity within the serotonergic system and the precise roles of brain 5-HT subsystems in modulation of specific behaviors and physiological processes.

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