Biochemical and Biophysical Research Communications 449 (2014) 412–418

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Pharmacological assessment of methamphetamine-induced behavioral hyperactivity mediated by dopaminergic transmission in planarian *Dugesia japonica*

Natsuka Tashiro [a,2], Kaneyasu Nishimura [a,b,1,2], Kanako Daido [a], Tomoe Oka [a], Mio Todo [a], Asami Toshikawa [a], Jun Tsushima [a], Kazuyuki Takata [a], Eishi Ashihara [a], Kanji Yoshimoto [c], Kiyokazu Agata [b], Yoshihisa Kitamura [a,*]

[a] Department of Clinical and Translational Physiology, Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan
[b] Department of Biophysics, Graduate School of Science, Kyoto University, Oiwake, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan
[c] Department of Food Sciences and Biotechnology, Faculty of Life Science, Hiroshima Institute of Technology, 2-1-1 Miyake, Saeki-ku, Hiroshima 731-5193, Japan

ARTICLE INFO

Article history:
Received 7 May 2014
Available online 22 May 2014

Keywords:
Planarian
Dopamine transporter
Spontaneous locomotion
Methamphetamine

ABSTRACT

The freshwater planarian *Dugesia japonica* has a simple central nervous system (CNS) and can regenerate complete organs, even a functional brain. Recent studies demonstrated that there is a great variety of neuronal-related genes, specifically expressed in several domains of the planarian brain. We identified a planarian *dat* gene, named it *D. japonica dopamine transporter* (*Djdat*), and analyzed its expression and function. Both *in situ* hybridization and immunofluorescence revealed that localization of *Djdat* mRNA and protein was the same as that of *D. japonica* tyrosine hydroxylase (DjTH). Although, dopamine (DA) content in *Djdat(RNAi)* planarians was not altered, *Djdat(RNAi)* planarians showed increased spontaneous locomotion. The hyperactivity in the *Djdat(RNAi)* planarians was significantly suppressed by SCH23390 or sulpiride pretreatment, which are D_1 or D_2 receptor antagonists, respectively. These results suggest that planarians have a *Djdat* ortholog and the ability to regulate dopaminergic neurotransmission and association with spontaneous locomotion.

1. Introduction

Dopamine transporter (DAT) is a presynaptic protein, which plays an important role in regulating extracellular dopamine (DA) concentration by reuptaking into presynaptic terminals after release [1–3]. DAT is also a target for psychoactive drugs, such as methamphetamine and cocaine [4,5]. Because *DAT*-knockout mice show hyperactivity, they have been used as a disease animal model of attention-deficit/hyperactivity disorder (ADHD), which is characterized by attention deficit, inappropriate hyperactivity, and impulsivity [6]. Many investigations have used a rodent model, but many issues remain unclear. Thus, planarians provide unique opportunities to investigate such issues, considering they have relatively simple nervous systems.

The freshwater planarian *Dugesia japonica* has a simple central nervous system (CNS) consisting of a brain and a pair of ventral nerve cords (VNCs) (Fig. 1D and E). After artificial amputation, planarians can regenerate into complete animals, including a functional brain [7–9]. This high regenerative capacity is maintained by pluripotent stem cells that are present in the mesenchymal space throughout the planarian body [10–13]. In our laboratory, some neurotransmitters such as DA, serotonin, γ-aminobutyric acid (GABA), octopamine, and acetylcholine, and genes coding these synthesizing enzymes were identified in planarians [14–18]. Thus, it is believed that planarians can regenerate a functional brain from adult pluripotent stem cells [19–21]. In this study, we isolated a planarian *dat* gene, named it *D. japonica dat* (*Djdat*), and analyzed its function by an RNA interference (RNAi) method and pharmacological approaches.

2. Materials and methods

2.1. Animals

In this study, planarians (*D. japonica*) of the SSP strain were used. They were maintained in autoclaved tap water at 24 °C, and fed chicken liver twice a week.

* Corresponding author. Fax: +81 75 595 4796.
E-mail address: yo-kita@mb.kyoto-phu.ac.jp (Y. Kitamura).
[1] Present address: Department of Clinical Application, Center for iPS Cell Research and Application (CiRA), Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
[2] These authors equally contributed to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.05.059
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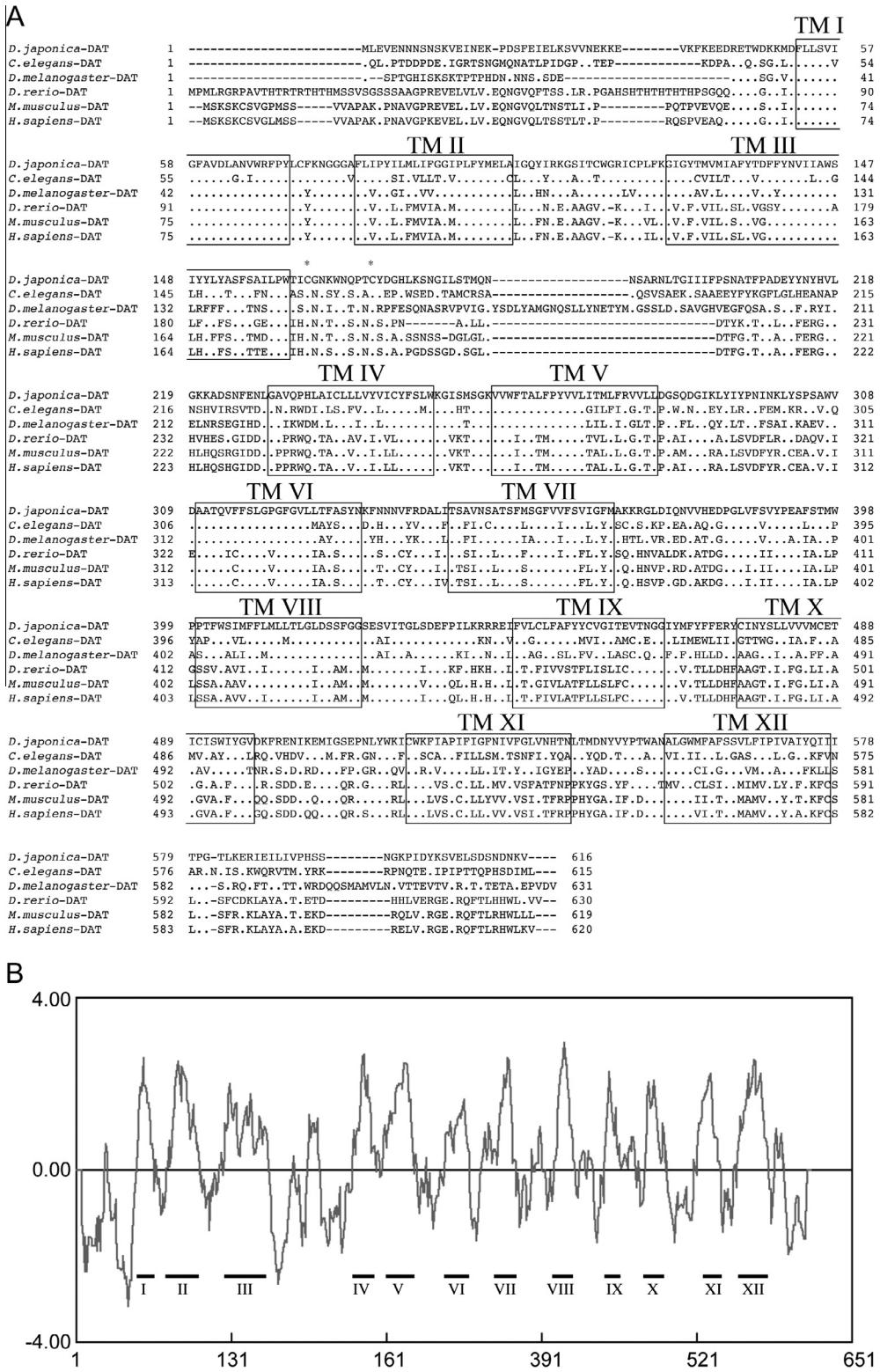

Fig. 1. Sequence analysis of DjDAT. (A) Comparison of amino acid sequences of DjDAT with DAT in other species, corresponding to DAT of *Caenorhabditis elegans* (Q03614), *Drosophila melanogaster* (AAF76882), *Danio rerio* (NP_571830), *Mus musculus* (CAB51926), and *Homo sapiens* (AAC50179). Twelve transmembrane domains are numbered (TM I–XII). Asterisks indicate the 164th and 173th Cys residue. (B) Hydrophobicity analysis of DjDAT. Hydropathy calculations were conducted according to the method by Kyte and Doolittle using GENETYX-MAC Ver.12.2.0. The 12 transmembrane domains are underlined and numbered with Roman numerals I–XII.

2.2. Cloning of Djdat cDNA

Total RNA was extracted from 100 planarians using Isongen-LS (Nippon Gene, Toyama, Japan). mRNA was purified from the total RNA using an Oligotex-dT30 <Super> mRNA purification kit (Takara, Kyoto, Japan). cDNA was synthesized from mRNA using a first-strand cDNA synthesis kit (Amersham Biosciences, Arlington Heights, IL, USA). Degenerate oligonucleotides were designed for

homologous regions of DAT protein conserved among various animals, and these primers were as follows:

DAT F1:5′-GGNTTYGCNGTNGAYYTNGCNGAY-3′
DAT R1:5′-RAARAANACYTGNGTNGCNGCRTC-3′
DAT R2:5′-NCCYTTCCANARNSWRAARTA-3′
(B = C/G/T, N = A/T/G/C, R = A/G, S = C/G, W = T/A, Y = C/T)

The polymerase chain reaction (PCR) was performed using these primers. A 591-bp cDNA fragment of DAT was isolated. Then, we performed the screening of a cDNA library constructed from poly $(A)^+$ RNA of planarian head pieces in a λZAPII vector (Stratagene, La Jolla, CA, USA) by the stepwise dilution method [22] using a specific primer set, and gene-specific primers (GSPs) were as follows:

GSP F1 5′-CCTTATCTTTGTTTCAAAAATGGAGGAGG-3′
GSP F2 5′-AGGGATCAATTACTTGTTGGGGA-3′
GSP F3 5′-AATAGTGCTACCAGTTTTATGTCGG-3′
GSP R1 5′-GCTGGAAATGTTGCATTGGATGGG-3′
GSP R2 5′-CCACAACTATTTAAAGCGCTTTTGT-3′
GSP R3 5′-TCTAATTGTACCCCATTCTAGCAATG-3′

Finally, a 2,100-bp cDNA fragment was isolated.

2.3. Whole-mount in situ hybridization

The plasmid pBluescript SK (−) containing *Djdat* cDNA was linearized with KpnI or NotI. Digoxigenin (DIG)-labeled RNA probe was synthesized using T7 RNA polymerase. Planarians were shaken vigorously in 5/8 Holtfreter's solution containing 2% HCl for 5 min at 4 °C. Then, they were fixed in 4% paraformaldehyde containing 5% methanol for 2 h at 4 °C. After bleaching by 5% H_2O_2 in methanol for 16 h at room temperature under fluorescent light, they were soaked with xylene:methanol (1:1) solution for 1 h at 4 °C. Next, they were rehydrated through methanol, followed by 75%, 50%, and 25% ethanol solution in series to 100 mM phosphate-buffered saline containing 0.3% Triton X-100. Hybridization was performed as previously described [18].

2.4. Generation of a rabbit polyclonal anti-DjDAT antibody

A rabbit polyclonal anti-DjDAT antibody was generated against three partial sequences of DjDAT protein. The following are the partial sequences for antigen of anti-DjDAT antibody:

EVKFKEEDRETWDKKMDC (35–51)
CYDGHLKSNGILSTMQNN (173–190)
CPIDYKSVELSDSNDNKV (600–616)

The fusion protein production and antibody generation was performed by Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan).

2.5. Whole-mount immunofluorescence

Planarians were fixed and pretreatment was performed as described above (Refer to Whole-mount *in situ* hybridization). They were blocked by 10% goat serum for 1 h, and incubated with a rabbit polyclonal anti-DjDAT antibody (diluted 1:500) and a mouse monoclonal anti-DjTH antibody (diluted 1:5000) [14] overnight at 4 °C. Then, they were incubated with either Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 546-conjugated goat anti-mouse IgG (diluted 1:500) (Invitrogen, Aurora, OH, USA).

2.6. Double-stranded RNA (dsRNA) synthesis and RNAi analysis

The plasmid pBluescript SK (−) containing the longest *Djdat* cDNA was linearized with KpnI and NotI. Antisense or sense RNAs were synthesized according to our previous report [14]. By microinjection using a Drummond Scientific Nanoject injector (Broomall, PA, USA), intact planarians were treated for 3 days (once per day) with injections of 100 nL (33 nL/injection, 3 times) dsRNA (1 μg/μL). Control planarians were injected with tap water instead of dsRNA. All planarians were amputated immediately anterior to the pharynx 3 h after the last injection. Head-regenerated animals from the tail pieces were used in these experiments.

2.7. Quantification of spontaneous locomotion

Planarians were placed singly in a 15-cm Petri dish containing 1% ethanol solution and located over a grid paper (gridlines spaced 5.0 mm apart). The spontaneous locomotion of each planarian was measured by counting the number of gridlines each planarian crossed per min during a 10-min observation period [23]. This behavioral assay was performed 30 min after methamphetamine hydrochloride treatment (0.03 μM; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). In addition, SCH23390 (10 μM; Sigma, St. Louis, MO, USA) or S (−)-sulpiride (100 μM; Sigma) pretreatment was performed for 30 min before methamphetamine treatment.

2.8. High-performance liquid chromatography (HPLC) analysis for DA content

Fifteen planarian heads were homogenized by sonication in 300 mM perchloric acid solution containing 20 mM ethylenediamine-N,N,N′,N′-tetraacetic acid and 3,4-dihydroxybenzylamine (as the internal standard). Quantitative analysis of the DA content was conducted according to a previous report [14].

2.9. Statistical analysis

Results were presented as the mean ± standard error of the mean (SEM). The significance of differences was determined by analysis of variance (ANOVA). Furthermore, *post hoc* comparisons were performed using Bonferroni/Dunn test or Tukey–Kramer test (Stat View; Abacus Concepts, Berkeley, CA, USA).

3. Results

3.1. Cloning of Djdat gene

A partial *Djdat* cDNA was obtained by a degenerate PCR method with primers designed for homologous regions of DAT protein conserved among various animals. Subsequently, *Djdat* cDNA was isolated by head cDNA library screening and the full-length of *Djdat* cDNA has 2100 bp. *Djdat* cDNA encoded a polypeptide composed of 616 amino acids, and hydrophobicity analyses predicted 12 transmembrane domains (TM I–XII) (Fig. 1A and B). Two highly conserved cysteine residues in the extracellular loop, which are critical for the functional expression and membrane trafficking of the monoamine transporters [24], were also conserved in DjDAT (Cys164 and Cys173) (Fig. 1A). The full-length sequence of *Djdat* cDNA has been submitted to DDBJ/EMBL/GenBank under accession number AB909444.

3.2. Distribution pattern of Djdat mRNA and protein in planarians

Whole-mount *in situ* hybridization revealed that a limited number of cells in the head region were stained with the *Djdat* RNA probe (Fig. 2A and B). *Djdat* mRNA-expressing neurons were detected in the brain, VNCs in the region anterior to the pharynx (Fig. 3A), and the head peripheral region (Fig. 2B). The expression pattern of *Djdat* mRNA was the same as that of *DjTH* mRNA (Fig. 2A–D). Co-expression of *Djdat* and *DjTH* was confirmed by double immunofluorescence with antibodies against DjDAT and DjTH, respectively (Fig. 2E–M). The DjDAT-immunopositive

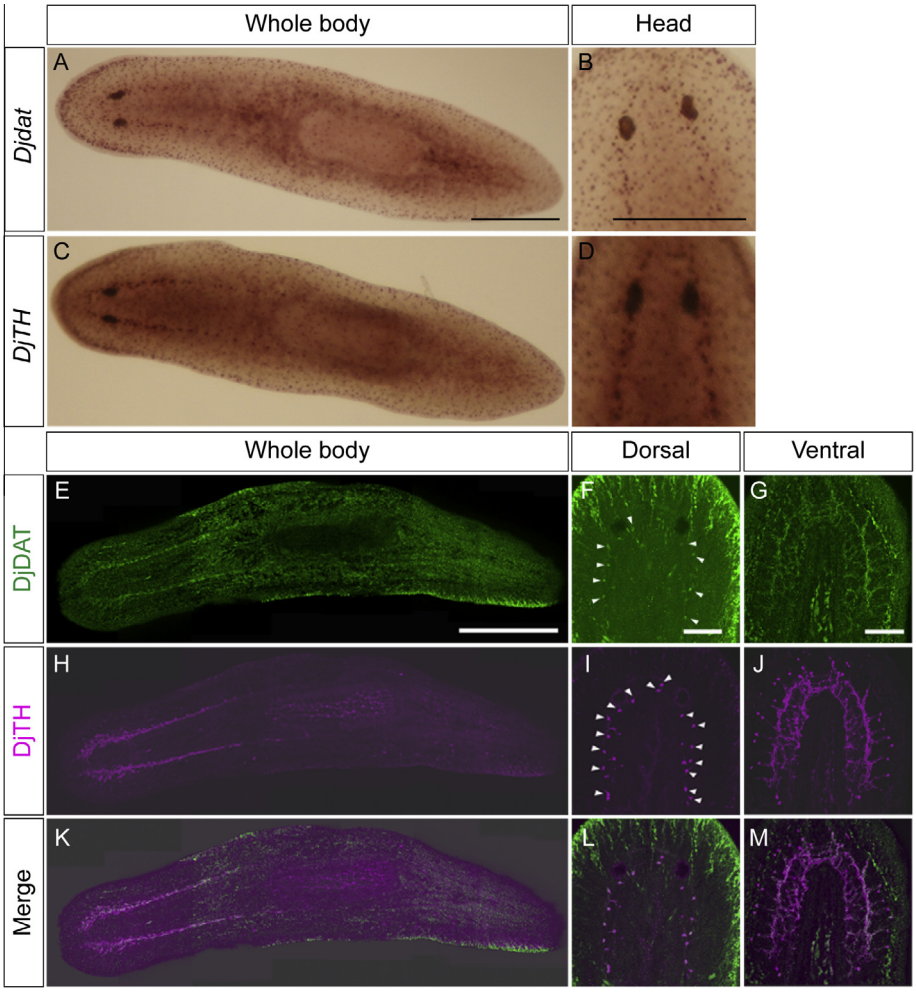

Fig. 2. Localization of *Djdat* and *DjTH* mRNAs and proteins. (A and B) Expression of *Djdat* mRNA. (C and D) Expression of *DjTH* mRNA. (E–G) Whole body (A and C) and magnified view of the head (B and D). Expression of DjDAT protein and (H–J) DjTH protein. (E, H and K) Ventral view of the whole body. (F, I and L) Dorsal view of head region. (G, J and M) Ventral view of head region. Scale bars: 1 mm (A, B and E), 100 μM (F and G).

neurons were co-located with DjTH protein in the planarian CNS (Fig. 2K–M), although DjDAT-immunoreactivity was weaker than DjTH-immunoreactivity (Fig. 2G, J and M).

3.3. Regeneration of brain dopaminergic neurons and spontaneous movement

When planarians are amputated into several pieces, their brain regenerates and reorganizes within 7 days, even from the non-brain tissue [25]. Previously, we revealed that planarians could regenerate and reorganize their dopaminergic neurons within 7 days [14,21]. We examined the regeneration process of dopaminergic neurons that were identified by antibodies against DjDAT and DjTH in planarians, which were amputated anterior to the pharynx (Fig. 3A). In the control planarians, although immunoreactivity of both DjDAT and DjTH was undetectable on the 1st day, on the 3rd day several DjDAT- and DjTH-immunopositive neural cell bodies and axons were weakly detected in the head region, which was newly regenerated from the amputated tail piece (Fig. 3A). During days 5–7, the morphological network of both DjDAT- and DjTH-immunopositive neurons were reconstructed, becoming similar to that in the intact planarian brain. Next, we investigated the regeneration process in both the *Djdat(RNAi)* and *DjTH(RNAi)* planarians. In the *Djdat(RNAi)* planarians, the regeneration process of DjTH-immunopositive neurons was almost the same as that of the control planarians, but DjDAT-immunopositive neurons were undetected during the observation period. Similarly, in *DjTH(RNAi)* planarians, the regeneration process of DjDAT-immunopositive neurons was almost the same as that of the control planarians, but that of DjTH-immunopositive neurons was undetected. These results indicated that both DjDAT and DjTH are expressed in dopaminergic neurons, and these transcripts are not affected by dopaminergic neuronal regeneration.

In tap water, planarians usually move smoothly by their cilia. When planarians were placed in 1% ethanol solution, they slowly twisted and moved like a looper. This twisting movement appears to be mediated by the muscle, not by the cilia, and spontaneous locomotion of *DjTH(RNAi)* planarians was significantly reduced. The measurement of spontaneous locomotion in 1% ethanol solution is a useful and simple tool to assess the dopaminergic phenotype [14]. First, we compared the activity of spontaneous locomotion among the *Djdat(RNAi)*, *DjTH(RNAi)*, and control planarians during the regeneration process. On every measurement day, the movement of *DjTH(RNAi)* planarians was decreased compared with that of the control planarians (Fig. 3B). The *Djdat(RNAi)* planarians showed the highest number of gridlines that each planarian crossed over during the 10-min observation period (Fig. 3B). Then, we compared the DA content in the regenerated heads of *Djdat(RNAi)*, *DjTH(RNAi)*, and control planarians. While the DA content in *DjTH(RNAi)* planarians was significantly

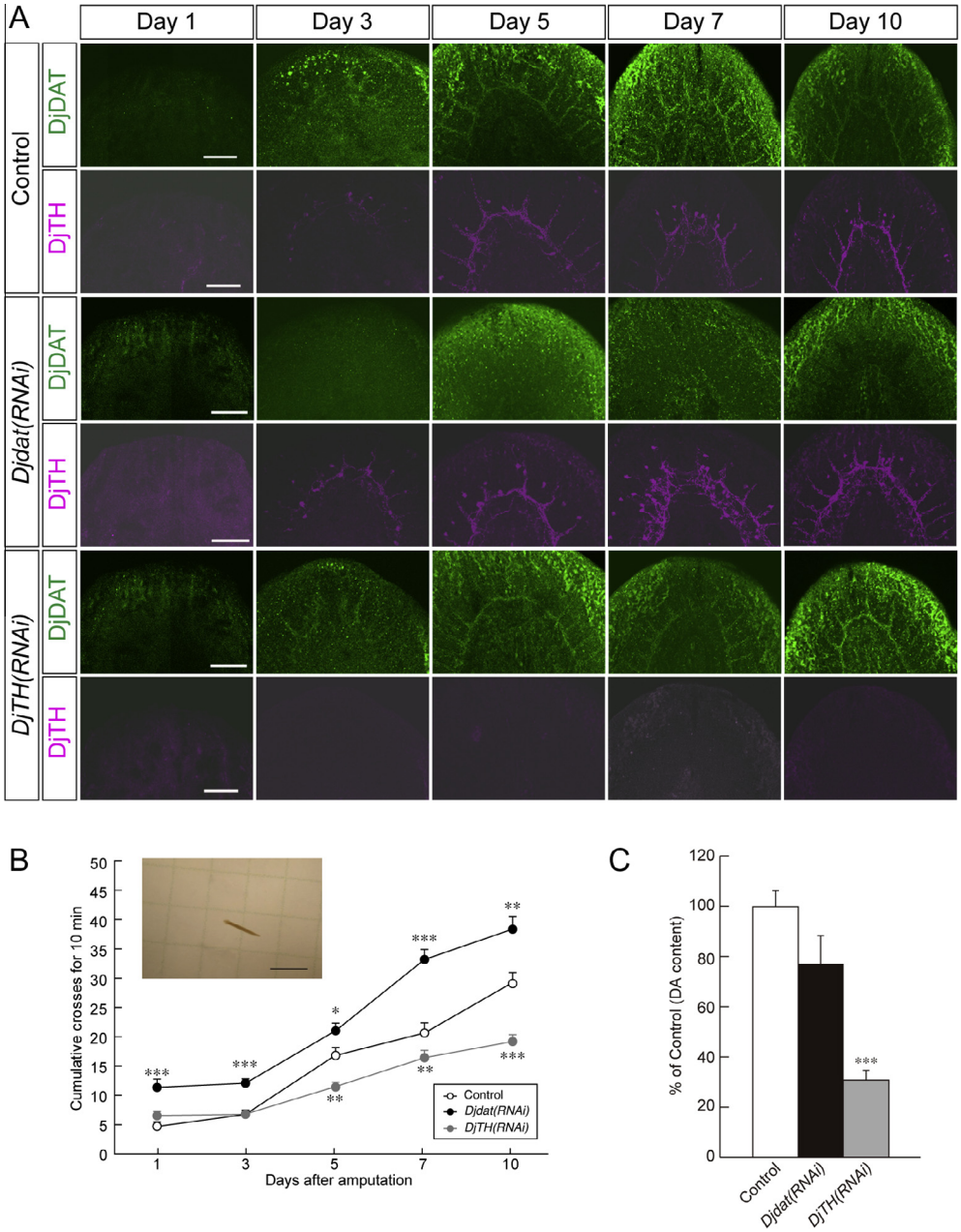

Fig. 3. Regeneration process and locomotion activity. (A) Regeneration process of DjDAT and DjTH in the *Djdat(RNAi)*, *DjTH(RNAi)*, and control planarians. Scale bar: 100 μM. (B) Changes in locomotion activity were measured for 10 min on each day during the 10-day regeneration process. Each value is mean ± SEM of 15 independent planarians. Significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the control group. (C) The DA content of in the head of the *Djdat(RNAi)* or *DjTH(RNAi)* planarian regenerates at day 10. Each value is the mean ± SEM of 11 independent samples (15 planarian heads/samples), based on vehicle-control as 100%. Significance: $^{***}p < 0.001$ vs. control.

decreased, the DA content in the *Djdat(RNAi)* planarians was slightly but not significantly decreased compared with that of the control planarians (Fig. 3C).

3.4. Pharmacological assessment and comparison of dopaminergic phenotype of Djdat(RNAi) or DjTH(RNAi) planarians

To investigate that the increase of spontaneous locomotion in the *Djdat(RNAi)* planarians was mediated by dopaminergic transmission, we assessed the spontaneous locomotion using SCH23390 (10 μM; D_1 receptor antagonist) or sulpiride (100 μM; D_2 receptor antagonist) pretreatment for 30 min. Both the pretreatments clearly suppressed the increase of spontaneous locomotion in the *Djdat(RNAi)* planarians, and reduced it to be the same as that in *DjTH(RNAi)* planarians (Fig. 4A).

DAT is a target for psychoactive drugs such as methamphetamine [26,27]. Previously, we demonstrated that high-dose methamphetamine (200 μM) treatment induced unusual hyperactivity such as screw-like and C-like hyperkinesia [14]. In this study, we examined spontaneous locomotion following low-dose methamphetamine (0.03 μM) treatment. Although low-dose methamphetamine (0.03 μM) treatment induced an increase of spontaneous locomotion, it did not induce screw-like and C-like hyperkinesia, which is observed in high-dose methamphetamine treatment (200 μM). Therefore, low-dose methamphetamine treatment is useful for the assessment of spontaneous locomotion in individual animals. Low-dose methamphetamine treatment induced an increase of spontaneous locomotion in the control planarians. Low-dose methamphetamine treatment did not induce an increase of spontaneous locomotion in *the Djdat(RNAi)* and *DjTH(RNAi)*

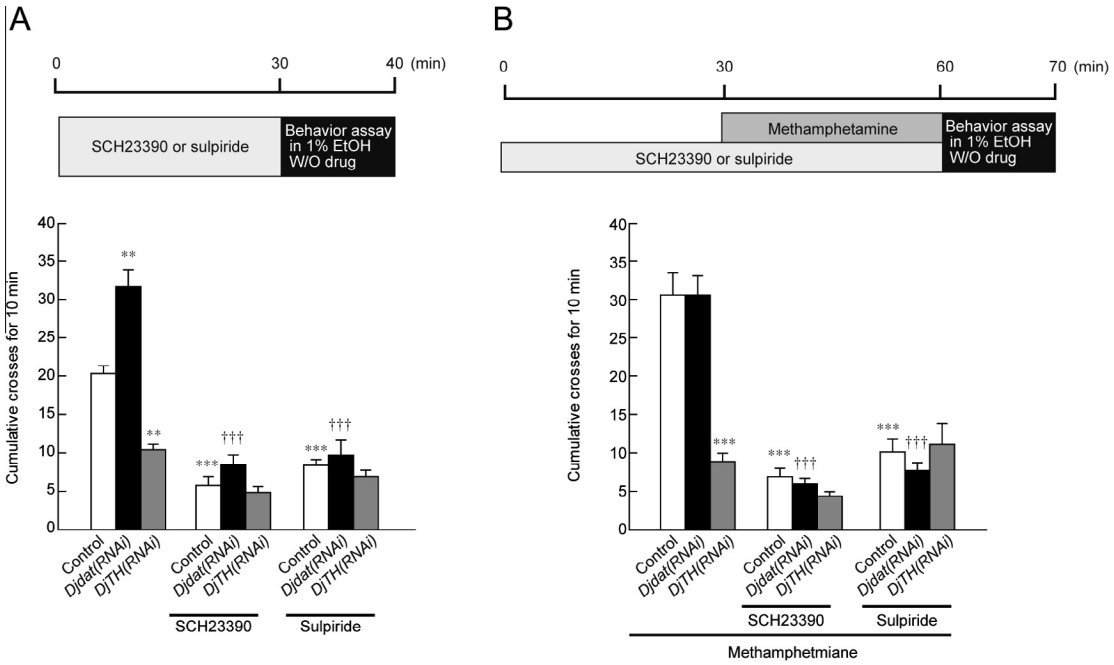

Fig. 4. Evaluation of spontaneous locomotion. (A) Locomotion activity in 1% ethanol solution, under conditions in which planarians were pretreated with/without 10 μM SCH23390 or 100 μM sulpiride for 30 min, compared with the *Djdat(RNAi)*, *DjTH(RNAi)*, and control planarians 7 days after amputation. Each value is mean ± SEM of 10 independent planarians. Significance: $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control without antagonist treatment. $^{\dagger\dagger\dagger}p < 0.01$ vs. *Djdat(RNAi)* planarians without antagonist treatment. (B) Locomotion activity with 0.03 μM methamphetamine treatment in 1% ethanol solution, under conditions in which planarians were pretreated with or without 10 μM SCH23390 or 100 μM sulpiride for 30 min. Each value is mean ± SEM of 10 independent planarians. Significance: $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control planarians. $^{\dagger\dagger\dagger}p < 0.01$ vs. *Djdat(RNAi)* planarians without antagonist treatment.

planarians compared with non-methamphetamine treatment (Fig. 4B). In addition, both SCH23390 and sulpiride pretreatment also suppressed the hyperactivity in the control and *Djdat(RNAi)* planarians, and reduced it to be the same as that in *DjTH(RNAi)* planarians. These results indicated that *Djdat*-knockdown mediated hyperactivity and methamphetamine-induced hyperactivity were mediated by dopaminergic transmission, and useful for simple assessment of dopaminergic phenotype in planarians.

4. Discussion

4.1. Identification of Djdat gene and distribution pattern of DjDAT

In this study, we first identified a full-length *Djdat* cDNA, which has 2100 bp and was predicted to encode 616 amino acid residues (Fig. 1A). By *in situ* hybridization, we demonstrated that *Djdat* mRNA was expressed in the brain, VNCs anterior to the pharynx, and head peripheral region (Fig. 2C and D). We observed that DjDAT-positive neurons, which were immunostained by the anti-DjDAT antibody, were expressed as an inverted U-shape in the brain and projected these axons/dendrites in VNCs (Fig. 2G, H and I). Double-immunofluorescence experiments using antibodies against DjDAT and DjTH revealed that DjDAT and DjTH were co-located in the same neurons (Fig. 2). These results suggested that DjDAT functions in the planarian dopaminergic neurons.

4.2. Changes in the DA content in the Djdat(RNAi) planarians

DAT is believed to play a central role in controlling the brain DA content by reuptake into the presynaptic terminals (Horn, 1990). To reveal the function of DjDAT, we performed several experiments combined with the gene knockdown RNAi method and pharmacological approaches. DjDAT-immunoreactivity was almost completely lost in regenerants in which the *Djdat* gene was knocked down by RNAi (Fig. 3A). Then, we measured the DA content in the regenerated heads of *Djdat(RNAi)* planarians. The DA content in *Djdat(RNAi)* planarians was decreased to approximately less than 23% of that in the control planarians (Fig. 3C). Similarly, the total DA content in the striatal homogenates in *DAT*-knockout mice was decreased to less than 5% of that in wild-type animals [28]. However, the ratio of extracellular to total DA was 10-fold greater in *DAT*-knockout mice compared with wild-type mice [29]. These data indicated that in mice the reserve pool of DA was acutely dependent on recycled extracellular DA, and the decrease in the number of DAT resulted in the diminution of the intracellular store of DA. On the other hand, the smaller reduction of the DA content in the *Djdat(RNAi)* planarians leads to the suggestion that the pool of the DA content are dependent on newly synthesized DA. DjDAT has a similar role in recycling extracellular DA in mammals. These results suggest that DjDAT mediates the fundamental functions of DAT.

4.3. Changes of spontaneous locomotion in the Djdat(RNAi) planarians

DA is a mediator of crucial functions such as movement, emotion, and cognition [30,31]. DAT plays a major role in the control of locomotor behavior by regulating dopaminergic tone in the basal ganglia and nucleus accumbens [1]. Therefore, we analyzed the changes in locomotion activity in the *Djdat(RNAi)* planarians. We evaluated the spontaneous locomotion for 10 days during the regeneration process. Until 3 days after amputation, there was no marked difference between the gene-knockdown and control planarians. However, 5–10 days after amputation, the *Djdat(RNAi)* planarians showed hyperactivity compared with the control planarians (Fig. 3B). These results indicate that the decrease of DjDAT expression is partly involved in the difference of movement between the *Djdat(RNAi)* and control planarians. Subsequently, we measured locomotion activity of the gene knockdown and control planarians 7 days after amputation, and revealed that the *Djdat (RNAi)* planarians showed hyperactivity during the 10-min observation period (Fig. 4A). In addition, we found the hyperactivity in

the *Djdat(RNAi)* planarians was suppressed by 10 μM SCH23390 or 100 μM sulpiride pretreatment, a D_1 receptor antagonist and D_2 receptor antagonist, respectively (Fig. 4A and B). Therefore, the lack of DA reuptake mediated by DjDAT caused poor regulation of the DA content and induced hyperactivity. Our results are comparable to the hyperactivity in *DAT*-knockout mice in a novel environment [32,33].

DAT is a target for psychoactive drugs [26,27]. Administration of amphetamine-derivatives to wild-type mice induces hyperactivity because these drugs induce an outflow of DA to extracellular space. A high dose of methamphetamine induces screw-like and C-like hyperkinesia in planarians [14,34,35]. However, the association between the effect of these drugs and DjDAT is unclear. In the present study, we tested the effect of methamphetamine on the *Djdat(RNAi)* and control planarians (Fig. 4B). The *Djdat(RNAi)* planarians did not show methamphetamine-induced hyperactivity during spontaneous locomotion (Fig. 4B). Administration of psychostimulants to wild-type mice induces hyperactivity. On the other hand, methamphetamine- [36,37], methylphenidate- [33], or cocaine- [4] dosed *DAT*-knockout mice do not show psychostimulants-induced hyperactivity. Similarly, the *Djdat(RNAi)* planarians lacked methamphetamine-induced hyperkinesia. These paradoxical inhibitory responses to psychostimulants are key characteristics of ADHD.

4.4. Conclusion and future prospects

In this study, we identified the orthologous gene of a *DA transporter* in planarians (*Djdat*). The *Djdat(RNAi)* planarians showed spontaneous hyperactive movement. It demonstrated that the controlled DA reuptake by DjDAT is important for normal spontaneous locomotion as in mammals. *Djdat(RNAi)* planarians also showed an absence of methamphetamine-induced hyperkinesia. Although it has been suggested that *DAT*-knockdown invertebrates can be used as ADHD model animals, these two phenotypes were consistent with the aspects of *DAT*-knockout mice. Therefore, our results speculated that the *Djdat(RNAi)* planarians, instead of mammals, can be practically used for pharmacological evaluation of dopaminergic phenotypes in primitive research.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Creative Scientific Research (K.A.); a Grant-in-Aid for Scientific Research on Innovative Areas (K.A.); a Grant-in-Aid for Global COE Program A06 (K.A.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT); and Grants-in-Aid from SRF (Y.K.), JSPS and MEXT-Supported Program for the Strategic Research Foundation at Private Universities (Y.K.).

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