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Pet-1 ETS Gene Plays a Critical Role in 5-HT Neuron Development and Is Required for Normal Anxiety-like and Aggressive Behavior

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Summary

The central serotonin (5-HT) neurotransmitter system is an important modulator of diverse physiological processes and behaviors; however, the transcriptional mechanisms controlling its development are largely unknown. The Pet-1 ETS factor is a precise marker of developing and adult 5-HT neurons and is expressed shortly before 5-HT appears in the hindbrain. Here we show that in mice lacking *Pet-1,* the majority of 5-HT neurons fail to differentiate. Remaining ones show deficient expression of genes required for 5-HT synthesis, uptake, and storage. Significantly, defective development of the 5-HT system is followed by heightened anxiety-like and aggressive behavior in adults. These findings indicate that Pet-1 is a critical determinant of 5-HT neuron identity and implicate a Pet-1-dependent program in serotonergic modulation of behavior.

Introduction

The 5-HT neurotransmitter system is an important modulator of neural circuitry that controls a wide range of behavioral and physiological processes including cognition, circadian rhythms, and mood (Jacobs and Azmitia, 1992). This system is comprised of a relatively small number of neurons that are clustered in nine phylogenetically conserved nuclei, B1–B9, in the midbrain and hindbrain (Dahlstrom and Fuxe, 1964; Steinbusch, 1981). Nearly all levels of the CNS receive serotonergic innervation through extensive collateralization of 5-HT axonal projections (Steinbusch, 1981). A large family of differentially expressed 5-HT receptor subtypes mediates synaptic and perhaps paracrine transmission upon binding of 5-HT released from presynaptic terminals (Barnes and Sharp, 1999; Kroeze et al., 2002). The broadly distributed neuromodulatory capacity of the 5-HT system is further reflected in the numerous human behavioral disorders that are thought to involve a dysfunction of the 5-HT system. A large body of data suggests that a deficiency of central serotonergic signaling is a major factor involved in the development of disorders such as aggression, impulsivity, anxiety, depression, suicide, and obsessive-compulsive disorder (Davidson et al., 2000; Lucki, 1998; Mann et al., 2001; Nelson and Chiavegatto, 2001). Recent evidence for an early postnatal role of forebrain 5-HT 1a receptors in the acquisition of normal adult anxiety-like behavior (Gross et al., 2002) supports the notion that a developmental deficit in 5-HT signaling may predispose individuals to mood disorders. Despite the prominence of the 5-HT system in central neuromodulation and psychiatric disorders, the genetic mechanisms governing the generation of 5-HT neurons are poorly understood and it is not known how these mechanisms are linked to eventual serotonergic control of behavior in adults.

5-HT immunoreactive cells first appear in the mantle layer of the embryonic hindbrain adjacent to the floor plate at about E13 in rat (Lidov and Molliver, 1982; Wallace and Lauder, 1983). These neurons form the rostral domain of the developing 5-HT system, which is positioned just caudal to the isthmus. The rostral domain gives rise to the B4–B9 nuclei, which provide innervation mainly to forebrain targets (Tork, 1990). One to two days later, a second domain of 5-HT immunoreactive cell bodies appears just caudal to the pontine flexure to form a caudal 5-HT neuron domain. This domain eventually gives rise to B1–B3 nuclei, which provide innveration mainly to the spinal cord (Tork, 1990). Coincident with the appearance of the transmitter, 5-HT neuron cell bodies begin to extend axons, which is followed by migration and aggregation of the cell bodies to form the B nuclei (Wallace and Lauder, 1983).

The regional restriction of 5-HT neuron induction is believed to result from early signaling events that pattern the hindbrain and thereby establish discrete organizing centers for 5-HT neuron specification (Hynes and Rosenthal, 1999). Gain-of-function and loss-of-function approaches have implicated sonic hedgehog (Shh), secreted from notocord and floor plate, FGF8 from the isthmus, and FGF4 from the primitive streak in the establishment of a hindbrain organizing center specifying rostral 5-HT neurons (Ye et al., 1998). Shh has also been implicated in specifying caudal 5-HT neurons, but additional signaling molecules that function with Shh to establish a caudal organizing center are unknown. The zinc finger protein Gli2 is an early downstream target of the Shh signaling pathway and has been implicated in specification of some 5-HT neurons (Matise et al., 1998). Nkx2.2, a homeodomain protein that defines certain neuronal and oligodendrocyte progenitor domains in the ventral spinal cord and hindbrain, is required for the appearance of caudal but not rostral 5-HT neurons (Briscoe et al., 1999; Zhou and Anderson, 2002). A second zinc finger protein, GATA-3, is thought to play an early role in the development of some 5-HT neurons in

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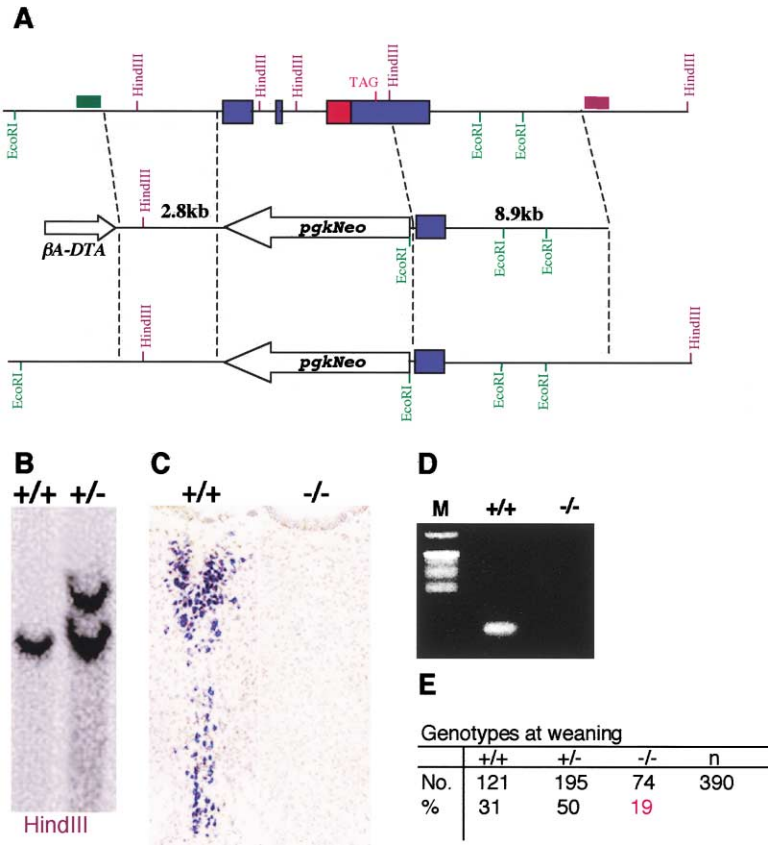

Figure 1. Generation and Analysis of the *Pet-1* Null Allele

(A) Targeting strategy. Top, map of mouse *Pet-1* with 5′ end at left. Exons are shown in blue except for DNA binding domain coding region shown in red. Middle, targeting vector designed to remove all but 470 bp of the 3′ untranslated region. Bottom, predicted structure of the *Pet-1* null allele. External 5′ probe (green box) and external 3′ probe (purple box) and respective restriction sites used to detect indicated genomic fragments in wild-type or targeted *Pet-1* locus are shown.

(B) Southern blot analysis of wild-type and targeted R1 ES cells using the 3′ probe on HindIII digested DNA. Confirmation of appropriate targeting was obtained with the 5′ probe and an EcoRI digest (data not shown).

(C and D) Verification of absence of *Pet-1* mRNA in the *Pet-1* null brain.

(C) In situ hybridization with a digoxigenin-labeled antisense probe to detect *Pet-1* mRNA in B6 dorsal raphe nucleus of wild-type and *Pet-1* null mice.

(D) Analysis of *Pet-1* mRNA by RT-PCR using hindbrain total RNA isolated from wild-type or *Pet-1* null mice.

(E) Genotypes at weaning from *Pet-1* heterozygous matings indicated a lower number of *Pet-1* null mice than the expected 1:2:1 Mendelian ratio.

caudal B nuclei (van Doorninck et al., 1999). However, each of these secreted signaling molecules and transcription factors have been implicated in the specification of numerous neuronal and nonneuronal cell types. Thus, it remains to be determined how a small subset of neuronal precursors is selected for specification to a serotonergic neuron phenotype.

In addition to these early steps of 5-HT progenitor cell specification, proper development and maintenance of the 5-HT transmitter system requires expression of numerous proteins that together define the mature phenotype of 5-HT neurons. Catalysis of the first and rate-limiting step in 5-HT biosynthesis by tryptophan hydroxylase (TPH) (McGeer and McGeer, 1973) and reuptake of the transmitter by the serotonin transporter (SERT) (Blakely et al., 1991) constitute two essential functions of 5-HT neurons that must be coupled for proper serotonergic synaptic transmission. Among mature central neurons, expression of the TPH and SERT genes is restricted nearly exclusively to 5-HT neurons (Hansson et al., 1998; Rattray et al., 1999; Rind et al., 2000). The more broadly expressed aromatic L-amino acid decarboxylase (AADC) and vesicular monamine transporter 2 (VMAT2) are required for the second and final step of 5-HT synthesis (McGeer and McGeer, 1973) and for packaging of 5-HT in synaptic vesicles (Weihe and Eiden, 2000), respectively. Transcriptional control of these genes is not well understood and therefore it is not known how their expression is coordinated for 5-HT neuron differentiation and maintenance.

ETS domain transcription factors play important roles in the specification of various hematopoietic cell types (Bartel et al., 2000; Bassuk and Leiden, 1997), but their roles in vertebrate nervous system development and function are only beginning to be appreciated (Arber et al., 2000; Livet et al., 2002). Previously, we identified an ETS domain transcription factor, Pet-1 (Fyodorov et al., 1998), whose expression pattern in the rat brain immediately suggested it is a key factor in a transcriptional mechanism that controls 5-HT neuron phenotype (Hendricks et al., 1999). Expression of *Pet-1* in the adult rat brain is striking, as it appears to mark all serotonergic neurons in all of the Dahlstrom and Fuxe B nuclei. However, its expression is not detected in nonserotonergic cell types intermingled in these clusters or elsewhere in the brain. The restricted expression of *Pet-1* begins at its onset, which is localized to the mantle layer of the E12.75 rat (Hendricks et al., 1999) and E11 mouse (Pfaar et al., 2002) rostral hindbrain. Interestingly, *Pet-1* expression precedes the appearance of 5-HT in both the rostral and caudal domains (Hendricks et al., 1999).

Here we have investigated the function of Pet-1 by generating *Pet-1*-deficient mice. Our findings indicate that proper development of the entire central 5-HT system is disrupted in the absence of *Pet-1*. Moreover, the defective embryonic development of the 5-HT system is followed by aggressive and anxiety-like behavior in adults. These findings indicate that Pet-1 is a critical determinant of 5-HT neuron identity and suggest the existence of a Pet-1-dependent transcriptional program

that selectively couples early steps in 5-HT neuron differentiation to serotonergic neuromodulation in the adult.

Results

Targeting of *Pet-1* and Germline Transmission of the Null Allele

To construct a *Pet-1* targeting vector, we screened a mouse 129sv genomic library with a probe made from a rat *Pet-1* cDNA (Fyodorov et al., 1998). Analysis of genomic insert DNA indicated that mouse *Pet-1* is about 4 kb. Therefore, we prepared a targeting vector designed to remove all of the *Pet-1* protein coding sequences and thereby generate a *Pet-1* null allele (Figure 1A). Southern blot analysis was used to identify targeted R1 ES stem cells (Figure 1B), and polymerase chain reaction (PCR) was used to detect germline transmission of the null allele. In situ hybridization (Figure 1C) and RT-PCR (Figure 1D) indicated that expression of *Pet-1* was eliminated in homozygous *Pet-1* null hindbrain. Mendelian ratios were obtained at embryonic ages 11.5 and 12.5. The percentage of homozygous *Pet-1* null mice at weaning, however, was somewhat lower than expected, suggesting that perhaps a small number of nulls may not survive (Figure 1E). Preliminary data suggest a window of vulnerability spanning approximately the first week after birth, after which no abnormal loss of nulls was evident (J. Erickson, L.J.W., T.J.H., and E.S.D., unpublished). Impaired ability to feed is not likely to account for this loss, as stomachs of *Pet-1* null neonates were filled with milk and this did not appear noticeably different from wild-type or heterozygous littermates.

Rostral and Caudal Hindbrain 5-HT Neuron Differentiation Is Disrupted in *Pet-1* Null Mice

The onset of *Pet-1* expression relative to the appearance of 5-HT in the developing hindbrain suggested that Pet-1 might play a role in differentiation of the 5-HT system. Thus, we began our analysis of the *Pet-1* null phenotype by determining whether the initial appearance of the transmitter was altered either in the rostral or caudal hindbrain domains or both. The earliest time at which significant numbers of 5-HT immunoreactive cell bodies can be detected in mouse is approximately E11.25 to 11.5 for the rostral domain and E12.5 for the caudal domain. At E11.5 (Figures 2A and 2B) and E12.5 (Figures 2C and 2D), we found dramatically reduced numbers of 5-HT immunoreactive cell bodies in the rostral domain of *Pet-1* null mice compared to that of wild-type littermates. Similarly, reduced numbers of 5-HT immunoreactive cell bodies were seen at E12.5 in the caudal domain of *Pet-1* null mice (Figures 2E and 2F). By contrast, analysis of heterozygotes failed to identify a deficiency of 5-HT immunoreactive cell bodies relative to wild-type littermates (Figures 2J and 2K). Counts of 5-HT immunoreactive cell bodies indicated an 80% deficiency in the rostral and caudal domains of null mice (Figure 2G). The remaining 5-HT immunoreactive cells of *Pet-1* null mice seemed to be appropriately positioned in the mantle layer, and at E12.5 rostral 5-HT-positive cell bodies were beginning to migrate (Figure 2D). Moreover, ascending and descending 5-HT immunoreactive fibers could be seen projecting away from the remaining null cell bodies in both rostral and caudal domains, respectively, suggesting that these cells initiate axon pathfinding (Figures 2D and 2F and data not shown).

To determine whether the failure to detect normal numbers of 5-HT immunoreactive cell bodies was merely because of a lack of transmitter, we stained hindbrain sections with anti-AADC antiserum. A similar deficit of AADC immunoreactive cell bodies was observed at E12.5 in the rostral 5-HT domain of *Pet-1* null embryos but not in the developing mesencephalic dopaminergic system rostral to the isthmus (Figures 2H and 2I). These data suggest that a large number of 5-HT neurons are either not generated or their development is arrested at a precursor stage.

To distinguish between these possibilities, we stained wild-type, heterozygous, and *Pet-1* null hindbrain sections with antibody raised against the neomycin phosphotransferase (*neo*) gene that functioned as the selectable marker during ES cell targeting of the *Pet-1* locus (Figure 1). As expected, no anti-NEO staining was present in the wild-type hindbrain (Figure 2M). However, analysis of adjacent sections from heterozygotes indicated similar patterns of staining for NEO and 5-HT in the rostral and caudal domains (Figures 2K and 2N). Thus, *neo* is properly expressed under the control of *Pet-1* regulatory elements and can be used to detect 5-HT neuron precursors in targeted mice. Interestingly, analysis of *Pet-1* null hindbrain revealed that while the number of 5-HT immunoreactive cell bodies was greatly reduced (Figure 2L), the pattern of NEO staining (Figure 2O) was indistinguishable from that seen in heterozygotes (Figure 2N). Moreover, costaining for these markers in the *Pet-1* null rostral domain revealed that most NEO-positive cells were 5-HT negative (Figure 2Q). These data suggest that in mice lacking *Pet-1*, 5-HT neuron precursors are generated in correct numbers but the majority of them fail to differentiate.

5-HT Neurons Are Missing in All *Pet-1* Null Dahlstrom and Fuxe B Nuclei

The viability of *Pet-1* homozygous null mice provided an opportunity to investigate the consequences of *Pet-1* deletion on the adult 5-HT system. As the positions of 5-HT neuron clusters in the adult spans several levels beginning rostrally in the midbrain central gray and extending to the most caudal portions of the medulla, a series of coronal sections were prepared for analysis of each B nucleus. Immediately evident was the diminished number of 5-HT immunoreactive cell bodies in *Pet-1* null B nuclei of the midbrain and medulla relative to wild-type littermates (Figures 3A–3F, 3K, and 3L). Similar to our finding in the embryonic hindbrain, no deficiency of 5-HT immunoreactive cell bodies was detected in heterozygous mice relative to wild-type controls (data not shown). Quantification of the deficiency summed for all nine B nuclei indicated a 70% loss of 5-HT immunoreactive cell bodies in *Pet-1* null brains (Figure 3K). Moreover, 5-HT cell body numbers were reduced to a similar extent in each of the *Pet-1* null B nuclei (Figure 3L). Interestingly, remaining *Pet-1* null 5-HT neurons ap-

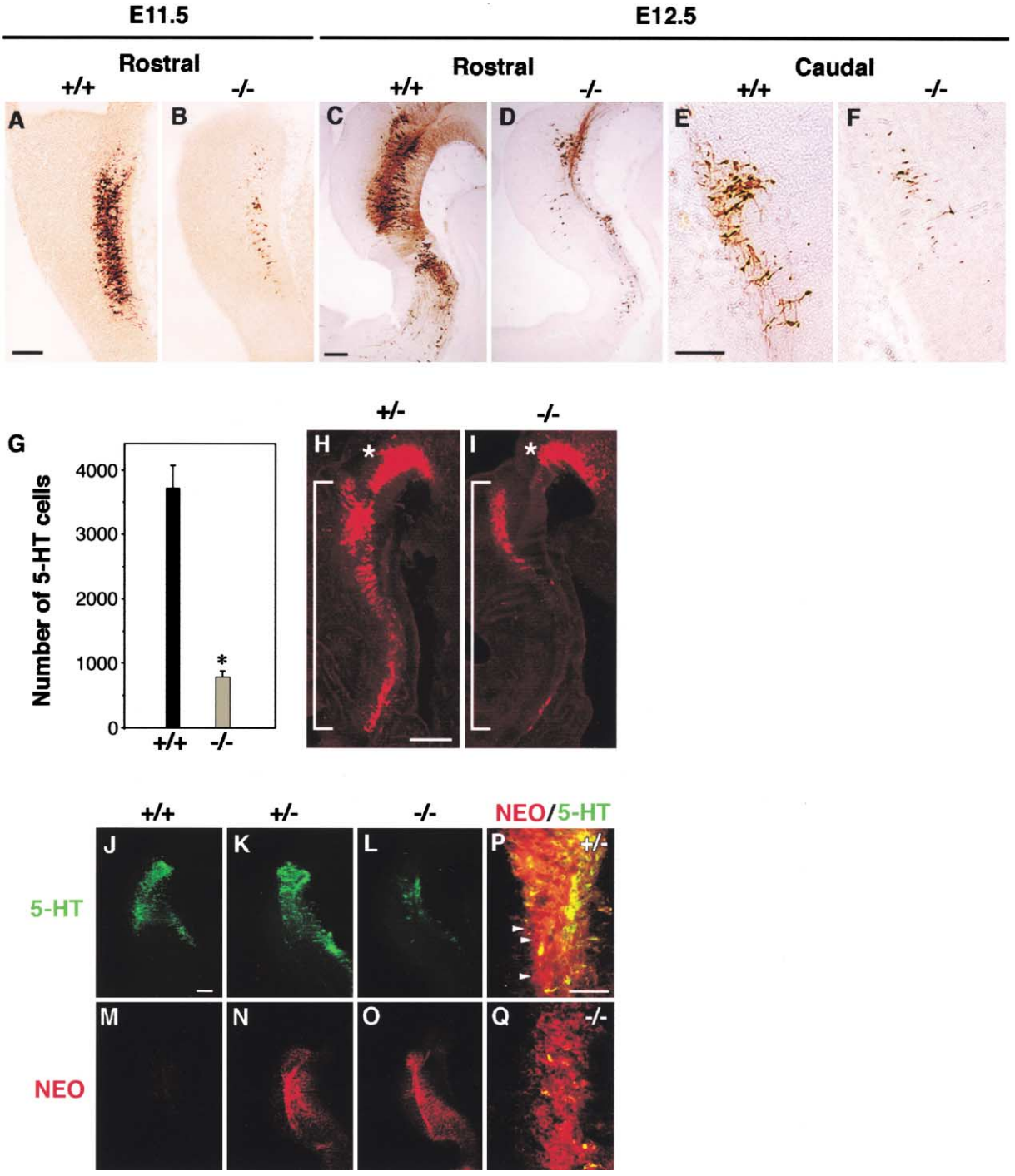

Figure 2. Early Differentiation Defect in the Developing 5-HT Rostral and Caudal Domains of *Pet-1* Null Mice

(A–F) Anti-5-HT immunoreactivity in wild-type (A, C, and E) and *Pet-1* null (B, D, and F) hindbrains at ages E11.5 (A and B) and E12.5 (C–F) in the rostral (A–D) and caudal (E and F) domains. A deficiency of 5-HT immunoreactive cells was evident at E11.5 and E12.5 in both the rostral and caudal domains. The numbers of 5-HT immunoreactive neurons in heterozygotes did not appear different from that of wild-type (data not shown).

(G) Counts of 5-HT immunoreactive cell bodies at E12.5 in wild-type ($n = 3$) versus *Pet-1* null ($n = 4$) mice. Data are presented as mean $\pm$ SEM; asterisk, $p \leq 0.001$.

(H and I) Anti-AADC immunostaining at E12.5 in heterozygous (H) and *Pet-1* null (I) hindbrains showed a deficit of immunoreactivity in the rostral domain (bracket) similar to that seen with anti-5-HT antisera. By contrast, the level of anti-AADC staining rostral to the isthmus (asterisks) in the null was not different from wild-type, indicating that the developing mesencephalic dopamine system was not altered.

(J–O) Anti-5-HT immunoreactivity (J–L) and anti-neomycin phosphotransferase (NEO) immunoreactivity (M–O) on adjacent sections of wild-type (J and M), heterozygous (K and N), and *Pet-1* null (L and O) E12.5 rostral hindbrain.

(P and Q) Overlay of double immunohistochemistry for 5-HT (Oregon green) and NEO (Cy3) in the rostral domain of E12.5 heterozygous (P) and *Pet-1* null (Q) mice. The ventricular zone is to the left in all sections. Arrowheads in (P) indicate NEO-positive, 5-HT-negative cells in the heterozygote, which is consistent with our previous observation that Pet-1 expression precedes 5-HT immunoreactivity in the developing 5-HT system (Hendricks et al., 1999). Littermate comparisons were used throughout. Scale bars in (A), (C), (E), (H), and (J) are 100 μm. The scale bar in (P) is 50 μm.

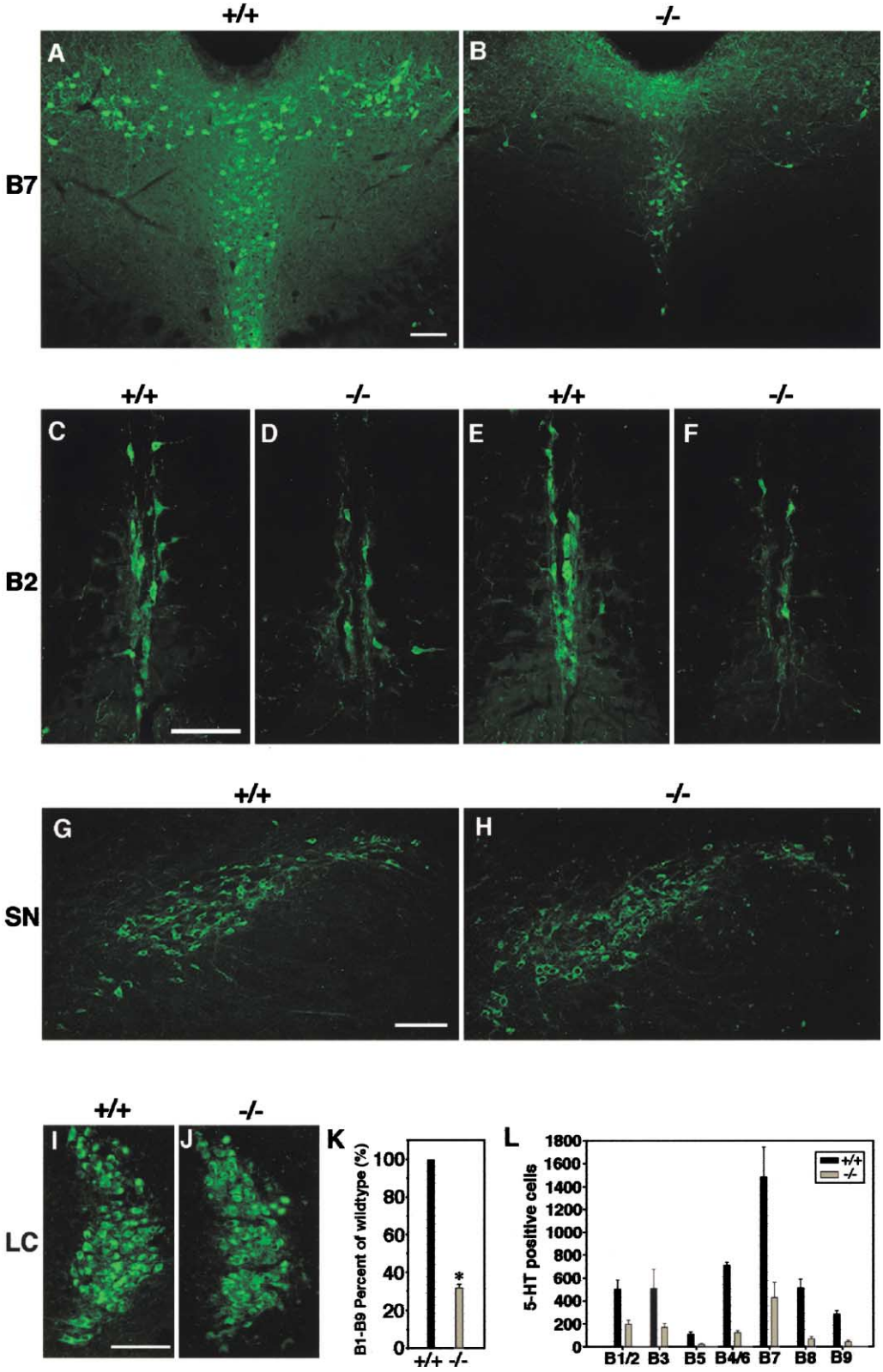

Figure 3. Numbers of 5-HT Immunoreactive Cell Bodies Are Dramatically Decreased in Each of the B Nuclei of Adult *Pet-1* Null Mice

(A–F) Anti-5-HT immunoreactivity in wild-type (A, C, and E) and *Pet-1* null (B, D, and F) adult B7 dorsal raphe nucleus (A and B) and B2 raphe obscurus nucleus (C–F). Two different rostro-caudal levels of B2 are shown in (C)–(F).

(G–J) Anti-VMAT2 immunoreactivity in wild-type (G and I) and *Pet-1* null (H and J) adult substantia nigra (G and H) and locus coeruleus (I and J). No defect was evident in these monaminergic nuclei of *Pet-1* null mice.

(K and L) Counts of 5-HT immunoreactive cell bodies in wild-type versus *Pet-1* null mice throughout the entire B1–B9 nuclei (K) and in individual B nuclei (L). This indicated that a defect in the number of 5-HT immunoreactive cell bodies in the adult was distributed across all B nuclei. Data are presented as mean ± SEM, $n = 5$ for both wild-type and null in (K) and $n = 4$ for both wild-type and null in (L); asterisk, $p \leq 0.001$. Scale bars in (A), (C), (G), and (I) are 100 μm. SN, substantia nigra; LC, locus coeruleus.

peared to be positioned normally in each B nucleus, and ectopic neurons were not seen (Figures 3A–3F).

We also investigated whether elimination of *Pet-1* had secondary effects on other monamine systems of the midbrain and medulla. However, analysis of the dopaminergic substantia nigra (Figures 3G and 3H) and noradrenergic locus coeruleus (Figures 3I and 3J) with anti-VMAT2 antisera failed to detect differences in neuron number for these systems. These findings together with anti-AADC staining in embryonic hindbrain and mesencephalon (Figures 2H and 2I) indicate that while the elimination of *Pet-1* dramatically affected the 5-HT system, no effect on other monamine systems was evident.

We next examined the distribution and density of cell bodies in specific B nuclei of wild-type and *Pet-1* null mice. Cell bodies of the B6 nucleus can be clearly delineated with cresyl violet in wild-type brainstem sections as indicated by the arrow in Figure 4A. In striking contrast, this nucleus appeared to be selectively missing in *Pet-1* null mice (Figure 4B). Costaining with the neuronal marker anti-NeuN and anti-5-HT antisera confirmed that nearly all cells in the wild-type B6 nucleus were 5-HT immunoreactive neurons (Figures 4C and 4E) and that the majority of these cells were missing in the null B6 nucleus (Figures 4D and 4F). Costaining with NeuN also showed that remaining 5-HT immunoreactive cells retained neuronal character (Figure 4F, inset). Similar results were obtained in other B nuclei (data not shown). These data show that the failure to detect normal numbers of 5-HT immunoreactive cell bodies in adult *Pet-1* null B nuclei is because the majority of 5-HT neurons are absent. Together with the NEO immunostaining data presented in Figures 2M–2Q, these findings suggest that a large number of 5-HT neuron precursors are either lost by apoptosis or are transfated to another cell type.

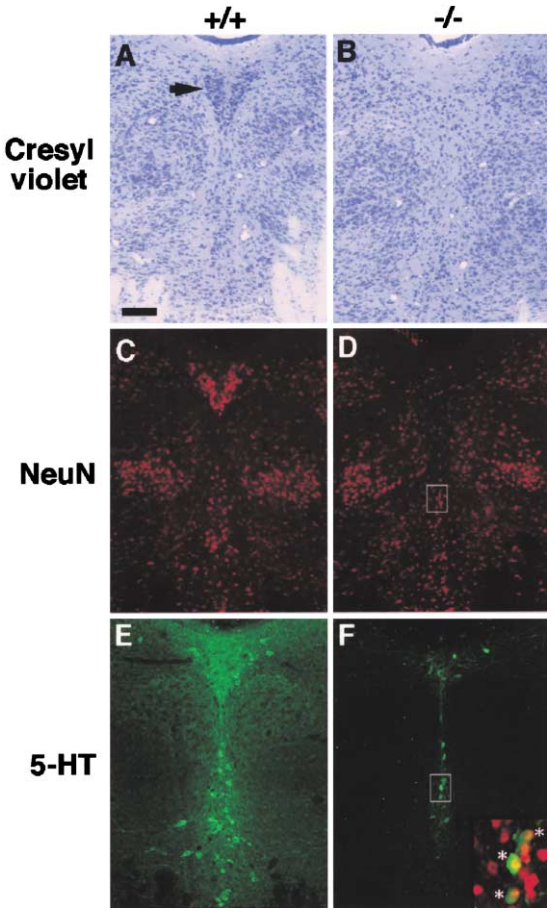

Figure 4. 5-HT Neuron Cell Bodies Are Selectively Missing from Adult *Pet-1* Null B Nuclei

(A and B) Cresyl violet staining of cell bodies in B6 dorsal raphe nucleus (arrow) of wild-type (A) and *Pet-1* null brain (B).

(C–F) Coimmunoreactivity for the neuronal nuclear marker, NeuN (C and D), and 5-HT (E and F) demonstrated a selective absence of most 5-HT immunoreactive neurons in the B6 nucleus of the *Pet-1* null mouse. Inset: an overlay of the boxed areas demonstrated that 5-HT immunoreactive *Pet-1* null cell bodies (asterisk) were NeuN immunoreactive, indicating that remaining 5-HT cells retained neuronal character. Scale bar in (A) is 100 μm for (A)–(F), inset 33 μm.

Serotonergic- and Monaminergic-Specific Gene Expression Defects in Remaining *Pet-1* Null 5-HT Neurons

5-HT neurons that remain in *Pet-1* null brains appeared normal in terms of their raphe locations, neuronal character, and transmitter content. However, we wondered whether these cells might show defects in serotonergic-specific traits, which could reveal further functions of the Pet-1 gene including its potential downstream targets. We previously identified Pet-1 binding sites in the upstream regions of *TPH* and *SERT*, raising the possibility that Pet-1 is involved in their regulation (Hendricks et al., 1999). To investigate expression of these markers, we used both immunohistochemistry and in situ hybridization. In wild-type dorsal raphe (B7) nuclei, costaining with anti-TPH and anti-5-HT confirmed the precise colocalization of these markers (Figures 5A and 5C). By contrast, costaining for these markers in *Pet-1* null B7 (Figures 5B and 5D) and B1–B3 nuclei (data not shown) revealed little or no anti-TPH staining in remaining 5-HT neurons. To confirm that TPH gene expression is indeed reduced in remaining *Pet-1* null 5-HT neurons, we prepared a TPH-specific digoxigenin-labeled antisense RNA probe for in situ hybridization. Consistent with the immunohistochemical results, we found that TPH mRNA was also dramatically diminished in *Pet-1* null B nuclei (Figures 5I and 5J). Similarly, SERT immunoreactive fibers were virtually undetectable in *Pet-1* null target fields (Figures 5M and 5N) despite the presence of some readily detectable 5-HT immunoreactive fibers (Figures 5O and 5P). Moreover, at all rostro-caudal levels of the 5-HT system, SERT mRNA was reduced in *Pet-1* null B nuclei (Figures 5K and 5L). In addition to these serotonergic-specific markers, we also detected diminished expression of VMAT2 (Figures 5E and 5F) but not AADC (Figures 5G and 5H) in *Pet-1* null B nuclei. Similar deficiencies of TPH, VMAT2, and SERT immunoreactivity were detected in *Pet-1* null embryos (data not shown). Thus, these defects in remaining 5-HT neurons suggest that *Pet-1* is involved in coordinating expression of several 5-HT neuron-specific traits.

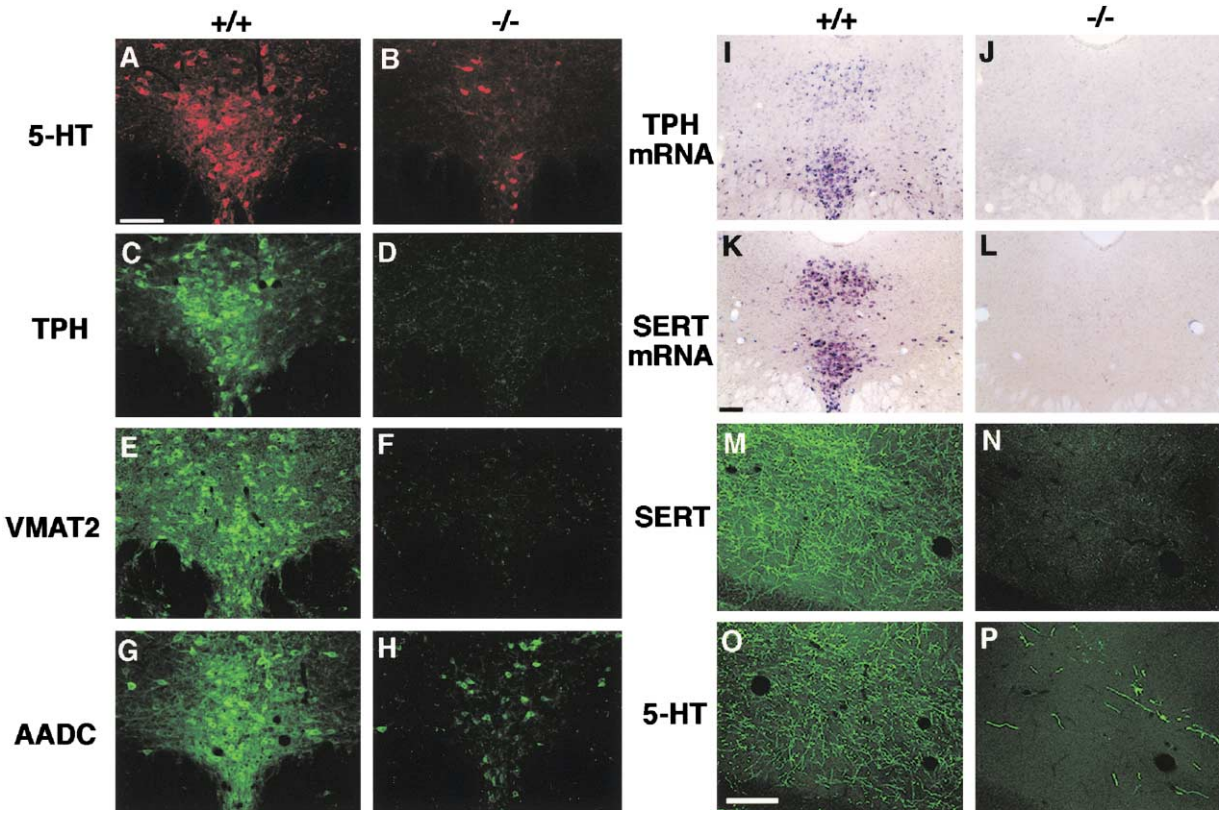

Figure 5. Serotonergic-Specific Gene Expression Is Disrupted in Remaining 5-HT-Positive Neurons of *Pet-1* Null Mice

(A–D) Coimmunoreactivity for 5-HT (A and B) and TPH (C and D) indicated greatly reduced expression of TPH protein in remaining *Pet-1* null 5-HT neurons (B and D).

(E and F) Anti-VMAT2 immunoreactivity was greatly reduced in remaining *Pet-1* null 5-HT neurons.

(G and H) In contrast, anti-AADC immunoreactivity remained easily detectable in these remaining null 5-HT neurons.

(I–L) In situ hybridization for TPH mRNA (I and J) and SERT mRNA (K and L) in the B7 dorsal raphe nucleus showed greatly reduced mRNA levels in *Pet-1* null brains.

(M–P) SERT immunoreactivity in hippocampus (M and N) was virtually undetectable in *Pet-1* null mice despite the presence of some remaining 5-HT immunoreactive fibers (O and P). Scale bar in (A) is 100 μm for (A)–(H), scale bar in (K) is 100 μm for (I)–(L), and scale bar in (O) is 100 μm for (M)–(P).

5-HT Transmitter Deficiency in Target Fields of *Pet-1* Null Mice

The significant reduction of 5-HT neurons in both the rostral and caudal domains of *Pet-1* null mice as well as defective TPH gene expression in remaining ones would be expected to result in a deficiency of the transmitter in target fields. To investigate this, we first used 5-HT immunohistochemistry to visualize 5-HT immunoreactive fibers in brain. Confocal imaging showed that the density of 5-HT immmunoreactive fibers in cortex (Figures 6A and 6B) and hippocampus (Figures 6C and 6D) was greatly diminished in *Pet-1* null mice compared to wild-type littermates. A similar paucity of fibers was found throughout the brain (data not shown). We then used HPLC to quantitate the levels of 5-HT. Analysis of cortex, hippocampus, and caudate confirmed the deficiency of the transmitter in *Pet-1* null brains and indicated that 5-HT and 5-HIAA levels were 10%–15% of those detected in wild-type brain regions (Figure 6E). Consistent with histological analyses, the levels of 5-HT in *Pet-1* heterozygotes was not significantly different from that of wild-type controls (Figure 6F). In contrast to 5-HT and 5-HIAA, the levels of dopamine and its major metabolites were not significantly different in the caudate of wild-type and *Pet-1* null mice (Figure 6G), which is consistent with anti-AADC and anti-VMAT2 staining of the developing (Figures 2H and 2I) and mature (Figures 3G and 3H) dopamine system, respectively. Thus, elimination of *Pet-1* resulted in a specific reduction of 5-HT and its major metabolite in target fields.

Given the severe deficiency of central 5-HT and the large literature implicating the 5-HT system in neuronal development (Whitaker-Azmitia et al., 1996), we investigated whether substantial defects in cytoarchitecture were present in the *Pet-1* null brain. A series of wild-type and *Pet-1* null cresyl violet-stained sections from both sexes were carefully examined using a blinded protocol for defects in nuclear groups of the cortex, hippocampus, amygdala, thalamus, hypothalamus, and spinal cord as well as the great commissures and major projection pathways. We were unable to find any obvious differences in any structure between the *Pet-1* null CNS and that of wild-type littermates (Figure 7). These results suggest that there are no major structural abnor-

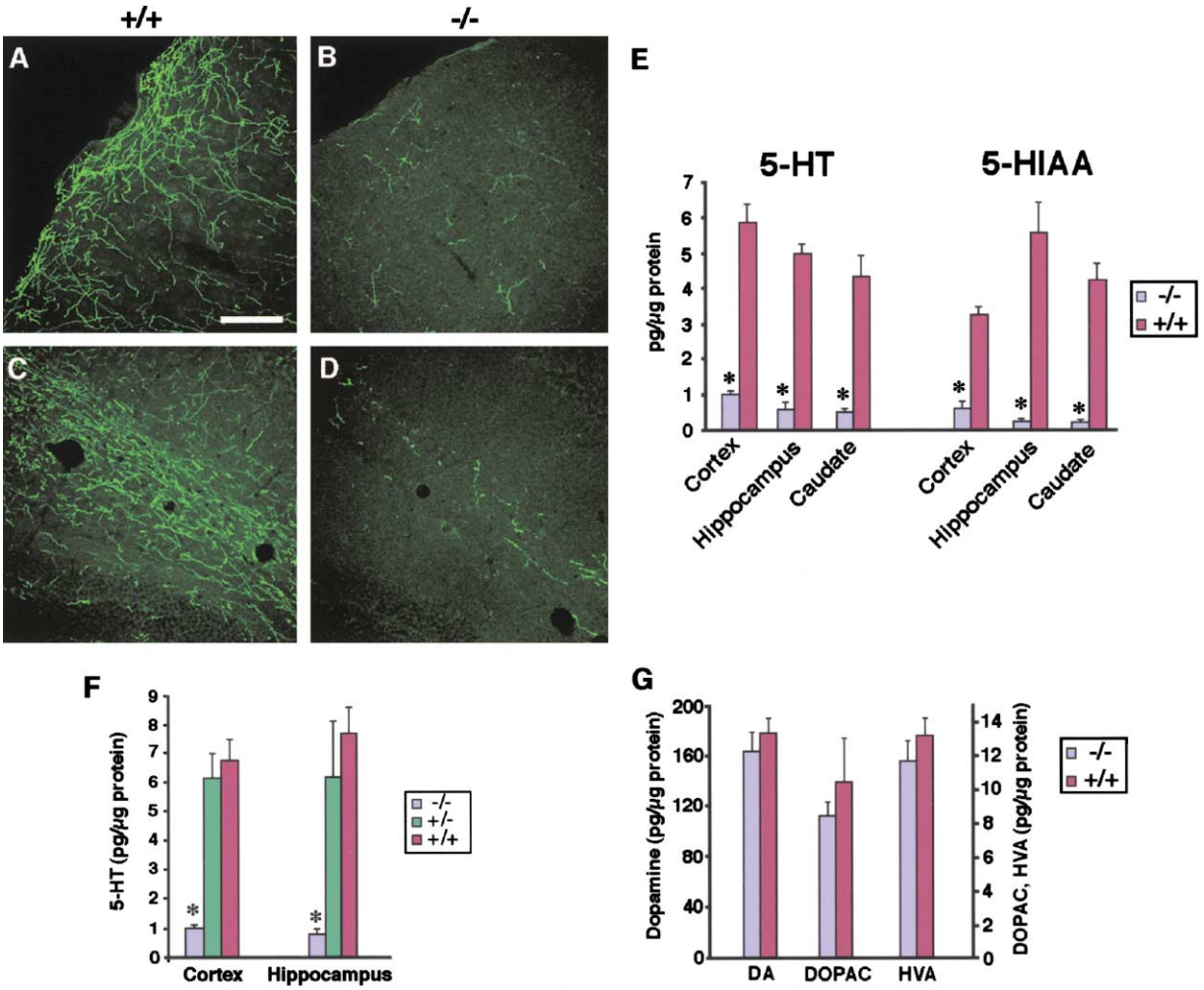

Figure 6. *Pet-1* Null Mice Have a Severe Deficiency of 5-HT in Target Fields

(A–D) Confocal images of anti-5-HT immunoreactive fibers in cortex (A and B) and hippocampus (C and D) in wild-type (A and C) and *Pet-1* null (B and D) mice.

(E) HPLC analysis of 5-HT and 5-HIAA levels in cortex, hippocampus, and caudate in wild-type (n = 6) and *Pet-1* null (n = 5) brain.

(F) HPLC analysis of 5-HT levels in wild-type (n = 6), *Pet-1* heterozygous (n = 6), and *Pet-1* null (n = 7) cortex and hippocampus. There was no statistically significant difference between wild-type and heterozygous for either 5-HT or 5-HIAA levels.

(G) The levels of dopamine (DA) and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in wild-type (n = 4) and *Pet-1* null (n = 5) caudate were not significantly different. Data are presented as mean ± SEM; asterisk, $p < 0.005$.

malities in the *Pet-1* null brain outside the B nuclei. However, we note that our analysis does not exclude the presence of subtle defects such as changes in neuronal substructures, gene expression, or small differences in neuron number.

Pet-1 Null Adults Display Heightened Anxiety-like Behavior and Aggression

The viability of the majority of *Pet-1* null mice provided an opportunity to investigate the behavioral consequences of deficient central 5-HT levels. We conducted several tests to assess general health, overall motor skills, motor learning, and activity of the null mice. We first determined prior to testing the body weights of adult *Pet-1* null mice and their wild-type littermates (Figure 8A). No significant differences were detected in either gender for the two genotypes, which suggests that feeding behavior in *Pet-1* null mice is normal. Balance and motor coordination were next examined using an accelerating rotorod test. Both *Pet-1* null mice and wild-type littermates showed an increase in rotorod performance on each subsequent day of testing, indicating normal motor learning and normal balance and coordination (Figure 8B). Although in early trials *Pet-1* null mice exhibited significantly shorter durations on the rotorod, these differences were eliminated by the end of testing. We then measured locomotor activity in a novel environment using the open field test (Figure 8C). General activity and exploratory behavior was evaluated by determining the total distance traveled during a 15 min testing session. *Pet-1* null mice tended to show a lower level of total activity compared to wild-type littermates (Figure 8C, left), but this difference was not statistically significant. Together, these data indicate that *Pet-1* null mice

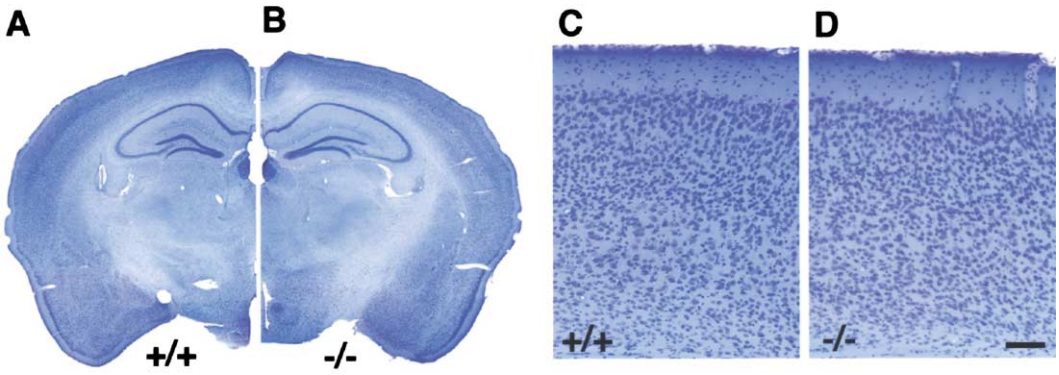

Figure 7. Gross Morphology and Cytoarchitecture of the *Pet-1* Null Forebrain

(A and B) Coronal vibratome (30 μm) sections obtained from wild-type (A) and *Pet-1* null (B) animals at the level of the crossing of the habenular commissure.

(C and D) High-magnification image of neocortex wild-type (C) and *Pet-1* null (D). No consistent differences between genotypes could be discerned, and thus perceived dissimilarities represent normal interindividual differences and variation in sectioning among samples. n = 4, for each genotype. Scale bar equals 550 μm for (A) and (B) and 100 μm for (C) and (D).

have generally normal health, activity, motor coordination, and learning.

We next investigated whether *Pet-1* null mice were abnormally aggressive. This behavior was of interest, as a large number of animal and human studies (Gingrich and Hen, 2001; Kamali et al., 2001; Nelson and Chiavegatto, 2001; Young and Leyton, 2002) point to a negative association between aggression and serotonergic function. We noticed from the outset of our phenotypic analyses that *Pet-1* null males relative to either their wild-type or heterozygous littermates were much more likely to attack each other and other wild-type or heterozygous cagemates. This behavior was also evident upon routine handling. To compare this behavior in *Pet-1* nulls versus controls, we used the standard resident-intruder assay of isolation-induced intermale aggression (Saudou et al., 1994). This assay revealed that for each of three consecutive trials, the latency to first attack of the intruder was significantly shorter for *Pet-1* null residents than for wild-type littermate residents (Figure 8E). Strikingly, *Pet-1* null mice frequently attacked intruders within 10 s of pairing (Figure 8F and see Supplemental Movies at http://www.neuron.org/cgi/content/full/37/2/233/DC1). By contrast, wild-type mice never attacked this quickly. Moreover, *Pet-1* null residents initiated a significantly greater number of attacks relative to controls (Figure 8G). Finally, although the number of attacks in the third trial did not differ between the genotypes (Figure 8G), *Pet-1* null residents produced significantly greater numbers of bite wounds in both the second and third trials (Figure 8H). Similar findings were obtained in a different experimental environment with a different cohort of mice (E.J.W. and J.D.S., data not shown). These findings indicate that isolation-induced intermale aggression is significantly elevated in *Pet-1* nulls.

While no significant differences between genotypes were found for overall locomotor activity, *Pet-1* nulls spent significantly less time than controls in the center of the test chamber relative to total distance traveled (Figure 8C, right). This increased avoidance of the central region reflects an augmentation of the natural tendency of mice to avoid open unprotected areas and suggests that *Pet-1* null mice exhibit increased anxiety-like behavior. To investigate this behavior further, we subjected the mice to an elevated plus maze test, which is a more sensitive measure of anxiety-like behavior. *Pet-1* null mice spent significantly less time in the central region of the plus maze at the start of the test and, strikingly, none of the nulls moved into the open arms of the maze for the entire 5 min test period (Figure 8D). Because *Pet-1* null males are highly aggressive and resident-intruder analyses preceded the plus maze test, there was concern of potential test order effect and social experience effect on anxiety-like behavior. However, several points argue against this potential confounding effect. First, the majority of plus maze test animals were not used in resident-intruder tests and none of the nulls spent time in the plus maze open arms. Second, open field testing was performed before resident-intruder tests. Third, animals used for these behavioral tests were individually housed shortly after weaning and therefore their social experience was minimal. Thus, in addition to heightened aggressive behavior, *Pet-1* null mice show an elevation of anxiety-like behavior.

Discussion

The findings presented here indicate that the 5-HT neuron-restricted ETS factor, Pet-1, plays a pivotal role in the transcriptional mechanisms that generate 5-HT neurons. Two key features of 5-HT neuron development were disrupted in *Pet-1* null mice. First, the majority of 5-HT neuron precursors failed to differentiate in both the rostral and caudal null hindbrain, and a corresponding number of 5-HT neurons were missing in each of the nine adult B nuclei. Second, multiple serotonergic-specific gene expression defects were identified in the developing and adult null 5-HT system, indicating that Pet-1 is required to coordinate expression of genes whose protein products together define the mature 5-HT neuron phenotype. This combination of defects resulted in low levels of transmitter in target fields, suggesting that the developing and adult null brain is largely devoid of

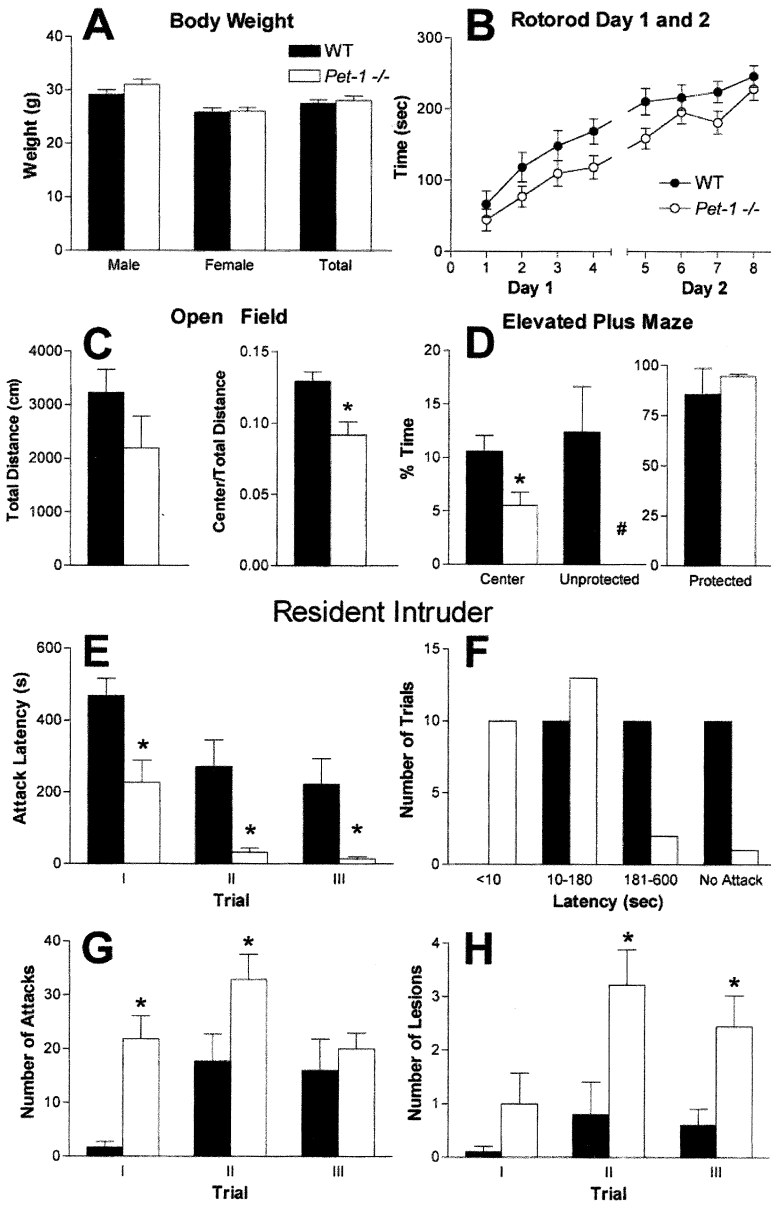

Figure 8. Elevated Anxiety-like Behavior and Aggression in Adult *Pet-1* Null Mice

(A) Body weights of animals before behavioral testing. No differences were observed in *Pet-1* null mice compared to controls. n = 11, male +/+; n = 10, female +/+; n = 6, male −/−; n = 9, female −/−. Black bars, +/+, and open bars, −/− in all relevant panels.

(B) Rotorod testing. Results shown are total time control mice or *Pet-1* null mice remained on the rotating rod per training period. Mice were tested over 2 consecutive days with four training trials given each day. *Pet-1* nulls showed a slight delay in latency during training; however, no difference was seen by the last trial on day 2 (+/+, n = 20; −/−, n = 16; p = 0.42).

(C) Open field behavior. General activity levels were monitored using a 15 min open field test. *Pet-1* nulls tended to exhibit decreased total distance traveled relative to wild-type littermates, but this did not reach statistical significance (+/+, n =15; −/−, n = 11, p = 0.16). In contrast, a significant difference was seen between genotypes for the center to total distance ratio (+/+, n = 15; −/−, n = 11; asterisk, p = 0.002).

(D) Elevated plus maze. Results represent the percent time spent in each sector over a 5 min testing period. When placed in the center of the plus maze, wild-type mice spent more time in the center sector before exploring than *Pet-1* null mice (+/+, n = 8; −/−, n = 8; asterisk, p = 0.019). None of the tested *Pet-1* null mice explored the unprotected arms of the plus maze (hatch mark, p value could not be determined).

(E–H) Resident-intruder assay.

(E) Latency to the first attack was reduced in *Pet-1* null mice, (Trial 1, asterisk, p = 0.006; Trial 2, asterisk, p = 0.008; Trial 3, asterisk, p = 0.002).

(F) Histogram of attack latencies in wild-type versus *Pet-1* null mice showed that in the vast majority of trials, *Pet-1* null mice attacked in less than 180 s and many attacks occurred in less than 10 s. Moreover, seven of the nine *Pet-1* null residents tested showed this short attack latency in at least one trial but this was never observed in wild-type residents.

(G) *Pet-1* null mice attacked intruder mice more frequently (Trial 1, asterisk, p = 0.005; Trial 2, asterisk, p = 0.04; Trial 3, p = 0.56).

(H) *Pet-1* null residents produced a greater number of skin lesions than wild-type residents (Trial 1, p = 0.36; Trial 2, asterisk, p = 0.016; Trial 3, asterisk, p = 0.018). +/+, n = 10; −/−, n = 9 for all trials.

5-HT presynaptic input. Despite low transmitter levels beginning early in development, no defects in gross brain morphology and cytoarchitecture were apparent in *Pet-1* null mice. However, the null mice developed abnormal anxiety-like and aggressive behavior as adults. We discuss our findings in terms of a model (Figure 9) in which a Pet-1-dependent transcriptional program links early 5-HT neuron development to serotonergic control of behavior in the adult.

Pet-1 Is a Cell Type-Restricted Determinant of Central 5-HT Neuron Identity

Early neuroepithelial patterning events executed by the Shh and FGF signaling pathways are required for development of the central 5-HT system. How these general ventral patterning signals are interpreted to specify a subset of progenitors to a 5-HT neuron fate remain largely unknown. Multiple lines of evidence suggest that Pet-1 performs a relatively late role in the specification of 5-HT neuron phenotype. First, as with 5-HT, *Pet-1* mRNA in the embryonic hindbrain is nearly exclusively limited to the mantle layer; very few *Pet-1*-positive cells can be found in the proliferative ventricular zone (Hendricks et al., 1999). Second, caudal 5-HT neurons are believed to be born before and during the period of rostral 5-HT neuron birth (Altman and Bayer, 1981); however, appearance of 5-HT in the caudal domain is delayed relative to that in the rostral domain (Hendricks et al., 1999; Wallace and Lauder, 1983). Thus, the similar delay in *Pet-1* expression in the caudal domain (Hen-

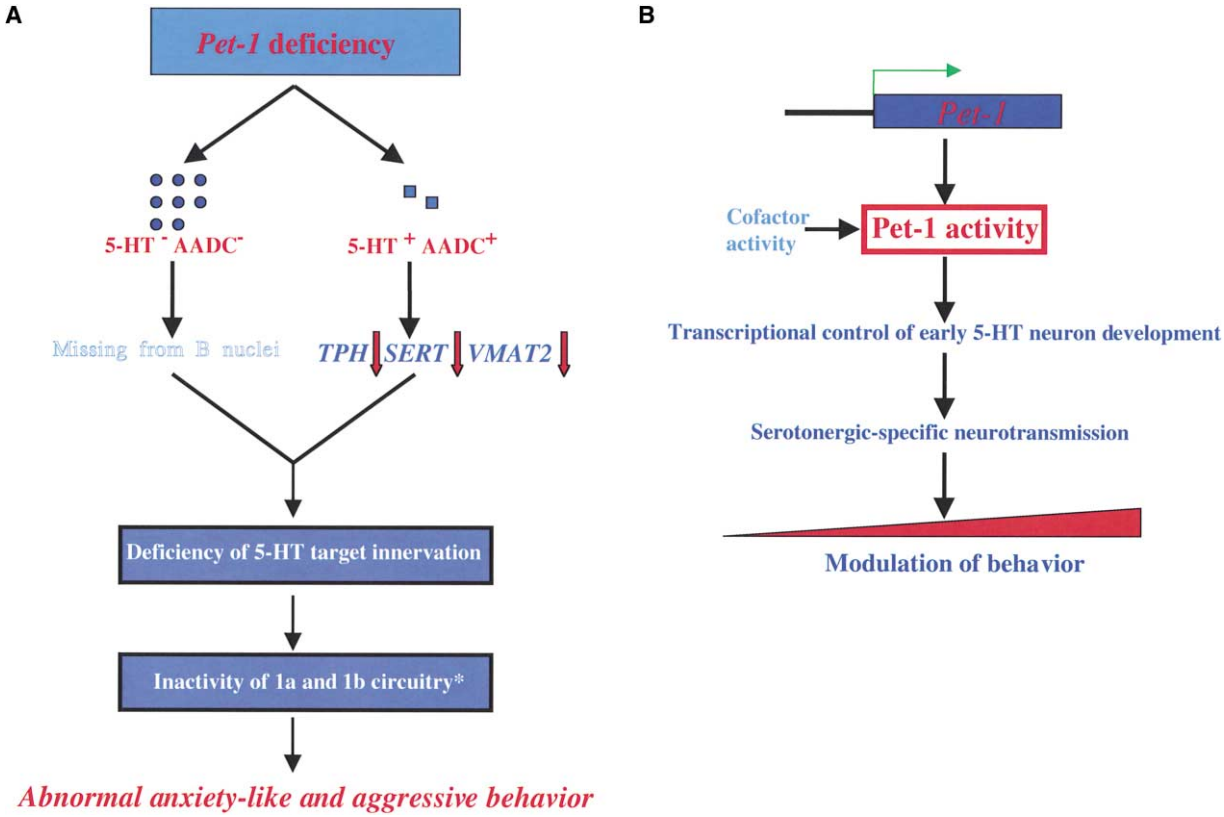

Figure 9. Summary and Model for Transcriptional Regulation of Serotonin-Modulated Behaviors

(A) *Pet-1* loss-of-function phenotype suggests that Pet-1 function is essential in the majority of 5-HT precursors, and in its absence mature 5-HT neurons that would normally arise from these cells fail to populate B nuclei. In the minor 5-HT-positive precursor population, Pet-1 function is not essential but is required for coordinating expression of genes encoding proteins required for biosynthesis, reuptake, and storage of 5-HT. This combination of early differentiation defects results in low transmitter levels throughout the developing and adult brain, and consequently target circuitry is never appropriately modulated. The heightened anxiety-like and aggressive behavior that follows suggests that 5-HT 1a and 1b receptor signaling pathways are largely silent.

*We note, however, that other 5-HT receptor subtypes may also contribute to these abnormal behaviors.

(B) Proposed Pet-1-dependent transcriptional program linking embryonic 5-HT neuron development to serotonergic control of behavior in the adult. We speculate that genetic variation in this program may lead to variation in serotonergic system output and thereby contribute to differences in heritable behavioral traits (see text for details).

dricks et al., 1999) corresponds well to the biphasic period of hindbrain 5-HT neuron differentiation. Third, 5-HT neuron precursors appeared to be generated in normal numbers in the null as assessed by anti-NEO staining but were 5-HT- and *AADC*-negative. Finally, diminished expression of *TPH*, *SERT*, and *VMAT2* in remaining adult null 5-HT neurons indicate further differentiation defects. Together, these data suggest that Pet-1 is a distant downstream effector of Shh/FGF signaling in which it performs a relatively late step in 5-HT neuron differentiation (Ye et al., 1998). The expression patterns of Nkx2.2 (Briscoe et al., 1999) and GATA-3 (van Doorninck et al., 1999) suggest that they may activate Pet-1. However, their functions appear limited to caudal 5-HT neurons (Briscoe et al., 1999; van Doorninck et al., 1999), and therefore candidate upstream regulators of Pet-1 in rostral progenitors remain to be identified. Nevertheless, these data suggest that the transcriptional mechanism that restricts expression of Pet-1 to a small number of neuronal precursors is a key event in the regional restriction of 5-HT neurons to the rostral and caudal hindbrain.

Coordinate Control of 5-HT Synthesis, Reuptake, and Storage by *Pet-1*

A second point to emerge from our study is that Pet-1 functions to coordinate the expression of several genes required specifically for serotonergic neurotransmission (Figure 9A). Both of the enzymes in the 5-HT biosynthetic pathway as well as proteins required for 5-HT uptake into the presynaptic terminal and transport into synaptic vesicles appear to be under Pet-1 genetic control. Regulation of multiple neurotransmitter-specific traits by a single transcription factor is reminiscent of the coordinate regulation of genes required for GABA synthesis and packaging into synaptic vesicles in *C. elegans* through direct control by the UNC-30 homeodomain protein (Eastman et al., 1999). The UNC-86 POU domain factor coordinates expression of tryptophan hydroxylase and a vesicular transporter in a subset of *C. elegans* 5-HT neurons (Sze et al., 2002). Similarly, both loss-of-function and gain-of-function approaches have shown that the dopamine β-hydroxylase (DBH) and tyrosine hydroxylase (TH) genes are coordinately regulated by the Phox2 homeodomain proteins in the noradrenergic

neurotransmitter system of the mammalian peripheral nervous system (Lo et al., 1999; Pattyn et al., 1999; Stanke et al., 1999). Analysis of the DBH and TH promoters strongly suggest that the Phox2 proteins transactivate these genes through direct interactions (Kim et al., 1998; Swanson et al., 1997). The simplest interpretation of our data is that Pet-1 also functions as an activator to directly coordinate expression of specific 5-HT neuron traits. The in vitro detection of Pet-1 binding sites in the promoter regions of *TPH*, *SERT*, and *AADC* offers an avenue to begin to test this idea (Hendricks et al., 1999). It is likely that additional genes are transcriptional targets of Pet-1 and their identification is important for determining whether the *Pet-1*-mediated genetic program in 5-HT neurons is limited to coordinating transmitter system-specific traits or whether Pet-1 also controls other characteristics of 5-HT neurons.

Based on two different experimental approaches, we found lower *TPH* expression in remaining 5-HT-positive *Pet-1* null neurons. How then can these remaining cells show 5-HT immunoreactivity? One likely explanation is that *TPH* expression is not completely eliminated and thus there may be sufficient TPH enzymatic activity for synthesis of 5-hydroxytryptophan. In support of this idea, weak but specific TPH staining of neurons could be detected in B7 dorsal raphe of *Pet-1* null mice when a very high concentration of anti-TPH antibody was used (data not shown). Moreover, compensatory changes in 5-HT neuron function, such as uptake of tryptophan, posttranslational regulation of TPH, 5-HT degradation, or biosynthesis of the tetrahydrobiopterin cofactor for TPH, may help to maintain a certain level of 5-HT and its biosynthesis. An additional possibility is that there is an unrecognized alternative pathway for 5-HT biosynthesis that operates in normal 5-HT neurons or emerges in response to a defective 5-HT system.

Pet-1 Loss of Function Suggests an Unexpected Heterogeneity in 5-HT Neurons

The large deficiency of 5-HT neurons and the gene expression defects seen in the remaining ones of null mice suggests the existence of two intermingled 5-HT neuron precursor populations, each of which may depend on Pet-1 in different ways (Figure 9A). In the predominant precursor population, Pet-1 appears essential, and in its absence precursors are unable to complete differentiation. Although not yet determined, it seems likely that the NEO-positive 5-HT-negative precursors in *Pet-1* null embryonic hindbrain are those that fail to differentiate and populate adult B nuclei and are eliminated by apoptosis or are transfated. The smaller number of remaining, albeit defective, 5-HT neurons suggests the presence of a *Pet-1*-positive precursor variant in both the rostral and caudal domains. In this population, Pet-1 may be required for proper expression of several serotonergic-specific genes but it is not essential for their appearance and maintenance in B nuclei. One possibility is that an alternative pathway partially compensates for Pet-1 so that part of the 5-HT neuron differentiation program is executed in a minority of *Pet-1* null precursors. This pathway could include another ETS factor or perhaps it is genetically distinct and operates in parallel. Whether the remaining 5-HT neurons seen in the null brain represent a previously unrecognized functional or anatomical subclass remains to be determined.

Pet-1 and Transcriptional Regulation of Serotonin-Modulated Behaviors

Despite a severe defect in the central 5-HT system, *Pet-1* nulls show no significant motor deficits and the majority of them survive to adulthood. Moreover, no gross morphological defects were evident in the null brain. By contrast, *Pet-1* null adults displayed dramatically increased anxiety-like and aggressive behavior. This is of particular interest as a large number of dietary, pharmacological, and genetic studies in humans, nonhuman primates, and rodents indicate that dysfunction of the 5-HT system is a critical anomaly underlying these aberrant behaviors (Davidson et al., 2000; Gingrich and Hen, 2001; Kamali et al., 2001; Nelson and Chiavegatto, 2001; Ressler and Nemeroff, 2000; Van Praag, 2001; Young and Leyton, 2002). While other 5-HT receptor subtypes are perhaps involved (Duxon et al., 1997; Naughton et al., 2000), both pharmacological and genetic approaches have implicated 5-HT1a receptors in modulating anxiety (Heisler et al., 1998; Menard and Treit, 1999; Naughton et al., 2000; Parks et al., 1998; Ramboz et al., 1998). Interestingly, heightened anxiety-like behavior of 5-HT1a receptor knockout mice can be rescued, genetically, by tissue-specific expression of the receptor in forebrain, suggesting that forebrain postsynaptic 1a receptors rather than those located presynaptically on 5-HT neuron cell bodies are critically involved in modulating this behavior (Gross et al., 2002). Thus, the increased anxiety-like behavior of *Pet-1* nulls is likely to result at least in part from a deficiency of serotonergic innervation of forebrain.

In support of the link between aggression and serotonergic hypofunction, a number of genes encoding proteins involved in synaptic function have been implicated in the control of aggressive behavior through 5-HT system signaling (Nelson and Chiavegatto, 2001). For instance, homozygous *nNOS* null mice (Chiavegatto et al., 2001) and heterozygous *BDNF* null mice (Lyons et al., 1999) are highly aggressive. In both the *nNOS* and *BDNF* strains, aggressive behavior could be returned to wild-type levels by pharmacological enhancement of serotonergic signaling, thus implicating defective 5-HT function in these mice. In contrast to 5-HT1a receptor null mice, 5-HT1b receptor null mice display heightened aggression but not anxiety (Saudou et al., 1994). The role of the 1b subtype in aggressive behavior is consistent with pharmacological studies showing that a class of 5-HT1 agonists, labeled serenics, has antiaggressive effects (Zhuang et al., 1999). Moreover, lesion studies have implicated postsynaptic 5-HT1 b receptors in modulating aggression (Sijbesma et al., 1991).

These studies together with 5-HT-specific expression of Pet-1 in the brain and the lack of gross behavioral and anatomic defects strongly argue that the increase in anxiety and aggression in *Pet-1* nulls results, at least in part, from inactivity of postsynaptic 1a and 1b signaling brought about through early disruption of presynaptic 5-HT function (Figure 9A). Perhaps the failure to adequately activate 1a receptors in forebrain during the critical early postnatal period rather than in adults ac-

counts for the anxiety-like behavior of the *Pet-1* null mice (Gross et al., 2002). As disorders of mood and aggression often accompany one another in humans (Van Praag, 2001), *Pet-1* null mice are a new model for investigating the cellular and molecular changes in neuronal circuitry that could lead to complex behavioral dysfunction.

Finally, our findings point to the existence of a Pet-1-dependent transcriptional program that can influence both aggression and anxiety-like behavior in the adult through its control of early 5-HT neuron development (Figure 9B). The lack of detectable Pet-1 expression elsewhere in the brain suggests that central Pet-1 function is dedicated to this program. Although not yet tested, the persistence of *Pet-1* expression in adult 5-HT neurons raise the possibility that Pet-1 is required to maintain 5-HT neuron phenotype and to maintain proper serotonergic modulation of behavior. It is intriguing that basic neurologic functions of adult *Pet-1* nulls are intact and Pet-1-dependent transcription is not absolutely required for viability. Therefore, individual variation in the function or regulation of Pet-1-dependent transcription may differentially impact central 5-HT system tone without being lethal or producing dramatic impairments in nervous system function. This could include differences in the activity of transcription factors that determine the level of Pet-1 transcription or the activity of cofactors that function with Pet-1. Additionally, output of the system might be influenced by naturally occurring allelic variants of *Pet-1* transcriptional regulatory elements or protein coding sequences, including nulls. Thus, genetically transmitted variation in this program might be imagined to contribute to individual differences in heritable behavioral traits related to anxiety and aggression. Elucidating the network of factors operating in this program offers a novel approach to better understand how the mechanisms governing early 5-HT neuron differentiation impacts adult behavior. Our findings raise great interest in determining the relevance of this program to genetic mechanisms underlying psychiatric disorders.

Experimental Procedures

Generation of *Pet-1* Null Mice

Mouse *Pet-1* genomic clones were obtained by screening a bacteriophage lambda library constructed with 129Sv DNA (Stratagene). Three overlapping clones were identified and the locus was partially sequenced. Comparison with the rat *Pet-1* cDNA sequence (Fyodorov et al., 1998) was used to deduce the intron-exon structure. Sequences upstream and downstream of the coding region were cloned into a targeting construct designed to remove the entire *Pet-1* protein coding sequence by homologous recombination using standard selection cassettes. Several rounds of electroporation and G418 selection were performed on R1 ES cells. A total of 459 colonies were isolated and screened by Southern blot analysis using an EcoRI restriction digest and a 5′ external probe (Figure 1A). Eleven positive clones were identified and rescreened using a HindIII digestion and 3′ external probe (Figures 1A and 1B). The 5′ probe hybridized to an 11.1 kb fragment in wild-type DNA and an additional 6.4 kb fragment in targeted DNA (data not shown). The 3′ probe hybridized to an 11.6 kb fragment in wild-type DNA and an additional 14.9 kb fragment in targeted DNA (Figure 1B). Two clones, 369 and 371, were chosen for blastocyst injection. All resulting chimeras displayed germline transmission and were bred to mice of both 129Sv and C57BL/6 backgrounds. The F1 mice from the C57BL/6 mating were interbred to produce *Pet-1* null mice on a mixed C57BL/6 and 129 background. These mice and their offspring were used for all analyses in this study. Genotyping of progeny was by Southern blot or by PCR analysis of tail DNA. The genotyping primers used were 5′-CGC ACT TGG GGG GTC ATT ATC AC-3′, 5′-CGG TGG ATG TGG AAT GTG TGC G-3′, and 5′-GCC TGA TGT TCA AGG AAG ACC TCG G-3′. PCR conditions were 35 cycles of 94°C for 50 s, 62°C for 30 s, and 72°C for 40 s. The PCR assay generated a 209 bp fragment for the wild-type allele and a 361 bp fragment for the *Pet-1* null allele.

Histochemistry

Standard procedures and 20 μm sections were used throughout except where noted otherwise. Antibodies used were rabbit polyclonal 5-HT (Diasorin), rat monoclonal 5-HT (Chemicon), rabbit polyclonal neomycin phosphotransferase (Cortex Biochem), mouse monoclonal NeuN (Chemicon), rabbit polyclonal AADC (Protos), mouse monoclonal TPH (Sigma), rabbit polyclonal VMAT2 (Chemicon), and rabbit polyclonal SERT (Diasorin). Detection fluorophores used were FITC, Texas red, Cy3, or Oregon Green. Cell counting was performed on alternate sections through the entire developing and adult 5-HT system. Fluorescent and brightfield photomicrographs were collected on an Olympus BX51 microscope using a Spot RT color digital camera. Confocal images were taken with a Zeiss LSM 410 confocal laser microscope, using an argon/krypton laser (excitation 488 nm) and a 25× Plan-Neofluar NA 0.81 mm Korr, oil objective.

In Situ Hybridization

In situ hybridization was performed as described (Hendricks et al., 1999). Probes used were rat *Pet-1*, full-length; rat *TPH,* 610 nucleotides; and mouse *SERT,* 636 nucleotides.

HPLC Determination of Monamines

Assays were performed with electrochemical detection as described (Yamamoto and Novotney, 1998).

Behavioral Testing

Body weight measurements were taken prior to testing at 17–21 weeks of age. The test order for behavioral analyses at Baylor was body weight measurements, rotorod, open field, resident-intruder (data not shown), and elevated plus maze. Mice shipped to Baylor were acclimatized for several weeks before testing. All tests were evaluated for statistical significance using either student's t test or Mann-Whitney rank sum test.

Rotorod

The accelerating rotorod test was utilized to assess overall balance and motor coordination. The test was performed on an accelerating rotorod apparatus (Ugo Basile) with a 3 cm diameter rod starting with an initial rotation of 4 rpm and accelerating to 40 rpm over 5 min.

Open Field Test

The open field domain consisted of a square area (43 cm × 43 cm) surrounded by Plexiglas walls with the field lit by overhead lighting. Through the use of eight photoreceptor beams on each side of the test arena, the field was divided into 16 quadrants by which the activity of an animal was determined and recorded with a PC-controlled Digiscan optical animal activity system (RXYZCM, Omnitech Electronics). The animal was released in the center of the field and allowed to roam the open field for 15 min. Activity was recorded from the number of photo-beam disruptions in each quadrant to give the total distance traveled. Center to total distance ratio was determined from dividing the center distance by the total distance.

Elevated Plus Maze

The elevated plus maze relies on the natural conflict between the tendencies of mice to explore a novel environment and to avoid open and/or brightly lit areas. The plus maze consisted of two open arms (30 cm × 5 cm) facing each other and two enclosed arms (30 cm × 5 cm × 15 cm) also facing each other. Each arm is attached to a common center platform (5 cm × 5 cm). The structure was constructed from white finished metal 1 mm thick and elevated 40 cm off the floor. Two of the runways were well lit and open and the other two were closed, providing protection. Two males and six females of each genotype were used. Each mouse was placed in the center of the maze facing an open arm. Testing took place during

the light phase under standard light. During the testing period of 5 min, the tester recorded the number of entries into each arm type, the time spent in each arm, and the amount of rearing and grooming. All four paws placed on an arm qualified as an entry.

Resident-Intruder Assay

Resident-intruder assays were performed essentially as described (Saudou et al., 1994). Resident male mice were singly housed for 4 weeks prior to the introduction of a wild-type male intruder mouse (C57BL/6J, 6–7 months old), which were housed in groups of five. Sessions were videotaped from overhead for 10 min. Skin lesions were counted on intruder mice after each session. Behavior was scored blindly for resident attack latency and total number of attacks by the resident; if no attack was observed, attack latency was scored as 600 s. An attack was defined as a single bite or flurry of rapid bites initiated by the resident. Animals used in this assay were littermates. Each resident was tested for 3 sessions with unique intruder pairings. Intruder mice were rested for 1 week between sessions and resident mice were rested 1–4 days between sessions.

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