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Transgene expression within the spinal cord of hTH-eGFP rats

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Abstract

The enzyme tyrosine hydroxylase (TH) is broadly expressed in catecholaminergic neurons. In the spinal cord, four components contain TH, including A11 diencephalospinal dopaminergic (DA-ergic) pathways, intraspinal DA-related neurons, supraspinal noradrenergic projections, and afferents of TH-expressing sensory neurons. A human TH-enhanced green fluorescent protein (hTH-eGFP) transgenic rat was recently developed to tag TH⁺ profiles in the nervous system for visualization. Using immunostaining, we found that only A11 pathways express GFP whereas the other 3 components do not in the spinal cord. Thus, this may suggest a genetic difference among these TH⁺ elements even though they produce the same protein.

Keywords

Transgenic rat; Diencephalospinal pathway; Spinal interneuron

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Introduction

Tyrosine hydroxylase (TH) is the rate-limiting enzyme for dopamine (DA) and adrenaline/noradrenaline synthesis in the central and peripheral nervous system. The enzyme catalyzes tyrosine to levodopa and the latter is further converted to DA by dopamine decarboxylase (DDC). If dopamine- β -hydroxylase (DBH) is present, it transforms DA to norepinephrine. Accordingly, noradrenergic neurons are TH⁺/DBH⁺ while DA-ergic neurons are featured with TH⁺/DBH⁻. In the mammalian spinal cord, TH⁺ elements consist of neurons and projections originating from 4 different regions of the nervous system, such as intraspinal DA-related neurons in the lumbosacral spinal cord (Hou et al., 2016), diencephalospinal pathways from the A11 cell group, supraspinal adrenergic/noradrenergic projections from nuclei (A5-7) in the brainstem (Sharples et al., 2014), and afferent fibers of TH-expressing sensory neurons in dorsal root ganglia (DRGs) throughout spinal levels (Brumovsky, 2016).

The diencephalic A11 neurons are the primary source of DA that emerges in the spinal cord. They project to the entire spinal levels, mainly the superficial dorsal horn and the intermediolateral cell column (IML). The structural characteristics suggest that these neurons are highly related to sensory and autonomic function. Indeed, dysregulation of the A11 pathway leads to painful syndromes, such as migraines and restless leg syndrome (Charbit et al., 2010; Clemens et al., 2006). In parallel, 3 nuclei in the brain, including A5, A6 (locus coeruleus) and A7, project and transport noradrenaline to the spinal cord. A5 neurons mostly project to thoracic sympathetic nuclei, and A6 projections accumulate in the dorsal horn while A7 fibers are dense in the ventral horn (Bruinstroop et al., 2012). This distribution indicates they are involved in autonomic, sensory, and motor functions, respectively. In the 1980s, a group of TH⁺/DBH⁻ neurons were found in the rat lumbosacral spinal cord. Our recent studies showed these neurons undergo plasticity and contribute to a low level of DA sustained in the lower spinal cord after spinal cord injury (SCI) interrupts DA transportation from A11 descending pathways (Hou et al., 2016). It is assumed that the spinal TH⁺ population serves as a compensatory DA source when supraspinal DA provision is insufficient. In addition, TH⁺/DBH⁻ neurons are also found in DRGs at entire spinal levels, which belong to the peripheral nervous system. Although these TH⁺ DRG neurons sense input from skin or visceral organs (Brumovsky, 2016), their functional details are never determined.

Recently, a transgenic rat line was generated by infusing a human TH promoter to upstream of an enhanced green fluorescent protein (eGFP) reporter gene. Theoretically, all cells that express TH should be tagged with GFP. The founder of the rat line has shown that the major DA-ergic neurons in the brain express GFP (Iacovitti et al., 2014). As it appears to be an ideal tool for genetic analysis and electrophysiologic study of TH⁺ neurons, we initially attempted to take advantage of this rat line to differentiate GFP-tagged TH⁺ neurons in the spinal cord and DRGs of naïve and SCI rats. Unexpectedly, a heterogeneous expression pattern of GFP transgene in the spinal cord was detected. This report provides important information about the transgenic line for researchers who will consider utilizing it in the future research.

Materials and methods

Adult hTH-eGFP transgenic rats were purchased from Taconic Biosciences (NTac:SD-Tg (TH-eGFP) 24Xen) and then bred in-house. A total of 8 rats were used, including 3 naïve and 3 SCI female adults (weighing 250-300 g) and 2 postnatal day 10 (P10) pups. The guidelines of the National Institutes of Health and the Institutional Animal Care and Use Committee at Drexel University on animal care and housing were followed to minimize the number of animals used and potential suffering.

House-bred transgenic pups were identified by PCR to amplify a 264 bp product. The following primers were used: CAGCAGACTTCTTCAAGTCC and GATCTTGAAGTTCACCTTGATGC (Iacovitti et al., 2014). Subsequent electrophoresis was applied to visualize the presence of the eGFP gene (Fig. 1A). We previously observed that the number of TH⁺ neurons in the lower spinal cord was increased after SCI. To investigate whether the newly emerged cells express GFP after SCI, we performed a complete spinal cord transection at the 10th thoracic (T10) level (n = 3), as previously reported (Hou et al., 2016). Briefly, rats were anesthetized with 4% isoflurane in oxygen and kept anesthetic in 2% isoflurane during the surgery. Rats were placed on the heating pad with a supine position, a skin incision about 2 cm was made in the midline of the back. The paraspinal muscle was removed by scissors to facilitate structural recognition of the T8-10 spine. Then, a laminectomy was performed at T8 vertebrae to expose the dorsal part of the T10 spinal cord segment. The spinal cord was completely transected using a No. 11 blade and the lesion was confirmed visually at the time of surgery and after dissection. Once the bleeding stopped, the muscle was sutured and the skin was closed by wound clips. Animals were subcutaneously administered with cefazolin (10 mg/kg, West-ward), 3 ml Lactated Ringer's solution (VEDCO), and buprenorphine hydrochloride (0.1 mg/kg, Par). Bladder care was manually performed at least three times per day until sacrifice. Animals survived for an additional 3-4 weeks. In addition, 3 naïve adults and 2 P10 pups were also sacrificed. P10 rats were anesthetized by euthasol. A sharp 25G needle was inserted into the left ventricle. We first infused 100 ml 0.1 M phosphate-buffered saline (PBS) by a peristaltic pump, then switched the solution to 100 ml 4% paraformaldehyde (PFA) in PBS. After perfusion, the lumbosacral spinal cord was collected. Adult rats were euthanized by euthasol followed by transcardial perfusion with 200 ml 0.1 M PBS, followed by 200 ml 4% PFA solution. The brain, lumbosacral spinal cord, lumbar DRGs (L1, L2, L5, L6) were dissected. All tissues were post-fixed in 4% PFA overnight and cryoprotected in 30% sucrose in PBS for at least 2 days at 4°C. Tissue samples were embedded in optimum cutting temperature (OCT) compound (Sakura Finetek). Longitudinal spinal cord sections (35 µm) and coronal brain sections (35 µm) were obtained as six series using a cryostat (Microm Laborgerate) and kept in order in 24 well plates at 4°C. DRGs were serially cryosectioned in the longitudinal plane at 20 µm and mounted on slides for subsequent histological analysis.

For immunostaining, free-floating sections of brain (210 µm apart), lumbosacral spinal cord (210 µm apart) or mounted DRGs (120 µm apart) were washed 3 times in tris-buffered saline (TBS) and then incubated with primary antibodies in blocking buffer (TBS containing 5% donkey serum and 0.5% Triton X-100). Primary antibodies against TH (rabbit, 1:1000, Millipore) and GFP (chicken, 1:1000, Aves Labs) were used to observe the co-expression.

DBH antibody (mouse, 1:500, Millipore) was used to identify noradrenergic profiles. Calcitonin gene-related peptide (CGRP, rabbit, 1:1000, Peninsula) and parvalbumin (PV, rabbit, 1:2,000, Abcam) were markers for peptidergic and proprioceptive DRG neurons, respectively. Double immunostaining for GFP/CGRP or GFP/PV was conducted to characterize GFP⁺ sensory neurons. Triple immunostaining for GFP/TH/DBH was used to detect supraspinal noradrenergic projections. Sections were incubated in primary antibody solution overnight at 4°C and then rinsed 3 times in TBS. Subsequently, sections were moved to secondary antibody solution containing Alexa-488, 594, or 647 conjugated donkey secondary antibodies (1:500, Invitrogen) in blocking buffer for 3 hours at room temperature. After washed, tissue sections were mounted on slides, air-dried, and coverslipped with mounting medium (SouthernBiotech). All slides were examined immediately and preserved in -20°C freezer. Imaging acquisition was processed on a Leica DM5500B microscope connect to a digital camera (Hamamatsu C11440-42U), and optimization of minimum and maximum display value was performed with SlideBook software (Intelligent Imaging Innovations). The far-red channel was pseudo-colored to blue. Adobe Photoshop 7.0 was used only to adjust contrast and construct image combinations.

Results

In the adult intact spinal cord, immunohistochemical analysis revealed many TH⁺ but DBH⁻ cells in the superficial dorsal horn, parasympathetic nuclei, and dorsal gray commissure (DGC) around the central canal at L6-S3 spinal levels, consistent with previous reports (Hou et al., 2016). However, none of these TH⁺ cells expressed transgene GFP (Fig. 1B-D). In both the white and gray matter, numerous supraspinal noradrenergic projections were double-labeled with TH and DBH, which were not co-labeled by GFP. Meanwhile, many TH and GFP double-labeled but DBH⁻ neuronal fibers distributed in the entire spinal segments, mainly within the dorsal and medial parts of the cord (Fig. 1E). In addition, there were axon terminals expressing GFP only but not TH that was considered to originate from ectopic expression of the transgene. No GFP and DBH double-labeled cells or axons were detected in the spinal cord (Fig. 1F).

Based on the preliminary results, intraspinal TH⁺ population may change dynamically during different life stages or conditions, such as postnatal, adult, and after SCI (unpublished data). To determine the potential alteration of GFP transgene expression, immunostaining was employed in the spinal cord tissue collected from P10 rats as well as adult rats with SCI. Notably, TH⁺ neurons in the lower spinal cord did not express GFP in these two groups. In the spinal cord of P10 rats, dense TH⁺ fibers without GFP co-labeling were seen (Fig. 1G, H), and some axon extensions were only positive for GFP. In the spinal cord of adult rats with SCI, TH⁺ neurons in the parasympathetic nuclei (Fig. 1I, J) and GFP⁺ axons in the dorsal spinal cord (Fig. 1K) were identified. No double-labeled profiles were detected.

Previous studies reported that DA-ergic but not noradrenergic neurons in the brain express GFP in this transgenic rat line (Iacovitti et al., 2014). In the intact spinal cord, we observed TH⁺/GFP⁺ but not TH⁺/GFP⁺/DBH⁺ axon extensions which supports the findings above. To confirm their origination, the brain sections were immunostained to recognize TH⁺ nuclei projecting to the spinal cord. The results showed that TH⁺ neurons in the A11 cell group

expressed GFP while A6 (locus coeruleus) noradrenergic neurons did not (Fig. 2A-D). Interestingly, very few noradrenergic neurons in the A5 region express GFP (Fig. 2C, E). This validates that the major descending TH⁺/GFP⁺ neuronal pathways stem from the A11 DA-ergic neurons. Additionally, some TH⁻ cells in the parabrachial nuclei adjacent to the A6 were labeled with GFP which could be caused by ectopic transgene expression (arrow, Fig. 2C).

It has been reported that plenty of TH⁺ sensory neurons are harbored in DRGs throughout the spinal cord level (Brumovsky, 2016). Immunostaining revealed single TH or GFP labeled sensory neurons in lumbar DRGs. However, these two categories did not have overlap (Fig. 2F). Further, GFP⁺ DRG neurons did not express CGRP and PV, suggesting that they are not nociceptive or proprioceptive neurons (Fig. 2G, H).

Discussion

In the spinal cord of this hTH-eGFP transgenic rat line, the reporter is mainly expressed in the A11 diencephalospinal DA-ergic pathways, whereas other TH⁺ components do not produce GFP, such as intraspinal TH⁺ neurons, supraspinal noradrenergic projections, and afferent fibers of TH⁺ DRG neurons. The results are in compliance with previous studies in the brain that described DA-ergic but not noradrenergic neurons expressing GFP.

Among TH⁺ populations projecting to the spinal cord, GFP was exclusively expressed in the A11 cell group. After SCI interrupts all descending pathways, some GFP⁺/TH⁻ terminals were still seen in the dorsal spinal cord. In line with detected GFP⁺/TH⁻ neurons in DRGs, these GFP⁺ terminals should be the central projections of those DRG neurons. Accordingly, GFP⁺ axons in the intact spinal cord are comprised of descending A11 projections and afferent fibers of GFP⁺ DRG neurons. In research of supraspinal pathways, the utilization of neuronal tracing techniques to identify A11 projections often has problems of specificity since tracers injected into the nuclei are easy to diffuse into adjacent areas and label neurons there. It is therefore plausible that the transgenic rat provides a useful tool to visualize the descending A11 DA-ergic projections in the spinal cord by double immunostaining for TH and GFP.

Why is the expression of GFP transgene different in these distinct groups of TH⁺ neurons? The founder of the rat line attributed the failure of GFP expression in noradrenergic neurons to the instability of the reporter. If this is the case, it may also be true in intraspinal and sensory TH⁺ neurons. It is unclear whether TH has different genetic regulation between different categories of neurons because this factor could influence genetic recombination. Another possible reason is that some essential genetic elements may be missing in the hTH promotor plasmid so that only a certain type of TH⁺ cells, e.g., DA-ergic ones, can recognize for translation (Iacovitti et al., 2014). It should be mentioned that similar transgene expression pattern was shown in the brain when researchers infused the same plasmid into mice single cell embryos (Kessler et al., 2003), indicating no species difference. This made us wonder whether there are discrepancies of TH regulation between human and murine. Indeed, it has been found that humans and mice share merely 46.4% homology in TH promotor sequence, and the degree is even less between humans and rats that is about 30%

(Jin et al., 2006). Among these three species, it was only identified 5 conserved regions in this 11kb human TH promotor, which even display functional differences. For example, Nurr1 is a transcription factor that is crucial for TH expression. A recent study unveiled that TH regulation does not depend on Nurr1 in humans but does in murine (Jin et al., 2006). Thus, the human TH promotor is genuinely different from that in rats and mice and this may explain the tissue-specific expression pattern of transgene.

Approximately 1% sensory neurons in lumbar DRGs are TH⁺ in rats (Price et al., 1983) and 15% in mice (Li et al., 2011). In the transgenic rat, DRG TH⁺ neurons were not tagged by GFP. Unexpectedly, we found a certain population of only GFP⁺ neurons. Two possibilities may explain this ectopic expression. Firstly, DRG neurons may have different repressive gene regulators from those in the brain since they belong to the peripheral nervous system. The binding sites for repressors may exceed the range of this 11kb hTH promotor. Another possibility is that high copies of transgene titrate out repressors in DRG neurons so that the transgene escapes from its specific regulators (Kessler et al., 2003). As a result, ectopic GFP expression occurs in a distinct sensory neuron population. These neurons do not express nociceptive marker CGRP or proprioceptive marker PV, and their identity remains to be determined.

Conclusions

A heterogeneous transgene expression pattern was revealed in the spinal cord of TH-eGFP rats. The reporter was mainly expressed in the A11 DA-ergic pathways whereas other TH⁺ components within the spinal cord were not tagged by GFP, such as intraspinal TH⁺ neurons, supraspinal noradrenergic projections, and afferent fibers of TH⁺ DRG neurons. This may suggest different genetic regulations among these TH⁺ elements even though they express the same product. Thus, it is pivotal to acknowledge this important information when researchers consider utilizing the transgenic line for spinal cord experiments.

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Abbreviations:

CGRP	calcitonin gene-related peptide
DA	dopamine
DBH	dopamine-βhydroxylase
DDC	dopamine decarboxylase
DGC	dorsal gray commissure

DRG	dorsal root ganglia
eGFP	enhanced green fluorescent protein
IML	intermediolateral cell column
L	lumbar spine
P10	postnatal day 10
PV	parvalbumin
RLS	restless leg syndrome
S	sacral spine
SCI	spinal cord injury
T	thoracic spine
TH	tyrosine hydroxylase
V	ventricle

References

- Bruinstroop E, Cano G, Vanderhorst VG, Cavalcante JC, Wirth J, Sena-Esteves M, Saper CB 2012 Spinal projections of the A5, A6 (locus coeruleus), and A7 noradrenergic cell groups in rats. *J Comp Neurol* 520, 1985–2001. [PubMed: 22173709]
- Brumovsky PR 2016 Dorsal root ganglion neurons and tyrosine hydroxylase--an intriguing association with implications for sensation and pain. *Pain* 157, 314–320. [PubMed: 26447702]
- Charbit AR, Akerman S, Goadsby PJ 2010 Dopamine: what's new in migraine? *Curr Opin Neurol* 23, 275–281. [PubMed: 20164768]
- Clemens S, Rye D, Hochman S 2006 Restless legs syndrome: revisiting the dopamine hypothesis from the spinal cord perspective. *Neurology* 67, 125–130. [PubMed: 16832090]
- Hou S, Carson DM, Wu D, Klaw MC, Houle JD, Tom VJ 2016 Dopamine is produced in the rat spinal cord and regulates micturition reflex after spinal cord injury. *Exp Neurol* 285, 136–146. [PubMed: 26655672]
- Iacovitti L, Wei X, Cai J, Kostuk EW, Lin R, Gorodinsky A, Roman P, Kusek G, Das SS, Dufour A, Martinez TN, Dave KD 2014 The hTH-GFP reporter rat model for the study of Parkinson's disease. *PLoS One* 9, e113151. [PubMed: 25462571]
- Jin H, Romano G, Marshall C, Donaldson AE, Suon S, Iacovitti L 2006 Tyrosine hydroxylase gene regulation in human neuronal progenitor cells does not depend on Nurr1 as in the murine and rat systems. *J Cell Physiol* 207, 49–57. [PubMed: 16252282]
- Kessler MA, Yang M, Gollomp KL, Jin H, Iacovitti L 2003 The human tyrosine hydroxylase gene promoter. *Brain Res Mol Brain Res* 112, 8–23. [PubMed: 12670698]
- Li L, Rutlin M, Abaira VE, Cassidy C, Kus L, Gong S, Jankowski MP, Luo W, Heintz N, Koerber HR, Woodbury CJ, Ginty DD 2011 The functional organization of cutaneous low-threshold mechanosensory neurons. *Cell* 147, 1615–1627. [PubMed: 22196735]
- Price J, Mudge AW 1983 A subpopulation of rat dorsal root ganglion neurones is catecholaminergic. *Nature* 301, 241–243. [PubMed: 6130474]
- Sharples SA, Koblinger K, Humphreys JM, Whelan PJ 2014 Dopamine: a parallel pathway for the modulation of spinal locomotor networks. *Front Neural Circuits* 8, 55. [PubMed: 24982614]

Highlights

- The reporter gene is mainly expressed in the A11 diencephalospinal dopaminergic pathway in the spinal cord of a hTH-eGFP transgenic rat
- Green fluorescent protein (GFP) is not expressed in tyrosine hydroxylase (TH)⁺ spinal interneurons, supraspinal noradrenergic projections, and afferents of TH⁺ sensory neurons
- Some dorsal root ganglionic (DRG) neurons that do not contain TH show ectopic GFP expression in cell soma and central projections

Fig. 1. Transgene expression in the spinal cord of hTH-eGFP rats.

A, Neonates containing GFP transgene are identified by DNA electrophoresis. **B**, A schematic diagram illustrates A9-11 DA-ergic neuronal groups (yellow), A5 and A6 noradrenergic groups (purple), spinal TH⁺ groups (red), and GFP ectopic expression (green). **C-F**, In a longitudinal section of rat intact lumbosacral spinal cord, TH⁺ neurons (red, arrowheads) do not express GFP (**D**). TH⁺/DBH⁺/GFP⁻ noradrenergic branches (purple) and TH⁺/DBH⁻/GFP⁺ DA-ergic fibers (yellow, arrowheads) are prominent in the gray and white matter (**E**). However, none of them express both GFP and DBH (**F**). **G-H**, In a longitudinal section of P10 rat spinal cord, many TH⁺ neurons/extensions and GFP⁺ fibers are detected. **I-K**, Three weeks after T10 spinal cord transection (T10-Tx), descending projections

degenerated in the spinal cord. TH⁺ neurons are shown to mainly reside in the parasympathetic nuclei from L6-S3 levels (**I, J**) while some GFP⁺ axon terminals are observed in the dorsal spinal cord (**K**). Scale bars: **C, G, and I**, 500 μm ; **F, H, J** and **K**, 100 μm .

Fig. 2. Transgene expression in the brain and DRGs of hTH-eGFP rats.

A, B, TH-immunolabeled A11 cell group express GFP in adult rats. **C-E,** In a coronal section of the brainstem, no TH⁺ noradrenergic neurons in the A6 are co-labeled with GFP (**D**) while very few of those in the A5 region are GFP⁺ (**E**). Additionally, ectopic GFP expression occurs in the parabrachial nuclei (arrow, **C**). **F-H,** In DRGs at L6 level, TH⁺ sensory neurons do not express GFP, whereas many GFP⁺ neurons are discovered (**F**), which are not co-labeled with CGRP or PV (**G, H**), suggesting they are not nociceptive or proprioceptive sensory neurons. Scale bars: **A** and **C**, 500 μ m; **B, E,** and **H**, 100 μ m; **F**, 200 μ m; V, ventricle.