

Ninjin'yoeito, a traditional Japanese Kampo medicine, suppresses the onset of anhedonia induced by dysfunction in the striatal dopamine receptor type 2-expressing medium spiny neurons

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Objective Recent studies have suggested that ninjin'yoeito (NYT), a traditional Japanese Kampo medicine, improves diminished motivation in humans and animals, rendering it a novel therapeutic option for impaired motivation. To better characterize the effect of NYT on motivation, we examined its effect on motivated behaviors in mice.

Methods Mouse models of neurodegeneration-related apathy, in which striatal dopamine receptor type 2-expressing medium spiny neurons (D2-MSNs) were progressively damaged by diphtheria toxin expression, were chosen.

Results The decrease in effort-based operant responding for rewards (sucrose pellets), indicative of the mouse's motivated behavior, in the affected mice was not suppressed by chronic treatment with NYT suspended in drinking water at 1% (w/v). Mice were then subjected to a sucrose preference test, wherein they freely chose to ingest tap water and a sucrose solution without being required to exert effort. The affected mice showed a decline in preference for sucrose over tap water, relative to nonaffected controls, indicating anhedonia-like traits. In contrast to the diminished operant behavior, the

anhedonic behavior in the affected mice was prevented by the NYT administration. Furthermore, NYT did not affect the size of *Drd2* mRNA disappearance in the striatum of affected mice, suggesting that the NYT effect was unrelated to DTA-mediated neurodegeneration.

Conclusion These results demonstrate that the beneficial effect of NYT on motivation is mediated, at least in part, through the potentiation of hedonic capacity by certain neuromodulatory pathways. *NeuroReport* 32: 869–874 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

The inability to experience pleasure and experience of a reduced level of passion for things previously moving or exciting are referred to as anhedonia and apathy, respectively, both of which are common syndromes associated with motivation [1]. Impaired motivation is often associated with neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease and Huntington's disease as well as psychiatric disorders such as depression and schizophrenia [1]. Despite being a frequent clinical manifestation associated with these disorders, diminished motivation is not satisfactorily treated. Although impaired motivation involves multiregional brain dysfunction, some clinical studies have revealed that the severity of

apathy in neurodegenerative disorders is correlated with impaired dopaminergic inputs to the striatum or with striatal atrophy itself [2–4]. Furthermore, a recent animal study demonstrated that dysfunction in dopamine receptor type 2-expressing medium spiny neurons (D2-MSN) in the striatum, which resembles the pathological findings from patients in the early stages of Huntington's disease, primarily results in a reduction in instrumental motivation expressed as decreased goal-directed operant behaviors in mice [5]. As such, meso-striatal dopaminergic malfunction may be attributable to the onset of reduced motivation in neurodegenerative disorders.

Ninjin'yoeito (NYT) is a traditional Japanese kampo medicine comprising 12 natural components (11 plants and a fungus). It has been approved by the Japanese government and indicated for declined constitution from disease, fatigue and malaise, anorexia, perspiration during sleep, cold limbs and anemia since 1986.

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An open-label pilot study recently indicated that Neuropsychiatric Inventory total scores and its subscale apathy scores in Alzheimer's disease patients were significantly improved by repeated treatment with NYT [6]. Additionally, combinatorial treatment with donepezil and NYT for Alzheimer's disease patients was more effective for their depressive symptoms than donepezil alone [7]. Furthermore, NYT was shown to prevent a stress-induced decline in nest-building behaviors, a proxy for mouse motivation [8]. These clinical and animal studies have suggested that NYT may be a potent therapeutic for neurodegeneration-related deficits in motivation. However, increasing attention has been paid to the mechanism of action underlying the effect of NYT on motivation.

Motivated behavior in mice consists of sequential behavioral components, including initiation of action, sustaining efforts, hedonic perception from rewards and reward learning, which are controlled by different neural pathways [1,9,10]. To better characterize the mechanism of action of NYT, we examined its effect on behavioral phenotypes of the D2-MSN dysfunction-induced apathy model mice, in which D2-MSN was progressively damaged by diphtheria toxin subunit A (DTA) expression based on the Tet-off system. Using this model, we investigated the following two items related to mouse's motivated behaviors: (1) goal-directed lever presses for obtaining sucrose pellets (instrumental motivation) and (2) preference for sucrose solution over tap water in a conventional two-bottle choice paradigm (hedonic capacity), attempting to narrow down the behavioral components of motivation that NYT acts on. Furthermore, the effect of NYT treatment on D2-MSN damage was evaluated by *in situ* hybridization to label *Drd2* mRNA in the striatum.

Methods

Animals

All animal experiments were conducted in accordance with the guidelines for the care and use of experimental animals set forth by Tsumura & Co. The study procedures were approved by the experimental animal ethics committees of Tsumura & Co. (approval number: 18-035). All mice ($n=50$) were singly housed in a temperature- (20–26 °C) and humidity- (30–70 %) controlled rooms according to a 12-h light/dark cycle. All mice were of a C57BL/6J, 129SvEvTac, and CBA mixed background that harbored a tetO-DTA (tetO-DTA) transgene (Jax stock number 008168). Sixteen out of 50 mice were single transgenic (DTA mice), which, by itself, did not result in DTA expression; the rest were bigenic mice for the tetO-DTA and *Drd2-tTA* [5] transgenes (D2-DTA mice), in which DTA expression was induced in *tTA*-expressing cells in the absence of doxycycline. Genotyping was performed as described in a previous study [5]. Mice were divided into the following three groups: water-administered DTA mice (DTA-Control, $n=16$), water-administered D2-DTA mice (D2-DTA-Control, $n=17$)

and NYT-administered D2-DTA mice (D2-DTA-NYT, $n=17$). All mice were fed 0.01 % doxycycline-containing food (CLEA Japan, Inc., Tokyo, Japan), except during doxycycline deprivation in the motivation test.

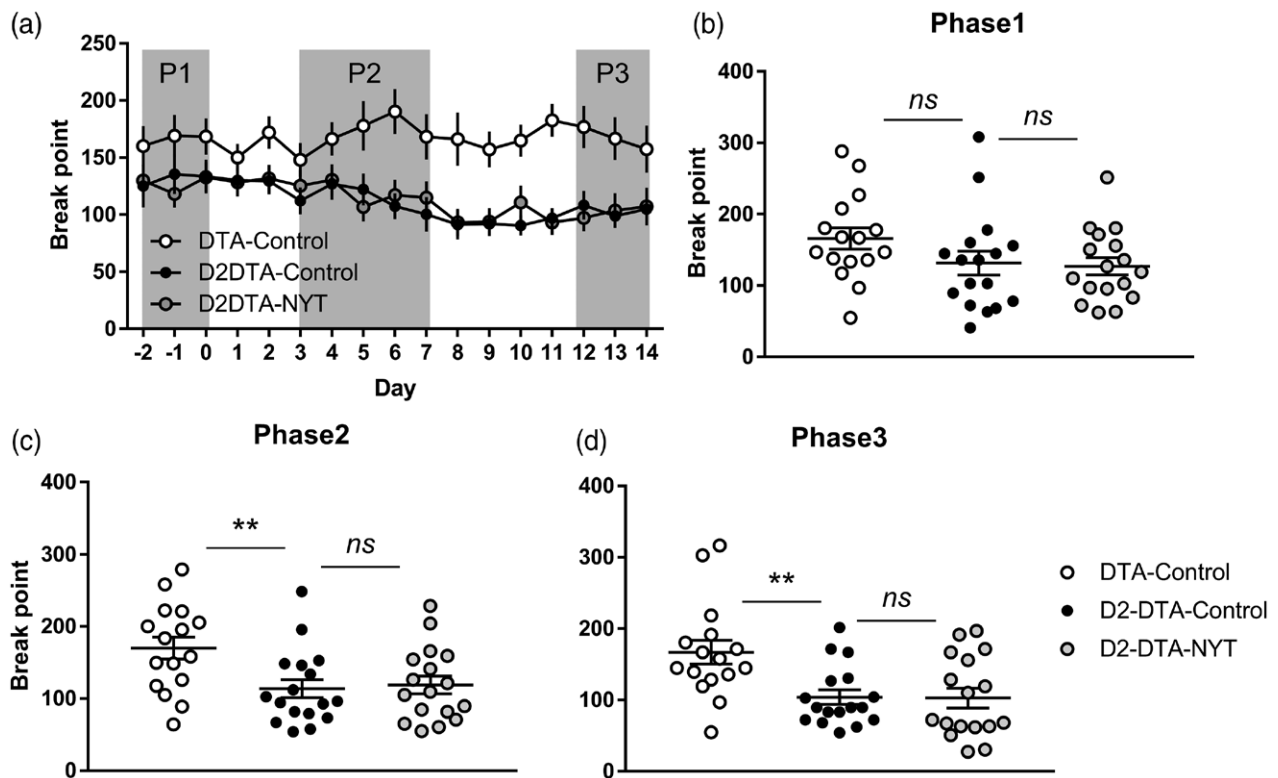
Drugs

NYT was supplied by Tsumura & Co. The formula to produce 6 g of NYT extract powder was composed of a mixture of 12 dried natural components: *Rehmanniae Radix* (4 g), *Angelicae Acutilobae Radix* (4 g), *Atractylodis Rhizoma* (4 g), *Poria* (4 g), *Ginseng Radix* (3 g), *Cinnamomi Cortex* (2.5 g), *Polygalae Radix* (2 g), *Paeoniae Radix* (2 g), *Citri Unshiu Pericarpium* (2 g), *Astragali Radix* (1.5 g), *Glycyrrhizae Radix* (1 g) and *Schisandrae Fructus* (1 g). The mixture was boiled for 1 h, after which the resultant extract was filtered, concentrated and spray-dried to produce the power of the NYT extract (lot number: 372176700). The powder was suspended in water at a concentration of 1% (w/v) in water bottles, which were provided to the D2-DTA-NYT group with ad libitum access. The administration of NYT was begun simultaneously with doxycycline deprivation and lasted until euthanasia, except during the sucrose preference test.

Instrumental motivation

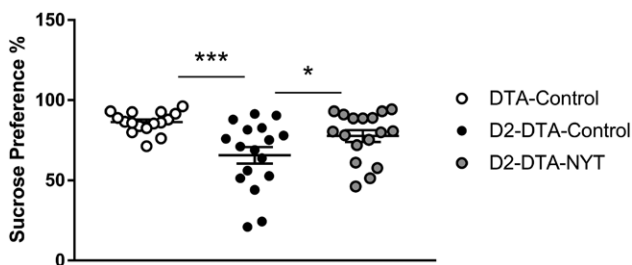
Operant conditioning was performed as described in a previous study [5]. Briefly, mice were trained to learn the instrumental condition in which lever pressing triggers the delivery of food rewards (sucrose pellets) in operant chambers (MED-NP9M-B1, Med Associates Inc., St. Albans, Vermont, USA), in a stepwise manner. All mice were first made to engage in fixed ratio tasks in which the number of lever presses required to obtain a reward was constant. After passing the fixed ratio tasks, mice proceeded to a progressive ratio task, in which the lever presses required were incrementally increased in a session every time a mouse obtained a reward. The response ratio in the progressive ratio task was defined as $5 \times \exp(0.2R) - 5$ (R was defined as 1 + the number of rewards already obtained), thus providing the lever presses required as 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, and so on, as the trials proceeded. Mice could obtain a reward in a trial, and each trial was passed within 5 min in the progressive ratio task. The maximum response ratio that mice attained in a progressive ratio session was defined as a 'breakpoint' and seen as an indicator of instrumental motivation (higher breakpoint represents higher motivation). During the progressive ratio tasks, mice underwent one session daily, and their body weight was controlled such as to remain within $85 \pm 5\%$ for each individual relative to that on the first day of the training by adjusting the weight of food daily presented, thereby equalizing appetite patterns for sucrose pellets among individuals. After determining the breakpoint baseline level for 3 days, doxycycline depletion was initiated (the day was referred to as Day 0) and continued for 14 days. After the operant test, doxycycline feeding was resumed for all mice and continued until euthanasia.

Fig. 1



Breakpoint comparison. (a) temporal change in breakpoints before and after doxycycline depletion. (b–d) Intergroup comparison of breakpoints analyzed in phase 1 (b), phase 2 (c) and phase 3 (d). Mean \pm SEM, $n=16, 17, 17$, **: $P<0.01$.

Fig. 2



Sucrose preference. D2-diphtheria toxin subunit A (DTA)-Control mice exhibited a significant reduction in sucrose preference, which was suppressed by Ninjin'yoeito (NYT) treatment. Mean \pm SEM, $n=16, 17, 17$, *: $P<0.05$, ***: $P<0.001$.

Sucrose preference

The sucrose preference test was performed for 2 consecutive days between days 15 and 20, based on the conventional two-bottle choice paradigm, as previously described [11]. Two bottles (one containing tap water and the other containing 1% sucrose solution) were placed in each cage, and mice were allowed *ad libitum* access to them. Mice were habituated to the condition for 24 h, and daily intake from each bottle was determined

by measuring the weight of the bottles for the following 24 h. Sucrose preference was defined as [intake of sucrose solution (g)/total liquid intake (g)] \times 100%.

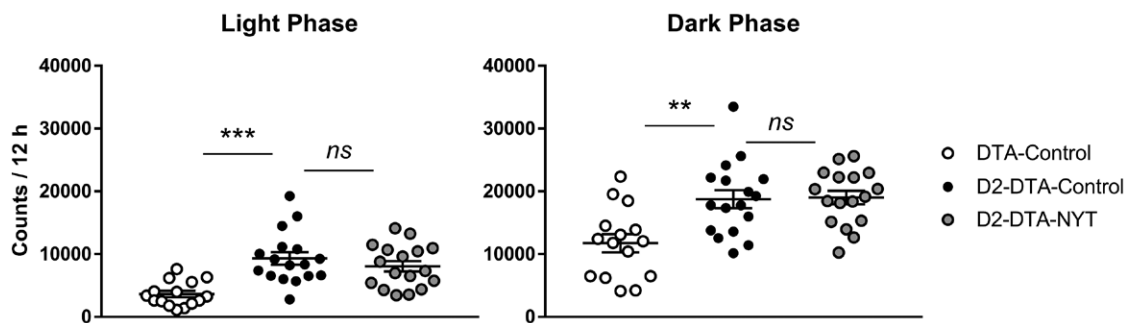
Home cage activity

Activity recording was started 1–4 days after the sucrose preference test. Home cage activity was measured under 24-h monitoring for 9 or 10 consecutive days between days 18 and 32 using infrared sensors (NS-AS01, Neuroscience, Inc., Tokyo, Japan) attached to each cage. The assessments of the mice's activity levels every 12 h were averaged over the entire period to compare the activity levels during both light and dark phases.

In situ hybridization

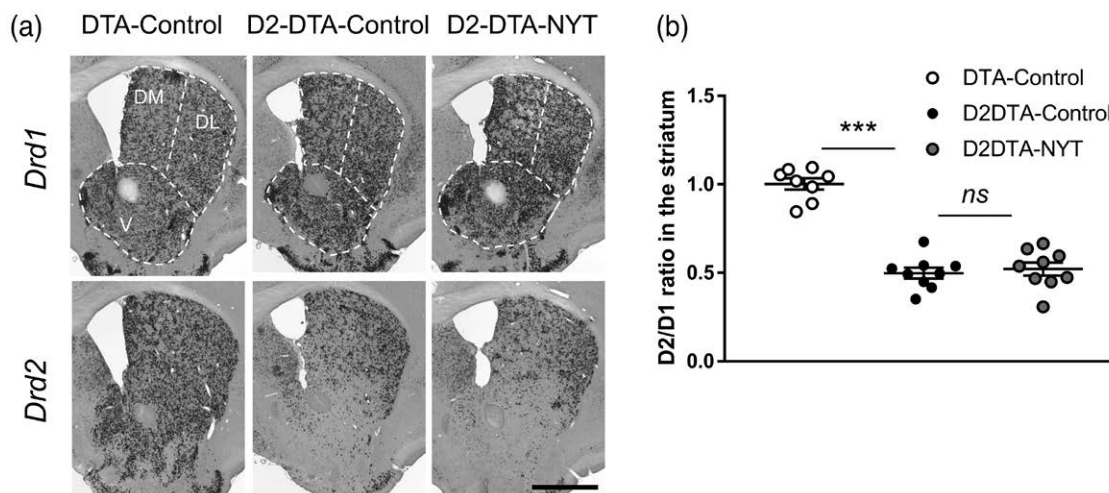
On day 29, randomly selected mice ($n=8-9$) were deeply anesthetized with isoflurane and perfused with 4% paraformaldehyde in PBS. The brains were postfixed in the same fixative overnight, cryoprotected in 20% sucrose in PBS, frozen, and sectioned into 25 μm -thick slices. Three sections from Bregma +0.74 mm to +1.10 mm were analyzed for each animal. We conducted in situ hybridization (ISH), as described previously [5]. Briefly, sections were treated with proteinase K (40 μgml^{-1} , Roche, Japan) for 30 min, followed by acetylation. The sections were incubated with digoxigenin -tagged complementary RNA

Fig. 3



Locomotor activity. Home cage activity was measured under 24-h monitoring. Activity counts in the light phase (left) and dark phase (right) are compared. Mean \pm SE, $n=15, 17, 17$; **, $P<0.01$, ***, $P<0.0001$. Note that data from one mouse in the DTA-Control group were excluded from the analyses due to difficulties with the sensor.

Fig. 4



In situ hybridization (ISH) analysis on the coronal brain sections. (a) ISH was performed using antisense probes for Drd1 (upper) and Drd2 mRNA (lower). DL: dorsolateral striatum; DM: dorsomedial striatum; V: ventral striatum. Scale bar: 1 mm (b) The signal ratio of Drd2/Drd1 for each mouse was compared between the groups. Mean \pm SEM, $n=8, 9, 9$; **, $P<0.01$, ***, $P<0.001$.

probes for Drd1 and Drd2 mRNA at 63 °C., followed by an anti-digoxigenin antibody conjugated with alkaline phosphatase (1:5000 dilution, Roche). Color development was performed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche), and sections were stained with nuclear fast red (Sigma). Images of the sections were captured using a light microscope (BZ-X710; Keyence, Osaka, Japan). Images were binarized to determine the number of positive pixels for both Drd1 and Drd2 slides, as previously described [5]. The signal ratio of Drd2/Drd1 for each individual was calculated as the disappearance value of Drd2 mRNA (lower value indicates greater disappearance of Drd2 mRNA).

Statistical analyses

For chronological breakpoint data, a two-way repeated-measures (RM) analysis of variance (ANOVA) was

used. For intergroup comparisons, Holm Sidak's multiple comparisons were performed using Prism 7 (GraphPad, San Diego, California, USA). Statistical significance was defined as $P<0.05$.

Results

Ninjin'yoieito failed to prevent a decline in operant motivation in D2-diphtheria toxin subunit A mice

A two-way RM ANOVA on chronological changes in breakpoint revealed significant main effects of the day [$F(16, 752)=3.309$; $P<0.0001$] and group [$F(2, 47)=6.962$; $P=0.0022$], with a significant interaction [$F(32, 752)=1.77$; $P=0.0059$] (Fig. 1a). For intergroup comparisons, breakpoints for each mouse were averaged over the following phases, as previously identified [5]: phase 1 (Days -2-0, before induction of DTA); phase 2 (Days 3-7, DTA mRNA can be detected) and phase

3 (days 12–14, cell death of D2-MSN can be observed). During phase 1, the breakpoints did not significantly differ between groups (DTA-Control vs. D2-DTA-Control: $t=1.642$; $P=0.2030$; D2-DTA-Control vs. D2-DTA-NYT: $t=0.2121$; $P=0.8329$) (Fig. 1b). As expected, D2-DTA-Control mice displayed lower breakpoints than DTA-Controls during phases 2 and 3 (phase 2: $t=2.968$; $P=0.0094$; phase 3: $t=3.222$, $P=0.0046$) (Fig. 1c and d). During these periods, the D2-DTA-NYT group showed similar breakpoints to those in the D2-DTA-Control group (phase 2: $t=0.2772$, $P=0.7828$; phase 3: $t=0.06713$, $P=0.9468$) (Fig. 1c and d).

Ninjin'yoeito treatment suppressed a decrease in sucrose preference in D2-diphtheria toxin subunit A mice

D2-DTA-Control mice exhibited a significantly lower preference for sucrose compared to DTA-Control mice ($t=3.746$; $P=0.0010$) (Fig. 2). D2-DTA-NYT mice had a significantly higher preference than D2-DTA-Control mice ($t=2.215$; $P=0.0317$) (Fig. 2).

Ninjin'yoeito did not affect D2-diphtheria toxin subunit A mice hyperactivity

D2-DTA mice were shown to exhibit hyperactivity 14 days after doxycycline deprivation [5], which is commonly observed with a broad ablation of D2-MSNs in the striatum [12]. D2-DTA-Control mice exhibited hyperactivity compared to the DTA-Control group during both light and dark phases (light phase: $t=4.811$; $P<0.0001$; dark phase: $t=3.781$; $P=0.0011$) (Fig. 3). NYT treatment did not alter the activity levels of D2-DTA mice in either phase (light phase: $t=1.093$; $P=0.2803$; dark phase: $t=0.1528$; $P=0.8793$).

Ninjin'yoeito had no effect on the disappearance of Drd2 mRNA in the striatum of D2-diphtheria toxin subunit A mice

We assessed whether NYT treatment attenuated the extent of DTA-induced disappearance of Drd2 mRNA in the striatum. ISH pointed to the loss of Drd2 mRNA signal in the ventral and dorsomedial striatal regions, with the most lateral part of the dorsolateral striatum remaining unaffected in both D2-DTA-Control and D2-DTA-NYT mice (Fig. 4a). The signal ratio of Drd2/Drd1 was significantly reduced in D2-DTA-Control mice compared to that in DTA-Control mice ($t=10.6$; $P<0.0001$) (Fig. 4b). There was no significant difference in the Drd2/Drd1 ratio between D2-DTA-Control and DTA-Control groups ($t=0.4952$; $P=0.6252$) (Fig. 4).

Discussion

In this study, we demonstrated that D2-DTA mice show anhedonia-like behaviors as well as a previously reported reduction in instrumental motivation. Interestingly, NYT, a traditional Japanese Kampo medicine, suppressed the anhedonia-like phenotype, but not the decrease in

effort-based operant behavior, in D2-DTA mice without affecting the DTA-mediated cell damage in the D2-MSNs.

Motivated behaviors are orchestrated by a presumable cycle of cost-benefit decisions, preparation for action, initiating and sustaining efforts, receiving hedonic impact and learning from outcomes [1,9,10]. In this study, D2-DTA mice showed a reduced sweet preference (deficit in hedonic impact) from days 15–20, unlike the previous study, in which D2-DTA mice exhibited an intact sweet preference on day 7 [5]. This may be explained by the striatal regional difference in D2-MSN dysfunction because DTA expression started on day 3 in the ventrolateral striatum and then gradually expanded to the dorso- and ventromedial regions by day 28 [5]. Considering that, together with the intact sucrose preference in D2-DTA mice on day 7, optogenetic inactivation of the D2-MSNs within the ventral striatum reportedly failed to reduce sucrose intake [13], the anhedonia-like phenotype in the present D2-DTA mice may be attributed to dysfunction in the D2-MSNs located in the dorsomedial striatum. To identify the responsible subregion for the anhedonic phenotype, a temporal assessment of sucrose preference and concomitant histopathological analysis between days 7 and 20 is required in future studies.

Although NYT previously improved the apathetic phenotype in animal and clinical studies [6,8], it failed to prevent the decline in goal-directed lever presses in this study. This may be reasonable in view of the previous finding that it exerts a promotional effect on motivation via D2 receptors [8]. Thus, the ineffectiveness of NYT on decreased motivation in the present D2-DTA mice, even at the intermediate phase of doxycycline depletion, suggests that the target cells of NYT that express D2 receptors may be the D2-MSNs in the ventrolateral striatum. However, to explore this hypothesis, whether D2-DTA mice are unresponsive to NYT under a condition able to promote motivation in DTA-Controls should be examined. In addition to the D2 pathway, given the possible inhibitory action of NYT on dopamine degradation enzymes [8], D1 signaling is another potential target of NYT, considering its well-known association with motivation [9,14]. This is also a future research topic regarding the effect of NYT on motivation.

Unlike amotivation, which is expressed as a reduction in operant response, NYT suppressed the anhedonia-like decline in sucrose preference in D2-DTA mice. Because neither the loss of Drd2 mRNA nor the hyperactivity in D2-DTA mice was suppressed, NYT did not have the potential to prevent the loss of function in the D2-MSNs *per se*. A major player in hedonic impact is opioid signaling; in particular, μ opioid receptors in the ventral striatum and ventral pallidum enhance hedonic perception by sucrose intake [14,15]. Although it remains unknown whether NYT potentiates opioid signaling, it is

noteworthy that catalpol and ginsenoside Rh2, ingredients in *Rehmanniae Radix* and *Ginseng Radix*, respectively, increase the endogenous levels of β -endorphin [16,17]. Furthermore, it is well known that hedonic deficits are evoked as a depressive-like phenotype by stress exposure and can be improved by antidepressants, in the form of selective serotonin reuptake inhibitors (SSRIs) [18], pointing to the serotonergic involvement in improving impaired hedonic capacity. However, several lines of evidence have revealed that SSRIs conversely reduce effort-based operant responding and nest-building behaviors in rodents [19–21], dismissing serotonergic enhancement as the target of NYT's action, considering the previously confirmed counteracting effects of NYT on diminished nest building [8]. Another important biological pathway, other than direct actions on neurotransmitter signaling to restore depression-related anhedonia, is neurotrophin signaling and downstream neurogenesis in the hippocampus [22]. Importantly, *Ginseng Radix* and *Polygalae Radix* extracts reportedly improved a stress-induced reduction in sucrose preference via neurotrophin signaling [23]. The antidepressant-like aspects of NYT should also be addressed in future studies.

Conclusion

We demonstrated that dysfunction in the D2-MSNs in massive striatal regions, excluding the dorsolateral area, evoked a decrease in sucrose preference in addition to that in effort-based operant responding. Furthermore, the inhibition of anhedonia-like traits, but not the diminished effortful behaviors, in D2-DTA mice characterized the effect of NYT on motivation, at least as a preventive measure for hedonic deficits.

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Conflicts of interest

H.O., R.I., Y.O. and K.M. are employees of Tsumura & Co. M.M. received a grant from Tsumura & Co. For the remaining authors, there are no conflicts of interest.

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