UNIVERSITY OF CALIFORNIA SAN DIEGO

Regulation of Vesicular Glutamate Transporter 2 Transcription in Dopaminergic Neurons by High Dosage of Amphetamine in Mouse *Substantia Nigra pars compacta*

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Biology

by

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2018

The Thesis of Xinyi Shen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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University of California San Diego

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ABSTRACT OF THE THESIS

Regulation of Vesicular Glutamate Transporter 2 Transcription in Dopaminergic Neurons by High Dosage of Amphetamine in Mouse *Substantia Nigra pars compacta*

by

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Master of Science in Biology

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Parkinson Disease (PD) is known as one of the most common neurodegenerative diseases and affects 1%-2% of the population (1). Previous research suggests that PD is caused by the loss of dopaminergic (DA) neurons in substantia nigra pars compacta (SNc) in midbrain areas (6). The ability of DA neurons in some midbrain areas, such as ventral tegmental area (VTA), to endogenously

express vesicular glutamate transporter 2 (Vglut2) and co-release glutamate has been proved to increase the dopamine uptake in DA neurons (11). The dynamic regulation of Vglut2 expression in SNc DA neurons has an important influence in the cell survival. Firstly, in mammalian and *Drosophila melanogaster* PD models and when SNc DA neurons are injured, the endogenous upregulation of vesicular glutamate transporter 2 (Vglut2) is proved to be neuroprotective to the SNc DA neurons (6). However, heterologous expression of Vglut2 in SNc DA neurons causes the loss of these DA neurons. To explore the mechanism governing Vglut2 regulation in SNc DA neurons, we analyzed the co-localization level between Vglut2 messenger RNA (mRNA) and tyrosine hydroxylase (TH) mRNA as a marker for DA neurons with high dosage of amphetamine (AMPH) to acutely deplete dopamine in SNc DA neurons. We hypothesize that dopamine stress caused by high dosage of amphetamine will upregulate Vglut2 expression potentially as a compensatory way to increase dopamine releasing in SNc DA neurons.

Here, our results indicate that the co-localization rate between Vglut2 mRNA and TH mRNA increases after treatment of high dosage amphetamine to DA neurons in the SNc. The increased co-localization rate suggests that dopamine depletion increases the number of SNc DA neurons co-expressing Vglut2. With knowing the potential mechanism governing Vglut2 dynamic regulation in SNc DA neurons, we might have a clearer understanding the role of Vglut2 expression in DA neuron loss in PD and thus provide potential new strategies in PD intervention.

Chapter 1: Introduction

1.1: Parkinson's Disease

Parkinson's disease (PD) is known as the second most common neurodegenerative disorder. While the genetic mutation only account for a small portion of all cases, the non-genetic factors plays a bigger part – though not excluding the chance interacting with susceptible genes- to cause PD (1). The primary reason to cause PD is the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) in midbrain area (2).

1.2: The Function of SNc Area

SNc is one of the two parts of substantia nigra, which contains the A9 group of DA neurons in midbrain area (3). The most prominent function of SNc is the regulation of locomotor activity, although in an indirect way: via D1 pathway, SNc sends excitatory input to striatum, which in turn release GABA to inhibit the inhibitory function of globus pallidus on thalamic nucleus (4-8). By lesion the SNc, the movement of animal subjects is largely influenced, which is evidenced by symptoms of PD (9-10).

1.3: The Role of Dynamic Regulation of Vglut2 in SNc DA Neurons Survival

Currently, there are two competing theories in explaining the loss of SNc DA neurons in PD: Lewy pathology and mitochondrial dysfunction (11). However, the dynamic regulation of vesicular glutamate transporter2 (Vglut2) and accompanied

glutamate co-releasing might have an important influence in the selective vulnerability of SNc DA neurons in PD (12, 18). It has been proved that a subset of midbrain DA neurons, such as DA neurons in VTA, has the ability to co-release glutamate and express Vglut2 in adult mammal models, including mouse, rate, primate, and human (12-14). More specifically, Vglut2 is present in a high level in ventral tegmental area (VTA) under normal condition, but absent in other areas in midbrain, including substantia nigra (15-17). The endogenous expression of Vglut2 is shown to increase dopamine uptake in DA neurons (12). Moreover, the endogenous expression of Vglut2 is proved to be neuroprotective to SNc DA neurons, because more SNc DA neurons are subject to lost in Vglut2 knock-out mouse compared to wild type mouse after SNc DA neurons are injured (18). However, heterologous expression of Vglut2 in SNc causes the loss of the DA neurons (18). Although the dynamic regulation of Vglut2 has been proved to have an important influence in SNc DA neurons, the mechanism that governs the regulation of Vglut2 still remain unclear.

1.4: Dopamine Depletion Mechanism of Amphetamine

Amphetamine (AMPH) is a central nervous system stimulant, which has been used to treat attention deficit hyperactivity disorder and narcolepsy, by depleting monoamines and increasing monoamines– including norepinephrine and dopamine-actions by blocking their reuptake pathway (19). In DA neurons, AMPH either directly diffuse through the membrane, or competes with endogenous dopamine for transporting back into the presynaptic terminals via dopamine transporter (DAT) (20-21). As a substrate for the presynaptic vesicular monoamine transporter2 (VMAT2), AMPH is

uptaken by VMAT2 into vesicles and due to its weak basicity, causes pH gradient collapse in the vesicle and thus result in dopamine release via VMAT2 (22-23). AMPH also activates trace amine-associate transporter1 (TAAR1) and thus phosphorylates DAT via protein kinase A (PKA) and protein kinase C (PKC) signaling (21). DAT phosphorylation by PKA results in DAT internalization, which leads the phosphorylated DAT ceases to reuptake dopamine from synaptic cleft (21, 25). DAT phosphorylation by PKC either causes DAT internalization, or reverses the dopamine transporting direction of DAT (21, 24, 25). Moreover, DAT phosphorylation also increases intracellular calcium level through Ca2+/calmodulin-dependent protein kinase II (CAMKII)-dependent pathway, resulting in dopamine efflux (25-29).

1.5: Hypothesis of Potential Mechanism Governing the Regulation of Vglut2 Expression

Since amphetamine significantly interfere the dopaminergic neurons functioning by causing dopamine stress, including acute dopamine depletion in DA neurons (18), we hypothesize that acute treatment of high dosage amphetamine will upregulate the Vglut2 expression potentially as a compensate method to increase dopamine releasing in the affected SNc DA neurons. Moreover, in order to further explore the influence of high dosage amphetamine on regulation of Vglut2 in SNc DA neurons on different time scales, we analyze the Vglut2 co-expression rate of DA neurons after 72 hours and 240 hours, respectively, after the last amphetamine injection.

Chapter 2: Results

2.1: Upregulated Expression of Vglut2 in Adult SNc DA neurons 72 hours after Amphetamine Treatment

2.1.1: Visualization of TH and Vglut2 mRNA in SNc Area and SNr Area

Amphetamine was injected in mouse abdomen 4 times with 2hours apart during each injection and given 72 hours since the last injection to let it fully function in mouse brain. We used RNAscope to detect TH and *Slc17a6* (Vglut2) transcripts in the midbrain (Figure 1-4). In SNc area, we detected three different signal patterns, which are: dopaminergic neurons with Vglut2 expression, dopaminergic neurons without Vglut2 co-expression, and only Vglut2 expression without TH signal (Figure 2). In SNr area, we only detected the Vglut2 signal without TH signal (Figure 4).

2.1.2: Distribution of Vglut2 Co-Expression Level in the SNc DA Neurons

The number of dopaminergic neurons was not significantly reduced with the treatment of amphetamine (Figure 5.1), but the percentage of dopaminergic neurons co-expressing Vglut2 increased by around 15% after treatment with amphetamine (Figure 5.2). The number of *Vglut2+/Th+* neuron increased in all the level of Vglut2 expression, except for the level of 20+ puncta (Figure 6). The average number of Vglut2 puncta per DA neuron is similar between the animals 72 hour after amphetamine injection or saline injection (Figure 7), but we excluded all the DA neurons expressing the number of

Vglut2+ puncta over 20, because of the impracticality to count for the actual number of puncta in each DA neurons. Furthermore, the distribution of *Vglut2*+ puncta per *Th*+ neurons has no significant shift (Figure 8).

2.1.3: Distribution of the Rate of *Vglut2+/Th*+ Neurons across Different Locations in SNc Area

Besides the distribution of Vglut2 co-expression level by counting the number of Vglut2 puncta overlapping within each DA neuron, we also analyzed the percentage of *Vglut2+/Th*+ neurons across different location in SNc area from rostral to caudal. The percentage of *Vglut2+/Th*+ neurons 72 hours after amphetamine treatment is higher than that of control group (saline injection), which indicates that acute amphetamine treatment increased the dopaminergic neurons co-expressing Vglut2 in all parts of SNc area (Figure 9). These data support the hypothesis that acute treatment of high dosage amphetamine leads to the transcriptional re-emergence of Vglut2 expression in adult SNc DA neurons.

2.2: Upregulated Expression of Vglut2 in Adult SNc DA neurons 240 hours after Amphetamine Treatment

2.2.1: Visualization of TH and Vglut2 mRNA in SNc Area and SNr Area

Amphetamine was injected in mouse abdomen 4 times with 2hours apart during each injection and given 240 hours since the last injection to let it fully function in mouse brain. We used RNAscope to detect TH and *Slc17a6* (Vglut2) transcripts in the midbrain (Figure 10-13). In SNc area, we detected three different signal patterns, which are: dopaminergic neurons with Vglut2 expression, dopaminergic neurons without Vglut2 co-expression, and only Vglut2 expression without TH signal (Figure 11). In SNr area, we only detected the Vglut2 signal without TH signal (Figure 13).

2.2.2: Distribution of Vglut2 Co-Expression Level in the SNc DA Neurons

The number of dopaminergic neurons remains similar with the treatment of amphetamine with no significantly increase or decrease (Figure 14.1), but the percentage of dopaminergic neurons co-expressing Vglut2 increased by around 15% after treatment with amphetamine (Figure 14.2). The number of *Vglut2+/Th+* neuron increased in all the level of Vglut2 expression (Figure 15). The average number of Vglut2 puncta per DA neuron is similar between the animals 240 hour after amphetamine injection or saline injection (Figure 16), but we excluded all the DA neurons expressing the number of *Vglut2+* puncta over 20, because of the impracticality

to count for the actual number of puncta in each DA neurons. Furthermore, the distribution of *Vglut2*+ puncta per *Th*+ neurons has no significant shift (Figure 17).

2.2.3: Distribution of the Rate of *Vglut2+/Th+* Neurons across Different Locations in SNc Area

Besides the distribution of Vglut2 co-expression level by counting the number of Vglut2 puncta overlapping within each DA neuron, we also analyzed the percentage of *Vglut2+/Th*+ neurons across different location in SNc area from rostral to caudal. The percentage of *Vglut2+/Th*+ neurons 240 hours after amphetamine treatment is higher than that of control group (saline injection), which indicates acute amphetamine treatment increased the dopaminergic neurons co-expressing Vglut2 in all parts of SNc area (Figure 18). These data support the hypothesis that acute treatment of high dosage amphetamine leads to the transcriptional re-emergence of Vglut2 expression in adult SNc DA neurons.

Chapter 3: Discussion

The RNAscope technique used in this experiment to visualize TH and Vglut2 signal function reliably, since TH signal is only detected in SNc area but not SNr area. Despite the minor decrease of the number of DA neurons in SNc after treated by amphetamine, we identified an absolute increase in the number of DA neurons co-expressing Vglut2 transcripts. These results indicate that amphetamine provokes a

transcriptional upregulation of cellular Vglut2 levels in adult SNc DA neurons, which might provide a hypothesis of the observed upregulation of Vglut2 in DA neurons in PD.

However, Vglut2 co-expression rate in this experiment is higher than the rate treated in 6-OHDA treatment (6). The Vglut2 co-expression rate in control group is around 10% higher and the Vglut2 co-expression rate treated by amphetamine is around 15% higher. The increased Vglut2 co-expression rate is possibly due to the counting criteria set up to distinguish real Vglut2 signal and background noise, given that even in control group, the co-expression rate is significantly higher than that of the 6-OHDA treatment.

There is no significant difference between the average numbers of puncta per DA neuron in the SNc area, which indicates that the Vglut2 co-expression level in each cell does not significantly increase. Furthermore, amphetamine did not cause significant distribution shift of Vglut2 co-expression level, which indicates that unlike 6-OHDA treatment cause the distribution of Vglut2 co-expression level shift rightward (6), amphetamine treatment cause an upregulation of Vglut2 co-expression in all the level of Vglut2 co-expression. Given the similar average number of Vglut2 puncta per neuron in the SNc area after amphetamine or saline injection, amphetamine causes an increase of the number of DA neurons co-expressing Vglut2 in the SNc, but without increasing the individual expressivity of Vglut2. This result that amphetamine treatment increases the number of DA neurons co-expressing Vglut2 is also consistent with the number of Vglut2 co-expression of DA neurons across different location in SNc area, which proves that the upregulation of Vglut2 co-expression of DA neurons take place in the whole SNc area. However, in 10-day waiting period, the Vglut2 co-expression level over 20

puncta for control group is significantly lower than that of 3-day waiting period. This significant lower amount of Vglut2 co-expression level over 20 puncta for control group in 240hr post-injection period might be caused by some uncontrolled variables, such as the specific time of the day when sacrificing the mouse, since there is no evidence to prove whether the Vglut2 expression within midbrain area is subject to circadian regulation. Besides, the difference in Vglut2 co-expression level over 20 puncta for control groups in two different post injection periods might be due to the different stress level the mouse had experienced. However, there is no evidence yet to prove that the stress level will affect the level of Vglut2 expression in SNc DA neurons.

Despite the fact that Vglut2 co-expression rate treated by amphetamine is significantly higher than the control group across the different location in SNc area, compared to the 3-day waiting period, amphetamine treatment caused the distribution of Vglut2 co-expression rate across different location of SNc area of the 10-day waiting period significantly more scattered. The more scattered distribution of Vglut2 co-expression rate might be also result from uncontrolled variables, including the time of the day sacrificing the animals and also possible different stress felt by each animal.

Generally, our results indicate that the acute treatment of high dosage amphetamine causes the upregulation of Vglut2 transcription in adult SNc DA neurons. Since the upregulation of Vglut2 to a high level in the adult is proved to be toxic to DA neurons in SNc area (Steinkellner et. al., 2018), the upregulation of Vglut2 co-expression caused by amphetamine might provide a hypothesis to explain the mechanism of Vglut2 upregulation. And since in human PD, the upregulation of Vglut2

is also observed, the upregulation caused by amphetamine might also contribute a potential explanation to the mechanism of Vglut2 upregulation in human PD.

Chapter 4: Methods

Mice. Mice were used in accordance with protocols approved by the UCSD Institutional Animal Care and Use Committee. Mice expressing Cre under control of DAT (*Slc6a3*^{IRESCre}, Jackson stock 006660) regulatory elements were obtained from The Jackson Laboratory and then bred in house. Mice were fully (>10 generations) crossed to C57BL/6. 9 females and 4 males were used; 5 of 9 females were used in experimental group and 2 of 4 males were used in experimental group. Mice were group-housed on 12-hour light/dark cycle, with food and water available ad libitum.

Amphetamine. The dosage of amphetamine was 10mg/kg of body weight. SIGMA D-amphetamine hemisulfate salt was used to dissolve in saline to prepare for 1.0mg/mL solution. The amphetamine solution was given in volume of 10mL/kg of body weight.

Amphetamine injections. DAT +/iCre or DAT iCre/iCre mice (8-10 weeks, n=7) were treated with amphetamine (Sigma D-Amphetamine; 2mg/ml), which was dissolved in saline via abdominal injection. Control mice (DAT +/iCre or DAT iCre/iCre, 8-10 weeks, n=6) were treated with saline in the volume of 10ml/kg of body weight. Each

animal is being injected 4 times with either amphetamine for experimental group or saline for control group. There was a 2-hour gap between each injection. After 4th injection, all animal was put back to vivarium and observed for 72 hours before brain extraction.

RNAscope. Mice were anesthetized with sodium pentobarbital with 1:10 dilution in saline. Brains were frozen in chilled isopentane. Brains were serially cut (20μm) on a cryostat with temperature of -20°C and mounted directly onto glass slides. Sections were stored at -80°C before starting of the RNAscope assay (Advanced Cell Diagnostics). The manufacture protocol was strictly followed for the RNAscope assay. Briefly, sections were fixed with 4% PFA for 15 minutes at 4°C followed by dehydration in increasing ethanol concentration from 50%, 70% to 100% twice and the protease treatment. RNA probes included antisense probes against mouse TH (317621-C3) and *Slc17a6* (319171-C1). After treated with probes, the signal was further amplified with amplifier 1, 2, 3, 4B. Slides were counterstained with DAPI dissolved in Fluoromount-G mounting medium. Images were taken at x20 magnification using a Zeiss Axio Observer Epifluorescence microscope.

Cell counting for fluorescent images. Sections covering the rostrocaudal extent of the substantia nigra pars compacta (SNc, bregma -3.88 to -2.54) were collected and hybridized for TH and Vglut2 (*Slc17a6*). Counts were made after the investigator was blinded. 20X Tiled images were acquired using a Zeiss Epifluorescence microscope

(Axio Observer, Zeiss) and used to count *Vglut2*$^+$, *Th*$^+$, and *Vglut2*$^+$*/ Th*$^+$ dopaminergic neurons by Zeiss. Counted sections were 320μm apart, and 4 sections were counted per animal. *Vglut2*$^+$*/ Th*$^+$ cells were further counted for the intensity of Vglut2 signals by counting the number of puncta of Vglut2 signal. The dopaminergic neurons co-localizing with Vglut2 was considered as any cell whose number of puncta of Vglut2 was no less than 4. The intensity of Vglut2 expression was further represented by the number of puncta, and any cell expressing more than 20 puncta was combined together for analysis.

Study Approval. All animal experiments were approved by the Institutional Animal Care and Use Committee of UCSD (La Jolla, California, USA), and NIH guidelines for laboratory animal care and safety were strictly followed.

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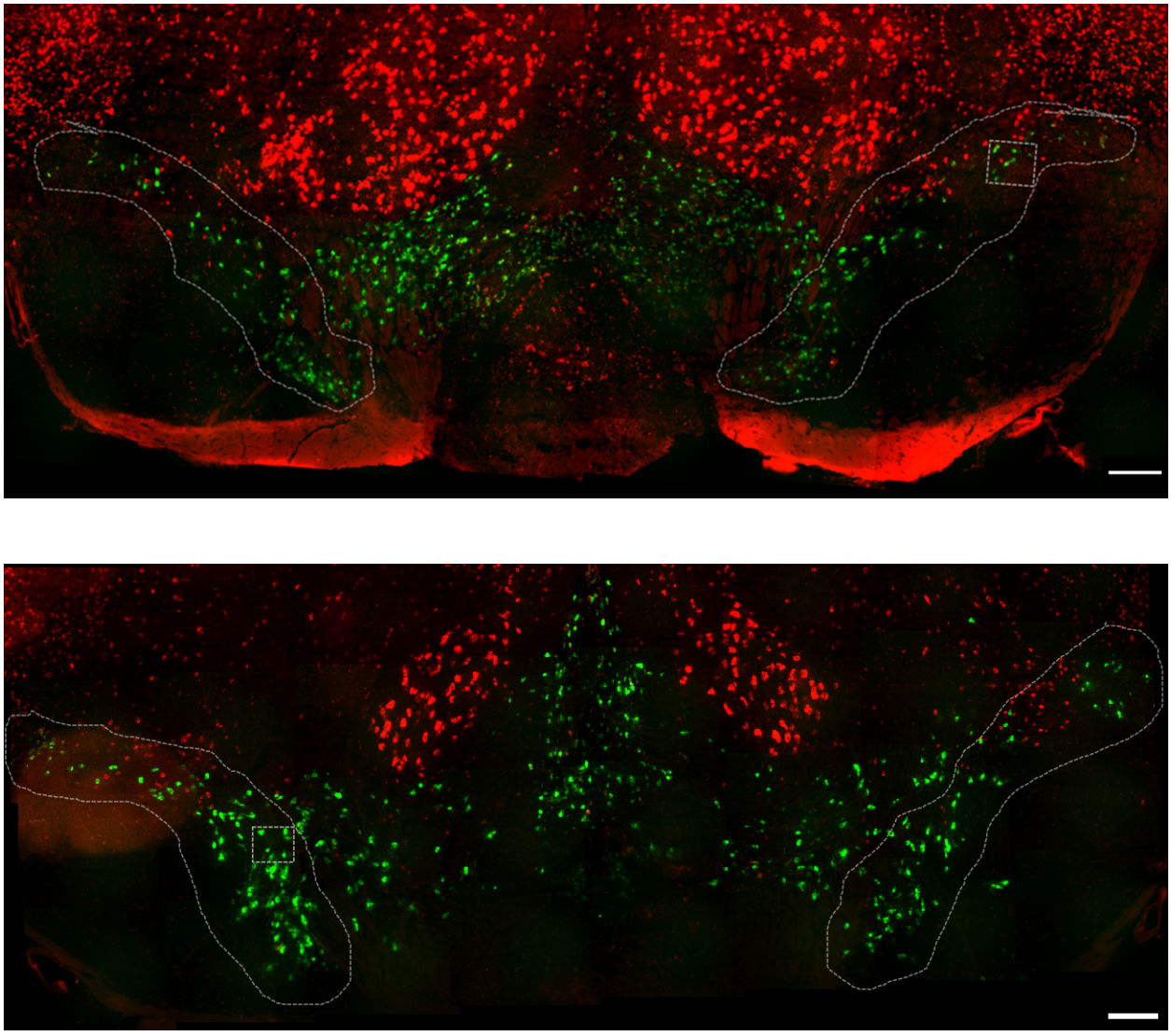

Figure1. SNc images labeled with probes against *Th* and *Vglut2* mRNA after 72 hours since the last abdominal injection of amphetamine (top panel) or saline (bottom panel). Scale bars: 200μm.

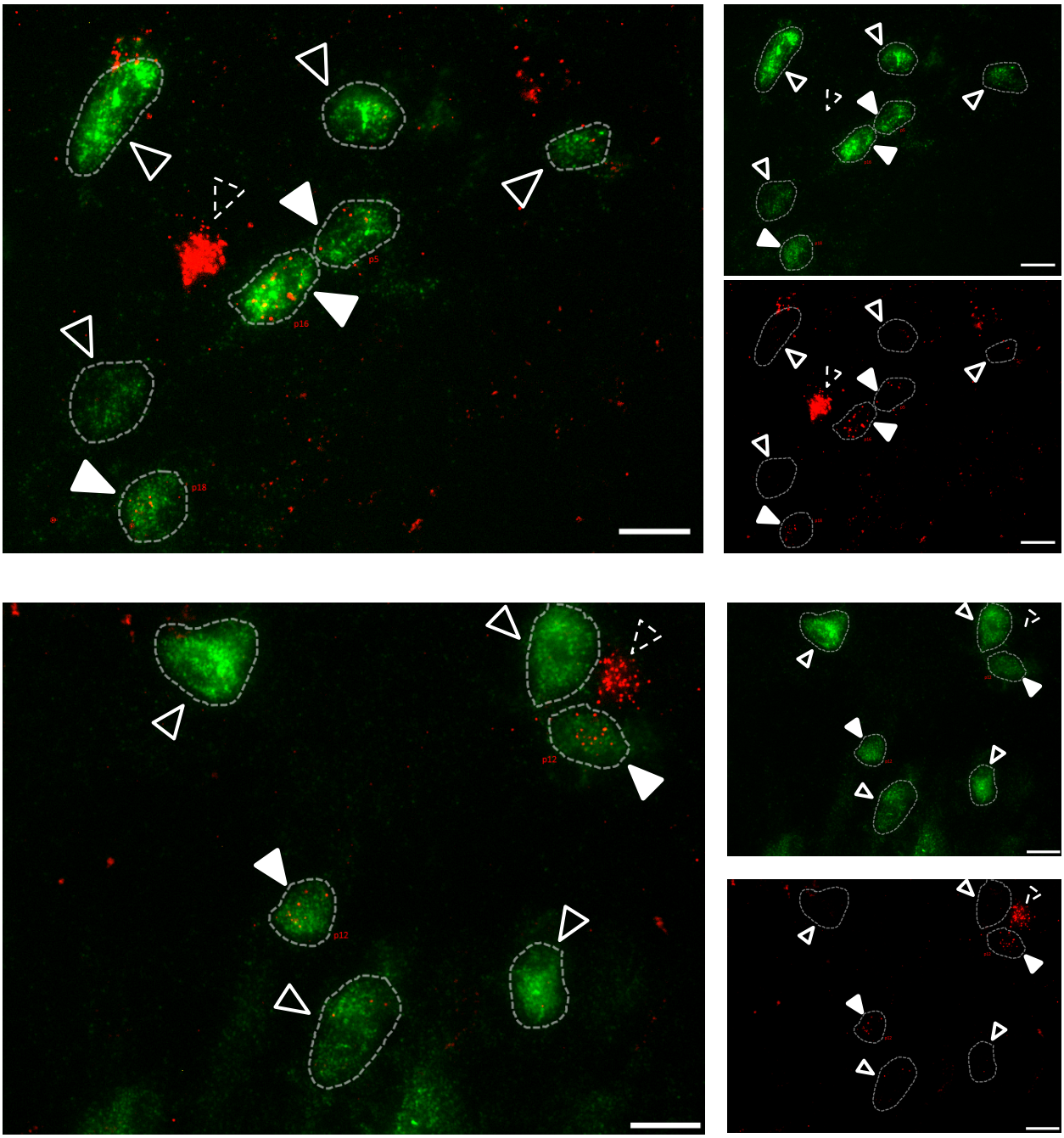

Figure 2. 20X magnification images of SNc neurons 72 hours after amphetamine injection (right panel) and saline injection (left panel). Solid arrows indicate *Th+/Vglut2+* neurons. Hollow arrows with solid border indicate *Th+/Vglut2-* neurons. Hollow arrows with dash line border indicate *Th-/Vglut2+* neurons. Scale bars: 20μm.

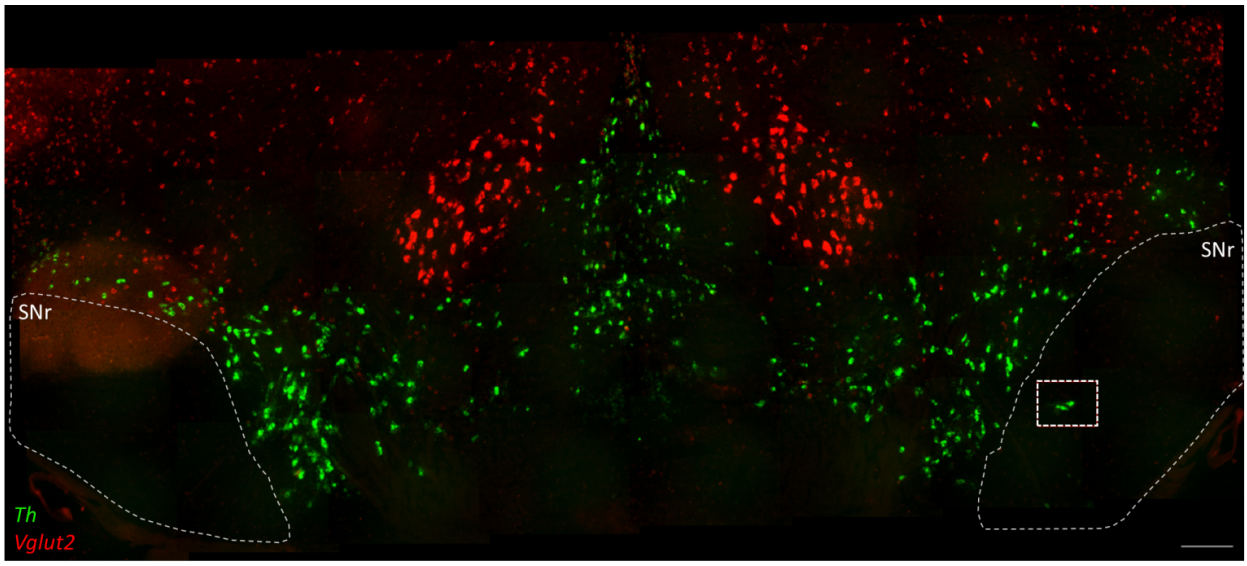

Figure 3. SNr images labeled with probes against *Th* and *Vglut2* mRNA after 72-hour waiting period. Scale bars: 200μm.

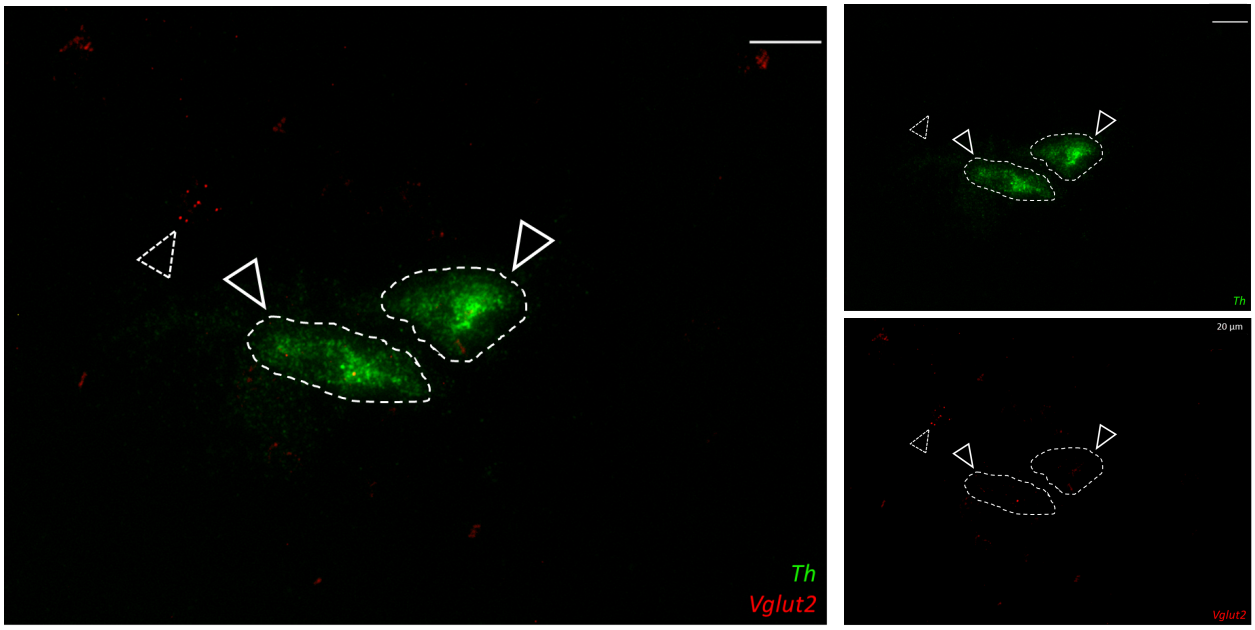

Figure 4. 20X magnification images of SNr neurons after 72-hour waiting period. Hollow arrows with solid border indicate *Th+/Vglut2-* neurons. Hollow arrows with dash line border indicate *Th-/Vglut2+* neurons. Scale bars: 20μm.

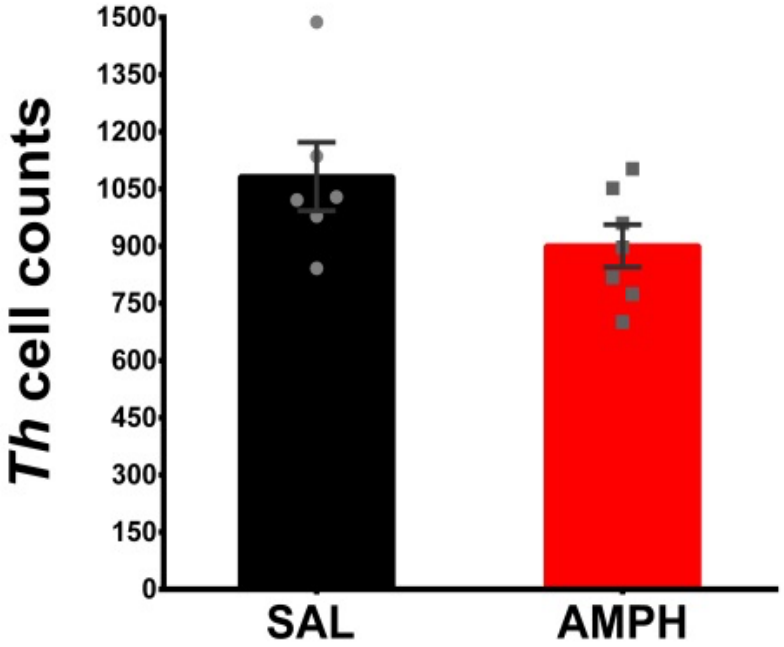

Figure 5.1. Quantification of Th+ neurons in SNc 72 hours after amphetamine injection or saline injection (left panel: unpaired *t* test; *t*=1.775, *df*=11, amphetamine *n*=7, saline *n*=6, *P*>0.05).

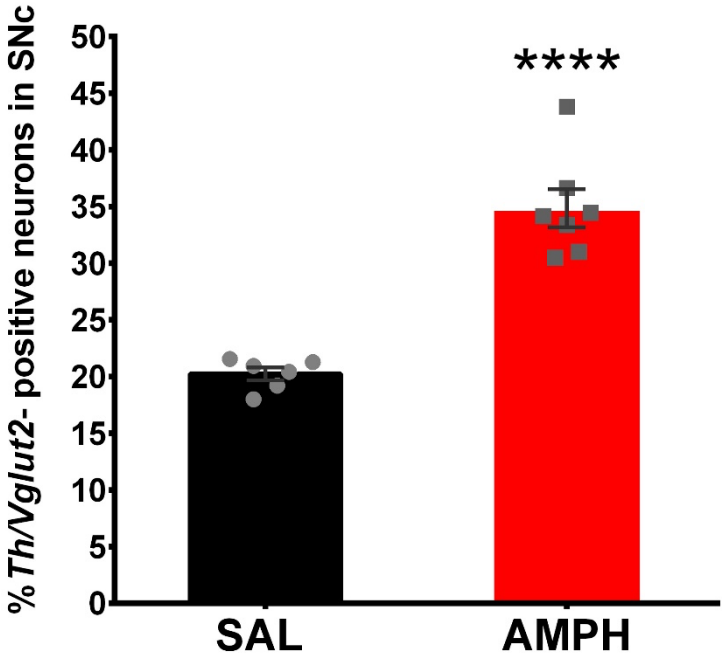

Figure 5.2. Fraction of Th-labeled neurons co-labeling for Vglut2 in SNc 72 hours after amphetamine injection or saline injection (right panel: unpaired *t* test; *t*=7.660, *df*=11, amphetamine *n*=7, saline *n*=6, *P*< 0.0001).

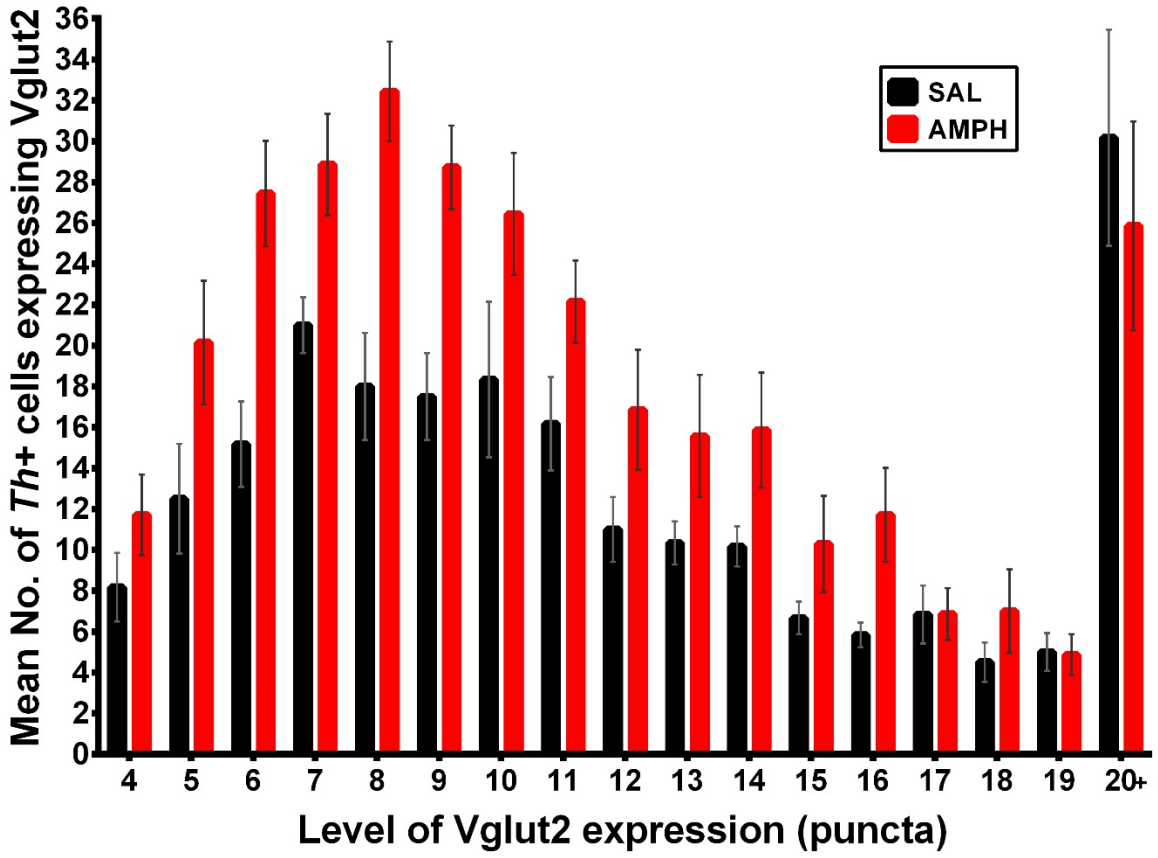

Figure 6. Histogram showing the distribution of *Vglut2* puncta in *Th*+ neurons in SNc 72 hours after amphetamine injection (red) or saline injection (black).

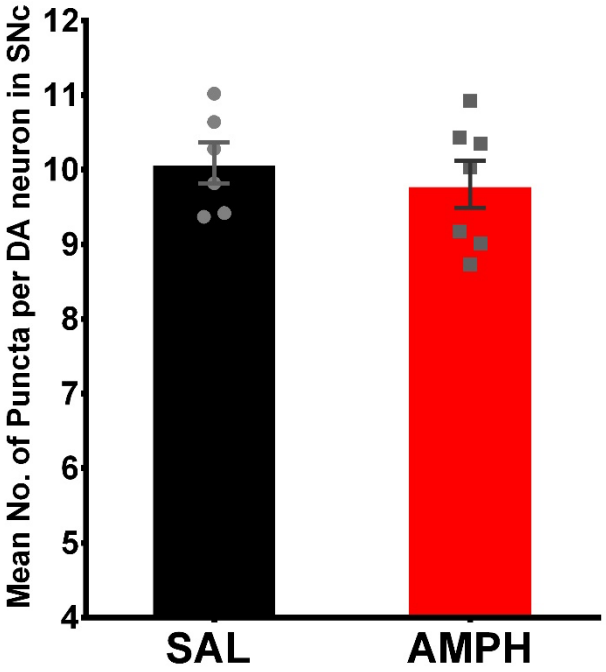

Figure 7. Quantification of mean number of puncta per DA neuron in SNc 72 hour after amphetamine injection or saline injection, with number of *Vglut2*+ puncta over 20 excluded (unpaired *t* test; t=2.007, df=11, amphetamine n=7, saline n=6).

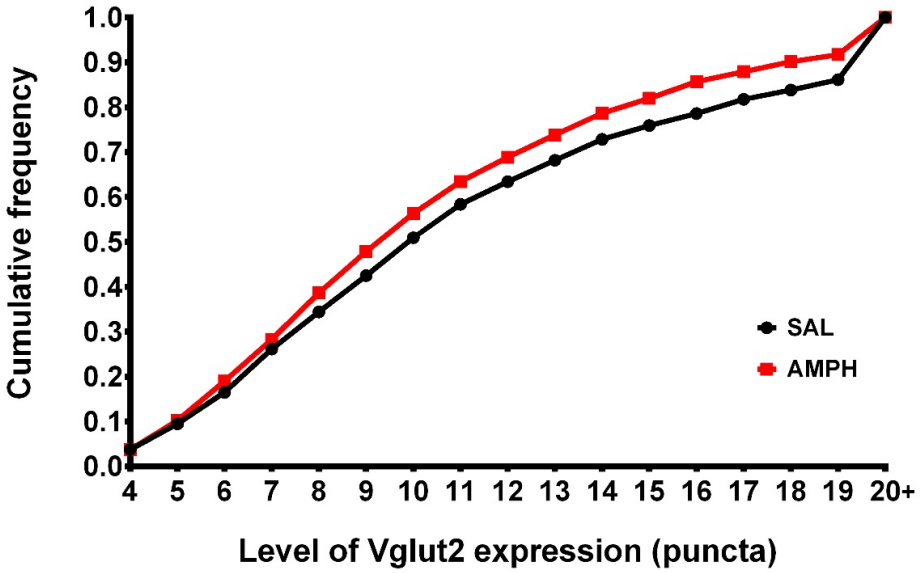

Figure 8. Cumulative probability blot comparing 72-hour post-injection amphetamine-induced increase (red) in number of *Vglut2*+ puncta in *Th*+ neurons in SNc with 72-hour post-injection saline increase (black) (saline n=6, amphetamine n=7; Kolmogorov-Smirnov [KS] test).

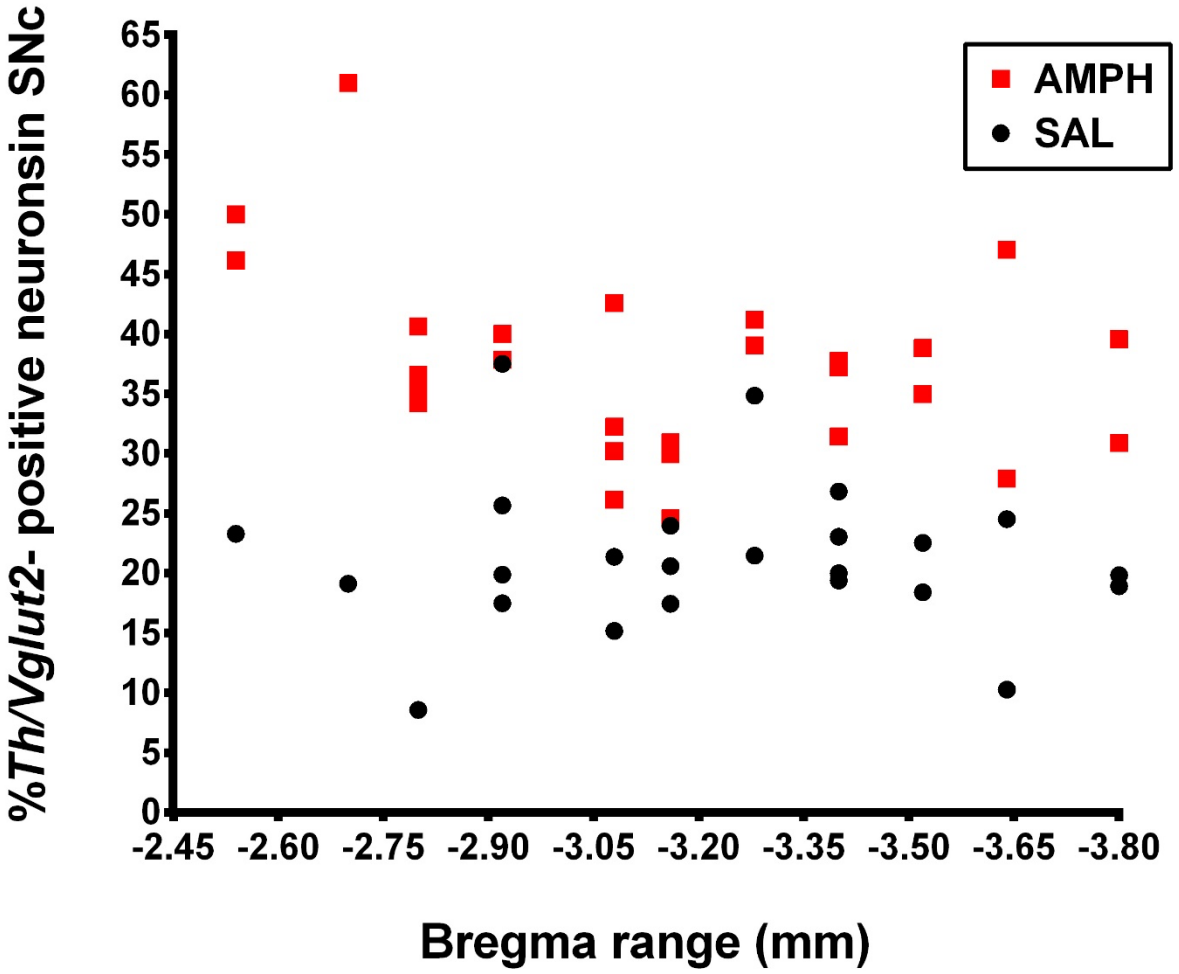

Figure 9. Distribution of *Vglut2* co-expression rate of *Th*+ neurons across different bregma points in SNc 72 hours after amphetamine injection (red) or saline injection (black).

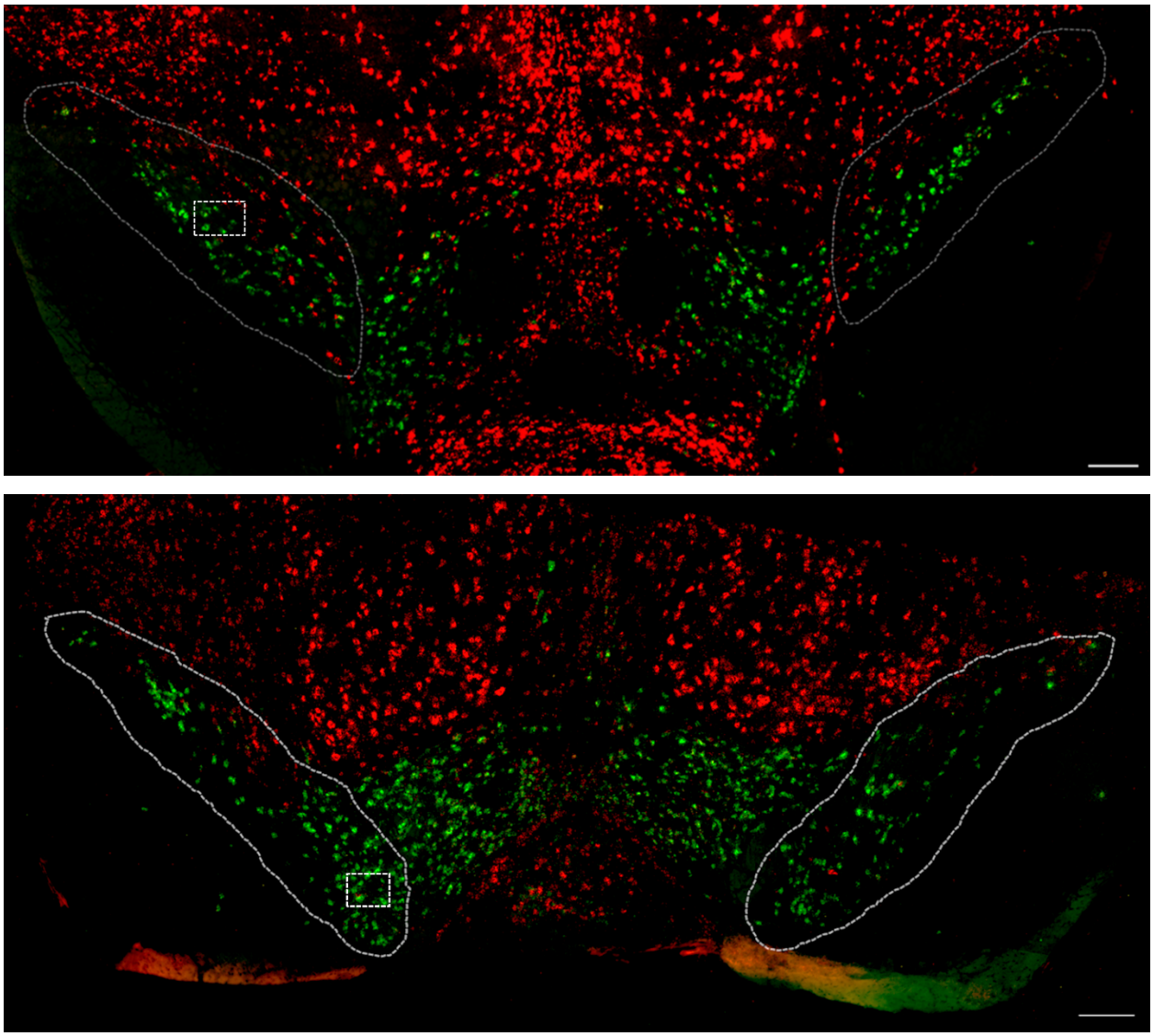

Figure 10. SNc images labeled with probes against *Th* and *Vglut2* mRNA after 240 hours since the last abdominal injection of amphetamine (top panel) or saline (bottom panel). Scale bars: 200μm.

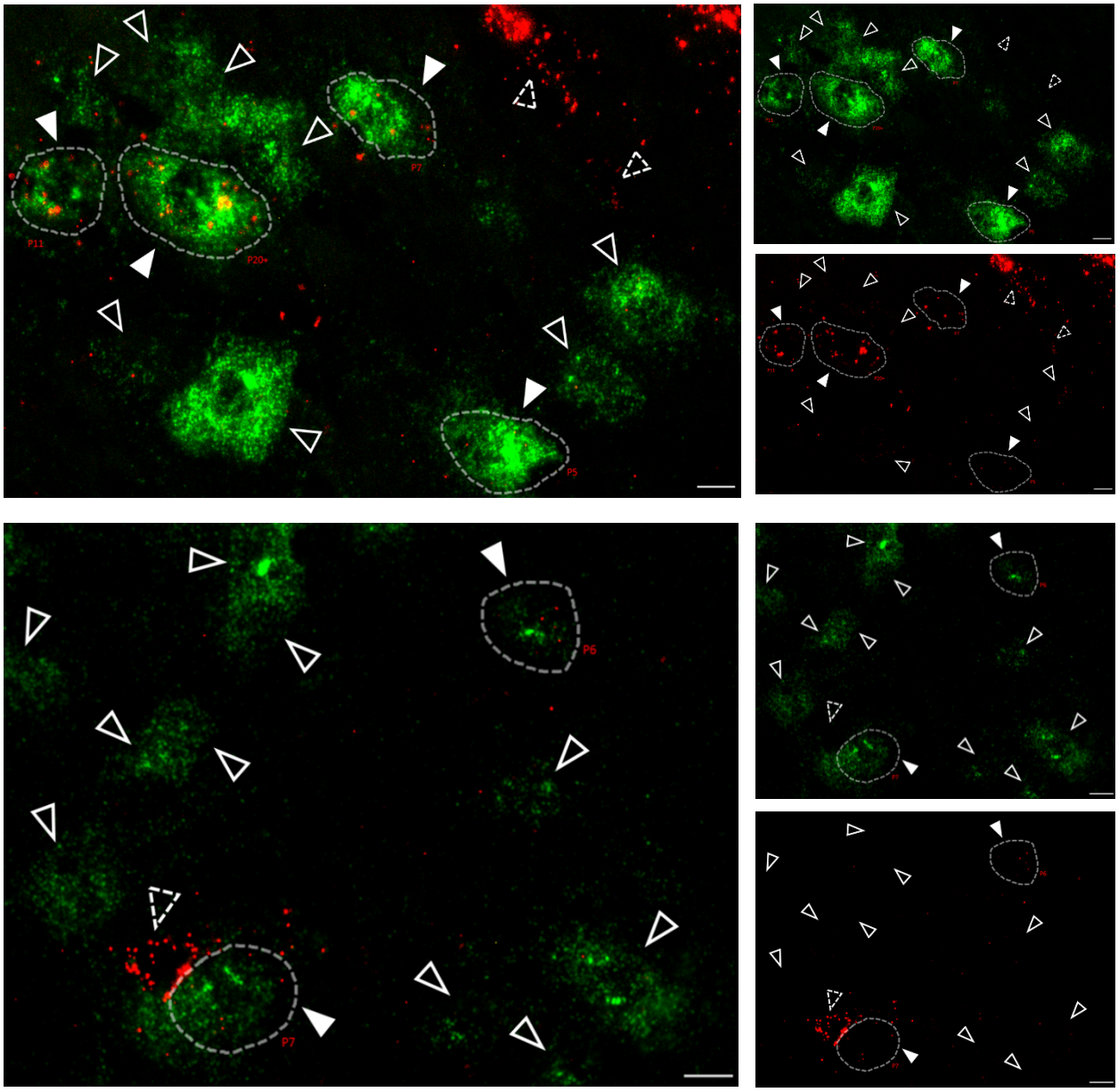

Figure 11. 20X magnification images of SNc neurons 240 hours after amphetamine injection (right panel) and saline injection (left panel). Solid arrows indicate *Th+/Vglut2+* neurons. Hollow arrows with solid border indicate *Th+/Vglut2-* neurons. Hollow arrows with dash line border indicate *Th-/Vglut2+* neurons. Scale bars: 20μm.

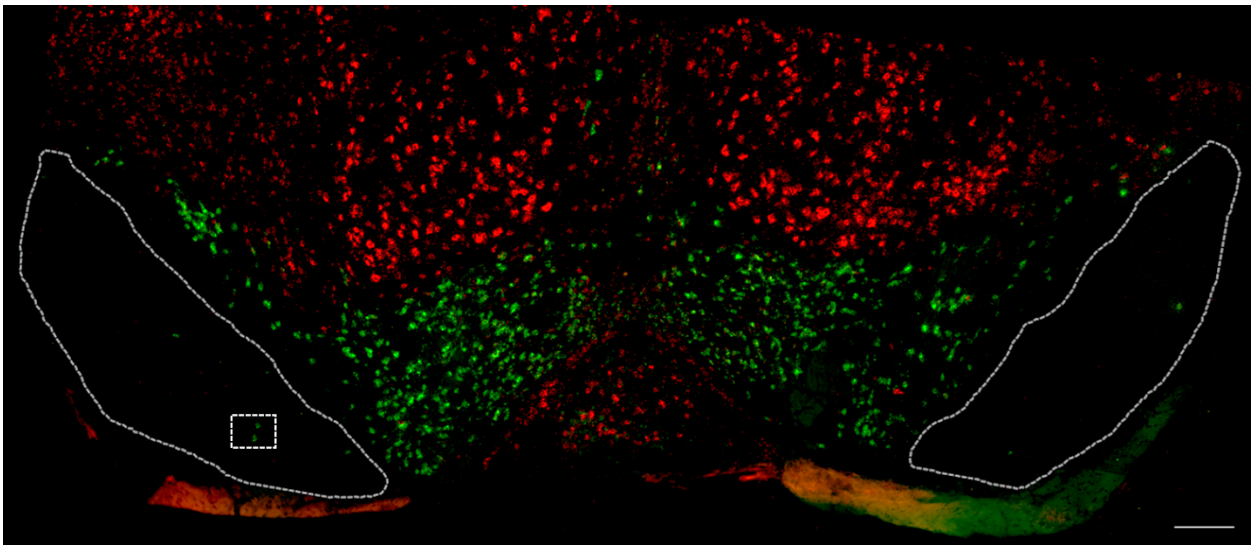

Figure 12. SNr images labeled with probes against TH and Vglut2 mRNA after 240-hour waiting period. Scale bars: 200μm.

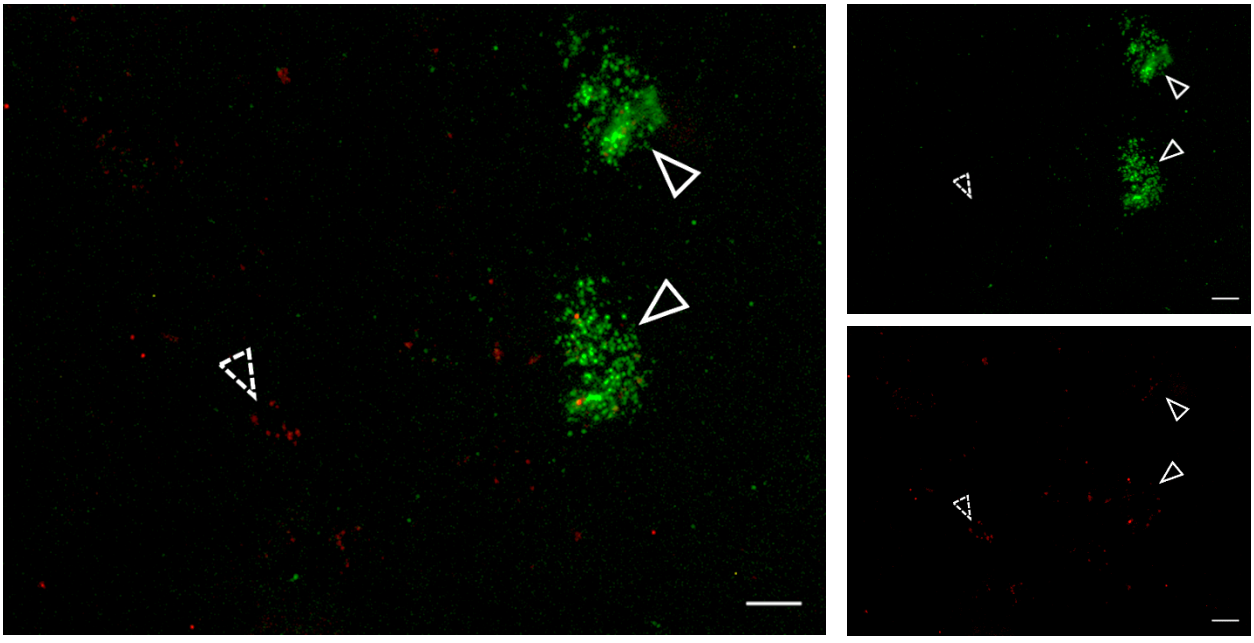

Figure 13. 20X magnification images of SNr neurons after 240-hour waiting period. Hollow arrows with solid border indicate *Th+/Vglut2-* neurons. Hollow arrows with dash line border indicate *Th-/Vglut2+* neurons. Scale bars: 20μm.

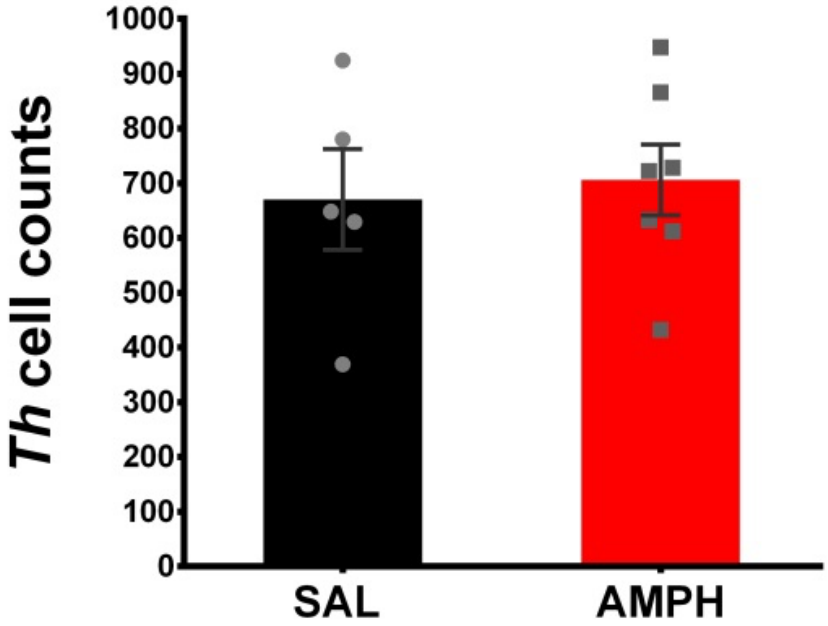

Figure 14.1. Quantification of *Th*+ neurons in SNc 240 hours after amphetamine injection or saline injection (top panel: unpaired t test; *t*=0.3304, *df*=10, amphetamine *n*=7, saline *n*=5).

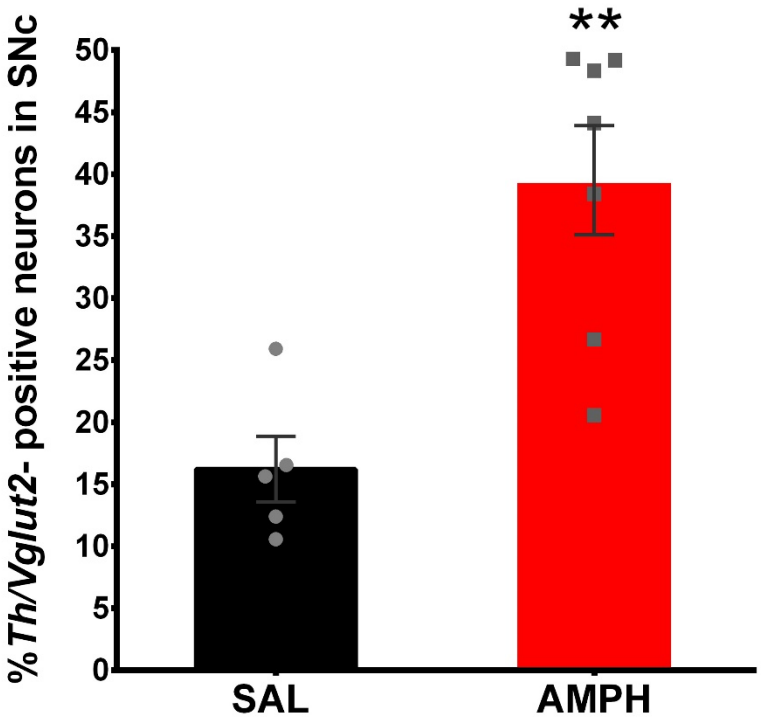

Figure 14.2. Fraction of *Th*-labeled neurons co-labeling for *Vglut2* neurons in SNc 240 hours after amphetamine injection or saline injection (bottom panel: unpaired *t* test; *t*=4.071, *df*=10, amphetamine *n*=7, saline *n*=5, *P*< 0.05).

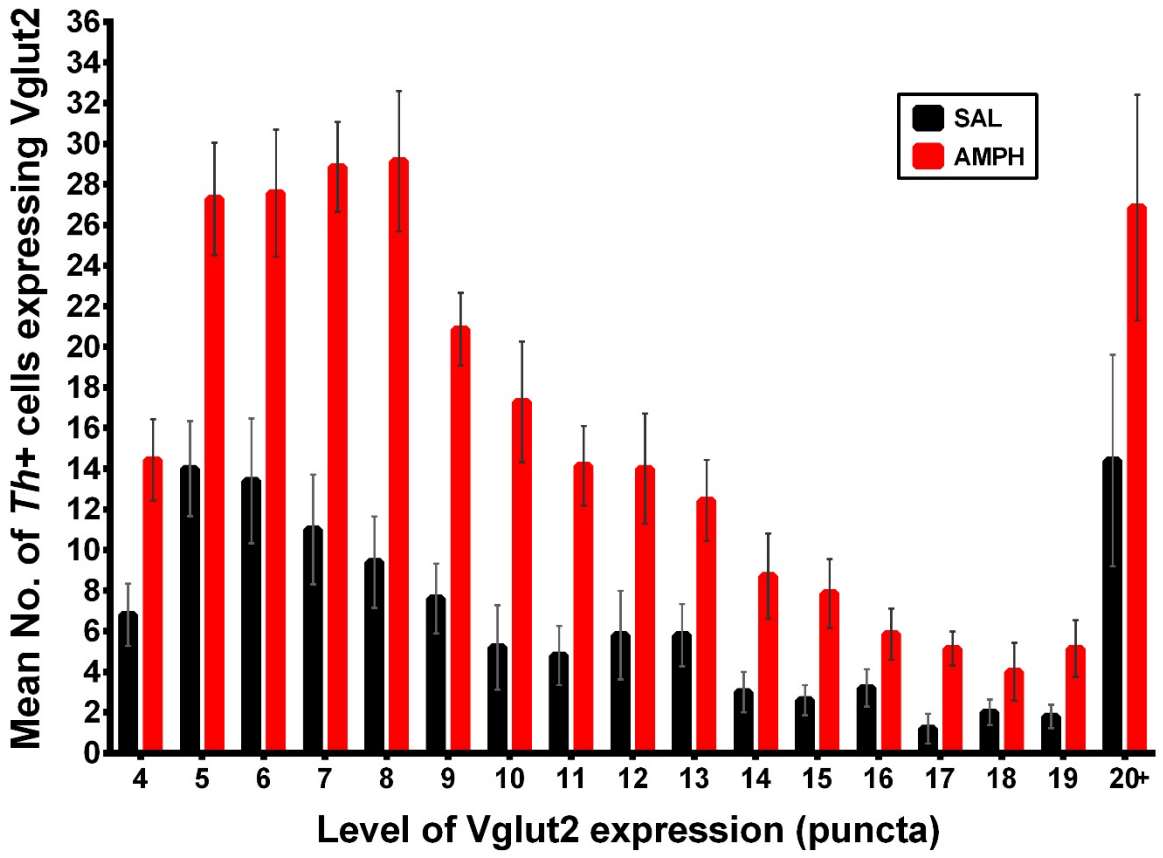

Figure 15. Histogram showing the distribution of Vglut2 puncta in Th+ neurons in SNc 240 hours after amphetamine injection (red) or saline injection (black).

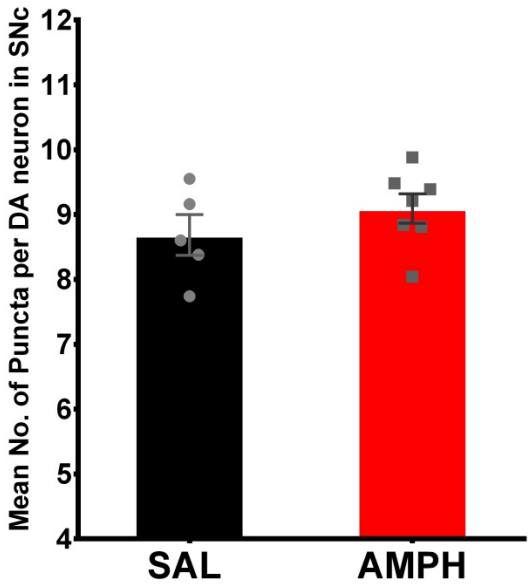

Figure 16. Quantification of average number of puncta per DA neuron in SNc 240 hour after amphetamine injection or saline injection with number of *Vglut2*+ puncta over 20 excluded (unpaired *t* test; *t*=0.7176, *df*=10, *n*=7 for amphetamine injection, *n*=5 for saline injection).

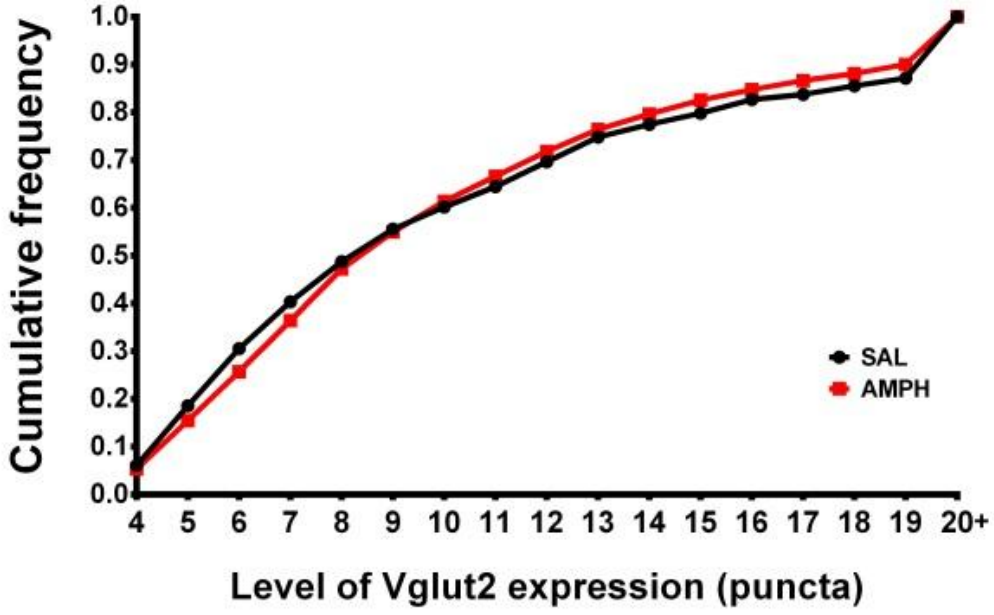

Figure 17. Cumulative probability blot comparing 240-hour post-amphetamine injection induced increase (red) in number of *Vglut2*+ puncta in *Th*+ neurons in SNc with 240-hour post-saline injection increase (black) (saline *n*=6, amphetamine *n*=7; Kolmogorov-Smirnov [KS] test).

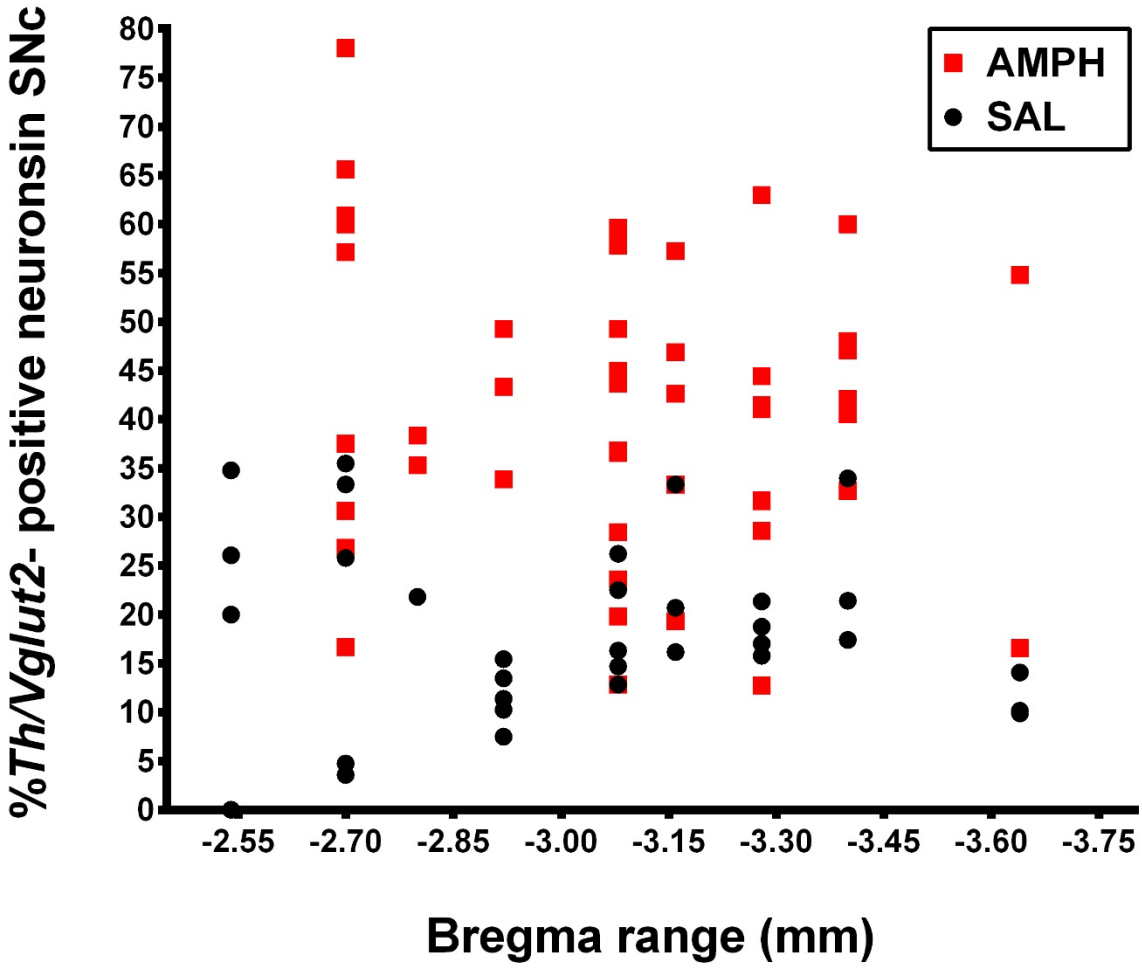

Figure 18. Distribution of Vglut2 co-expression rate of Th+ neurons across different bregma points in SNc after amphetamine injection (red) or saline injection (black).