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Title:

Behavioural and pharmacological effects of fluvoxamine on decision-making in food patches and the inter-temporal choices of domestic chicks

Running head:

Serotonin and foraging decisions

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Highlights

- SSRI makes chicks stay longer at a gradually depleting food patch.
- SSRI makes chicks commit less impulsive choices.
- SSRI makes chicks invest smaller efforts for food.
- SSRI makes chicks emit distress calls less frequently.
- SSRI increases 5-HT and DA levels in the medial striatum / nucleus accumbens.

Abstract

Behavioural effects of fluvoxamine (FLV, selective serotonin reuptake inhibitor) were examined in 1–2 week old domestic chicks. Chicks were tested in an I-shaped maze equipped with a feeder (*ON* feeder) that served 1 or 2 grains of millet at gradually increasing intervals, so that a depleting food patch was mimicked. By leaving the feeder, the food delivery program was reset, and chicks gained food at short intervals only after a travel to a dummy feeder (*OFF* feeder) placed on the opposite side of the maze. Chicks quickly learned to actively shuttle between the *ON* and the *OFF* feeders. FLV (intra-peritoneal injection, 20 mg/kg BW) acutely caused chicks to stay longer at the gradually depleting *ON* feeder. Inter-temporal choices were also tested, whereby two colored beads were simultaneously presented, each associated with a small/short-delay reward or a large/long-delay alternative. FLV suppressed the choice of the short-delay option. It is suggested that an enhanced level of serotonin (5-HT) makes chicks more tolerant of the delayed food item in both behavioural paradigms. Furthermore, the decision to leave a depleting patch cannot be equated to choosing the long-delay option of the choice paradigm. Furthermore, FLV suppressed work efforts (velocity and running distance) in uncued shuttle and number of distress calls. *In vivo* microdialysis experiments revealed that FLV enhanced the extracellular concentration of 5-HT as well as dopamine (DA) locally in the medial striatum/nucleus accumbens. Underlying neuromodulatory mechanisms of behavioural control are examined in relation to locomotion, behavioural tolerance and interval timing.

Keywords:

serotonin, dopamine, optimal foraging behaviour, impulsiveness, effort, medial striatum, nucleus accumbens, decision making

1. Introduction

Because food items are unevenly distributed in nature, animals often forage by sequentially visiting one patch after another. As the food density of a patch gradually declines, the energy intake rate should inevitably decrease. Foragers should thus leave the depleting patch at an appropriate point, even though some food items are remaining. By adopting marginal value theorem, Charnov [1] argued that optimal foragers leave a patch at the point when the instantaneous gain rate matches the average gain rate available in the environment. The optimal patch-use model, as formulated by Stephens & Krebs [2], gained strong empirical support through studies of diverse animals in the wild. For example, wasps [3] and starlings [4] have been shown to behave optimally so that the long-term gain rate was maximized in the central-place resource collection.

Some studies have tried to examine patch-use behaviours as psychological tasks performed in the laboratory. Agetsuma [5] simulated a depleting food patch by gradually decreasing reinforcements, and found that the subject monkeys stopped responding in a manner that matched the optimal patch-use model. On the other hand, Hayden et al. [6] simulated a patch-use condition and analyzed neural activities of the dorsal anterior cingulate cortex. In Hayden's protocol, monkeys made choices between an option after a fixed short delay (gradually smaller drops of juice, mimicking next food in the patch) and an alternative option after a variable "travel time" (reset of the diminished return, mimicking new patch). In that study, however, the prospective "travel time" was explicitly presented, and the monkeys could make decisions in a manner similar to the inter-temporal choice paradigm. In the concurrent choices, animals generally adopt a short-sighted strategy such as maximization of instantaneous gain rate [7] or profitability [8], in contrast with the paradigm of patch-use behaviour.

The discrepancy between these two paradigms was clearly pointed out by Stephens and Anderson [9], who examined captive blue jays under two different conditions, one mimicking patch-use behaviour ("stay or leave" choices on single options), and the other inter-temporal choice (concurrent choices of "small-immediate" and "large-delayed" options). Even though the economic consequences of these two conditions were set as identical, the jays behaved differently and showed a considerable far-sightedness in the patch-use task. The authors thus argued that behavioural data obtained in the inter-temporal choice paradigm are not an "accurate guide" for understanding patch-use behaviour. However, it remains unanswered whether the neural substrates are distinct or shared between these two paradigms of behaviour.

In the present study, we examined if these paradigms could share serotonin (5-HT) as a common neuro-modulator. In inter-temporal choices tested in rats, antidepressant drugs (imipramine-like drugs and serotonin reuptake inhibitors) have been shown to acutely improve impulse control, suggesting the involvement of noradrenergic / serotonergic neuro-modulation [10, 11]; null results [12, 13] should also be noticed. However, 5-HT depletion by 5,7-dihydroxytryptamine infusion to raphe nuclei caused rats to commit more impulsive choices than controls [14,15], supporting the critical role of 5-HT. Functional involvement of 5-HT has also been shown by Denk et al. [16], where a blockade of 5-HT synthesis caused rats to choose an immediate reward more frequently without effects on effort-based choices. Taking these experimental findings into account, Doya [17] proposes an integrated framework in which neuromodulators (5-HT, dopamine and noradrenalin) affect economical decision making. However, the involvement of modulators has not been studied in patch-use behaviour, except for a recent genetic study in *Caenorhabditis elegans* [18].

Fluvoxamine maleate (FLV) was used as the selective serotonin reuptake inhibitor (SSRI) in this study, because FLV binds to a chicken's 5-HT transporter (SERT) at a high affinity, comparable with the human SERT [19]. If 5-HT level is increased by FLV application and if choice impulsiveness is suppressed by FLV, then FLV may also influence patch-use behaviour. FLV-treated chicks may leave a depleting food patch earlier, so that they gain a larger amount of food after a longer delay (or a new patch after a long travel time) as illustrated in Fig 1. In addition, the effects of FLV on work efforts and distress calls were also measured.

Pharmacological effects of FLV on the striatal 5-HT level were examined by *in vivo* microdialysis. Striatal nuclei (neostriatum and nucleus accumbens [NAc] in particular) have been shown to play a critical role in inter-temporal choices, because localized lesions caused impulsive choices in rats [20] and in domestic chicks [21]. Dense 5-HT innervation of the caudate putamen by the dorsal raphe is reported in rats [22]. In chicks, similarly, NAc and the surrounding striatum are similarly innervated by 5-HT positive fibers [23].

2. Materials and methods

2.1.Animals

A total of 193 male domestic chicks (*Gallus domesticus*, white leghorns) were used. Each chick was used once and never reused in other experiments. Thirteen chicks were discarded because they heavily emitted distress calls, therefore the present study was based on data obtained from the remaining 180 individuals; 36 chicks (**experiment-1**), 70 (**-2**), 20 (**-3**), 16 (**-4**), 18 (**-5**), 12 (**-6**), and 8 (**-7**), respectively. Five additional chicks served as companion individuals in the study of distress calling. New hatchlings

(post-hatch day 1) were purchased from a local supplier (Hokuren Central Hatchery, Iwamizawa, Hokkaido) and were communally housed in transparent plastic cages (15 × 28 × 12 cm) that were thermo-controlled at *ca.* 30°C under illumination by white light bulbs (12L: 12D, the light period starting at 08:00). On days 2–4, chicks were fed with 1–3 g of food (per day per chick; mixture of millet and chick mash food). On day 5 and afterwards, chicks were supplied with a daily 4 g ration in addition to 0.5–1.0 g of millet gained during experiments. Water was freely available. Body weight steadily increased, maintaining *ca*. 80% of the freely-fed body weight. After the end of the experiments, chicks were sacrificed by carbon dioxide if not processed for brain histology. Experiments were conducted under the guidelines and approval of the Committee of Animal Experiments of Hokkaido University. The guidelines are based on the national regulations for animal welfare in Japan (Law for the Humane Treatment and Management of Animals; after a partial amendment No.68, 2005).

2.2.Apparatuses and drugs

2.2.1. I-shaped maze for measurement of patch residence time

An I-shaped maze was used for recording the residence time (**experiment-1** and **-2**, Fig. 2A). The maze was composed of a pair of feeders connected by a runway (60 cm long and 13 cm wide). Each feeder was equipped with a mechanical sensor and a colored door that was painted in blue or red. One of the feeders served as *ON* feeder that supplied grains of millet. Color assignment was counter-balanced among individuals in each group. The sensor detected the arrival of a chick and triggered the door to open for the chick to approach the food tray. The *ON* feeder delivered food (1 or 2 grains of millet at a time) at pre-programmed intervals shown up to 25 times. Fig 2B (a and b)

illustrate the patterns of gradually diminishing food supply by plotting the cumulative gain (number of millet pellets) against the residence time (sec) during which the chick stayed at the *ON* feeder. Open and filled circles denote the time at which food of 1 (a) or 2 (b) grains was delivered. For further explanation of the procedures of behavioural tasks, see below (section 2.3.1.).

The opposite (*OFF*) feeder did not deliver food. Visits to the *OFF* feeder terminated the food supply at the *ON* feeder, and caused the interval between grain deliveries at the *ON* feeder to be reset to the minimum value. Programmed food supply at the *ON* feeder was restarted when the chick revisited the *ON* feeder (Fig. 2C). Chicks quickly learned to forage food by shuttling between the two feeders. The I-maze was placed in a dark room, illuminated by white light bulbs, and kept at *ca.* 26–30°C. Behaviour was monitored by a video camera placed above the maze, so that experimenters observed chicks without being seen.

Figures 2 and 3 around here

2.2.2. Operant chamber for binary choice tests

An operant chamber was used for recording behaviours in the inter-temporal choice paradigm (**experiment-3**). A thermo-controlled box (21 × 19 × 25 cm, kept at *ca.* 27–30°C and illuminated by light bulbs) was used [24-26]. One of the surrounding walls was equipped with a pair of holes placed side by side (separated by 3 cm, 4 cm above floor level), through which one or two colored beads (white, blue or red) were presented for 1 sec. When a chick pecked one bead, both beads were withdrawn and millet food (1 or 6 grains) was supplied to the central food tray after a delay (0 or 1.5 sec). Assignment of colors to small/short-delay food (red for 1 grain after 0 sec; ***SS***) and large/long-delay food (blue for 6 grains after 1.5 sec; ***LL***) was fixed among individuals. A white bead

was always assigned to a non-rewarding option.

2.2.3. I-shaped maze for studying work efforts

An I-shaped maze (88 cm long and 12 cm wide) was used for testing each chick's foraging efforts (**experiment-4**); see [27] for details. Briefly, the maze was equipped with a pair of terminal feeders, each of which supplied a grain of millet at variable intervals (mean = 15 sec, uniformly ranging 10–20 sec) for 30 times (therefore 60 grains in total). Note the low reinforcement rate, which did not deplete during the trial that lasted for 8–9 min. The maze was not equipped with doors, and chicks freely shuttled between the two feeders and gained food if available. No visual cues were delivered but distracting motor sounds were given so that chicks were unable to associate any sensory cues with food delivery. Behaviour was recorded by a video camera placed above the maze, and trajectories of the shuttling chick were analyzed off-line by using Move-tr/2D (ver 7.0) software (Library Co., Japan) according to the position of a fluorescent marker attached to the head.

Other behavioural tests (distress calls; **experiment-5**) and *in vivo* microdialysis (**experiment-6** and **-7**) were conducted in the operant chamber, but chicks did not perform any specific behavioural tasks.

2.2.4. Drugs

Fluvoxamine maleate (FLV) was kindly supplied by Abbott Japan Co., Ltd. (former Solvay Pharmaceuticals B.V.) / Meiji Seika Pharma Co., Ltd. FLV was dissolved in a 0.01 M phosphate-buffered saline (pH 7.2) at concentration of 1.5 mg or 3.0 mg per ml, and intra-peritoneally administered at 1 ml/15 g body weight (BW), yielding 10 or 20 mg/kg BW, respectively. For *in vivo* microdialysis experiments (**experiment-6** and **-7**), we used an artificial cerebrospinal fluid (aCSF) composed of: KCl 2.7 mM, NaCl 140

mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 1.0 mM, NaH_2PO_4 0.03 mM, Na_2HPO_4 1.7 mM, pH 7.2. In the reverse microdialysis (**experiment-7**), FLV was added to the aCSF at concentrations of 2 or 20 μM.

2.3.Procedures of behavioural tasks

2.3.1. Patch residence time (experiment-1 and -2)

On post-hatch day 8, chicks were initially given 3 consecutive blocks of habituation to the I-maze. In each block, a communal group of 3 individuals was placed in the maze with 25 grains of scattered millet for 15 min. On days 9–11, individual chicks were tested in a block of behavioural sessions per day, and a block was composed of 20 cycles of shuttling between the *ON* and *OFF* feeders. Chicks were discarded if they stopped midway continually emitting distress calls for a period longer than 15 min.

We recorded behaviours under 6 different patterns of food supply (Fig. 2B). To avoid effects of preceding experiments, each chick was examined under one condition and not reused. In 4 patterns, the *ON* feeder delivered 1 grain of millet at a time, and up to 25 grains were supplied. In 2 other conditions, 2 grains were delivered at a time, so that the gain was 50 grains in total.

Intervals between delivery was either kept constant ({2.0, ×1.0} and {14.0, ×1.0} conditions) or gradually increased ({2.0, ×1.1} and {0.5, ×1.2} conditions). Figures in parentheses {α, xβ} indicate that the initial interval (between the 1st and 2nd delivery) was α sec, and increment rate was β. The last interval (between the 24th and the 25th) of the depleting conditions was therefore 16 and 22 sec for {2.0, ×1.1} and {0.5, ×1.2}, respectively. Predictions of these experiments are schematically illustrated in Fig. 3A and B.

Four parameters (periods of time) were measured based on the sensor signals recorded by a PC using the LabView system (National Instruments Co., USA). (1) ***Residence time*** in the *ON* feeder: period from the arrival at the *ON* feeder until the arrival at the *OFF* feeder. (2) ***Leave-point interval***: interval between the last two deliveries at the point when the chick left the *ON* feeder (Fig. 4C). (3) ***Travel time***: period from the arrival at the *OFF* feeder until the arrival at the *ON* feeder. (4) ***Waiting time***: period from the leave point until the point of time at which the next grain should have been delivered in the *ON* feeder (Fig. 4D).

As the first step, behaviours were recorded without any pharmacological treatments in 6 groups of chicks (**experiment-1**; under 4 diminishing conditions and 2 constant interval conditions, as described above). Behavioural tasks were conducted for 3 days from post-hatch day 9 to day 11. Chicks were examined in one block (20 shuttles) per day.

As the second step, the effects of FLV on *residence time* were examined in 6 other groups of chicks (**experiment-2**); vehicle control, 10 and 20 mg/kg BW in each of the 2 diminishing conditions. Behavioural tasks were conducted for 4 days from post-hatch day 9 to day 12. On day 9, one block (composed of 30 shuttles) was given, and chicks were pseudo-randomly allocated into 3 groups (vehicle, 10 and 20 mg/kg BW); 3 groups were thus counter-balanced based on the recorded residence time. On days 10–12, chicks received one block (composed of 20 shuttles) per day, 1 hour after the FLV or vehicle solution was intra-peritoneally injected.

2.3.2. Inter-temporal choices

Effects of FLV on inter-temporal choices (**experiment-3**) were examined according to the procedure described previously [25]. Briefly, after initial habituation to the

operant chamber on day 5, chicks were individually trained to associate color cues with food rewards as described above. One block of single-cue training consisted of 12 pseudo-randomly arranged trials for ***SS*** (small/short-delay reward; red), 12 trials for ***LL*** (large/long-delay reward; blue), and 24 trials for no reward (white), respectively. Chicks received 2 blocks of training on day 6. On days 7–9, chicks were thereafter trained in binary choices between a rewarding bead (red or blue) and a non-rewarding white bead in one block per day. One block consisted of 12 pseudo-randomly arranged trials for ***SS*** (red) paired with white, 12 trials for ***LL*** (blue) paired with white, and 24 trials for a pair of non-rewarding white beads. The side of presentations was randomised in each trial. On day 10, the remaining chicks were allocated to two groups in a counter-balanced manner based on body weight; one group for FLV and another for vehicle treatment. At 60 min after the injection, chicks were tested in one block composed of 10 test trials of choices between ***SS*** (red) and ***LL*** (blue), which were pseudo-randomly arranged with trials for a pair of non-rewarding white beads (10 trials), a pair of ***SS*** (red; 10 trials) and a pair of ***LL*** (blue; 10 trials).

2.3.3. Work efforts in uncued shuttle tests

Effects of FLV on work efforts (running for feeders) were examined (**experiment-4**) according to the procedure described elsewhere [27]. Briefly, chicks were habituated to the I-maze on days 7 and 8, and then allocated to two groups in a counter-balanced manner based on body weight; one group for FLV and another for vehicle treatment. On days 9–11, chicks were individually tested 75 min after the FLV or vehicle solution was intra-peritoneally injected. In each test session, the two terminal feeders supplied millet grains according to the programmed food delivery that lasted for 8–9 min in total. After the food supply was terminated and all grains were consumed,

chicks were left in the maze for an additional 2 min before the test session of the day was terminated.

Five parameters were measured. (1) ***Total Residence time*** (sec) in each feeder (*i.e.*, areas < 10 cm from the terminal walls). (2) ***Number of visits*** (times) to each feeder. (3) ***Residence time per visit*** (sec/times) (*i.e.*, the ***residence time*** divided by the ***number of visits***). (4) ***Running distance*** (cm) and (5) ***velocity*** (cm/s) during the 8 min period of food delivery; ***Velocity*** was measured in the midway after excluding the areas near (< 10 cm) the feeders.

2.3.4. Distress calls

Effects of FLV on distress calls were examined at 3 doses (vehicle, 10, and 20 mg/kg BW, **experiment-5**). To induce distress calls, the subject chick was socially isolated from companion chicks for 3 min, as reported previously [28], and the number of distress calls were counted. Calls were also counted before and after the isolation period (the average yielded baseline level, each for 3 min), and the difference (***Δ distress calls***) was calculated. Calls were counted 20 min after the FLV or vehicle injection. FLV or the vehicle was injected every day from day 8 to day 10, and calls were measured on days 8 and 10.

2.3.5. Statistical analysis

For the data obtained in **experiments-1** to **-4**, generalized linear mixed models (GLMMs) were constructed to estimate the effects of behavioural factors and the drug application by using R (ver. 2.6.0 [29]) for the platform of statistical calculations. See Tables 1-3 and supplementary tables for details. For the distress calls (**experiment-5**), ANOVA was used after Bartlett's test for homogeneity of variance with the significance level set at $p < 0.05$.

2.4.Microdialysis

2.4.1. Surgical operation for chronic implantation of cannulae

Effects of systemic injection of FLV on centrally released serotonin (5-HT) and dopamine (DA) were examined in **experiment-6** by microdialysis. To confirm its direct effects on the central nervous system, the effects of locally infused FLV through the dialysis microprobe (reverse microdialysis) was examined in **experiment-7**.

A guide cannula was chronically implanted on the day before the microdialysis experiment. Chicks were anaesthetized by intra-muscular injection of ketamine and xylazine (0.24 ml of a 1:1 mixture of 10 mg/ml ketamine (Sankyo Co., Japan) and 2 mg/ml xylazine hydrochloride (Sigma, USA). Supplementary injections (0.05 ml each) were given to maintain a stable anaesthesia if necessary. Chicks were then fixed on a stereotaxic apparatus. Skin over the skull was incised, and the skull bone and dura matter were cut out to expose the brain surface. A guide cannula (AG-8, Eicom Co., Japan) was inserted into the medial striatum (mSt) of the left hemisphere. Coordinates of the probe tip were; 1.20–1.75 mm anterior from the bregma, 1.2–1.5 mm lateral from midline, and 4.3–5.3 mm from brain surface. Incised skull was covered with cotton, and the cannula was secured to the skull with dental cement. In **experiment-6**, a polyethylene tubing (1.0 mm o.d.) was further inserted into the peritoneal body cavity for systemic injection of FLV. The tubing was implanted under the skin and connected to a 1 ml syringe.

2.4.2. Sampling perfusate and high performance liquid chromatography (HPLC) measurement of 5-HT and DA

On the next day, an *in vivo* microdialysis experiment was conducted in a

freely-behaving condition without any behavioural tasks. A dialysis probe (A-I-8-02, Eicom Co.) was inserted into the brain through the guide cannula, and sampling started immediately. The active membrane of the probe (0.22 mm o.d., 2 mm long) was exposed to brain tissue. The internal space of the probe was perfused with aCSF at a rate of 1 μl/min using a syringe pump (ESP-32, Eicom Co.). Contents of 5-HT and DA in the perfusate were measured by HPLC with a reverse-phase column (PP-ODS, Eicom Co.) and an electrochemical detector (ECD-300, Eicom Co.). The mobile phase consisted of: 2.1 mM sodium 1-decansulfonate, 0.1 mM EDTA-2 Na, 0.1 M phosphate buffer and 1% (v/v) methanol (pH 6.0). A working electrode was maintained at +400 mV, and the flow pump rate was set at 0.5 ml/min. A typical example of HPLC chromatogram is shown in supplementary Fig. S1; dopamine and 5-HT were clearly separated without overlapping. Standard curve (supplementary Fig. S2) indicated that the measured signal (AUC; area under cover, in min x nA) was proportionate to the concentration of dopamine and 5-HT in a range of 0.01 to 1 ng/ml, which covered the range of perfusate samples obtained in this study. For statistical comparisons, non-parametric tests were used at a significance level set at $p < 0.05$.

2.4.3. Histological verification

After the microdialysis experiment, the probe location was marked by injecting dye (0.1% pontamine sky blue, ca.10 μl). The dialysis probe was replaced with another probe with a small hole at the tip, and the dye was injected by pressure. Chicks were anaesthetized by an injection of ketamine – xylazine cocktail (0.5 ml of a 1:1 mixture), and transcardially perfused with a fixative (4% paraformaldehyde in 0.1 mM PB, pH 7.2, 50 ml). Brains were dissected out of the surrounding tissue, post-fixed at 4°C for 1–7 days, embedded in egg yolk, and cut into 50 μm thick frontal sections by using a

vibrating microslicer (DTK-1000, Dosaka EM, Japan). Sections were mounted on aminopropylsilane (APS)-coated slide glasses, dried, stained with cresyl violet and imaged by a digital scanner.

3. Results

3.1. Behavioural effects of FLV

3.1.1. *Residence time* in naïve chicks (experiment-1)

The mean ***residence time*** was shorter in {0.5, ×1.2} than that recorded in {2.0, ×1.1} irrespective of the food amount (1 grain or 2 grains) (Fig. 4A, B). Without interval increments, chicks left the *ON* feeder after the food supply was completed (vertical dashed line, {2.0, ×1.0}) or immediately after the first or second grain ({14.0, ×1.0}). Statistical analyses were thus performed for the other 4 groups of chicks, namely {0.5, ×1.2} and {2.0, ×1.1} for 1 and 2 grains per delivery. Based on mean residence time, AICs (Akaike Information Criteria) were compared among the 4 models in which the variables of *pattern* ({0.5, ×1.2} or {2.0, ×1.1}) and food amount (1 or 2 grains) were considered (Table 1). The model with the *pattern* variable was chosen for its smallest AIC (72.95), and the model with the second smallest AIC (73.44) contained both *pattern* and *grain* variables, although the coefficient of *grain* (as indicated in parentheses) could contain 0 value at considerably high probability (p>0.05), thus the variable *grain* was not supposed to be significant. . The null model gave rise to the 3rd smallest AIC (86.88), and the model with the variable grain yielded an even larger AIC (88.12), indicating that the variable *grain* should not be considered. The present results are therefore compatible with one prediction of the optimal patch-use model (Fig. 3A), that the chicks leave earlier in the higher increment rate (×1.2 rather than ×1.1).

However, the results did not match with another prediction (Fig. 3B), that chicks leave earlier in the larger gain rate (2 grains rather than 1 grain). In accordance with mean ***residence time*** (Fig. 4B), the mean ***leave-point interval*** (Fig. 4C) was shorter in {0.5, ×1.2} than in {2.0, ×1.1} irrespective of the food amount.

Figure 4 and Table 1 around here

The mean ***travel time*** (or the time spent in the *OFF* feeder) was also compared with the respective ***waiting time*** (or the time to the next delivery at the *ON* feeder) (Fig. 4D); symbols denote mean values in individuals. In both 1 grain and 2 grain trials, the ***waiting time*** tended to be longer than the ***travel time***, even though the more profitable option (the food available by revisit after a travel time) was temporally more proximate. Chicks waited longer for the next grain particularly in the condition of {0.5, ×1.2}. Most probably, chicks behaved retrospectively based on the time after the last grain was delivered, rather than based on prospective food gains.

3.1.2. Effects of FLV on *residence time* (experiment-2)

To examine if the enhancement of endogenous 5-HT level could acutely or chronically affect patch-use behaviour, we examined FLV effects for 3 successive days. A FLV solution (or vehicle) was injected (i.p.) 1 hour before the test of each day. We found that FLV elongated the mean ***residence time*** in both conditions acutely from the first day, but only at a high dose (20 mg/kgBW) (Fig. 5A). At a low dose (10 mg), however, significant effects were not found. We did not examine higher doses (40 mg or higher), because pilot trials revealed that higher FLV doses often caused chicks to sleep. In the following experiments, we therefore examined the effects of 20 mg FLV. Based on residence time, AICs were compared among the 4 models in which the variables of *drug* (vehicle, 10 or 20 mg/kg BW) and *day* (10–12) were considered (Tables 2 and 3).

Of these models, the full model yielded the smallest AIC (375.9 and 630.1 for the {0.5, ×1.2} and {2.0, ×1.1} conditions, respectively), and the coefficients of both variables (*drug* and *day*) included 0 value at probability $p<0.05$, thus the *drug* effect was supposed to be significant. On the other hand, the models composed only with the *day* variables yielded a larger AIC (380.3 and 633.3, respectively), and AICs of the null models were even larger (394.7 and 645.0, respectively); significant drug effects were thus confirmed in both conditions of {0.5, ×1.2} and {2.0, ×1.1}.

Figure 5, Tables 2 and 3 around here

3.1.3. Effects of FLV on inter-temporal choices (experiment-3)

FLV suppressed choice impulsiveness (Fig. 5B), as the number of choices of the large reward (***LL***) were higher in the 20 mg group than in the vehicle control group. Based on the number of choices of the large reward, AICs were compared between the two models in which the variable *drug* (vehicle or 20 mg) was included or not (Table S1). The *drug* model had an AIC (46.74) that was smaller than the null model (49.09), and the *drug* coefficient included 0 value at $p<0.05$, thus the drug effect was supposed to be significant.

3.1.4. Effects of FLV on work efforts in shuttles (experiment-4)

FLV decreased running distance and running velocity, but the number of visits, total residence time and residence time per visit were not influenced (Fig. 5C). For each parameter, AICs were compared among the 4 models in which the variables *drug* (vehicle or 20 mg/kg BW) and *day* (9–11) were considered (Tables S2–S6). For *total residence time*, the null model was chosen for its smallest AIC. For *residence time per visit*, the model with the *day* variable was chosen. On the other hand, for *running distance* and *velocity*, full models were chosen with significant coefficients for the *drug*

variable. For the *number of visits*, the full model was chosen. Taking all of these estimations into account, it is concluded that FLV suppressed the *total running distance* by mainly slowing down the *velocity*, but the decision to leave the uncued food patches was not influenced, in a clear contrast to FLV effects on *residence time* at the diminishing patch (**experiment-2**).

3.1.5. Effects of FLV on distress calls (experiment-5)

FLV suppressed the number of distress calls, suggesting a general soothing effect. Two-way ANOVAs revealed significant effects on ***Δ distress calls*** (Fig. 5D) of dose (F_{dose} (2, 30) = 15.11, $p < 0.0001$), but not of day of experiment (F_{day} (1, 30) = 3.47, $p > 0.05$) without interaction ($F_{dose\ x\ day}$(2, 30) = 0.27, $p > 0.05$).

3.2. Pharmacological effects of FLV on 5-HT and DA in the striatum

3.2.1. Location of the microdialysis probes

Histological reconstruction of tracks revealed that the microdialysis probes were located in the mSt and surrounding tissues in all of the chicks studied (Fig. 6). These locations extended over the core and shell regions of the nucleus accumbens [30,31]. In some chicks, the stained track was found in the lateral part of the bed nucleus of the stria terminalis and the ventral pallidum sub-regions of the basal ganglia ventromedial to the mSt [32]. These regions in the basal ganglia are densely innervated by 5-HT positive fibers [23].

Figures 6 and 7 around here

3.2.2. Microdialysis and systemic application of FLV (experiment-6)

Intra-peritoneal injection of FLV (20 mg/kg BW) elevated concentrations of both 5-HT and DA in the perfusate. Samples were collected from chicks housed in an

illuminated chamber not equipped with feeders. Notice that the subject chicks did not forage food, nor did they actively walk around. Fig. 7Aa and Ac indicate the time-course of relative concentrations measured as % of the reference level (sample #0 indicated by an arrowhead). AUCs did not significantly differ between groups (Fig. 7Ab and Ad); $U = 17$, $p > 0.05$ for 5-HT; $U = 13$, $p > 0.05$ for DA (Mann–Whitney's U-test, $n_1 = n_2 = 6$). After the injection (samples #1 to #4), on the other hand, significant differences were found; $U = 0$, $p < 0.005$ for 5-HT; $U = 0$, $p < 0.005$ for DA. It is thus suggested that FLV could have an inhibitory effect on 5-HT re-uptake, but the effect was not specific, as the DA level was also elevated. The injected FLV could indirectly increase the DA level via unspecified pathways, or otherwise FLV (or the elevated 5-HT) directly enhanced DA release (or re-uptake mechanisms) in the striatum. We therefore conducted a reverse dialysis experiment, in which FLV was applied to the perfusing aCSF solution.

3.2.3. Reverse microdialysis and local application of FLV (experiment-7)

Similar effects were found in the reverse microdialysis. FLV was added to the perfusate twice at 2 different doses (2 and 20 μM at sample #1 and #5, respectively), and considerable increases were found in 5-HT and DA after 20 μM (Fig. 7Ba and Bc). AUCs were compared among 3 phases of sampling, namely, samples #-4 to #-1 (aCSF), #1 to #4 (2 μM), and #5 to#8 (20 μM) by using Friedmann's tests ($k = 3$ blocks of treatments, $m = 8$ individuals); $S = 86$ ($p < 0.01$) for 5-HT, and $S = 78$ ($p < 0.01$) for DA, respectively. We therefore conclude that FLV elevated 5-HT and DA locally in the striatum.

4. Discussion

We designed the present experiments to mimic optimal patch-use behaviour in the field. The residence time was dependent on the pattern of food supply (***pattern***) as expected, but not on the amount (***grain***) (**experiment-1**; Fig. 4A-C). Furthermore, the ***travel time*** was generally shorter than the ***waiting time*** at the feeder (Fig. 4D); clearly, chicks did not consider the larger gratification obtainable by leaving the patch as an alternative option. We therefore agree with Stephens and Anderson [9] that inter-temporal choice data do not give a good account for understanding patch-use behaviour.

We expected to find that chicks with a systemic FLV application would leave the depleting feeder earlier than chicks with a vehicle control (Fig. 1), under the premise that the decision to leave a food patch is identical to the choice of the large/long-delay option in the inter-temporal choices. FLV actually suppressed choice impulsiveness (**experiment-3**; Fig. 5B), confirming previous studies (see below for further discussion). However it is to be noticed that the effect of FLV on choices might be ascribed to altered sensitivity to delay or to amount of the food reward. We should have examined whether FLV modified the choices solely based on food amount, while the delay was identical. Further experiments should be added in future. FLV elongated residence time, contrary to our initial expectation (**experiment-2**; Fig. 5A). FLV also suppressed the running distance and velocity in the work effort test, as well as the distress calls induced by social isolation (**experiment-4** and **-5**; Fig. 5C, D), suggesting a spectrum of soothing effects. *In vivo* microdialysis revealed an increase in both 5-HT and DA by systemic and local application of FLV (**experiment-6** and **-7**; Fig. 6, 7), suggesting the possibility that the systemic FLV directly affected the medial striatum. It is notable however that the dialysis experiments were performed without any tasks, whereas the

behavioural effects of FLV were examined in chicks that were actively foraging in the maze. The increases in 5-HT and DA by FLV may appear differently under the behavioural conditions.

It is therefore concluded that (1) the present behavioural experiments only partially reproduced optimal patch-use behaviour. (2) FLV suppressed impulsiveness in the inter-temporal choices, and (3) it also made chicks tolerant of the delay in the next food gained in the patch. Finally, (4) these behavioural effects of FLV might be mediated by enhanced action of either or both of 5-HT and DA in the medial striatum.

4.1. 5-HTergic control of motor activities and residence time

FLV suppressed running distance and velocity in this study (Fig. 5C), suggesting that acutely enhanced 5-HT level decreases motor activities. In accordance with this finding, a previous report showed that a $5HT_{1}$ and $_{2}$ agonist (lysergic acid diethylamide) decreased the exploratory locomotor activity of rats (see review by Geyer [33]). However, behavioural effects of 5-HT may be dependent on the context. SSRI induced hyperlocomotion in mice when tested in a novel environment, but not in a habituated environment [34]. In chicks, FLV effects should be examined in other contexts such as spontaneous motor activity in an open field without food.

On the other hand, FLV effects on residence time and the inter-temporal choices (Fig. 5A and B) may be explained differently, whereby 5-HT enhances the tolerance for delay of the forthcoming reward. The present findings are in concert with previous studies of behavioural pharmacology ([10,11] for acute effects of 5-HT uptake inhibitor, [11,14,15] for 5,7-DHT infusion to dorsal raphe) and a recent neurophysiological analysis of raphe neurons ([35]; also see [36] for a microdialysis study in the dorsal

raphe nucleus). It must however be noticed that the acute effects of SSRIs have not been reproduced in inter-temporal choice tests using rats, in which subjects made choices by lever-pressing [12,13]. In these studies, the discrepancy with Bizot et al. [10] has been ascribed to different task conditions.

Acute effects of SSRIs have also been questioned in a study using pigeons [37]. They adopted behavioural titration for inter-temporal choices and examined a series of SSRIs (fluoxetine, citalopram and paroxetine; systemic application), but found only chronic effects that appeared after consecutive application for 17 days. The present results, on the other hand, revealed acute effects of FLV in chicks (Fig. 5B). The discrepancy may be ascribed to the different affinities of these SSRIs to the avian SERT [19]. While FLV strongly inhibits the chicken SERT (1.2 times in terms of K_i compared to human SERT), the SSRIs used [37] had lower levels of inhibition (fluoxetine 5.6, citalopram 41 and paroxetine 41, respectively).

4.2. 5-HT and interval timing (or retrospective / immediate sense of time)

Involvement of DA in the control of the internal clock has gained substantial experimental support (see reviews by Meck [38,39]). Haloperidol, a D_2 receptor antagonist, is reported to delay the internal clock [40]. It is assumed that the corticostriatal circuit responsible for the internal clock is under speed-control by DA actions from the substantia nigra. However, significant action of 5-HT on the timing mechanism is questioned. Ho et al. [41] review recent studies of 5-HT actions on interval timing. It is notable that 5-HT depletion by 5,7-DHT application increases the Weber fraction of the timing behaviour. However, they argue that 5-HT does not change the speed of the internal clock, but is involved in behavioural switching. Ho et al. [42]

actually report that systemic FLV does not influence indices of behavioural timing tested by a fixed-interval peak procedure.

It might be argued that the FLV effect on residence time (Fig. 5A) is explained by its action on interval timing, particularly through the unexpected increase in DA level by FLV (Fig. 7). However, the enhanced DA level should shorten the residence time by over-estimation of the perception of time, if its action is similar to amphetamine [39]. It is thus not plausible that the observed effects are due to the accompanying DA increase. In conclusion, we may suppose that the perception of interval timing was not influenced by FLV in the present study.

4.3. DA release through 5-HT enhanced by FLV

Systemic application of FLV increased not only the 5-HT but also the DA level in the striatum (Fig. 7A), which was reproduced in the reverse dialysis experiment (Fig. 7B), indicating that FLV increased DA locally in the striatum. As reviewed by Navailles and Deurwaedere [43], it is possible that the enhanced 5-HT could directly act on the dopaminergic terminals, making the DA transporter (DAT) reverse the direction of dopamine release. A similar mechanism may underlie the DA increase by FLV found in the present study. Alternatively, it is possible that FLV directly acted on the DAT and inhibited the DA uptake. Although data are not available on the avian DAT, a biochemical study has revealed that binding of FLV to human DAT is negligibly small [44]. The cellular mechanisms underlying the DA release by FLV need further investigation.

Co-release of 5-HT and DA by a 5-HT reuptake blocker (alaproclate) has already been reported in an *in vivo* experiment [45] and also in *in vitro* preparations of striatum

using an SSRI (fluoxetine, [46]), but the functional significance remains unknown. Further detailed experiments are needed to reveal the functional significance of the cross talks between 5-HT and DA.

Conclusions

Systemic application of FLV caused chicks to stay longer at a gradually depleting food patch. FLV also suppressed impulsiveness in inter-temporal choices, suggesting that an enhanced 5-HT level made chicks more tolerant of the delayed food reward in both behavioural paradigms. On the other hand, the decision to leave a depleting patch cannot be equated to the choice of the large/long delay option of the inter-temporal choice paradigm. The microdialysis study revealed that FLV enhanced the extracellular concentration of 5-HT as well as DA in the medial striatum/nucleus accumbens. In addition to 5-HT, DA in the striatum may be involved in tolerance control.

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Figure legends

Figure 1. Predicted effects of the fluvoxamine (FLV) on inter-temporal choice and patch-use behaviour

If the common self-control mechanism underlies the inter-temporal choice and the patch-use behaviour, the FLV-treated chicks will leave a depleting patch earlier (or, a shorter residence time; right diagram) to gain food from the next patch. This prediction comes from the assumed effect of FLV on the inter-temporal choice (left diagram), suggesting that the FLV-treated chicks will prefer a large and delayed reward to a small and immediate reward.

Figure 2. Apparatus and timing of food delivery for the study of patch-use behaviours

(A) Schematic illustration of the apparatus. An I-shaped maze was composed of two feeders; *ON* feeder (red in this figure) served as a food patch, and *OFF* feeder (blue) did not supply food. As the chick arrives, the *ON* feeder starts to deliver food (1 or 2 grains of millet for each delivery) in a programmed manner as shown in (B). The *OFF* feeder similarly responds to the chick and terminates the food delivery at the *ON* feeder on the opposite side, but food is not delivered. (B) Cumulative gain (number of grains of millet) is plotted against the residence time at the *ON* feeder. Figures in parentheses {α, xβ} indicate the initial interval α (sec), and the increment rate β. Four patterns of depletion were programmed up to a total of 25 (a) or 50 (b) grains. Two other patterns of a constant rate (β = 1.0) were also examined at short (2.0 sec) or long (14.0 sec) intervals. (C) Time course of food deliveries and chick's behaviour. Each ball represents delivery of a grain of millet; note the gradually increasing intervals. *ON* and *OFF*

denotes the *ON* feeder and the *OFF* feeder, respectively. Leaving the *ON* feeder stopped the food deliveries, and visiting the opposite *OFF* feeder reset the interval of the *ON* feeder to the minimum value (0.5 sec or 2.0 sec).

Figure 3. Patch-use behaviour and inter-temporal choice

If chicks behave optimally, we will find that the residence time is shorter in {0.5, ×1.2} than that in {2.0, ×1.1}(A), and shorter in 2 grains than in 1 grain (B), depending on the travel time that is assumed to be 30 sec in these graphs.

Figure 4. Behaviours in depleting food patches (experiment-1)

(A) Residence time at the food patch distributed with large variance. Histograms of residence time under different conditions are shown. Residence time was measured in 20 consecutive counts for each chick, and the total 120 counts (=20 × 6 chicks) were merged; data obtained on the last day (day 12) are shown. Vertical dashed lines indicate the terminal point at which the last grain was supplied. (B) Mean residence times of the four patterns are compared; error bars indicate S.E.M. (C) Leave-point interval. (D) Travel time (time until the next visit to the *ON* feeder) is plotted against waiting time (time to the next grain the chick could have gained if it stayed at the feeder); symbols represent individuals.

Figure 5. Behavioural effects of FLV (fluvoxamine) on residence time (A), inter-temporal choices (B), work efforts (C), and distress calls (D)

(A) Systemic injection of FLV resulted in a longer residence time. (B) FLV suppressed impulsiveness in the inter-temporal choices, but the difference was only suggestive (see

text for statistics). (C) FLV decreased running distance and running velocity, but residence time per visit, total residence time and number of visits were not influenced. Means (±S.E.M.s) are plotted for the day of experiment. (D) Increase in the number of distress calls (per 3 min) elicited by social isolation.

Figure 6. Location of microdialysis probes in the striatum

(A) Representative photomicrograph of a track on a Nissl-stained section. Arrow heads point to dorsal and ventral edge of the probe stained by post-experimental injection of dye (0.1% pontamine sky blue). (B, C) Superimposed locations of probes are shown on frontal sections of telencephalon. N: nidopallium; lSt: lateral striatum; GP: globus pallidus; mSt: medial striatum.

Figure 7. Pharmacological effects of FLV on 5-HT and DA in the striatum

(A) Systemic injection of FLV (20 mg/kg BW) elevated the level of (a) 5-HT and (c) DA. Values are expressed as percent change relative to baseline (sample #0) as mean ± S.E.M. FLV (or vehicle) was intra-peritoneally injected at the beginning of the sample period #1 (arrowhead). The percent concentrations in 4 consecutive samples (namely 80 min) were summated to yield the area under curves (AUC) in b and d (mean ± S.E.M.); horizontal dashed lines indicate the baseline. AUCs were significantly different between FLV and vehicle only after the injection. (B) Local application of FLV through the probe revealed similar effects on extracellular 5-HT DA, suggesting direct action in the striatum. Time course and AUC of extracellular 5-HT and DA levels are similarly shown. AUCs were compared among three different phases of samples; samples #-4 to #-1 (aCSF), #1 to #4 (2 μM), #5 to #8 for (20μM), and significant differences were

found among these phases. Note also that the effects on DA were reproduced.

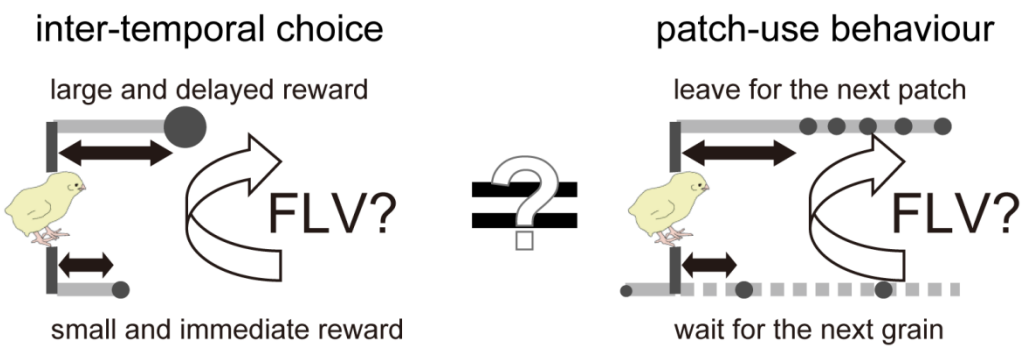
inter-temporal choice
large and delayed reward
FLV?
small and immediate reward
patch-use behaviour
leave for the next patch
FLV?
wait for the next grain

Fig. 1.

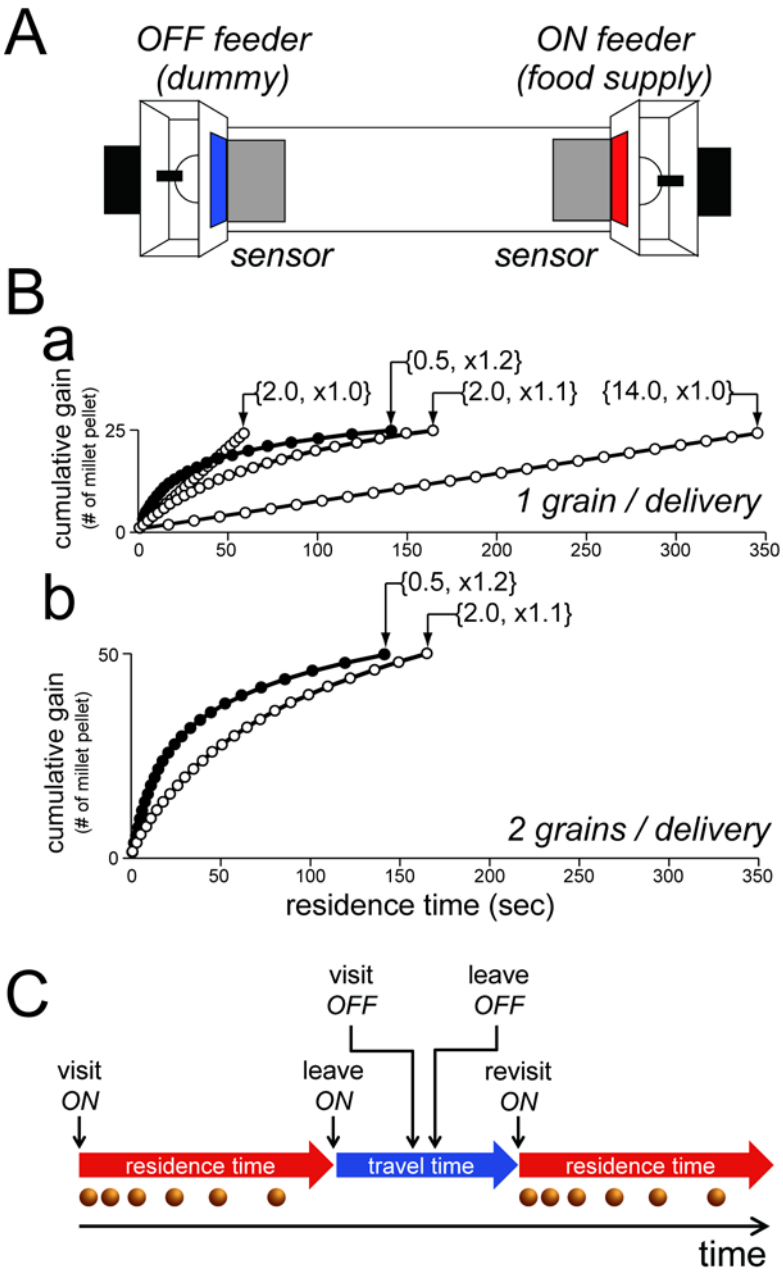
A
OFF feeder
(dummy)
ON feeder
(food supply)
sensor
sensor
B
a
cumulative gain
(# of millet pellet)
{2.0, x1.0}
{0.5, x1.2}
{2.0, x1.1}
{14.0, x1.0}
1 grain / delivery
b
{0.5, x1.2}
{2.0, x1.1}
2 grains / delivery
residence time (sec)
C
visit
ON
visit
OFF
leave
ON
leave
OFF
revisit
ON
residence time
travel time
residence time
time

Fig. 2.

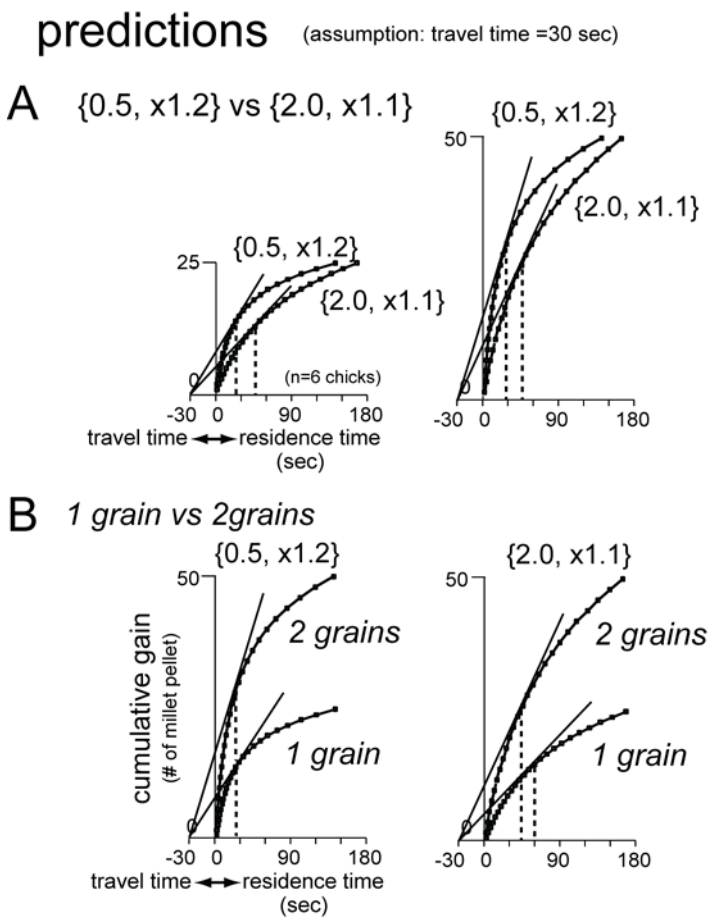
predictions
(assumption: travel time =30 sec)
A
{0.5, x1.2} vs {2.0, x1.1}
{0.5, x1.2}
{2.0, x1.1}
25
0
(n=6 chicks)
-30
0
90
180
travel time
residence time
(sec)
{0.5, x1.2}
{2.0, x1.1}
50
0
-30
0
90
180
B
1 grain vs 2grains
{0.5, x1.2}
{2.0, x1.1}
50
cumulative gain
(# of millet pellet)
2 grains
1 grain
-30
0
90
180
travel time
residence time
(sec)

Fig. 3.

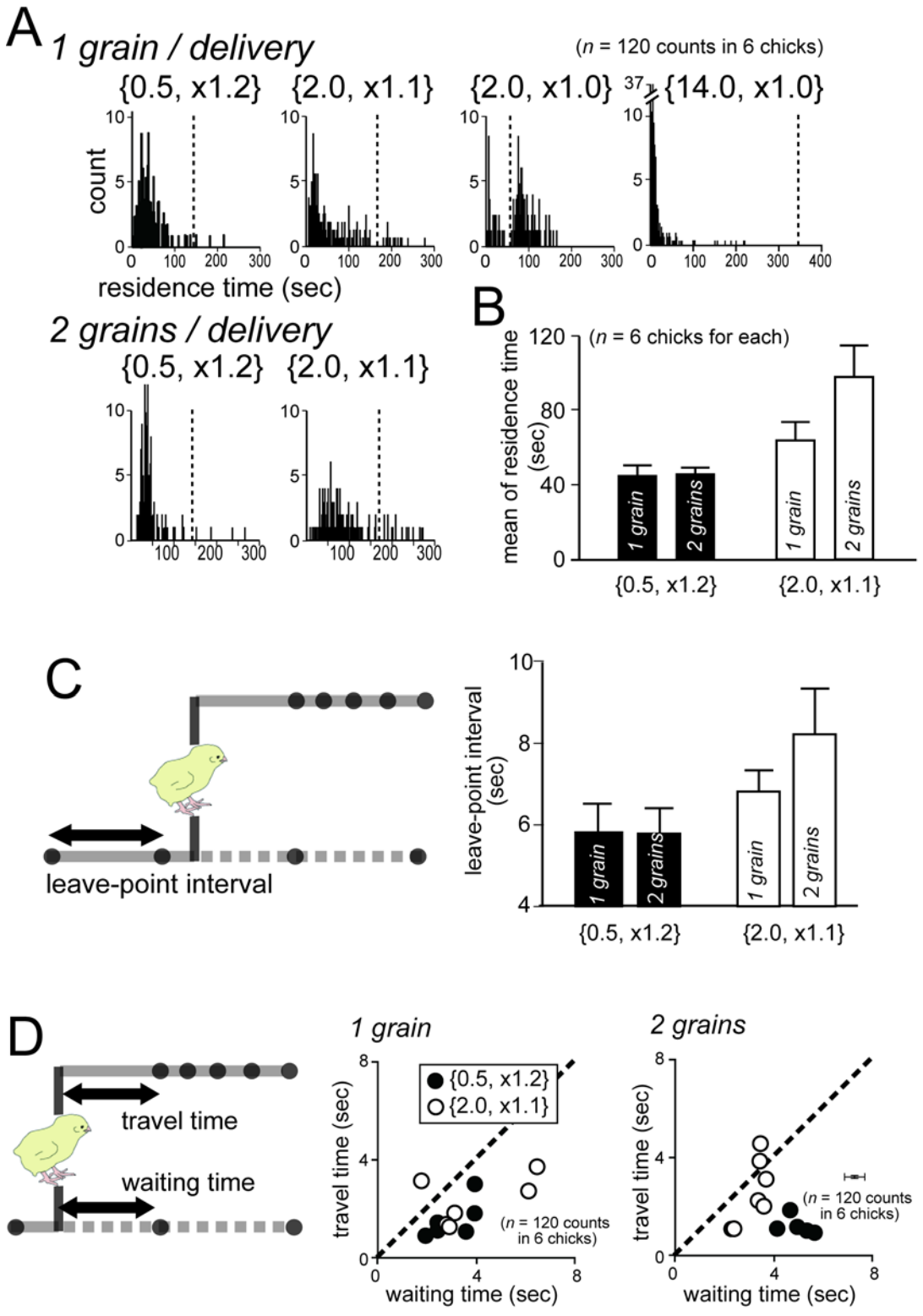
A
1 grain / delivery
(n = 120 counts in 6 chicks)
{0.5, x1.2}
{2.0, x1.1}
{2.0, x1.0}
{14.0, x1.0}
count
residence time (sec)
2 grains / delivery
B
(n = 6 chicks for each)
mean of residence time (sec)
1 grain
2 grains
C
leave-point interval
D
travel time
waiting time
1 grain
2 grains
travel time (sec)
waiting time (sec)

Fig. 4.

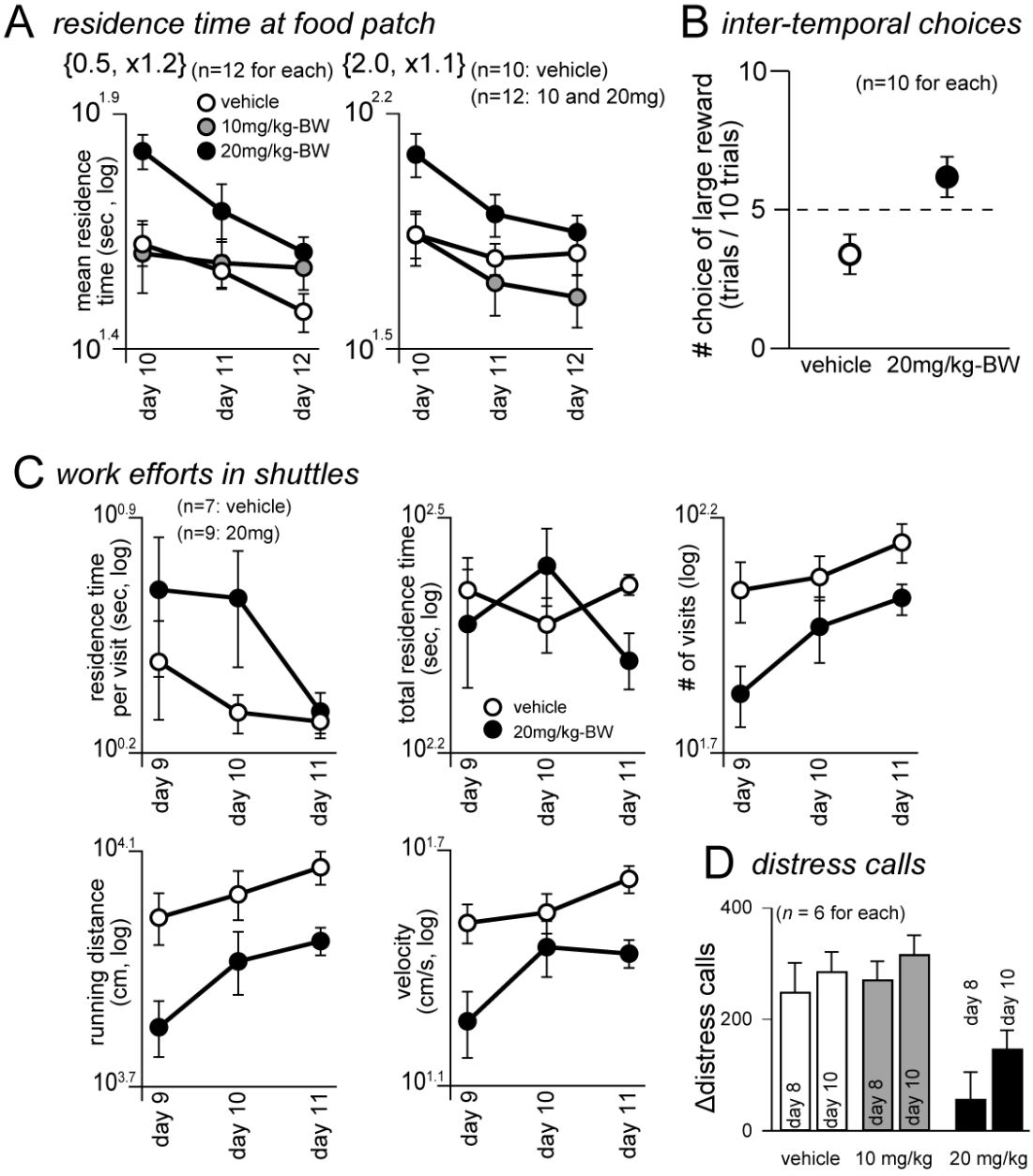
A residence time at food patch
{0.5, x1.2} (n=12 for each)
{2.0, x1.1} (n=10: vehicle)
(n=12: 10 and 20mg)
vehicle
10mg/kg-BW
20mg/kg-BW
mean residence time (sec , log)
day 10
day 11
day 12
B inter-temporal choices
(n=10 for each)
choice of large reward (trials / 10 trials)
vehicle
20mg/kg-BW
C work efforts in shuttles
(n=7: vehicle)
(n=9: 20mg)
residence time per visit (sec, log)
total residence time (sec, log)
# of visits (log)
running distance (cm, log)
velocity (cm/s, log)
day 9
day 10
day 11
D distress calls
(n = 6 for each)
Δdistress calls
day 8
day 10
vehicle
10 mg/kg
20 mg/kg

Fig. 5.

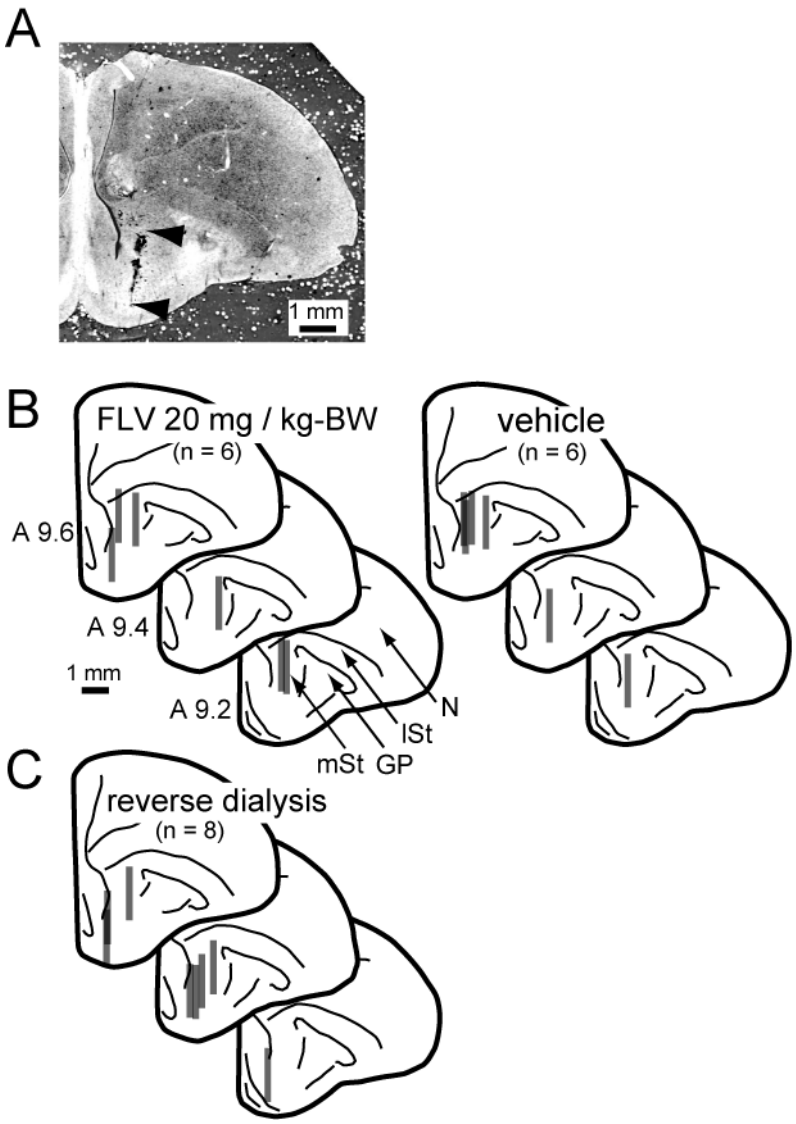
A
1 mm
B
FLV 20 mg / kg-BW
(n = 6)
vehicle
(n = 6)
A 9.6
A 9.4
A 9.2
1 mm
N
lSt
mSt
GP
C
reverse dialysis
(n = 8)

Fig. 6.

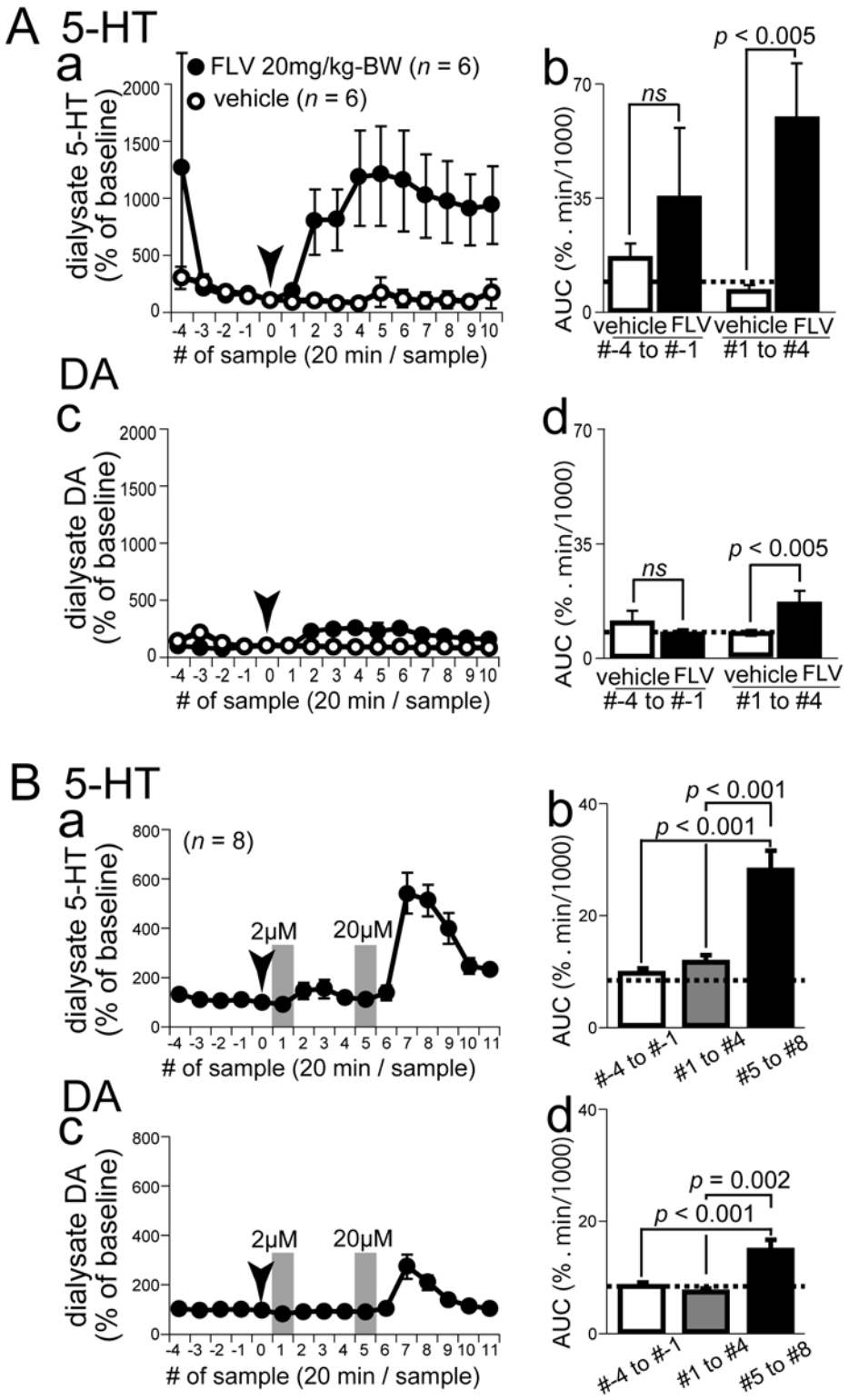
A 5-HT
a
FLV 20mg/kg-BW (n = 6)
vehicle (n = 6)
dialysate 5-HT (% of baseline)
# of sample (20 min / sample)
b
p < 0.005
ns
AUC (% . min/1000)
vehicle FLV vehicle FLV
#-4 to #-1 #1 to #4
DA
c
dialysate DA (% of baseline)
# of sample (20 min / sample)
d
ns
p < 0.005
AUC (% . min/1000)
vehicle FLV vehicle FLV
#-4 to #-1 #1 to #4
B 5-HT
a
(n = 8)
2μM
20μM
dialysate 5-HT (% of baseline)
# of sample (20 min / sample)
b
p < 0.001
p < 0.001
AUC (% . min/1000)
#-4 to #-1
#1 to #4
#5 to #8
DA
c
2μM
20μM
dialysate DA (% of baseline)
# of sample (20 min / sample)
d
p = 0.002
p < 0.001
AUC (% . min/1000)
#-4 to #-1
#1 to #4
#5 to #8

Fig. 7.

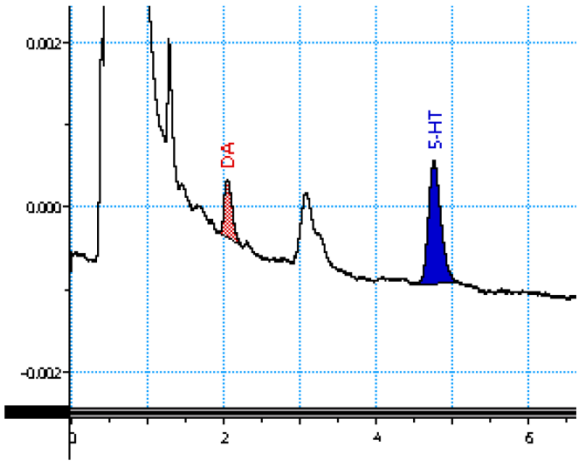

Fig. S1.

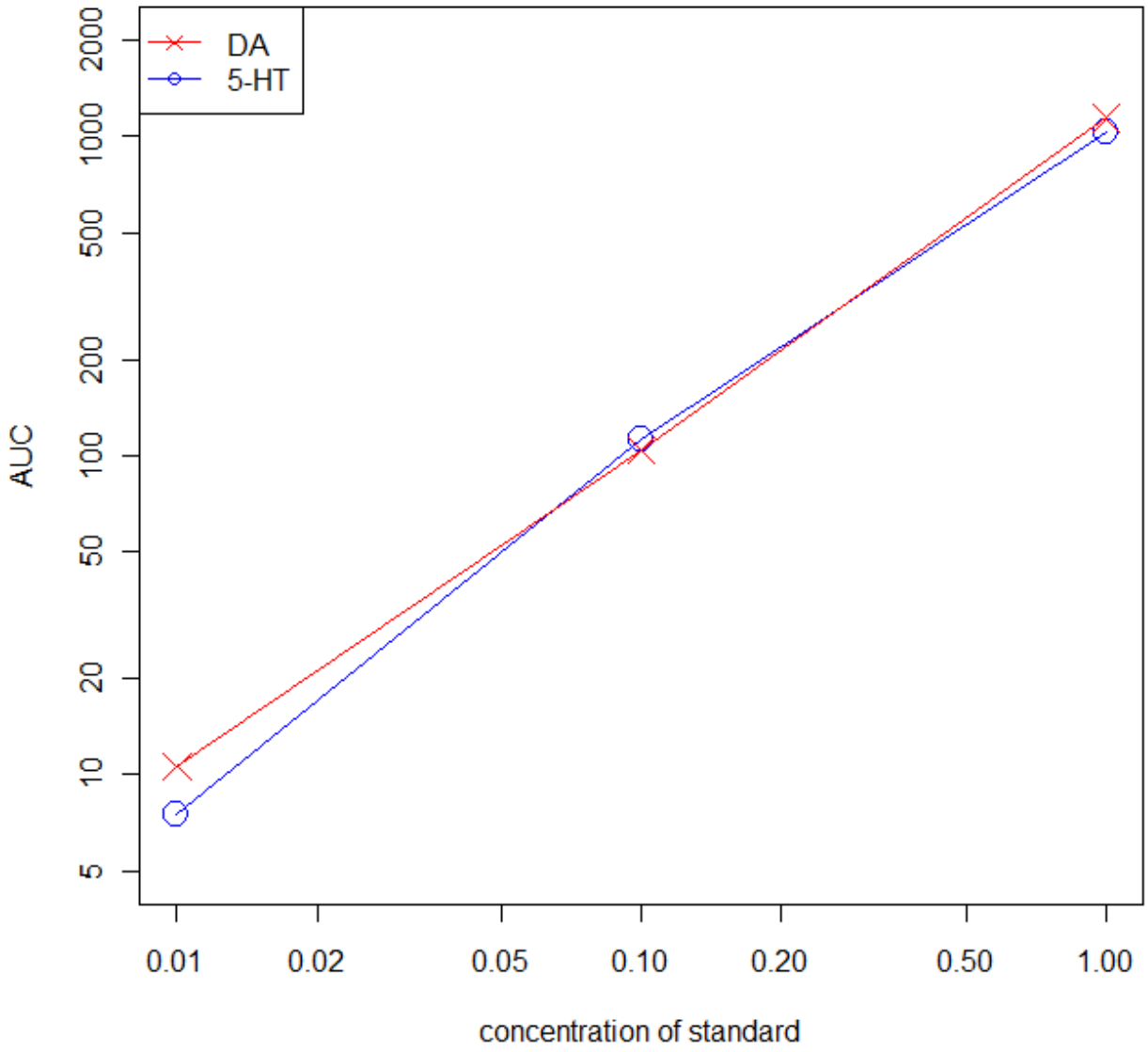
DA
5-HT
AUC
5
10
20
50
100
200
500
1000
2000
0.01
0.02
0.05
0.10
0.20
0.50
1.00
concentration of standard

Fig. S2.