Video Article

Rapid *In Situ* Hybridization using Oligonucleotide Probes on Paraformaldehyde-prefixed Brain of Rats with Serotonin Syndrome

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Abstract

3,4-Methylenedioxymethamphetamine (MDMA; ecstasy) toxicity may cause region-specific changes in serotonergic mRNA expression due to acute serotonin (5-hydroxytryptamine; 5-HT) syndrome. This hypothesis can be tested using *in situ* hybridization to detect the serotonin 5-HT_{2A} receptor gene *htr2a*. In the past, such procedures, utilizing radioactive riboprobe, were difficult because of the complicated workflow that needs several days to perform and the added difficulty that the technique required the use of fresh frozen tissues maintained in an RNase-free environment. Recently, the development of short oligonucleotide probes has simplified *in situ* hybridization procedures and allowed the use of paraformaldehyde-prefixed brain sections, which are more widely available in laboratories. Here, we describe a detailed protocol using non-radioactive oligonucleotide probes on the prefixed brain tissues. Hybridization probes used for this study include *dapB* (a bacterial gene coding for dihydrodipicolinate reductase), *ppiB* (a housekeeping gene coding for peptidylprolyl isomerase B), and *htr2a* (a serotonin gene coding for 5-HT_{2A} receptors). This method is relatively simply, cheap, reproducible and requires less than two days to complete.

Video Link

The video component of this article can be found at https://www.jove.com/video/53165/

Introduction

Serotonin (5-hydroxytryptamine; 5-HT) syndrome is an acute neurologic disorder caused by 5-HT-promoting drugs such as antidepressants[1], while also occurring in situations of MDMA use for recreational purposes[2]. Molecular mechanisms responsible for mood swings, learning and memory deficits that occur in association with the acute syndrome are not well understood[3,4]. *In situ* hybridization is a powerful research tool allowing the detection and quantification of specific mRNAs expressed potentially at a single-cell level. The conventional way to perform *in situ* hybridization is to utilize a radioactive-labeled riboprobe that specifically hybridizes the gene of interest. However, a major drawback is that the method requires complicated and time-consuming probe preparation and hybridization steps, as well as access to fresh frozen tissues maintained in an RNase-free environment[5,6].

Oligonucleotide probes have been recently developed to hybridize shorter RNA fragments than those required for riboprobes[7]. Furthermore, the probes produce a low background signal without sacrificing specificity[8]. This newly-developed probe technology can be used *in situ* hybridization on paraformaldehyde-prefixed brain tissues commonly available in immunocytochemical laboratories.

Here, we describe a protocol for *in situ* hybridization using oligonucleotide probes on paraformaldehyde-prefixed rat brain and compare findings with those noted in a fresh frozen brain[5,6]. This protocol is used to test the hypothesis that MDMA substance abuse causes changes in 5-HT_{2A} receptor gene *htr2a* mRNA in the brain. We began the procedure with MDMA treatment followed by paraformaldehyde tissue perfusion of the animal, *in situ* hybridization of the *thr2a* probe, and data analysis. Note that *dapB* (the bacterial gene coding for dihydrodipicolinate reductase) is used as a negative control, and *ppiB* (housekeeping gene coding for peptidylprolyl isomerase B) as a positive control.

Protocol

Animal use procedures described below were approved by The Institutional Animal Care and Use Committee (IACUC) at Ross University School of Veterinary Medicine and Florida Atlantic University. Although sterile techniques and gloves are required, the RNase-free environment is not necessary while using this protocol.

1. Preparation

1. Tissue preparation and sectioning

1. Assign rats randomly into three groups: fresh/saline (SAL; 0.9% NaCl), prefixed/SAL and prefixed/3,4-methylenedioxymethamphetamine (MDMA; see details in **table 1**).
2. Administer three doses of 1 ml/kg SAL and 10 mg/kg MDMA intraperitoneally, respectively, to the SAL and MDMA groups at 2 hr interval. Allow animals to survive the treatment for 6 days.
3. On day 7, administer 100 mg/kg ketamine in combination of 5 mg/kg xylazine intraperitoneally to the rats for a deep anesthesia (*i.e.*, loss of corneal blink and tail pinch reflexes).
4. For the fresh/SAL group, quickly decapitate rats and remove the brain for the fresh brain test (see details in the reference[5]).
5. For the prefixed/SAL and prefixed/MDMA groups, make a cut along the sternum about 10 -12 cm and then expose the end of the sternum.
6. Hold the end of the sternum with a hemostat and cut the diaphragm laterally on both sides with sharp scissors and then cut upward across the ribs and parallel to the lungs.
7. With one hand, hold the ventral tip of the heart with small forceps. With the other hand, pierce the left ventricle with a 18 G syringe needle (note the adapter side of the needle is connected with a Y-shaped silicon hose to ice-cold SAL and paraformaldehyde containers and peristaltic pumps; see the reference[9]).
8. Turn on the SAL pump at the medium level of the flow rate (~5 ml/min) and puncture the right atrium with the forceps to allow the escape of return circulation. Maintain SAL perfusion for 30 min.
9. Turn on the paraformaldehyde pump at the medium level and allow the ice-cold 4% paraformaldehyde to perfuse the animal for 30 min.
10. Remove perfusion instrument and decapitate the animal. Make a posteroanterior cut through the midline of the skull, and then flap the skull to expose the skull cavity [10].
11. Remove the brain with a spatula from the skull cavity and place the brain in a 50 ml centrifuge tube containing 25 ml of 4% paraformaldehyde. Keep the brain within the paraformaldehyde-containing tube at 4 ºC refrigerator for 24 hr.
12. Move the brain into a 50 ml centrifuge tube containing 25 ml of 30% sucrose solution at 4 ºC for 3 days. Finally, store the brain within plastic bags at -80 °C till use.
13. Mount the brain on a chuck with embedding media and freeze the embedding media containing brain in a cryostat at temperature of -26 ºC. Cut the fresh frozen brain into 20 µm sections and paraformaldehyde-prefixed brain into 10-µm sections. Transfer the section to a RT microscope slide by touching the slide to the section.
14. Air-dry at RT for 4 hr. Store the section slides in a tight sealed bag at -80 °C till use.

2. Dehydration
    1. Take slides from the -80 °C freezer and assign sections into one of three tests: *ht2a*, *ppiB* (positive control) or *dapB* (negative control); mark and label sections with a ball pen (do not use a pencil). Submerge slides in 100% alcohol at RT for 5 min. Dry the slides for 5 min in a fume hood.

3. Pretreatment
    1. Pipette 20 µl of the Pretreatment-1 reagent (inactivation of alkaline phosphatase) to the sections on slides. Place the slides on a rack rail in a moisture tray (note that the tray is covered with a lid to maintain a high humidity for preventing evaporation from the reaction solution).
    2. Gently shake the moisture tray on a horizontal shaker at a low speed for 10 min. Wash the slides twice with double-distilled water (ddH_2O) for 2 min. Submerge the slide in a 200 ml beaker containing 150 ml of the Pretreatment-2 reagent (breaking of the cross-linkages induced by paraformaldehyde fixation). Boil the slides for a total of 5 min at 100 ºC (heat-induced restoration of RNA structure[11]).
    3. Wash the slides twice with ddH_2O for 2 min. Place the slides in 100% alcohol for 5 min. Dry the slides for 5 min in the fume hood. Use a hydrophobic pen to draw a circle around the selected area in the tissue section (*e.g.*, non-cortical structures, including thalamus and hypothalamus as outlined in **Figure 2**).
    4. Pipette 10 µl of the Pretreatment-3 reagent (increases in probe penetration) to each selected area, and cover the moisture tray with the lid. Place the tray in the hybridization oven equipped with the horizontal shaker and set the oven temperature at 40 ºC; gently shake the tray for 30 min. Wash the slides twice with ddH_2O for 2 min.

2. Hybridization and Amplification

1. Pipette 10 µl of *htr2a*, *ppiB* and *dapB* probe reagents, respectively, on the selected areas. Cover the moisture tray with the lid to prevent evaporation of the reagent. Gently shake on the horizontal shaker set at a low speed. Incubate the slides at 40 ºC in the oven for 2 hr.
2. Wash the slides twice with a washing buffer for 2 min. Pipette 10 µl of the Amplification-1 reagent on each selected area, shake and incubate the slides at 40 ºC for 30 min.
3. Wash the slides twice with a washing buffer for 2 min. Pipette 10 µl of the Amplification-2 reagent on each selected area, shake and incubate the slides at 40 ºC for 15 min.
4. Wash the slides twice with a washing buffer for 2 min. Pipette 10 µl of the Amplification-3 reagent on each selected area, shake and incubate the slides at 40 ºC for 30 min.
5. Wash the slides twice with a washing buffer for 2 min. Pipette 10 µl of the Amplification-4 reagent on each selected area, shake and incubate the slides at 40 ºC for 15 min.
6. Wash the slides twice with a washing buffer for 2 min. Pipette 10 µl of the Amplification-5 reagent on each selected area, shake and incubate the slides at RT for 30 min.
7. Wash the slides twice with a washing buffer for 2 min. Pipette 10 µl of the Amplification-6 reagent on each selected area, shake and incubate the slides at RT for 15 min. Wash the slides twice with a washing buffer for 2 min.

3. Signal Detection

1. Pipette 10 µl of the Red reagent to each selected area (Note that the reagent is a mixture of Red-B and Red-A at a 1:60 ratio and should be used immediately). Place the slide in the moisture tray on the horizontal shaker at RT and shake gently at a low speed for 10 min.
2. Wash the slides twice with (ddH_2O) for 10 min. Dry the slides at 60 ºC in the oven for 15 min. Place the slides in xylene under the fume hood for 5 min. Pipette 20 µl of xylene-based mounting media on each selected area and cover with a coverslip.

4. Image Capture

1. Take digital microphotographs of the selected areas (*e.g.*, hypothalamus, **Figures 1 and 3**) with a magnification of 4× and 20× objective lenses. Save the image file.

5. Image Particle Analysis

1. Drag and drop the image file into the main ImageJ window. Go to the menu bar and select 'Image', next select 'Type' from the drop-down menu, and select '8-bit'.
2. Select 'Adjust', then select 'Threshold' to open the dialog box. Adjust the lower bar value in the dialog box so that the unwanted background is removed. Go to the menu bar and select 'Analyze', then select 'Analyze Particles' to open a second dialog box.
3. Set particle size at 10, next select 'Show outlines' and select 'Display results" and 'Summarize' boxes. Lastly, click 'OK' to view particle data in the dialog boxes.

6. Spreadsheet Calculation

1. Enter the data into the Excel sheet and average the numbers of particles in the SAL group as a baseline. Calculate the percentage level of each section and make a graph accordingly (**Figure 4**).

Representative Results

Using the oligonucleotide RNA probes (<25 nt), hybridization can be detected as red dots in hypothalamic cells prepared from the paraformaldehyde-prefixed and fresh frozen brains. The *htr2a* mRNA molecules are present in some cells (indicated by solid arrows), but not others (open arrows). We observed that there is no difference between the paraformaldehyde-prefixed and the fresh frozen tissues (**Figure 1**). A successful hybridization can be evaluated first by the naked eye (**Figure 2**). We found that the selected area (marked with circles) hybridized with the *dapB* probe did not show a visible signal to the naked eye (**Figures 2A-B**). In contrast, the area hybridized with the *ppiB* probe showed signals homogenously distributed throughout the region examined (**Figures 2C-D**). While in the *htr2a* test, signals are present in certain nuclei (indicated by arrows; **Figures 2E-F**), although all the circled area has been hybridized with the *htr2a* probe. Interestingly, the signals in MDMA slides were relatively weak compared to SAL slides. Substantial details of hybridization signals can be evaluated under a microscopic instrument. There is no red dot (**Figure 3A-B**) in the cells hybridized with the *dapB* probe whereas signals can be seen throughout the microscopic field hybridized with the *ppiB* probe (**Figure 3C-D**). The *htr2a* signals are mainly located in certain nuclei of the hypothalamus (**Figure 3E-F**). We observed a reduction in *htr2a* signals in the brain tissues previously treated with systemic MDMA at 10 mg/kg, which can be quantitatively determined using the ImageJ analysis. As shown in **Figure 4**, we found a 20% reduction in *htr2a* expression.

In conclusion, our results showed that using oligonucleotide probes is specific, and suitable for the prefixed brain tissue. Furthermore, protocol procedures, including *in situ* hybridization, signal detection and data analysis can be completed within two days.

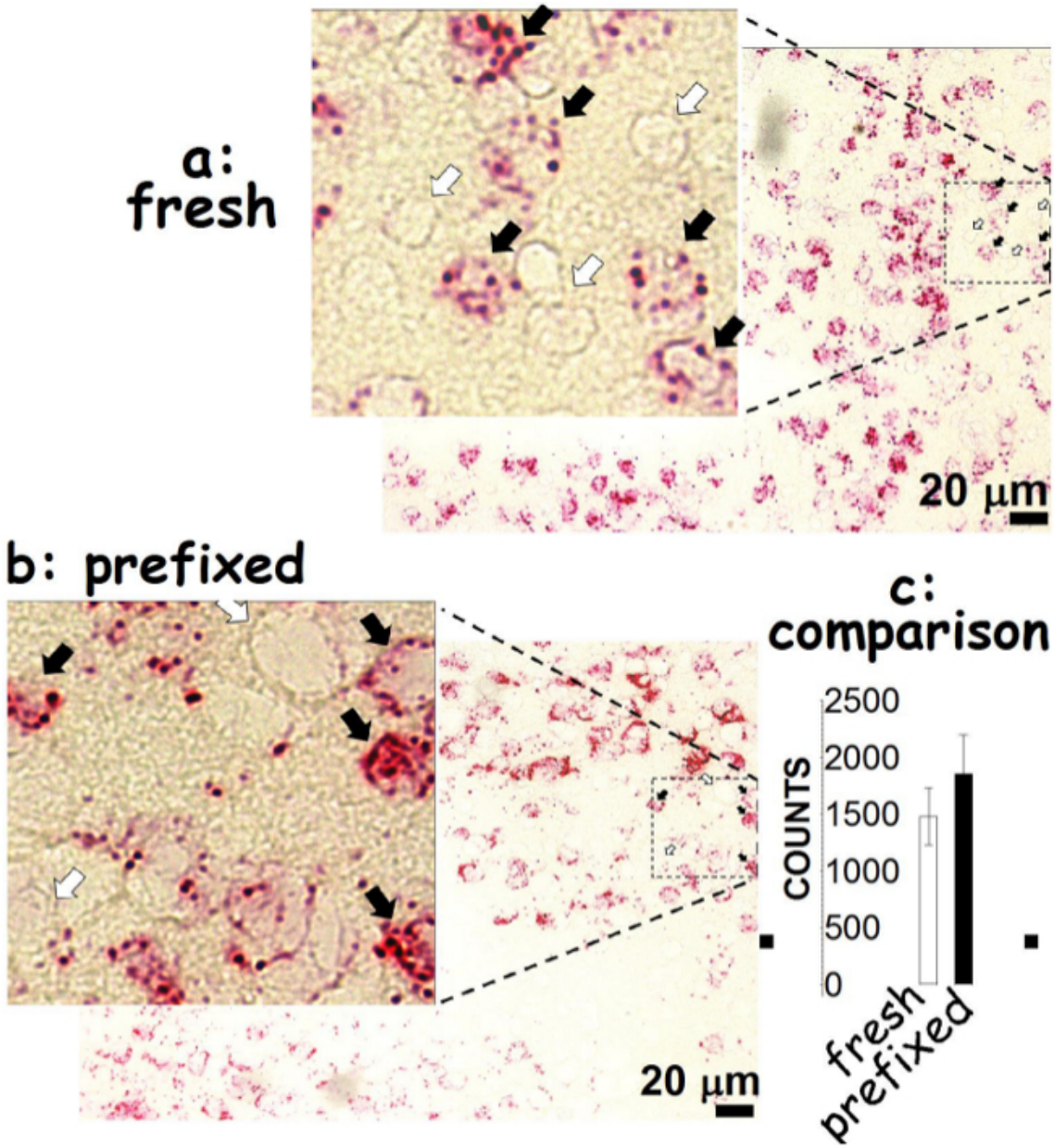

Figure 1. Comparison of the fresh and prefixed frozen tissues. Procedures used for fresh frozen brains have been described elsewhere[5]. Note that the fresh frozen sections were cut at 20 µm in thickness while the fixed frozen sections at 10 µm. The oligonucleotide probe was *htr2a* that hybridizes the 5-HT_{2A} receptor mRNA in the hypothalamus. Solid arrows indicate cells containing *htr2a* mRNA molecules in the fresh frozen (**A**) and prefixed tissues (**B**). Open arrows, no *htr2a* mRNA molecules detected. Bar: 20 µm. Using NIH ImageJ analysis, *htr2a* was counted. Numbers of *htr2a* counts are not different between the fresh and paraformaldehyde-prefixed frozen tissues (**C**). Please click here to view a larger version of this figure.

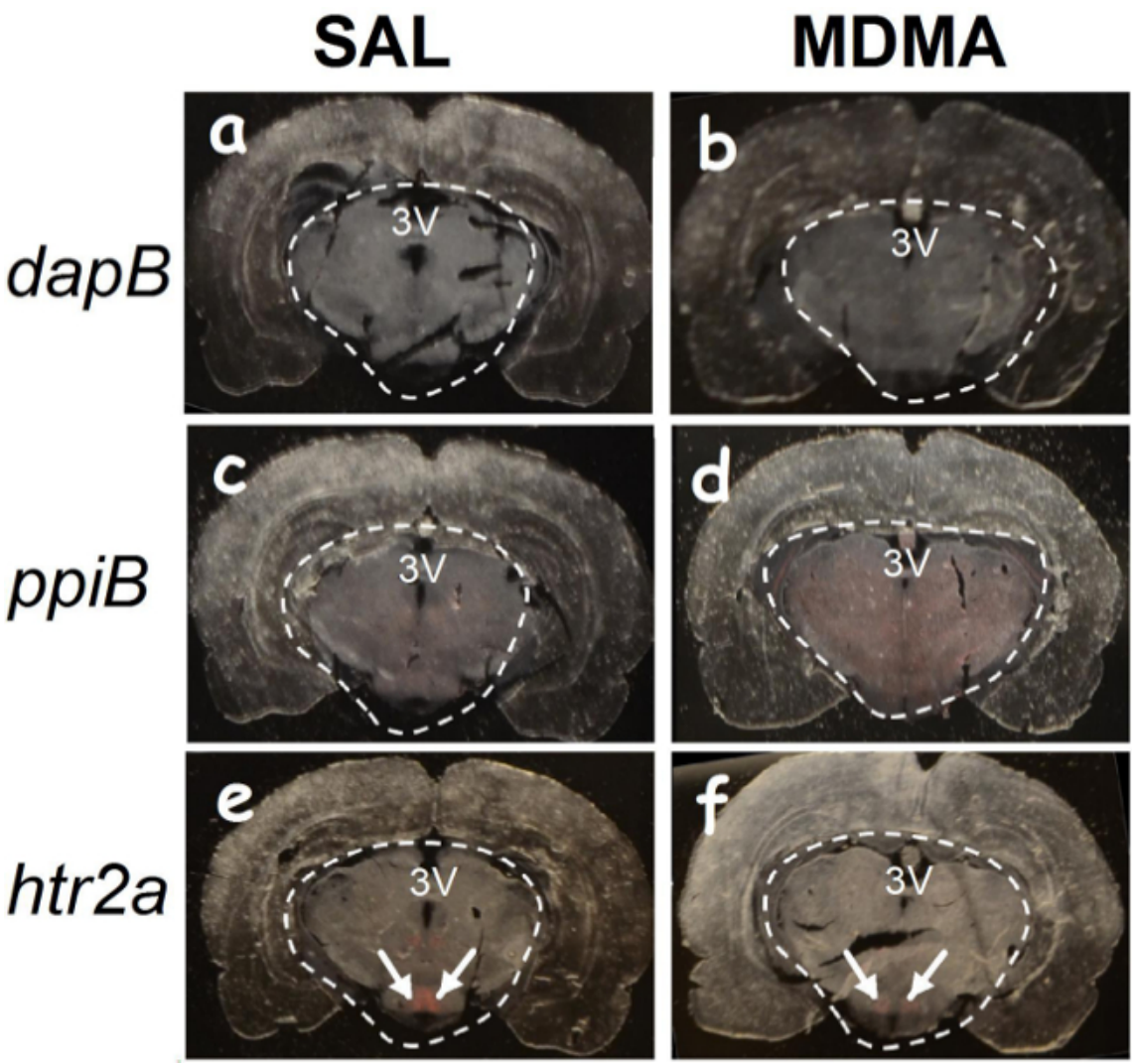

Figure 2. A naked-eye view of hybridization signals. Tissues were prefixed with paraformaldehyde in deeply anesthetized rats previously treated with saline (SAL) and 3,4-methylenedioxymethamphetamine (MDMA). The white-dashed lines indicate the region hybridized with oligonucleotide probes. The red color denotes mRNA signals to the naked eye. No red signal is observed while using the *dapB* probe hybridized to sections of SAL-pretreated (**A**) or MDMA-pretreated rats (**B**). In contrast, red signals are homogeneously distributed throughout the hypothalamic regions while using the *ppiB* probe (**C-D**). Interestingly, red signals produced by the *htr2a* probe are only in some regions (**E-F**; Arrows indicate the hypothalamic area). Stereotaxic coordinates: -3.50 ~ -3.80 relative to the bregma. 3V, the third ventricle. Please click here to view a larger version of this figure.

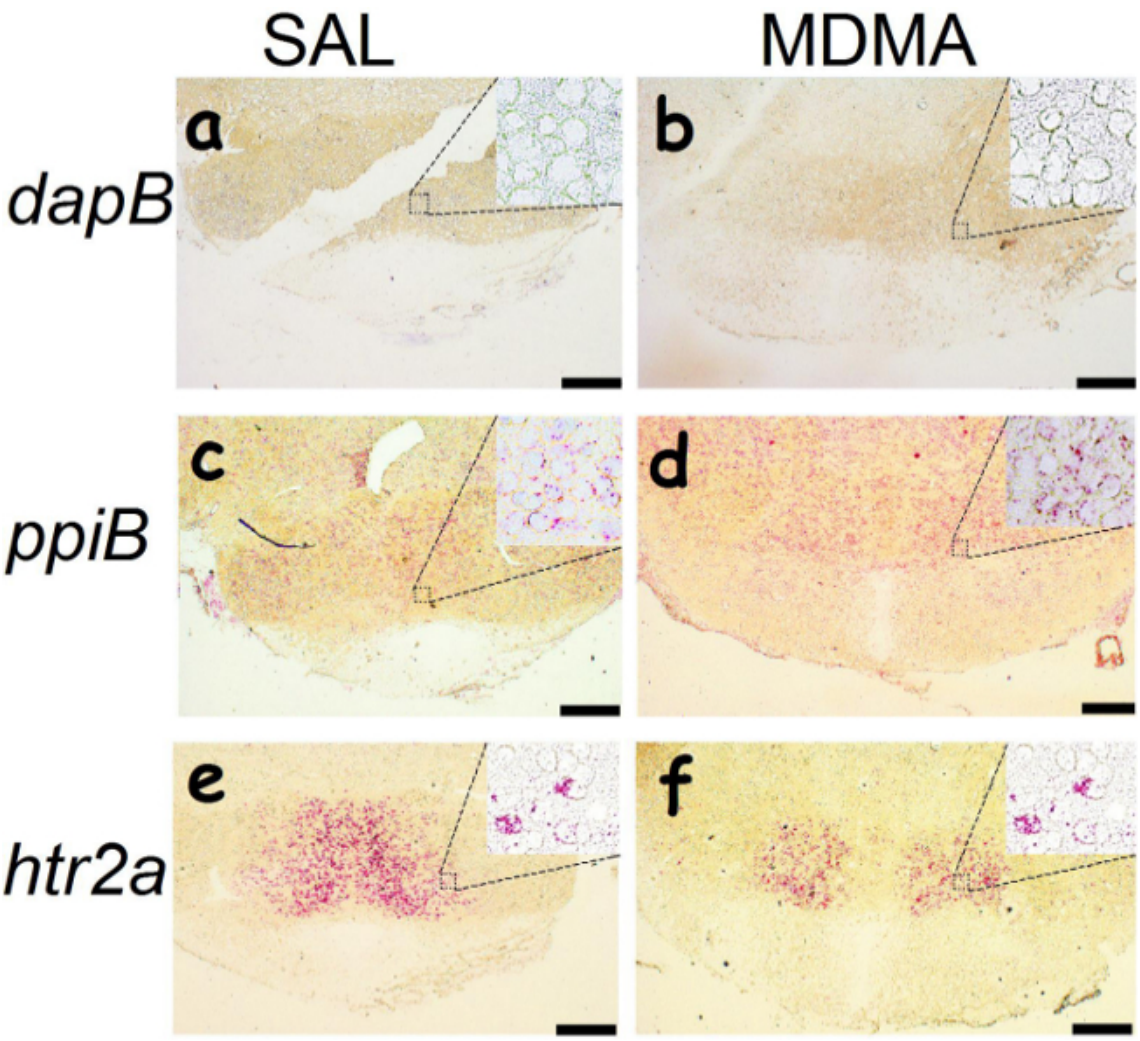

Figure 3. A light microscopic view of hybridization signals. Tissues were prefixed with paraformaldehyde in deeply anesthetized rats previously treated with saline (SAL) and 3,4-methylenedioxymethamphetamine (MDMA). Oligonucleotide probes are *dapB* (**A-B**), *ppiB* (**C-D**) and *htr2a* (**E-F**). Hybridization signals are identified as red dots using objective magnification powers ranging from 4× (Bars: 200 µm) to 20× (inset microphotographs). Please click here to view a larger version of this figure.

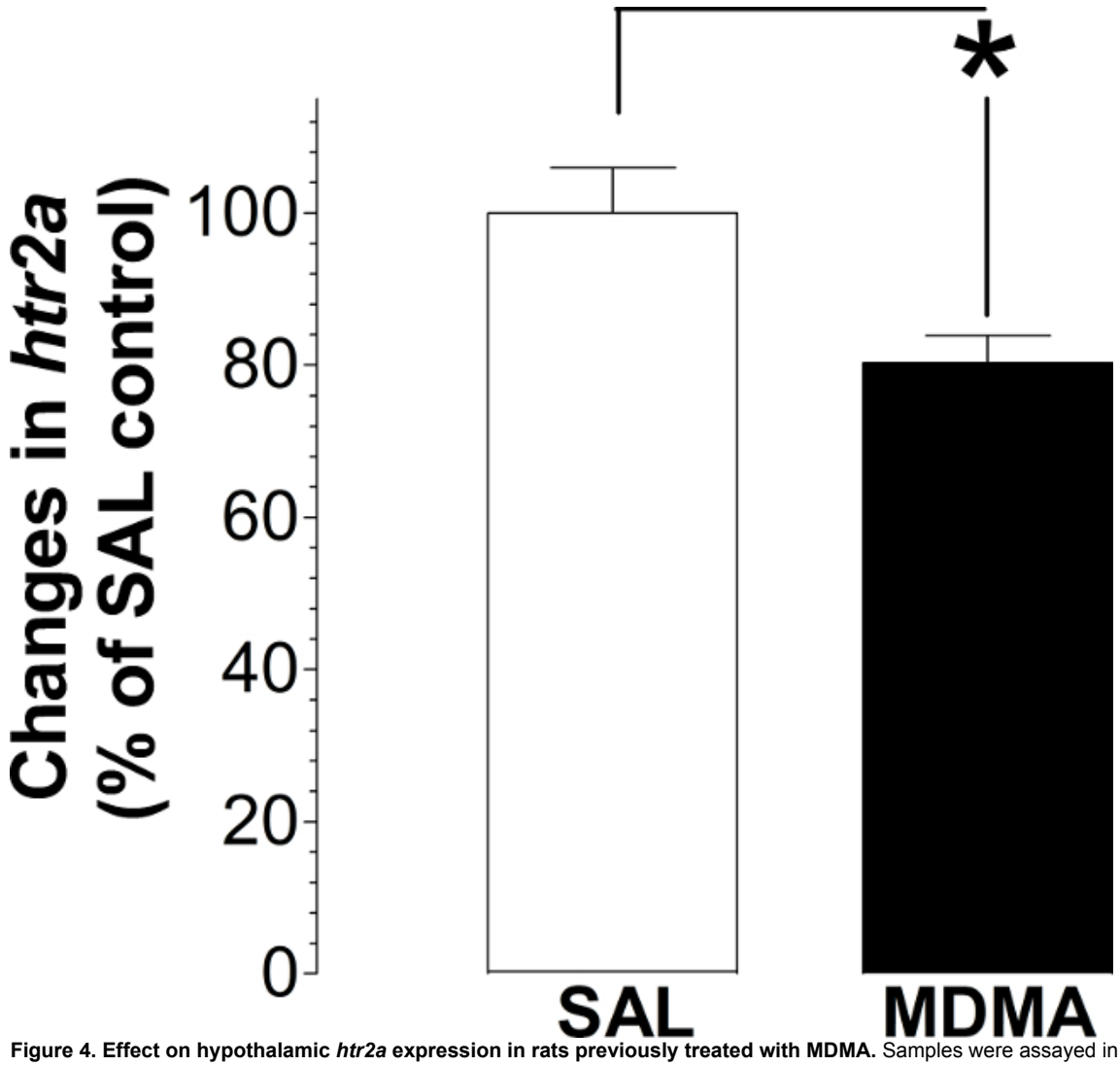

Figure 4. Effect on hypothalamic *htr2a* expression in rats previously treated with MDMA. Samples were assayed in duplicates and repeated three times. Data are expressed as a mean percentage ± s.e.m. of the SAL group (a total of 8 rats used in this study). *P <0.05 *vs*. the SAL group using Student's t test.

	Fresh		Paraformaldehyde-prefixed[a]	
	SAL[b]		SAL[b]	MDMA[c]
Target probe (*htr2a*)	√		√	√
Positive probe (*ppiB*)	–		√	√
Negative probe (*dapB*)	–		√	√

√, indicates the group examined with oligonucleotide probe in this study.
–, not examined;
[a], each group contains 4 different animals;
[b], the group pretreated intraperitoneally with physical saline (SAL; 0.9% NaCl);
[c], pretreated intraperitoneally with 10 mg/kg MDMA.

Table 1. Experimental design

Discussion

One of the major concerns of *in situ* hybridization test is to choose appropriate techniques used for RNA preservation because of RNase enzymes. It is well known that these enzymes are widely present in the cells and environment which can affect the results. However, enzyme activity can be quickly distinguished by placing the tissue in the dry ice/alcohol solution[5,12]. Although quick preservation is critical for hybridization using a riboprobe[13], little is known about experimental conditions for oligonucleotide probes. In this study using oligonucleotide probes, we found that there was no difference between the paraformaldehyde-prefixed and fresh brains quickly preserved in the ice/alcohol solution. This suggests

that brain RNA prefixed by paraformaldehyde had no significant effect on the quality of hybridization using the oligonucleotide probe. Since the prefixed brain is easily sectioned compared to the fresh brain, this protocol provides an alternative tissue preparation for the hybridization test.

We believe that keeping the brain tissue sections inside the sealed moisture tray with constant shaking on the horizontal shaker is essential for successful hybridization. Failure to follow those steps may result in uneven hybridization or high background. To eliminate possibilities of false-negative or false-positive results, tests should include necessary control slides. We found that the *dapB* probe did not show a visible signal. This is not surprising since *dapB* is a bacterial gene that is unlikely found in mammalian cells. In contrast, the area hybridized with the *ppiB* probe showed signals homogenously distributed throughout the region examined. This is predictable since the housekeeping gene should be present in any kind of mammalian cells. It has been suggested that 5-HT_{2A} mRNA is distributed heterogeneously in the brain nuclei, including the hypothalamus[14,15]. As expected, *htr2a* signals are present in certain nuclei in the region of the hypothalamus although the probe has been applied to all of the selected areas. It should be kept in mind that the use of alcohol should be avoided after the Red reagent (step 3.1) since red signals can be easily decolored by alcohol. We found that, after cellular RNA stained with the Red reagent, cells cannot be counterstained with hematoxylin or crystal violet although the exact cause of counterstaining failure is unknown. In summary, a characteristic distribution of *htr2a* mRNA supports the idea that these oligonucleotide probes are gene-specific despite the shorter sequence relative to other probes[16,17].

Background signals are another major concern, which likely affects the quality and faithfulness of data analysis. Unlike the high background produced by riboprobes[13], oligonucleotide probes normally show a very low background. Thus, image can be easily and reasonably quantified using publicly-available NIH ImageJ software. In this study, we showed an example of this technique by examining changes in *htr2a* expression in response to systemic MDMA at 10 mg/kg. We demonstrated that MDMA caused a 20% reduction in *htr2a* expression, supporting previous reports[18].

In addition to the three genes outlined above, this protocol has been validated with two other gene probes (*trh*, thyrotropin-release hormone gene, and *trhr*, thyrotropin-releasing hormone receptor gene; data not shown), demonstrating a high sensitivity and accuracy in detecting mRNA levels. Since most reactions require only 10 µl of reagents, the cost of an assay is also significantly reduced, compared to radioactive riboprobes. Furthermore, it is important to note that this protocol does not require the RNase-free environment. In conclusion, this protocol improves the speed of *in situ* hybridization assay, and increases reproducibility with relatively low cost.

Disclosures

The authors declare that they have no competing financial interests.

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