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Projections and interconnections of genetically defined serotonin neurons in mice

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Abstract

Brain serotonin neurons are heterogeneous and can be distinguished by several anatomical and physiological characteristics. Toward resolving this heterogeneity into classes of functional relevance, subtypes of mature serotonin neurons were previously identified based on gene expression differences initiated during development in different rhombomeric (r) segments of the hindbrain. This redefinition of mature serotonin neuron subtypes based on the criteria of genetic lineage, along with the enabling genetic fate mapping tools, now allows various functional properties, such as axonal projections, to be allocated onto these identified subtypes. Furthermore, our approach uniquely enables interconnections between the different serotonin neuron subtypes to be determined; this is especially relevant because serotonin neuron activity is regulated by several feedback mechanisms. We used intersectional and subtractive genetic fate mapping tools to generate three independent lines of mice in which serotonin neurons arising in different rhombomeric segments, either r1, r2 or both r3 and r5, were uniquely distinguished from all other serotonin neurons by their expression of enhanced green fluorescent protein. Each of these subgroups of serotonergic neurons had a unique combination of forebrain projection targets. Typically more than one subgroup innervated an individual target area. Unique patterns of interconnections between the different groups of serotonin neurons were also observed and these pathways could subserve feedback regulatory circuits. Overall, the current findings suggest that activation of subsets of serotonin neurons could result in topographic serotonin release in the forebrain coupled with feedback inhibition of serotonin neurons with alternative projection targets.

Keywords

dorsal raphe; feedback inhibition; median raphe; rhombomere; serotonin; serotonin1A receptor

Introduction

Across the brain, extracellular serotonin (5-HT) levels dynamically change with behavioral and physiological state (Kirby & Lucki, 1997; Bland *et al.*, 2003; Singer *et al.*, 2004; Kranz *et al.*, 2010). Underlying, or at least contributing to, these highly orchestrated patterns of serotonin levels and consequent effects on behavior is the topographic organization of innervating serotonergic axons that regulate the release and uptake of serotonin. Yet decoding this system of serotonergic projections at the cellular level has been a daunting task: projections are numerous, widespread and cover long distances; individual serotonergic neurons can have multiple targets including other serotonergic neurons to regulate feedback inhibition; and different genetically defined subtypes of serotonergic neurons often populate the same anatomical region (Steinbusch, 1984; Barnes & Sharp, 1999; Jensen *et al.*, 2008; Kiyasova *et al.*, 2011; Vasudeva *et al.*, 2011; Waselus *et al.*, 2011). Tools to decipher this remarkable neural system are being developed, including several that offer the resolution of combinatorial or intersectional genetics (Dymecki *et al.*, 2010).

In the current study, we applied intersectional genetic tools to delineate the connectivity relationships characterizing three serotonergic neuron subtypes. These subtypes were defined by developmental gene expression associated with origin in the serotonergic primordium contained within different rhombomeric (r) segments of the embryonic hindbrain. Serotonin neurons were identified by their expression of *Pet1* and the subset of these that originated from r1 was distinguished by developmental expression of *En1*. Likewise, expression of *Pet1* and *Hoxa2* identified serotonin neurons originating in r2, and expression of *Pet1* and *Egr2* identified those originating in r3 and r5. In the adult, these three subgroups contribute to serotonin neurons located in the dorsal raphe (DR), median raphe (MR), B9 area and the rostral portion of raphe magnus (RMg) (Jensen *et al.*, 2008).

To visualize and selectively map the axon projections associated with each of these three different serotonin neuron subtypes, we used the same intersectional and subtractive genetic approach as in Jensen *et al.* (2008), based on a set of Cre recombinase transgenics – *En1::cre*, *Rse2::cre* or *Egr2::cre* – each separately partnered with a *ePet1::Flpe* transgene, but now with the indicator allele *RC::FrePe* (R. Brust, B. Seri & S.M. Dymecki, unpublished data) to selectively identify the intersection neurons with enhanced green fluorescent protein (eGFP) expression. Using this approach we separately mapped target brain regions served by each of these three serotonin neuron subtypes. In addition, we examined how each of these eGFP-containing subgroups of serotonin neurons provided recurrent innervation to the remaining serotonin neurons that lacked eGFP, as a means to explore potential inter-system feedback inhibition. Such feedback collaterals could, for example, serve to inhibit serotonin neuron subtypes that have different projections, akin to a ‘lateral inhibition phenomenon’. This could greatly enhance the dynamic and differential changes in extracellular serotonin concentrations across brain regions.

Materials and methods

Generation, breeding and genotyping of triple transgenic mice

Transgenic mice expressing eGFP in specific r-derived serotonin neurons were obtained by combining three alleles. The first was the indicator allele *RC::FrePe* (*FRT*-disrupted red *mCherry* and *loxP*-disrupted *eGFP*; R. Brust, B. Seri & S.M. Dymecki, unpublished data) responsive to two recombinases (Cre, Flpe), such that when both Cre and Flpe are present, excisional recombination of *RC::FrePe* results in eGFP expression identifying the ‘intersectional population’. The Flpe recombinase was expressed via the *ePet1::Flpe* transgene, which yields expression selectively in serotonin neurons (Jensen *et al.*, 2008). Virtually all serotonin neurons express *Pet1* during development (Hendricks *et al.*, 1999)

and thus would be identified by this strategy, although it is known that *Pet1* is not required for the development of a subset of serotonin neurons (Hendricks *et al.*, 2003; Kiyasova *et al.*, 2011). The second recombinase, Cre, was provided by one of three alleles used to identify rhombomeric origin. *En1::cre* (*En1*^{Cki}; Kimmel *et al.*, 2000) was used to identify serotonergic neurons originating in r1. *Rse2::cre*, which was developed using a specific enhancer element from *Hoxa2*, was used to identify serotonin neurons arising from r2 (Awatramani *et al.*, 2003). Finally, *Egr2::cre* (*Egr2*^{Cki}; Voiculescu *et al.*, 2001) was used to identify neurons arising from either r3 or r5. The resulting eGFP-expressing neurons in each of these three lines of mice are referred to as 'r1-*Pet1*', 'r2-*Pet1*' and 'r3 / 5-*Pet1*' neurons, respectively, throughout the text.

PCR genotyping with the following primers allowed for identification of the required triple transgenic (*RC::FrePe*, *ePet1::Flpe*, *cre*) animals: *RC::FrePe* (eGFP) forward – 5'-TACGGCAAGCTGACCC TGAAGTTC-3', *RC::FrePe* (eGFP) reverse – 5'-AAGTCGATGCCC TTCAGCTCGATG-3', *Flpe* forward – 5'-GCATCTGGGAGATCACTGAG-3', *Flpe* reverse – 5'-CCCATTCCATGCGGGGTATCG-3', *cre* forward – 5'-GGCATGGTGC AAGTTGAATAACC-3' and *cre* reverse – 5'-GGCTAAGTGCCTTCTCTACAC-3'.

Mice were maintained on a 12 / 12-h light / dark cycle with food and water available *ad libitum*. All procedures were approved by the Harvard Medical School Standing Committee on Animals, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Three male and three female mice and three littermate controls lacking either cre and / or Flpe recombinase for each genotype combination were analysed per group.

Tissue preparation

Mice at postnatal day 28 were anesthetized with Avertin and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) (pH 7.3) followed by 4% paraformaldehyde (PFA) in PBS. Following overnight post-fixation in 4% PFA at 4 °C, brains were rinsed in PBS and equilibrated in a solution of 30% sucrose in PBS. Cryoprotected brains were embedded in tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC, USA), and 40-µm free-floating serial coronal cryosections were collected in PBS.

Immunohistochemistry

For the highest-sensitivity detection of eGFP in axons, we used immunoperoxidase labeling methods and examined tissue sections using both brightfield and darkfield illumination. For immunoperoxidase labeling, free-floating sections were rinsed with 0.1 M PBS and incubated with 0.3% H₂O₂ in PBS for 30 min at room temperature to quench endogenous peroxidase activity. Sections were rinsed with PBS, blocked with 5% normal goat serum (NGS) in 0.1% Triton-X-100 PBS (PBS-T) for 1 h at room temperature, followed by incubation with a chicken polyclonal anti-GFP (1 : 10 000; ab13970; Abcam, Cambridge, MA, USA) in PBS-T with 1% NGS for 48 h at 4 °C. Sections were rinsed with PBS-T three times, and incubated with a biotinylated goat anti-chicken secondary antibody (1 : 500; BA-9010; Vector Laboratories, Burlingame, CA, USA) in PBS-T with 1% NGS for 30 min at room temperature. Following three rinses with PBS-T, immunoreactivity was detected using the Vectastain Elite ABC kit and either DAB or the DAB- Nickel Substrate Kit according to the manufacturer's instructions (Vector Laboratories). Sections were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA), dehydrated and cleared through a series of alcohols and xylenes, and coverslipped with Permount (Fisher Scientific).

To evaluate the interconnections between different serotonin neuron subtypes, double immunofluorescence labeling was used. r-Specific subgroup neurons were detected by immunolabeling for GFP and remaining serotonin neurons were detected by immunolabeling for tryptophan hydroxylase-2 (TPH2). For double immunolabeling, free-floating sections were processed similarly except anti-GFP antibody was diluted 1 : 1000 and combined with anti-TPH2 antibody raised in rabbit (NB100-74555; Novus Biologicals, Littleton, CO, USA) diluted 1 : 1000. The anti-TPH2 antibody identifies cells exclusively in areas known to contain serotonin neurons, and does not identify cells in dopaminergic or noradrenergic nuclei (P. Jensen & K. G. Commons, unpublished observations). After rinsing, primary antisera were detected using Cy2-conjugated donkey anti-chicken and Cy3-conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Sections were then mounted onto glass slides and coverslipped with 90% glycerol-containing mounting media. Imaging of the fluorescent samples was performed using an inverted spinning-disc confocal microscope (Olympus IX81-DSU, Tokyo, Japan) with conventional mercury bulb illumination. All images of immunofluorescence labeling shown are projections of stacks of 10 images, photographed at 1- μ m Z-steps with either 20 $\times$ or 60 $\times$ objectives.

Data analysis

Analysis of eGFP-containing axons throughout the brain focused on the areas with the highest density of innervation, typically areas in the forebrain and pons. While there are additional spinal and medullary patterns of innervation, they were not examined in detail, with the exception of areas containing serotonin neurons in the brainstem. To examine the interconnections between groups of serotonin neurons within the brainstem, r1-*Pet1* axons, due to their abundance, were amenable to density quantification. However, r2-*Pet1* and r3 / 5-*Pet1* axons were fewer and therefore were subjected only to qualitative analysis. For quantification, the density of innervation of serotonin neurons from the r1-*Pet1* population was analysed using NIH ImageJ software. Regions of interest within the raphe included DR, MR, RMg, parapyramidal nucleus (Ppy) located lateral to RMg, raphe obscurus (ROb), raphe pallidus (RPa), and lateral paragigantocellular reticular nucleus (LPGi) located lateral to ROb and RPa.

On average three fields were photographed for each region of interest in each of three mice of the same genotype at 4, 10, 20 and 60 $\times$ magnification. For quantification, each field was visualized at 20 $\times$ magnification as a maximal projection of a Z-stack with ten Z-steps at 1- μ m intervals. The images containing r1-*Pet1* eGFP labeling (green) were converted to grayscale and a threshold function was manually applied to identify puncta corresponding to eGFP-immunolabeling while minimizing background detection. Using the polygon selection tool, the area of TPH2-labeled neurons and dendrites was outlined, and this area was selected on the eGFP-threshold image. Within the selected area, the number of particles representing eGFP puncta was counted and the total selected area (μ m²) was measured. The number of eGFP particles per 1000 μ m² was then calculated per image and averaged per region per subject. Individual subject means were averaged per group and standard error of the mean for each region was calculated. The density of r1-*Pet1* eGFP-labeled axons in each region of interest was statistically analysed with one-way analysis of variance (anova; spss 14.0; Chicago, IL, USA) with REGION as a factor. *Post hoc* comparisons using Fishers' least significant difference (LSD) test were followed with a threshold for significance of $P < 0.05$ where appropriate.

Results

We initially evaluated tissues from each of the three different genotypes of mice to confirm that the distribution of identified serotonin neurons (Fig. 1) was consistent with previous

analyses using a different indicator allele, where serotonin neuron subtypes were detected by expression of β -galactosidase (Jensen *et al.*, 2008). As found in the previous study, r1-*Pet1* neurons populated the DR and a portion of the MR and B9 area while r2-*Pet1* neurons resided exclusively within the MR and B9. r3 / 5-*Pet1* neurons populated MR and B9, probably corresponding to r3-derived neurons, as well as the rostral pole of the RMg, probably associated with r5 as the intervening r4 does not produce serotonin neurons and thus creates a gap separating the rostral and caudal serotonin systems.

Major efferent projections

Consistent with the observation that r1-*Pet1* neurons account for many serotonin neurons, their axons were widespread and detected in several brain areas. r1-*Pet1* neurons were the major source of cortical innervation, where they were found in all regions and through all lamina (Fig. 2A–D). r1-*Pet1* axons were typically fine with periodic undulations in diameter along their course or had small ($< 1 \mu\text{m}$ in diameter) varicosities, consistent with their majority origin in the DR (Kosofsky & Molliver, 1987; Jensen *et al.*, 2008). r1-*Pet1* axons innervated superficial layers of cortex where their axons typically had small varicosities (Fig. 2B and D). Larger varicose axons from r1-*Pet1* neurons were found in the lateral entorhinal cortex.

r1-*Pet1* axons also provided a major innervation to the hippocampal formation, where axons with both small and large varicosities were found (Fig. 3A–D). r1-*Pet1* axons with large beaded morphology were particularly concentrated in the base of the granule cell layer in the dentate gyrus, in stratum radiatum of CA3 and at the border between strata lacunosum-moleculare and radiatum in CA1. Fine axons were found more abundantly in the molecular layer of the dentate gyrus and stratum radiatum of CA1.

Subcortically, r1-*Pet1* axons were highly abundant in the septum, shell of the nucleus accumbens, basolateral amygdala, lateral globus pallidus, substantia innominata, ventral pallidum, hypothalamus, ventral tegmental area and substantia nigra. Dense tracts of axons were also visible passing through the lateral hypothalamus, lateral preoptic area, nucleus of the diagonal band and medial septum (Figs 4D–F and 5A).

In contrast to the widespread projections of r1-*Pet1* neurons, r2-*Pet1* neurons provided selective and often dense innervation of a handful of regions. In the cortex, r2-*Pet1* axons were identified in the medial prefrontal, the parietal cortex and, to a lesser extent, the perirhinal cortex (Figs 2A'–D' and 5B). In cortical areas, r2-*Pet1* axons were found more often in layers III–VI than in superficial layers I / II. The morphology of r2-*Pet1*-derived axons was more consistently that of large beaded axons. In the hippocampus, large beaded axons were found at the base of the granule cell layer and within the hilus of the dentate gyrus, as well as in the stratum lacunosum-moleculare / radiatum border of area CA1, and to a lesser extent the stratum radiatum of CA3 (Fig. 3A'–D').

In subcortical regions, r2-*Pet1* axons were detected in the medial septum, suprachiasmatic nucleus and the periventricular nucleus of the thalamus (Figs 4D'–F' and 5B). Additional but sparse r2-*Pet1* axons were identified in the basolateral amygdala (BLA), and generally throughout the hypothalamus.

r3 / 5-*Pet1* neurons had distinct sets of axon projections in a unique pattern. In frontal and parietal cortices, only rare individual fibers were visible from r3 / 5-*Pet1* neurons (Fig. 2A''–D''). However, r3 / 5-*Pet1* axons were detected in the piriform and amygdalar cortices as well as in the hippocampal formation (Fig. 3A''–D''). Within the hippocampus, r3 / 5-*Pet1* axons were distinctly beaded and found in similar areas as beaded axons arising from r1 or

r2, i.e. within the sub-granule cell layer and hilus of the dentate gyrus and the border between strata lacunosum-moleculare and radiatum in area CA1 (Fig. 3A''–C'').

r3 / 5-*Pet1* axons were rare in some subcortical areas heavily innervated by r1-*Pet1* and r2-*Pet1* axons such as the periventricular nucleus of the thalamus and the suprachiasmatic nucleus (Fig. 4D'' and E''). Sparse fibers from r3 / 5-*Pet1* neurons were detected within midline thalamic areas as well as throughout the hypothalamus. More caudally, r3 / 5-*Pet1* axons had all but exclusive innervation of the ventral, anterior and dorsal tegmental nuclei, also known as the tegmental nuclei of Gudden, as well as rich preference for the rostral–dorsolateral medulla including locus coeruleus and zones within the dorsal lateral parabrachial nucleus (Figs 4F'' and 5C). These areas also received some innervation from r1-*Pet1* axons (Fig. 5A), but were poorly innervated by r2-*Pet1* axons (Fig. 5B).

Projections from r1-*Pet1* neurons to remaining serotonin cell groups

We specifically examined how each of these genetically defined and eGFP-containing groups of serotonin neurons innervated the remaining unmarked serotonin neurons in the pons and medulla. Such projections between different serotonin neuron subtypes have the capacity to engage feedback inhibitory regulation of serotonin cell activity through serotonin release and subsequent signaling via the 5-HT_{1A} autoreceptor (reviewed by Barnes & Sharp, 1999). To assess this inter-system connectivity, we examined tissue from mice of each of the three different genotypes [*En1::cre* (r1), *ePet1::Flpe*, *RC::FrePe*; *Rse2::cre* (r2), *ePet1::Flpe*, *RC::FrePe*; and *Egr2::cre* (r3 / 5), *ePet1::Flpe*, *RC::FrePe*] where immunofluorescence labeling for eGFP identified the particular genetic subgroup of serotonin neurons, while labeling for TPH2 identified all remaining serotonin neurons. In this analysis, no assumptions were made regarding the rhombomeric origin or molecular signature of serotonin neurons lacking eGFP.

r1-*Pet1* axons (GFP+) innervated neurons immunolabeled with TPH2 but which were GFP-negative and thus not derived from r1. The densest ramification of such axons was visible in those portions of the MR populated by non-r1-derived serotonin neurons (Fig. 6A and B). Strings of r1-*Pet1* axons were often detected either overlapping with or in close proximity to non-r1-derived serotonin cell bodies or dendrites in the MR (Fig. 6B). r1-*Pet1* axons were also detected in both RMg (Fig. 6C) and ROb (Fig. 6D). Most of the r1-*Pet1* axons in the medulla were visible as short segments aligned along the dorsoventral axis overlying RMg and ROb. In contrast to these areas, very few r1-*Pet1* axons were detected in the RPa (Fig. 6E). The Ppy and LPGi areas received a few more axons than RPa (Fig. 6F).

Regionally selective innervation patterns of r1-*Pet1* axons were statistically demonstrable using ANOVA (Fig. 7), which revealed a significant effect of REGION ($F_{5,28} = 9.84$, $P < 0.001$) on the density of r1-*Pet1* axons. *Post-hoc* analyses with LSD tests indicated that r1-derived serotonin axon densities in MR are greater than those in any other regions of interest in the raphe ($P < 0.05$; Fig. 7). The axon densities within RMg are significantly greater than those in RPa and LPGi ($P < 0.05$), but were not significantly different from the axon densities detected in ROb and Ppy. The ROb has lower axon densities than MR, and greater densities than RPa, Ppy and LPGi ($P < 0.05$). The axon densities within Ppy and LPGi were not significantly different ($P > 0.05$).

Projections from r2-*Pet1* neurons to remaining serotonin cell groups

r2-*Pet1* axons were detected in the vicinity of non-r2-derived serotonin neurons nearby within the MR (Fig. 8). Notably, r2-*Pet1* axons were largely undetected in any other raphe nucleus, except for an individual truncated fiber occasionally detected in darkfield images

(Fig. 9A and A'). Thus, *r2-Pet1* projections are highly restricted within the MR, proximal to the location of their cell bodies.

Projections from *r3 / r5-Pet1* neurons to remaining serotonin cell groups

In contrast to *r2-Pet1* axons, the *r3 / r5-Pet1* axons provided innervation to other anatomically defined groups of serotonin neurons. In particular, *r3 / 5-Pet1* axons were clearly distributed throughout the rostral–caudal and dorsal–ventral extent of the DR (Fig. 9B and B'). Within the DR, there was a mild regional bias such that the axons appeared slightly more concentrated in the dorsal portion at the rostral level, and in the lateral parts at the mid-rostrocaudal level of the DR. Strings of double-labeled axons with varicosities overlying serotonin neurons were detected in the DR (Fig. 10A and B) as well as the MR (Fig. 10C).

In the caudal raphe nuclei, *r3 / 5-Pet1* axons were also detected in the lateral medulla including the Ppy and LPGi where they intermingled with non-*r3 / r5* derived serotonin neurons (Fig. 10D and E). However, the appearance of *r3 / 5-Pet1* axons was negligible in the ROb and RPa (Fig. 10F).

Thus overall while *r2-Pet1* neurons did not substantively innervate other groups of serotonin neurons, axons arising from either *r1-Pet1* or *r3 / r5-Pet1* neurons selectively innervated distinct subsets of serotonin neurons.

Discussion

In the present study, we show that subgroups of serotonin neurons distinguished by their positional and genetic code of origin have unique fingerprints of efferent projections in the adult, combined with differential propensities to innervate other groups of serotonin neurons. These observations are consistent with the idea that there is the capacity for subcomponents of the serotonin system to influence serotonin release in a region-specific manner. Interconnections between each group of genetically defined *Pet1* neurons and remaining serotonin neurons were not ubiquitous, nor did each of these cell groups exclusively innervate near neighbors. Rather, there were particular patterns of interconnections among distinct groups of serotonin neurons. These findings suggest that pathways regulating feedback inhibition are neither exclusively autoregulatory nor equally distributed across different groups of serotonin neurons, but rather may have a hierarchical organization.

Methodological considerations

Projections of serotonin neurons have been well studied using conventional tract-tracing methods, which have shown an ordered, albeit complex, topography of projection patterns (reviewed by Vasudeva *et al.*, 2011; Waselus *et al.*, 2011). However, conventional tract tracing is limited by the location and variability of injection sites, which may not map to functional groups, as well as the potential uptake of tract tracer by neurochemically heterogeneous cell types. The current methods are unique in identifying distinct groups of neurons based on their developmental origin and associated gene expression history, which may directly impact their final functional role. Furthermore, this approach sets the stage for manipulation of selective subsets of neurons (Kim *et al.*, 2009). An important technical consideration for the study of projections, however, is the sensitivity of detecting the indicator allele, as the size of axon terminals reduces the potential accumulation of eGFP. With the *RC::FrePe* indicator allele (R. Brust, B. Seri & S. M. Dymecki, unpublished data), we were able to detect eGFP-expressing axons in multiple areas, supporting the sensitivity of the methods.

Topographic projections of genetically defined serotonin neurons

The influence of rhombomeric origin on the development of serotonin neuron projections appears to be complex. r1-*Pet1* neurons account for a large number of serotonin neurons that probably can be further subdivided. Indeed, within the DR, subgroups of neurons have previously been identified by morphological criteria and gene expression patterns (reviewed by Hale & Lowry, 2011), and different subregions of the DR have been associated with differential projection patterns (reviewed by Vasudeva *et al.*, 2011; Waselus *et al.*, 2011). Therefore, in this case, the importance of r1 origin on the final developmental endpoint of serotonin neurons is not obvious. However, in comparing all three lines of mice, serotonin neurons arising from more caudal rhombomeres tended to have more caudal sets of projections, albeit with exceptions. In addition, there were particularly striking differences between the projections of r2- and r3 / 5-*Pet1* neurons in that r2-*Pet1* neurons heavily innervated the suprachiasmatic nucleus and periventricular nucleus of the thalamus, but r3 / 5-*Pet1* neurons did not. Taken together, these observations would suggest that rhombomeric origin does have an influence on patterns of projections, but that this choice is accompanied by, particularly in r1, a series of events that further shape the final developmental and functional niche of serotonin neurons.

Each r-defined group innervated a particular combination of forebrain areas and thus has the capacity to influence serotonin release in specific regions. However, each group typically shared innervation targets with other cell groups. That is, the majority of areas examined received innervation from more than one rhombomere. r2- and r3 / 5-*Pet1* neurons typically shared targets with r1-*Pet1* neurons and sometimes all three groups innervated a particular area, such as in the case of the hippocampal formation. It may be the case that these convergent axons release serotonin to have functionally common effects. Supporting this possibility, stimulation of either the DR or the MR, which both innervate the suprachiasmatic nucleus (Yamakawa & Antle, 2010; Kawano *et al.*, 1996), can elicit nonphotic phase shifts in circadian rhythm (Meyer-Bernstein & Morin, 1999; Glass *et al.*, 2000). However, it could also be possible that activation of different sets of serotonin projections could have functionally divergent effects in a target region, particularly if different sets of serotonergic axons innervated distinct cell types or were proximal to different serotonin receptor populations; this possibility has not yet been examined.

Previous studies reported two morphologically distinct serotonin fibers – fine axons with small varicosities and beaded axons with large, spherical varicosities (for a review, see Törk, 1990) – that have differential vulnerability to the neurotoxic effects of certain amphetamine derivatives (O'Hearn *et al.*, 1988). The fine and beaded axons are known to arise from the DR and MR, respectively (Kosofsky & Molliver, 1987; Hornung & Celio, 1992), and have different regional and laminar distributions in the forebrain (Mamounas *et al.*, 1991; Wilson & Molliver, 1991). Consistent with these studies, r1-*Pet1* neurons, primarily gave rise to axons with smaller varicosities, although large beaded axons were also found. In addition, axons from r2-*Pet1* and r3 / 5-*Pet1* neurons more consistently had a large-beaded morphology. Interestingly, in some cases axons with similar morphology but different developmental origin converged in a target area, such as within the same lamina of the hippocampus. However, in other cases, each group of axons had preferential termination zones, for example within the cortex. Indeed the current results, taken together with earlier studies in rat, would argue for highly organized topographic innervation of the cortex by serotonin neurons (Waterhouse *et al.*, 1986; Mamounas *et al.*, 1991).

Patterns of interconnections among different serotonin neurons

We studied in particular detail the innervation between each identified subgroup of serotonin neurons and the remaining serotonin neurons because these interconnections comprise

potential pathways for feedback control circuits that can have a profound effect on activity state. All groups of genetically defined serotonin neurons provided innervation to adjacent neurons. This suggests that nearby neurons are likely to coordinate their activation states. Consistent with this finding, individual neurons in the DR are known to have local axon collaterals ramifying within the DR (Li *et al.*, 2001). If functionally similar neurons cluster together, proximally localized axon collaterals are likely to participate in autoregulatory feedback networks, potentially playing a homeostatic function.

However, interconnections between serotonin neurons do not seem to be dictated by proximity and there was evidence for selective projections between distant serotonin neurons. For example, r1-*Pet1* neurons showed particular preferences for sending axons to the midline medullary RMg and ROb. These medullary areas are thought to have largely separate projection targets compared with r1-*Pet1* neurons, and are major contributors to serotonin neurotransmission within the spinal cord (Skagerberg & Bjorklund, 1985). Previous tract-tracing studies in the rat have suggested that only a selected group of serotonin neurons located in the lateral wings of the DR have descending projections to the ventral medial medulla (Beitz, 1982). Showing similar selectivity in target areas, r2- and r3 / 5-*Pet1* neurons contrasted in their innervation of the DR. The DR is known to be a target of axons from the MR (Vertes *et al.*, 1999), and our data would suggest only a certain subset of MR neurons contribute to this pathway (not r2-*Pet1* neurons). These observations suggest that there is the selective capacity of some serotonin neurons to communicate to other groups. While the complete logic of these interconnections remains to be resolved, the regional specificity of these interconnections is consistent with our previous observations that 5-HT1A-receptor-mediated feedback inhibitory processes appear to influence certain subgroups of serotonin neurons more than others, and depends on the behavioral state of the animal (Commons, 2008; Sperling & Commons, 2011).

Conclusions

In this study, we exploited genetic tools that identify subgroups of serotonin neurons based on genetic lineage to probe the organization of serotonin neuron projections. Our results suggest that rhombomeric origin is a contributor to organizing not only the location of serotonin cell bodies (Jensen *et al.*, 2008), but also the organization of their projections, and thus genetic and developmental process may provide key insights into resolving aspects of the functional heterogeneity within the serotonin system. Moreover, genetic access to refined subsets of serotonin neurons to manipulate their function can then be interpreted within the context of this insight into their projection patterns. In addition, the results show that there are very selective patterns of interconnections among different groups of serotonin neurons. These patterns of projections and interconnections have the potential to topographically control serotonin release in the brain. A fuller understanding of the functional subgroups of serotonin neurons and how they communicate with each other will have importance for understanding the neurobiological basis of the many pathological conditions associated with dysfunction of serotonin neurotransmission.

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Abbreviations

DR dorsal raphe

eGFP	enhanced green fluorescent protein
LPGi	lateral paragigantocellular reticular nucleus
MR	median raphe
NGS	normal goat serum
Ppy	parapyramidal nucleus
r1	rhombomere 1
r2	rhombomere 2
r3 / 5	rhombomere 3 or 5
RMg	raphe magnus
ROb	raphe obscurus
RPa	raphe pallidus
TPH2	tryptophan hydroxylase 2

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Fig. 1.

The distribution of serotonin neurons with developmental origin in rhombomere (r) 1, 2 or either 3 or 5 identified by eGFP expression was consistent with prior fate mapping (Jensen *et al.*, 2008). (A–C) Darkfield illumination of immunoperoxidase labeling for eGFP at similar levels through the pons of each of the three mouse lines studied. In each panel, the medial longitudinal fasciculus (mlf) is visible. (A) r1-*Pet1* neurons are visible in the dorsal raphe (DR) and dorsal portion of the median raphe (MR). (B) r2-*Pet1* neurons constitute a smaller population of neurons visible in the middle third of the MR. (C) r3 / 5-*Pet1* neurons are visible in the ventral MR, and their axons in the ventral tegmental nucleus of Gudden (arrowhead). Scale bar = 150 μ m.

Fig. 2.

Projection patterns of r1-*Pet1* neurons (left column), r2-*Pet1* neurons (center column) and r3 / 5-*Pet1* neurons (right column) in the prefrontal and parietal cortices. (A–A'') In the prelimbic cortex, parallel lines designate the layer V pyramidal cell layer. (A) r1-*Pet1* axons are enriched in lamina I / II (arrow) and found in deeper layers (arrowhead). (A') r2-*Pet1* axons are found through layers III–VI (arrowheads). (A'') Only an occasional, individual axon (arrowhead) arising from r3 / 5-*Pet1* neurons was found in the prelimbic cortex. (B–B'') High-magnification images show that r1-*Pet1* neurons (B) have typically fine axons (arrow) and those with minute beads (arrowhead) while r2- *Pet1* (B') and r3 / 5- *Pet1* (B'') neurons have axons with more pronounced, larger beads (arrowheads). (C–C'') Innervation

of parietal cortex by r1- (C), r2- (C') and r3 / 5- (C'') *Pet1* neurons. (C) r1-*Pet1* neurons were found in superficial and deeper layers, but without obvious laminar distributions. (C') r2-*Pet1* neurons also innervated the parietal cortex (arrowheads). (C'') Axons from r3 / 5-*Pet1* cells were not detected in parietal cortex. (D) Higher magnification of r1-*Pet1* axons in lamina 1 of prefrontal cortex shows small beaded morphology (arrow), although occasional varicosities are larger (arrowhead). (D') Higher magnification of r2-*Pet1* axons in parietal cortex shows large varicosities (arrowheads). (D'') Higher magnification of r3 / 5-*Pet1* axon located in prefrontal cortex shows a single ramification with large beaded morphology (arrowhead). Scale bar in A = 100 μm ; B = 50 μm ; C = 150 μm ; D = 50 μm .

Fig. 3. Hippocampal projections from r1-*Pet1* neurons (left column), r2-*Pet1* neurons (center column) and r3 / 5-*Pet1* neurons (right column). (A–A'') Innervation of the dentate gyrus; boxed areas shown at higher magnification in B through B''. (B) Beaded axons pertaining to r1-*Pet1* neurons (arrowhead) are visible below the granule cell layer (GCL) extending into the hilus (H). In addition, axons with smaller varicosities (arrow) are visible in the molecular layer (ML). Beaded r2-*Pet1* axons (B') and r3 / 5-*Pet1* axons (B'') are most prominent subjacent to the granule cell layer (arrowheads). (C–C'') Innervation of the border between strata lacunosum-molecular (L–M) and radiatum (Rad) of area CA1. Each group of cells provides axons with large varicosities to this area (arrowheads in each panel). r1-*Pet1* cells,

however, also provide finer axons (arrow in C), particularly extending into stratum radiatum. In stratum radiatum of CA3, (D) r1-*Pet1* axons are both smooth with modest periodic enlargements or 'small varicosities', but a few very large boutons are also seen (arrowhead). (D') A segment of thin axon (arrow) associated with larger boutons that are spaced farther apart (arrowheads) pertaining to r2-*Pet1* neurons. (D'') r3/ 5-*Pet1* axons tend to be sparse but with large varicosities (arrowheads). Scale bar in A = 100 μm ; B = 50 μm ; C and D = 35 μm .

Fig. 4.

Subcortical projection pattern of r1-*Pet1* neurons, r2-*Pet1* neurons and r3 / 5-*Pet1* neurons. (A–C) Boxed areas within line drawings adapted from Paxinos mouse atlas plates (Paxinos & Franklin, 2001) indicate the location of the photomicrographs in the same row. (D–D'') The periventricular nucleus of the thalamus located bilaterally at the base of the third ventricle (3V). r1-*Pet1* (D) and r2-*Pet1* (D') neurons densely innervate this area (arrows) while r3 / 5-*Pet1* neurons (D'') send a few scattered axons (arrow). (E–E'') Darkfield images of innervation within the suprachiasmatic nucleus (SCN), where the midline is designated with a vertical line. r1-*Pet1* and r2-*Pet1* neurons contribute to innervation of the SCN (arrows in E and E', respectively), whereas r3 / 5-*Pet1* axons are undetected above the optic chiasm (arrowheads in E''). (F–F'') In the rostral medulla, at the base of the fourth ventricle (4V), different patterns of innervation can be seen. (F) r1-*Pet1* neurons (r1, arrow) send very few axons to the dorsal tegmental nucleus of Gudden (double arrowheads) and sparsely innervate the rostral pole of the locus coeruleus (LC, arrow) and parabrachial nucleus (arrowhead). (F') r2-*Pet1* neurons (r2, arrow) have very sparse innervation of the same areas. (F'') In contrast, r3 / 5-*Pet1* neurons richly innervate Gudden's tegmental nucleus (double arrowheads), the rostral LC (arrow) and the lateral parabrachial nucleus (arrowhead). A few dendrites from r3 / 5-neurons are visible at the bottom of the field (r3 / 5, arrow). Scale bar = 100 μ m.

Fig. 5.

Schematic mapping, based on the flat maps of Swanson (2004), showing areas innervated by r1-*Pet1* neurons (A), r2-*Pet1* neurons (B) or r3 / 5-*Pet1* neurons (C). (A) r1-*Pet1* neurons have widespread axons including all of the targets of the DR as well as some targets of the MR and B9. Particularly dense innervation (dark blue) was noted in the medial septum (MS), the nucleus of the diagonal band (DB), nucleus accumbens shell region (SH), globus pallidus (GP), ventral pallidum (VP), substantia innominata (SI), basolateral amygdala (BLA), throughout the hypothalamus, ventral tegmental area (VTA), substantia nigra pars compacta (SNc) and pars reticulata (SNr). Areas with relatively less innervation are indicated in light blue. (B) Areas with dense (dark orange) innervation from r2-*Pet1* neurons include the nucleus of the diagonal band (DB) and medial septum, suprachiasmatic nucleus (SCN), and periventricular nucleus of the thalamus (PV). Areas with less dense innervation from r2-*Pet1* neurons (light orange) include prefrontal cortex including prelimbic (PL) and infralimbic (IL) areas, parietal cortex, lateral septum (LS), hippocampus, basolateral amygdala (BLA), hypothalamus and median raphe (MR). (C) r3 / 5-*Pet1* neurons shared some targets with r2-*Pet1* neurons, but also had distinctive projection sites with either dense (dark pink) or moderate (light pink) density of axons. Dense innervation targets included the medial septum (MS) and nucleus of the diagonal band (DB), ventral, anterior and dorsal tegmental nuclei of Gudden (Tg), parabrachial (PB) and locus coeruleus (LC). Less dense innervation was detected in the median and dorsal raphe nuclei (MR, DR), hypothalamus, lateral septum (LS), piriform (PIR) and amygdalar cortex (COA) as well as the capsular part of the central nucleus of the amygdala (CeC) and hippocampus. For interpretation of color references in figure legend, please refer to the Web version of this article.

Fig. 6. Projections of r1-*Pet1* neurons (green) to other groups of serotonin neurons, identified by immunolabeling for TPH2 (red). (A) r1-*Pet1* axons, detected by immunolabeling for eGFP (green), are visible coursing dorsal-ventral (arrows) overlying non-r1-derived serotonin neurons (red) in the caudal and ventral portions of the MR. Scale bar = 20 μ m. (B) Higher magnification of another section of the MR reveals thin and varicose r1-*Pet1* (green) axons in the vicinity of serotonin cell bodies (arrow). Inset – a single optical section at the area indicated by the arrow. Scale bar = 50 μ m. (C) Caudally, r1-*Pet1* axons (green) are visible on the midline through raphe mangus (RMg) and raphe obscurus (ROb; D) intermingling with serotonin neurons (arrows). Raphe pallidus (E; RPa) is sparsely innervated by a few r1-

Pet1 axons, one indicated with an arrow. (F) An intermediate density of r1-*Pet1* axons (green, arrows) is visible in the vicinity of laterally displaced serotonin neurons in the paragigantocellular area (LPGi). For interpretation of color references in figure legend, please refer to the Web version of this article.

Fig. 7.
r1-Pet1 axons have a differing propensity to innervate other serotonin cell groups. Asterisks denote significant differences between groups.

Fig. 8. Maximal projections of Z-stack images showing axons of r2-*Pet1* neurons abundant only in the MR, proximal to the location of r2-*Pet1* cell bodies. (A) MR neurons rostral and dorsal to r2-*Pet1*-derived neurons intermix with axons from r2-*Pet1* neurons, detected by immunolabeling for eGFP (green). Scale bar = 25 μ m. (B) In another section of the same part of the MR, varicose axons (arrows) are visible in proximity to serotonin neurons (arrows). Scale bar = 10 μ m. For interpretation of color references in figure legend, please refer to the Web version of this article.

Fig. 9.

Comparison of projections to the DR by r2-*Pet1* (A, A')- and r3 / 5-*Pet1* (B, B')- neurons. (A) Using darkfield illumination, r2-*Pet1* neurons are visible in the MR (arrows) ventral to the DR (bracketed region) at the base of the aqueduct (Aq) (scale bar = 200 μ m). Higher magnification (scale bar = 100 μ m) of bracketed area in A' reveals a single fiber (arrow). (B) In a similar section through the DR, r3 / 5-*pet1* cell bodies lie ventrally (arrow). Very dense innervation is seen in the anterior tegmental nucleus (or ventral tegmental nucleus of Gudden; arrowhead), while a plexus of fibers overlies the DR. Bracketed region shown at higher magnification in B' shows many varicose fibers in the DR.

Fig. 10.

Projections of r3 / 5-*Pet1* neurons to other serotonin neuron groups. (A) In the DR, axons of r3 / 5-*Pet1* neurons (arrow) intermingle with serotonin neurons. Scale bar = 50 μ m. (B) Another section of the DR at higher magnification (scale bar = 20 μ m) shows varicose r3 / 5-*Pet1*-GFP labeled axons (arrow). (C) In the MR, ventral to the anterior tegmental nucleus of Gudden (double-headed arrow), r3 / 5-*Pet1* axon varicosities (arrows) are in the same area as serotonin neurons. (D) Axons from r3 / 5-*Pet1* neurons are visible tracking lateral to serotonin neurons in the medulla (arrowheads), and at a lower density, nearer to serotonin neurons (arrow). (E) Serotonin neurons in the paraventricular area with adjacent r3 / 5-

Pet1 boutons (arrow). Inset – both axon and cell body within a single optical plane. (F) Very few axons from r3 / 5-*Pet1* neurons are detectable in raphe obscurus and pallidus.