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Serotonergic transcriptional networks and potential importance to mental health

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Summary

Transcription regulatory networks governing the genesis, maturation and maintenance of vertebrate brain serotonin (5-HT) neurons determine the level of serotonergic gene expression and signaling across the lifespan. Recent work suggests alterations in these networks can cause behavioral and physiological pathogenesis in mice. Here, we synthesize findings from vertebrate loss and gain of function studies to build a new model of the transcriptional regulatory networks that specify 5-HT neurons during fetal life, integrate them into CNS circuitry in early postnatal life and maintain them in adulthood. We then describe findings from animal and human genetic studies that support possible altered activity of serotonergic regulatory networks in the etiology of mental illness. We conclude with a discussion of the potential utility of our model, as an experimentally well-defined molecular pathway, to predict and interpret the biological impact of rare human variation that may be discovered in the network.

... “it is possible that the 5-HT in our brains plays an essential part in keeping us sane...”

Sir John Gaddum, 1954

Ever since the heady days of Sir John Gaddum’s animal and self-administration studies of the serotonergic hallucinogen, lysergic acid diethylamide, there has been an incessant quest for an understanding of brain serotonin (5-HT) system function and its importance in behavior and neuropsychiatric disease. An extensive literature that began to appear soon after Gaddum’s and others’ groundbreaking studies of 5-HT^{1,2} has implicated altered 5-HT signaling in the etiology of an apparently disparate group of neurodevelopmental disorders including depression, anxiety, obsessive compulsive disorder, disorders of energy balance, autism, schizophrenia and sudden infant death syndrome³. The wide range of disorders linked to serotonergic dysfunction is not surprising as the brain 5-HT transmitter system innervates nearly all cytoarchitectonic regions of the brain and spinal cord and has been implicated in the modulation of seemingly every human behavior and physiological process orchestrated by the nervous system. But what is quite striking, in view of this impressive breadth of influence, is that the 5-HT system originates from an exceedingly small proportion of neurons genetically programmed to make and use 5-HT as a neurotransmitter. 5-HT neuron numbers are estimated to be 26,000 in the mouse brain⁴ and roughly 300,000 in the human brain^{5,6}. The prevailing hypothesis of the role of 5-HT neurons in mental health disorders is that genetic determinants and their interaction with environmental factors program too little or too much 5-HT signaling emanating from these small numbers of neurons at critical developmental stages, which consequently disrupts the developmental trajectories of CNS circuit formation and thereby imposes susceptibility to mental illness⁷. As the genetic determinants of early 5-HT system function, it is

straightforward to understand why the regulatory programs that implement serotonergic neurogenesis and consequently determine the level of brain 5-HT signaling have gained considerable attention over the past decade for their potential biological relevance to neuropsychiatric disorders^{8–11}

Here, we review recent progress in understanding the regulatory programs governing 5-HT neuron development and function. We assemble from recent loss and gain of function findings a model of the transcriptional network constituting the 5-HT regulatory programs that direct the generation, maturation and maintenance of brain 5-HT neurons. We then discuss the potential utility of the network, as an experimentally well-defined molecular pathway, in understanding the elusive mechanisms of 5-HT system-related neuropsychiatric disease pathogenesis.

5-HT neuron identity

The small numbers of brain 5-HT neurons express hundreds of genes that encode generic molecular and cellular characteristics shared by all types of neurons such as the ion channels necessary for action potential generation and proteins required for release of neurotransmitter at synapses. However, it is the selection for expression of a serotonergic-type gene battery comprising *tryptophan hydroxylase 2* (*Tph2*), *aromatic amino acid decarboxylase* (*Aadc*), plasma membrane *serotonin transporter* (*Slc6a4* or *Sert*), *vesicular monoamine transporter* (*Slc18a2* or *Vmat2*), *monoamine oxidase A and B* (*MaoA, B*), *5-HT1a autoreceptor* (*Htr1a*), *5-HT1b autoreceptor* (*Htr1b*) and the several genes encoding the synthetic and recycling enzymes for tetrahydrobiopterin cofactor (BH₄) synthesis that endows these cells with the specialized capacity to synthesize, transport, autosecrete, inactivate through reuptake and metabolize 5-HT; hence establishing its physiological function as a neurotransmitter (Fig. 1). Although each of these 5-HT type genes is essential for serotonergic function none of them uniquely define brain 5-HT neuron identity as each of them, even the rate-limiting 5-HT synthetic enzyme, TPH2, is expressed in other neural and non-neural¹² cell types. What determines 5-HT neuron identity, therefore, is the integration of aggregate serotonergic characteristics encoded by the serotonergic-type gene battery with generic neuronal characteristics⁸. The set of genetic instructions that coordinates expression of serotonergic-type and neuronal-type characteristics constitutes an intrinsic regulatory program directing brain 5-HT neuron identity. Cell fate regulatory programs comprise complex networks of interacting transcription factors, associated signaling proteins, and the cis regulatory sequences through which combinations of transcription factors control target genes¹³. A particular regulatory program specifies and then progressively transforms proliferating neural progenitors into neurons of a particular identity, such as one able to synthesize 5-HT. The regulatory program controlling serotonergic neurogenesis, however, is not sufficient to equip 5-HT neurons with the capacity to exert widespread neuromodulatory influence on CNS circuitry. Additional intrinsic programs, therefore, likely regulate 5-HT neuron maturation during which these neurons are physiologically connected with 5-HT receptor bearing post-synaptic circuitry in widely distributed serotonergic axonal termination zones. Recent findings to be discussed below, also demonstrate that intrinsic serotonergic regulatory programs act in adulthood to maintain serotonergic signaling and plasticity.

Rhombencephalic 5-HT neuron development

The neuroanatomical development and distribution of vertebrate 5-HT neurons is well described in rats, primates and zebrafish with immunohistochemical approaches^{14–18} and more recently in mice with genetic based fate mapping¹⁹ (Fig. 2). 5-HT neuron development comprises neurogenesis and maturation. During the relatively brief period,

E9.5 to E12, of neurogenesis mouse 5-HT neurons are specified and born in the ventral rhombencephalon (Fig. 2a). These newly born 5-HT neurons will ultimately cluster in several disparate regions of the midbrain, pons, and medulla to form the raphe nuclei (Fig. 2b). The period of 5-HT neuron generation, like that of all neurons, comprises several experimentally recognizable stages (Fig. 2c). During the first stage, the secreted signaling molecules, sonic hedgehog (Shh), and fibroblast growth factors 4 and 8 regionally pattern the mid-hindbrain neuroepithelium along the doroventral and anterior-posterior (AP) axes, respectively, to specify serotonergic progenitors in the ventral hindbrain. The reader is referred to several articles that provide an up to date review of these early patterning events⁷⁻⁹. The first progenitors committed to a serotonergic fate appear in rhombomere (r) 1 where they generate 5-HT neurons from about E9.5 to E10.5. 5-HT neurons are born about a day later in r2 and r3 because progenitors at these longitudinal levels initially generate visceromotor neurons (vMN) before becoming competent to generate 5-HT neurons²⁰. A similar motorneuron to 5-HT neuron switch in progenitor fate occurs with similar temporal characteristics in r5-r8 such that caudal 5-HT neurons are born virtually simultaneously with those in the rostral domain. However, there is a 1-2 day delay in the synthesis of 5-HT in these newly born caudal cells²¹. The switch to serotonergic neurogenesis is never launched under normal circumstances in r4 so that motor neurogenesis persists in this rhombomere²⁰. During the next stage progenitors exit the cell cycle to become postmitotic neuronal precursors that do not yet possess serotonergic identity. Serotonergic identity is then acquired through coordinate expression of serotonergic-type gene battery. The newborn 5-HT neurons are phenotypically immature (Fig. 2c) and have not yet been integrated into neural circuitry. Therefore, beginning immediately after the birth of 5-HT neurons and extending at least to the end of the third postnatal week mouse 5-HT neurons undergo a series of complex maturational events^{15, 21}. These events include cell body migration, dendritic growth, expression of 5-HT autoregulatory pathways, formation of highly collateralized axonal pathways and synaptogenesis.

Intrinsic serotonergic transcription regulatory network

Loss and gain of function studies in mouse and chick have greatly illuminated the idiosyncratic intrinsic roles of eight transcriptional regulators and their complex interactions that progressively restrict the fate of progenitors to a 5-HT phenotype. The proneural bHLH factor, *Asc11* (also known as *Mash1*), and the homeodomain protein, *Nkx2.2*, are expressed at the vMN and 5-HT progenitor stages at all rhombomeric levels of the hindbrain and their induction is likely an early response to Shh signaling²²⁻²⁴. Their expression is extinguished as progenitors exit the cell cycle to become postmitotic precursors. The forkhead box factor, *Foxa2*, is expressed at a very low level in vMN progenitors but is induced as these progenitors switch to serotonergic neurogenesis²⁵. It is not expressed in r4 where 5-HT neurons are not generated. Next, expression of the zinc finger proteins *Gata2*, *Gata3*, and *Insm1* is initiated as serotonergic progenitors transit to the postmitotic precursor stage^{24, 26, 27}. Finally, expression of the Lim homeodomain, *Lmx1b*, and then the ETS factor, *Pet-1* are induced in postmitotic precursors several hours before 5-HT or TPH2 become detectable^{16, 28, 29}.

Few of the factors in the network are sufficient by themselves to induce 5-HT cell fate. Misexpression of either *Nkx2.2* or *Gata2* are sufficient to specify a 5-HT transmitter phenotype but only when they are misexpressed in chick r1²⁴. In contrast, combined misexpression of *Lmx1b* and *Pet-1* is able to induce ectopic 5-HT-containing cells in the ventral spinal cord. If *Nkx2.2* is included with *Lmx1b* and *Pet-1* then ectopic 5-HT-containing cells can be generated in the dorsal cord³⁰.

Specification of serotonergic progenitors

Nkx2.2

Loss of *Nkx2.2* does not affect 5-HT neurons in r1 but 5-HT neuron production is substantially reduced, although not eliminated in all of the posterior rhombomeres^{19, 22}. The role, if any, of *Nkx2.2* in r1 is not clear and it might be compensated by the coexpressed paralog *Nkx2.9*. However, analysis of *Nkx2.2* and *2.9* compound mutants has not been reported. *Nkx2.2* regulates 5-HT neuron production in rhombomeres posterior to r1, first, by controlling a temporal switch (Fig. 3) in progenitor fate from one of vMN to 5-HT neurons²⁰. It does so by regulating the timing of expression of another homeodomain protein, *Phox2b*, which functions as a proneural factor in vMN generation except in r1 where 5-HT neurons but not vMN are generated. In *Phox2b* mutants, vMN genesis fails throughout the hindbrain³¹ and instead 5-HT neurons are prematurely generated in r2–r3, r5–r8 and ectopic ones are generated in r4²⁰. In addition to playing an essential role in the vMN/5-HT temporal switch *Nkx2.2* is also required to switch on the downstream transcriptional network that directly induces the 5-HT-type gene battery (Fig. 4). In *Nkx2.2* mutants, expression of *Gata3*, *Lmx1b*, and *Pet-1* are greatly reduced posterior to r1³⁰. Misexpression of *Nkx2.2* in the chick hindbrain induces *Gata2*, *Gata3*, and 5-HT transmitter phenotype in r1²⁴. In addition, *Nkx6.1* is required for development of 5-HT in chick r1²⁴.

Foxa2

Foxa proteins are well known for playing multiple roles within single cell lineages³² and the serotonergic lineage is no exception. Foxa2 acts in a temporally regulated manner to establish serotonergic progenitor domains in the ventral hindbrain and then to activate expression of transcription factors that are directly involved in 5-HT neuron precursor differentiation²⁵. Foxa2 represses the homeodomain protein, *Phox2b*, in r1 (Fig. 3) and in so doing permanently suppresses vMN fate while promoting serotonergic fate of the resident progenitors. In more posterior rhombomeres except r4, *Phox2b* initially represses *Foxa2* hence directing a motor neuron fate of resident progenitors up to about E10.5 (Fig. 3). After E10.5 a reciprocal repression occurs in which Foxa2 now represses *Phox2b* to switch progenitors to a serotonergic fate. In r4, *Phox2b* permanently represses *Foxa2* hence preventing serotonergic neurogenesis (Fig. 3). The mechanism underlying the cross repressive switch is not understood. Ongoing Foxa2 function is required in the same progenitors for activation of *Gata2*, *Lmx1b* and *Pet-1* (Fig. 4)²⁵. The loss of Foxa2 at this later stage results in about a 50% loss of 5-HT neurons in the hindbrain.

Ascl1

Despite being expressed in motor neuron progenitors during vMN genesis, loss of *Ascl1* does not appear to cause defects in motor neuron number or phenotype³³. *Ascl1* function emerges only after *Phox2b* is shut off in motor neuron progenitors and at this stage it has been shown to perform multiple functions²³. First, *Ascl1* performs a proneural role to regulate serotonergic neurogenesis, as expression of all markers (*Gata2*, *Gata3*, *Pet-1*, *Lmx1b*, 5-HT) of newly generated 5-HT postmitotic precursors is nearly abolished throughout the hindbrain in *Ascl1*^{-/-} mutants. Second, *Ascl1* is required together with *Nkx2.2*, *Foxa2*, to induce the inferred (see below) parallel *Gata2* and *Gata3*-dependent 5-HT neuron differentiation networks²³ (Fig. 4). A third function for *Ascl1* is to directly activate the expression of the zinc finger protein, *Insm1*, as mitotically active serotonergic progenitors are exiting the cell cycle²⁶. Chromatin immunoprecipitation (ChIP) studies show a direct interaction of *Ascl1* with a conserved binding site located 4.5kb upstream of the *Insm1* transcription start site (Fig. 4). Moreover, *Insm1* expression is greatly reduced or eliminated in *Ascl1* mutant serotonergic precursors throughout the ventral hindbrain²⁶.

Insm1

Direct regulation of *Insm1* by *Ascl1* suggests a critical role for *Insm1* in acquisition of the serotonergic phenotype. In general, loss of function studies confirmed this prediction but also reveal some unexpected nuances that illuminate unique characteristics of *Insm1* function²⁶. First, widely varying deficiencies of 5-HT-positive neurons were detected in *Insm1* mutants along the AP axis of the hindbrain with levels in r2–r3 being severely affected. Second, although *Insm1* loss of function did not change progenitor specification, *Lmx1b* expression was uniformly and severely reduced. However, deficits in *Gata2* and *Pet-1* expression were variable along the AP axis. Third, initial *Aadc* expression was not affected in *Insm1* mutants but *Tph2* expression was greatly reduced along the entire AP axis. However, by E16.5 *Aadc* expression was reduced, consistent with the deficit in *Pet-1* expression at this stage. These findings suggest a complex network role for *Insm1* in which it perhaps directly regulates *Tph2* expression while reinforcing *Ascl1*, *Foxa2*, *Nkx2.2*–directed *Gata2* expression in a feedforward manner (Fig. 4). Alternatively, because *Insm1* plays a critical role in *Lmx1b* expression²⁶ and loss of *Lmx1b* results in virtually complete loss of 5-HT and *Tph2* expression^{30, 34} perhaps *Insm1* indirectly regulates *Tph2* through direct regulation of *Lmx1b* expression (Fig. 4).

Gata2

Studies of *Gata2*'s role in 5-HT development are complicated because all *Gata2*^{−/−} embryos die between E9.5 and E11.5 from severe hematopoietic defects³⁵. However, Craven et al., 2004 were able to show that induction of *Pet-1* at E10.5 fails to occur in the rostral hindbrain of *Gata2*^{−/−} embryos. Furthermore, in contrast to wild type or *Gata2*^{+/-} explants, no 5-HT can be detected in ventral neural tube explants taken from E8 *Gata2*^{−/−} embryos and analyzed after 5 days in culture²⁴. At all longitudinal levels, *Gata2* is absolutely required for induction of *Pet-1* (and presumably *Lmx1b*) but not *Gata3*²⁴. Thus, it can be inferred that *Nkx2.2*, *Ascl1*, and *Foxa2* induce a *Gata3*–dependent network and a parallel *Gata2*–dependent network, which the latter in turn induces *Lmx1b* and *Pet-1* (Fig. 4). Several lines of evidence suggest that *Gata2* directly induces *Pet-1* expression at the postmitotic precursor stage. First, as described above, *Pet-1* expression is completely eliminated in explants of *Gata2* mutant neural tubes²⁴. Second, ChIP studies indicate that *Gata2* occupies highly conserved *Gata* binding sites in the upstream *Pet-1* enhancer region. Third, point mutation of these sites greatly diminishes expression of a *Pet-1* enhancer regulated transgene reporter in mouse brain 5-HT neurons³⁶.

Differentiation of postmitotic precursors

Coordinate induction of the serotonergic-type gene battery encoding 5-HT synthesis, reuptake, vesicular transport and metabolism is the final step in serotonergic neurogenesis. *Lmx1b*, *Pet-1* and *Gata3* are induced in postmitotic precursors just before initiation of 5-HT synthesis and all are necessary for induction of the battery. None of these factors are dependent on each other for their induction and therefore, they provide parallel transcriptional inputs to induce serotonergic-type gene expression (Fig. 4). In addition, the loss of these factors does not affect progenitors. However, expression of all three of these factors is maintained across the lifespan, which suggests they may be terminal selectors³⁷ that have distinct functions at different stages of life. Indeed, recent findings summarized below have shown this to be true for *Pet-1* and *Lmx1b*.

Lmx1b

Lmx1b plays numerous critical roles in CNS development including development of the isthmus organizer that induces the mid–hindbrain boundary, mesencephalic dopaminergic neurons and spinal dorsal horn neurons³⁸. In homozygous *Lmx1b* mutants 5-HT neuron

precursors are generated in normal numbers but virtually all of them fail to activate expression of *Tph2*, *Sert*, *Vmat2* and 5-HT synthesis^{30, 34}. Despite being induced normally in *Lmx1b* mutants, *Pet-1* expression begins to decline at E12.5 and by E14.5 *Pet-1* expression is no longer detectable³⁴. Thus, *Lmx1b* is needed to maintain *Pet-1* at later stages of embryogenesis (Fig. 4). *Lmx1b* and *Pet-1* share many targets and therefore *Lmx1b*'s regulation of *Pet-1* suggests that these factors operate in a feedforward manner to regulate the serotonergic-type gene battery.

Neonatal death precluded postnatal analyses of constitutively targeted homozygous *Lmx1b* mutant mice. However, survival was improved when *Lmx1b* was conditionally targeted in 5-HT neurons, which permitted study in the postnatal period of the impact of a serotonergic *Lmx1b* deficiency. Analyses of these targeted mice showed that although mutant 5-HT neuron cell bodies were present in the fetal hindbrain, nearly all of them were missing postnatally³⁹. These findings verified a cell autonomous function of *Lmx1b* in 5-HT neurons and demonstrated that it is uniquely required, among the known network factors, for postnatal survival of these cells. The SIM1 transcription factor may also be required for the maintenance or survival of a small subset of 5-HT neurons in the DRN⁴⁰.

Although genetic programs appear to specify 5-HT neuron progenitors independent of activity-responsive signaling pathways recent studies in the embryonic *Xenopus* hindbrain have shown that a portion of 5-HT neuron precursors are dependent on calcium spike activity for serotonergic differentiation⁴¹. Demarque and Spitzer showed using overexpression of sodium channels or potassium channels that increased or decreased calcium spike activity led to almost 30% increases or decreases in the numbers of *Lmx1b*+ and *tph*+ neurons, respectively. *Lmx1b* was identified as a node for the activity-dependent regulation of 5-HT neuron number since these alterations were prevented when *Lmx1b* expression was suppressed. The alterations in 5-HT neuron numbers and *Lmx1b* expression also led to changes in swimming behavior of *Xenopus* larvae⁴¹. Together these findings indicate activity-dependent plasticity of the serotonergic transcriptional network and suggest that an activity-dependent homeostatic mechanism may transcriptionally adjust serotonergic neuromodulation.

Gata3

In *Gata3* deficient mice, most precursors fail to initiate 5-HT synthesis in caudal rhombomeres. In contrast, 5-HT neuron differentiation is only modestly affected in the rostral hindbrain suggesting a diminishing caudal to rostral gradient in *Gata3* dependency^{23, 27}. These findings suggest that *Gata3* directly regulates *Tph2* expression although *Gata3* binding sites in the *Tph2* regulatory region have not been identified (Fig. 4). Conditional targeting of *Gata3* in 5-HT neurons has shown that ongoing *Gata3* is also required to maintain normal levels of *Tph2*, *Sert* and to a lesser extent *Aadc* and *Vmat2*⁴².

Pet-1

Pet-1 is the only factor in the transcriptional network whose expression in the brain is restricted to 5-HT neurons^{28, 29}. *FEV* (Fifth Ewing Variant), the human orthologue of *Pet-1*, is expressed specifically in the rhesus macaque⁴³ and human^{44, 45} dorsal and median raphe. Similar to *Gata3* and *Lmx1b* loss of function, constitutive loss of *Pet-1* function does not alter the generation of 5-HT neurons precursors⁴⁶. However, about 70% of these *Pet-1*^{-/-} cells fail to initiate 5-HT synthesis. The defect is roughly uniform across all raphe nuclei and leads to an 80% deficiency of 5-HT in the adult ascending and descending 5-HT subsystems⁴⁶. These *Pet-1* mutant precursors also fail to induce *Tph2*, *Aadc*, *Sert*, *Vmat2*, and *Maob* and therefore, *Pet-1* is required for induction of all of the genes in the serotonergic-type gene battery (Fig. 4). It is not clear why *Pet-1* is largely dispensable for

serotonergic differentiation in 30% of 5-HT neuron precursors although recent findings show that the *Pet-1* resistant 5-HT neurons constitute a phenotypically distinct ascending serotonergic subsystem that continues to innervate circuitry involved in neuroendocrine and autonomic control of stress responses⁴⁷. While other ETS transcripts were detected in dissected adult human raphe tissue⁴⁴ whole genome expression profiling failed to identify enriched expression of other ETS genes in flow cytometry sorted E12.5 mouse embryonic 5-HT neurons⁴⁸, which if detected would have raised the simple explanation of paralogue compensation in *Pet-1* resistant 5-HT neurons.

Consistent with its ongoing expression in 5-HT neurons, conditional *Pet-1* targeting with an *ePet-Cre* transgene⁴⁹ that is active at the postmitotic stage and causes loss of *Pet-1* 1–2 days after serotonergic neurogenesis⁴², showed that *Pet-1* continues to function during the prolonged stage of 5-HT system maturation. First, although *Pet-1* resistant 5-HT⁺ neurons have normal axonal growth characteristics in culture⁴⁷, conditionally targeted *Pet-1* deficient neurons that lose the ability to synthesize 5-HT have altered axonal innervation patterns in the somatosensory cortex⁴². Although these results indicate a role in axonal development it is not yet clear what specific role *Pet-1* performs in regulating innervation patterns. The evolutionarily conserved role of different ETS factors in circuit formation suggests several possibilities: AST1 directed axon navigation in *C. elegans*⁵⁰, mouse *Pea3* directed axonal branching⁵¹ and mouse ER81 control of target connectivity⁵². Second, *in situ* hybridization analyses and whole cell electrophysiological recordings have shown that during the maturation stage, *Pet-1* is required to induce the 5-HT1a and 5-HT1b autoreceptor pathways^{42,53}, which do not begin to be expressed in the mouse brain until about E14.5^{42,54}. The 5-HT1a autoreceptor pathway was not altered in conditionally targeting *Gata3* mice, which places special importance on *Pet-1* in this physiologically and clinically relevant regulatory event⁵⁵. Conditional targeting of *Pet-1* in adult 5-HT neurons with a tamoxifen inducible *ePet-CreER* transgene showed that *Pet-1* is required for maintenance of *Tph2* and *Sert* expression but not expression of other genes whose induction depends on *Pet-1* during serotonergic neurogenesis and maturation. Likewise, Ding and colleagues recently reported tamoxifen-induced targeting of *Lmx1b* in adult 5-HT neurons and showed that *Lmx1b* is also needed to maintain *Tph2* and *Sert* expression but not *Pet-1* and *Aadc*⁵⁶. In contrast to *Pet-1*, however, *Lmx1b* was also required to maintain *Vmat2* expression suggesting distinct regulatory roles for *Pet-1* and *Lmx1b* in adulthood (Fig 4).

With the exception of *Pet-1*/DNA interactions, very little is known about the cis-regulatory modules through which factors in the network directly activate transcription of the serotonergic type gene battery. Conserved *Pet-1* binding sites have so far been investigated in the upstream regions of *Sert*, *Tph2*, *Htr1a* and *Pet-1*^{28,42,53}. *In vitro* DNA binding assays showed that bacterially expressed *Pet-1* can bind conserved ETS-like sites present in the upstream regions of *Sert* and *Htr1a* genes²⁸. Further analysis revealed multiple conserved *Pet-1* binding sites upstream of the human and rodent *Htr1a* genes⁵³. Reporter assays in the HEK293 cell line showed that activity of a 1.5kb *Htr1a* promoter construct was substantially reduced when the conserved GGAA core of any of the *Pet-1* binding sites were destroyed. However, destruction of a single distal *Pet-1* ETS site at –1406 completely abolished promoter activity suggesting a critical role for this site in the regulation of *Htr1a*⁵³. Chromatin immunoprecipitation studies with chromatin isolated from embryonic hindbrain have shown that *Pet-1* binds, *in vivo*, to the *Tph2*, *Sert*, and *Pet-1* upstream regulatory regions where conserved *Pet-1* binding sites are located⁴². In addition, *Pet-1* enhancer activity is dramatically reduced in 5-HT neurons lacking *Pet-1* expression in adulthood and therefore ongoing *Pet-1* binding is required to maintain its own expression. Together these findings support a model (Fig. 4) in which induction of *Pet-1* by the serotonergic transcription regulatory network is subsequently reinforced and stabilized

through direct positive autoregulation to maintain *Pet-1*, *Tph2*, and *Sert* expression across the lifespan⁴².

In summary, the parallel transcriptional inputs of Gata3, Lmx1b and Pet-1 to the serotonergic-type gene battery constitute a combinatorial code that determines 5-HT neuron identity (Fig. 4). Pet-1 functions as a terminal selector³⁷ defined as an autoregulated gene that induces and maintains expression of a cell-type specific gene battery across the lifespan through direct interaction with a common DNA regulatory motif. Pet-1, Lmx1b, and Gata3 likely function together as combinatorial terminal selectors, although the latter two factors have not been shown to autoregulate or to directly control the serotonergic-type gene battery. The essential role of Lmx1b but not Gata3 and Pet-1 in all 5-HT neurons suggest Lmx1b forms a distinct combinatorial code with other transcriptional inputs in a subset of 5-HT neurons. In light of the requirement for Lmx1b and Pet-1 in the maintenance of adult 5-HT synthesis and reuptake together with the activity dependent regulation of *Lmx1b*⁴¹ it will be interesting to determine whether environmental input such as stress or drug exposure alters Pet-1 and/or Lmx1b dependent regulation of serotonergic function in the mammalian brain.

Network alterations and pathogenesis

As alluded to at the beginning of this review, evidence from decades of studies in humans, non-human primates and rodents strongly support an association of altered serotonergic function with behavioral and physiological pathogenesis^{55, 57, 58}. It is now well understood that genetically engineered alterations in the levels of *Sert*, *Tph2*, or *Htr1a* expression are not compatible with normal emotional and stress-related behaviors, at least in mice^{59–61}. Many other physiological processes are also disrupted in mice deficient for these genes including growth trajectories, respiration, body weight maintenance, stress susceptibility, cardiovascular function, sleep patterns and maternal behavior^{59, 62}. These genetic-based animal studies provide a compelling impetus for the quest to find gene variation that impacts serotonergic signaling and risk for neuropsychiatric disease. Numerous variants thought to impact *presynaptic* serotonergic signaling through intrinsic alterations in brain 5-HT neuron function have been discovered and investigated for association with neuropsychiatric disease or their influence on the function of neural circuitry shaping emotional responses^{55, 58, 59, 63}. Most of these disease associated 5-HT neuron variants have been tested for functional impact in cultured cell lines and not in brain 5-HT neurons. Yet, an intriguing commonality among the variants with functional impact, *in vitro* and in some cases *in vivo* is that they all affect the level of expression or activity of *TPH2*, *SERT*, or *HTR1A*. Widely studied common polymorphisms in the human *SERT* transcriptional regulatory region affects the level of *SERT* transcription in cell culture^{64, 65} and appears to moderate the impact of stressful life events on risk for adult depression⁶⁶. An orthologous polymorphic region in rhesus macaques, likewise, influences stress reactivity in subjects exposed to adverse early rearing experiences⁶⁷. Perhaps the most convincing example of disease associated variation in *SERT* are the multiple rare non-synonymous variants creating gain of function alleles that produce elevated levels of the transporter or enhanced trafficking-independent, 5-HT transport activity. Furthermore, these alleles encode transporters that are no longer sensitive to intracellular signaling pathways that control SERT trafficking and activity⁶⁸. Importantly, in a large sample of multiplex autism families, it was shown that these variants in aggregate are significantly associated with autism and rigid compulsive behaviors⁶⁹. Despite its apparent very rare frequency⁷⁰, another notable example of variation that affects the level of serotonergic activity is a non-synonymous *TPH2* single nucleotide coding variant, G1463A⁷¹. The encoded R441H TPH2 variant, exhibits an 80% loss of 5-HT biosynthetic capacity in cell culture and has been associated with late life depression⁷¹. An orthologous mouse knock in allele causes severely

reduced brain 5-HT synthesis, increased aggression and anxiety-like behaviors⁶⁰. Importantly, the effect of this allele recapitulated in the mouse many of the alterations in the levels of serotonergic biomarker indices that are associated with depression in humans⁷², reinforcing the idea that the altered human indices do indeed reflect reduced brain 5-HT levels and neuropsychiatric disease.

In view of the human genetic evidence and abundant animal studies that support altered levels of 5-HT signaling in disease susceptibility a natural but largely unexplored question is whether variation in the serotonergic transcription regulatory network (Fig. 4) impacts the level of 5-HT neuron gene expression and susceptibility to mental illness. So far, variation with such an impact has not been discovered. However, several findings in non-human primates and mice highlight network variation as a potentially important mechanism in neuropsychiatric disease pathogenesis. First, studies of female cynomolgus monkeys have defined a stress sensitive (SS) subgroup, in which reproductive function is impaired, versus highly stress resilient (HSR) individuals who showed no reproductive suppression⁴³. Interestingly, in SS individuals the level of *FEV* expression in the dorsal raphe is significantly reduced compared to *FEV* expression in the HSR dorsal raphe⁴³. Moreover, the level of expression of *TPH2*, *SERT*, and the *HTR1A* autoreceptor genes were correspondingly decreased in SS DRN compared to the HSR DRN suggesting potentially altered *FEV*-dependent transcriptional regulation of 5-HT synthesis, reuptake and autosensing in response to stress in female macaques^{43, 73}. Second, *Lmx1b* and *Pet-1* deficient mice show several emotion and stress-related behavioral abnormalities that parallel those observed in *Tph2*, *Sert*, and *Htr1a* targeted mice including increase aggression, anxiety-like behavior and fear responses^{46, 47, 74}. Importantly, *Pet-1* dependent transcription is required into adulthood to maintain normal anxiety-like behaviors⁴² raising the possibility that behavioral pathogenesis might arise from adult onset disruption, genetically or environmentally, of serotonergic transcription. In addition, neonatal growth, respiration, thermoregulation, autonomic control of neonatal cardiac function, nociception and circadian rhythms are disrupted in *Lmx1b*^{-/-} and *Pet-1*^{-/-} mice⁷⁵⁻⁸⁴ suggesting potential broad impact of network alterations. Third, transgenic experiments showed that the extent of transgenic rescue of 5-HT neuron molecular defects and maternal behavior deficits in *Pet-1*^{-/-} mice is dependent on the level of expression of a *FEV*BAC transgene⁸⁵. These later findings provide evidence in support of the idea that the expression level of serotonergic regulatory network determines the levels of 5-HT neuron gene expression with attendant quantitative effects on 5-HT-modulated behaviors.

Given the direct regulation of *Tph2*, *Sert*, and *Htr1a* genes by *Pet-1* and probably also by *Lmx1b*, naturally occurring human alleles of these two network components are particularly intriguing as a source of potential disease relevant variation. The restricted expression of *FEV* in the brain suggests that different functional *FEV* alleles, if they exist, may have 5-HT system specific effects. Positive or negative epistasis⁸⁶ with other network alleles may determine whether or not *FEV* alleles cause deleterious effects on 5-HT system activity. However, naturally occurring functional variation within the serotonergic regulatory network would be expected, in most instances, to exact variable pleiotropic effects because all the others factors in the network are broadly expressed in multiple unrelated lineages within the brain and peripheral tissues. Human genetic studies of *LMX1B* variation provide an instructive example. Over 100 distinct heterozygous autosomal dominant *LMX1B* missense, nonsense and deletion mutations have been identified in different cases of Nail Patella Syndrome, a disorder of highly variable expressivity and penetrance that can include nail dysplasia, skeletal malformations, renal defects, sensory deficits, and ocular malformations^{38, 87, 88}. In support of *LMX1B* loss of function in NPS, *Lmx1b* deficient mice display several defining features of the syndrome⁸⁹. Many of the human mutations encode homeodomain missense and disrupt sequence specific DNA binding suggesting

direct effects on network interactions and downstream transcriptional targets. The likely haploinsufficiency of *LMX1B* in NPS ⁹⁰ suggests *LMX1B*–dependent transcriptional networks are particularly sensitive to the level of *LMX1B* activity. Given its place, functionally in the serotonergic regulatory network we imagine altered network function in at least some NPS individuals. Although not yet studied in sufficient depth to establish a clear link to neuropsychiatric disease, disrupted *LMX1B* binding may promote susceptibility to ADHD and major depressive disorder in NPS cases ⁹¹. Further study of this important possibility, including quantitative analysis of disease–associated serotonergic biomarker indices in NPS individuals, is of significant interest in the search for the genetic basis of serotonergic dysfunction in neuropsychiatric disorders.

Conclusions and future directions

A detailed understanding of the spatiotemporal expression patterns and functions of eight transcription factors has enabled the construction of a model for the dynamic regulatory network that specifies brain 5-HT neurons (Fig. 4). The network comprises temporally distinct regulatory states each corresponding to a particular stage in the development of 5-HT neuron identity and its maintenance in adulthood. Despite the well–studied effects of specific network perturbations on 5-HT neuron identity as well as a growing understanding of its effects on behavior and physiology several critical gaps in knowledge remain. For example, despite numerous obvious signs of network heterogeneity our understanding of it is insufficient to explain why the phenotype of substantial numbers of 5-HT neurons are resistant to loss of individual transcription factors and how diverse axonal trajectories, cell body migratory routes and physiological properties of different groups of 5-HT neurons are programmed. In addition, nothing is known about the layer of epigenetic transcriptional control within the network and how it responds to environmental signals that may help to shape 5-HT–modulated behaviors. Further study of the network with new genetic–based approaches that provide greater access to 5-HT neurons ^{49, 92} should help fill in these gaps and provide a better understanding of how it controls 5-HT function.

An additional theme of this review is the potential utility of our network model in helping to advance an understanding of the importance of 5-HT system dysfunction in neuropsychiatric disease pathogenesis. Given the likely essential role of the orthologous human network in the induction of the disease–associated genes, *TPH2*, *SERT*, and *HTR1A*, genetically or epigenetically–driven alterations in expression or function of the network may program altered levels of 5-HT synthesis, reuptake, or autoreceptor function and establish a vulnerability for mental illness. The identification of multiple biologically relevant rare disease–associated variants in *SERT*, *TPH2*, and *LMX1B* is consistent with the now widely recognized fact that significant heterogeneity exists in the allelic architecture of complex human disorders in general and in particular for neuropsychiatric disorders ^{93–95}. As an experimentally well–defined cell–type specific biological pathway the serotonergic regulatory network is a potentially rich source of rare heritable or *de novo* human variation that, if discovered, can be investigated for functional impact in the context of brain 5-HT system development and neuromodulation. The utility of our model is its power to help interpret the biological significance of network variants within an ensemble of functionally interacting serotonergic transcriptional determinants. Orthologous mutations can be investigated to study their impact on serotonergic differentiation of cultured embryonic stem cells ^{96, 97} and introduced into the mouse genome to determine their impact on 5-HT modulated behaviors and physiology. Functionally–defined network variants can then be investigated to determine whether they are present in unrelated individuals with particular neuropsychiatric diagnoses ⁹⁸ as has been accomplished for *LMX1B* variants in NPS and with *SERT* variants in autism.

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Figure 1. Serotonergic-type neuron identity

5-HT neurons coexpress a gene battery encoding 5-HT synthetic (*Tph2*, *Aadc*, *Gch1*, *Gfrp*, *Ptps*, *Qdpr*), reuptake (*Sert*), vesicular transport (*Vmat2*), autoreceptor signaling (*Htr1a*, *Htr1b*) and metabolism (*Maoa*, *Maob*) proteins. Tetrahydrobiopterin (BH4), an essential cofactor for Tph2 in the synthesis of 5-HTP, is synthesized (red pathway) *de novo* from guanosine triphosphate (GTP). It is also recycled through a regeneration pathway (brown). Aldehyde dehydrogenase (Aldh) converts 5-Hydroxyindolealdehyde into 5-Hydroxyindoleacetic acid (5-HIAA). Following release, 5-HT modulates 5-HT neuron firing through somatodendritic Htr1a autoreceptors, 5-HT release from the pre-synaptic terminal through Htr1b autoreceptors and stimulates neurotransmission through post-synaptic 5-HT receptors (5-HT1–7). Tph2, Tryptophan hydroxylase 2; Aadc, Aromatic L-amino acid decarboxylase; Sert, Serotonin transporter; Vmat2, vesicular monoamine transporter 2; Mao A,B, Monoamine oxidases A and B; Gch1, GTP cyclohydrolase 1; Gfrp, GTP cyclohydrolase I feedback regulator; Ptps, 6-pyruvoyl-tetrahydropterin synthase; Spr, sepiapterin reductase; Pcbd, pterin-4 alpha-carbinolamine dehydratase; Qdpr, quinoid dihydropteridine reductase. BH4 synthetic intermediates: H2NTP, 7,8-dihydroneopterin triphosphate; Ptp, 6-pyruvoyl-5,6,7,8-tetrahydropterin; BH4C, tetrahydrobiopterin 4α-carbinolamine; qBH2, Quinoid-dihydrobiopterin.

Figure 2. Neuroanatomical features of 5-HT neuron development

a) Schematic sagittal view of the developing rodent brain. All rodent 5-HT neurons are born caudal to the mid-hindbrain organizer (MHO) in two longitudinal domains, rostral and caudal, on either side of the floor plate in the ventral rhombencephalon (hindbrain). b) Schematic sagittal view of the adult midbrain, pons and medulla depicting the location of 5-HT neurons clusters (raphe nuclei). Intersectional/subtractive fate mapping has shown¹⁹ that all 5-HT neurons in the mature dorsal raphe nucleus (DRN, B4, B6 and B7 groups) are born in r1 (green highlight in a and b). 5-HT neurons in the median raphe nucleus (MRN, B8 and B5 groups) and laterally extending supralaminar 5-HT neurons, designated the B9 cluster, are derived from serotonergic progenitors in r1, r2 and r3 (green, yellow, blue highlight in a and b). 5-HT neurons born in r5–r8 migrate to form the raphe pallidus (RPa, B1), raphe obscurus (ROb, B2), raphe magnus (RMg, B3) and cell bodies in the ventrolateral medulla (B3). 5-HT neurons in the rostral portion of B3 are born in r5 (red highlight). c) Stages of 5-HT neuron development. Postmitotic 5-HT neuron precursors do not yet possess overt serotonergic identity. Induction of the serotonergic-type gene battery generates immature 5-HT neurons. These newborn neurons are integrated with CNS circuitry during a prolonged maturation stage that produces adult 5-HT neurons.

Figure 3. Serotonergic progenitor specification

In rhombomere 1 (r1), *Foxa2* continually suppresses *Phox2b* expression to block production of visceral motor neuron (vMN) progenitors and promotes production of 5-HT progenitors. In r2–3, r5–r8, *Nkx2.2* initially induces *Phox2b*, which in turn represses *Foxa2*. This subcircuit establishes a motoneuron fate of ventral progenitors in r2–r3 and r5–r8. These progenitors produce motor neurons from E9.5 to about E10.5. Starting at about E10.5 a reciprocal temporal cross-repression is initiated whereby *Foxa2* now shuts off *Phox2b* expression. *Foxa2* and *Nkx2.2* mediated repression of *Phox2b* switches the fate of ventral progenitors so that a wave of serotonergic neurogenesis follows motor neurogenesis. In r4, *Phox2b* continually represses *Foxa2* thus suppressing the vMN to 5-HT fate switch and prolonging the production of vMNs.

Figure 4. Serotonergic transcription regulatory network

Stage-specific subcircuits in the network are depicted in colored rectangles with enclosed transcriptional factor gene symbols and arrows or blocked lines leading to other network constituents. *Foxa2* is required for *Gata3* expression only in r1. In mouse *Nkx6.1* and *Nkx6.2* do not appear to be required for 5-HT neuron generation but in r4 they suppress 5-HT fate. *Nkx6.1* has been shown to be required for *Gata2* and *Pet-1* expression and production of 5-HT neurons only in chick r1. Chromatin immunoprecipitation experiments indicate direct *Gata2* binding (solid blue circle), *in vivo*, to the *Pet-1* upstream regulatory region where conserved *Gata2* binding sites are located. *Pet-1* induces *Htr1a* and *Htr1b* to initiate formation of the two 5-HT autoreceptor pathways in maturing 5-HT neurons (red highlighted box, E14.5). ChIP indicates that *Pet-1* binds (solid red circles), *in vivo*, to the *Tph2*, *Sert*, and *Pet-1* upstream regions where conserved *Pet-1* binding are located. In adult 5-HT neurons (beige highlighted box) *Lmx1b* and *Pet-1* maintain *Tph2* and *Sert*. *Lmx1b* also maintains *Vmat2* expression. *Pet-1* expression in adult 5-HT neurons is stabilized through direct positive autoregulation (curved red arrow). Dashed arrows indicates that *Gata2* is likely required for *Lmx1b* expression but this has not been formally demonstrated and *Insm-1* may also directly regulate *Tph2* but this has not been shown with ChIP.