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# Preparation of Dopamine Transporter-specific Antibodies Using Molecular Cloned Genes

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Dopamine transporter (DAT) plays the most important role in terminating the actions of dopamines released into the synaptic cleft. DAT is also the target of various psychotropic drugs such as cocaine and amphetamine. In this study we prepared DAT-specific antibodies using the 2nd extracellular loop of rat DAT as an antigen. The 2nd extracellular loop of the rat DAT was expressed in bacteria as a fusion protein with glutathione-S-transferase, and injected into rabbits to raise antibodies. Produced antibodies clearly recognized the rat DAT in ELISA, immunoblotting, and immunoprecipitation. As expected from the high sequence homology between the rat and human DAT, the antibodies raised for the rat DAT cross-reacted with the human DAT in the immunoblotting. Considering the specificity for DAT with wide range of applications such as ELISA, immunoblotting, and immunoprecipitation, these antibodies would be valuable tool for understanding the pharmacological actions of dopamine transporter and drug addiction.

**Key words :** Dopamine transporter, Antibodies, Drug addiction

## INTRODUCTION

Dopamine transporter (DAT) belongs to the plasma membrane neurotransmitter transporter family in which they have characteristic twelve transmembrane domains. It re-uptakes synaptic dopamines released from the presynaptic neurons by utilizing the energy of sodium gradient generated by $Na^+/K^+$-ATPase (Shimada *et al.*, 1991; Kilty *et al.*, 1991). Dopamine transporter plays a major role in clearing the synaptic dopamines and the mice lacking this protein show markedly increased locomotor activity (Giros *et al.*, 1996). Furthermore, DAT is known as the target of various psychoactive agents and the substances of abuses such as anti-depressants, cocaine, and amphetamine (Robinson and Becker, 1986; Snyder, 1973).

Dopamine transporter has been studied on SDS gels after photoaffinity labelling with the radiolabelled ligands (Grigoriadis *et al.*, 1989; Vaughan, 1995). This approach requires the presence of an active protein moiety capable of ligand binding and thus is not suitable for all experimental applications. In this study, we raised the antibodies specific for the second extracellular domain of DAT by taking advantage of cloned DAT.

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## MATERIALS AND METHODS

### Cell cultures

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Cultures were kept at 10% $CO_2$ atmosphere, 37°C in saturated humidity.

### Bacterial expression of fusion proteins

For the preparation of antigen, the second extracellular loop and the N-terminal tail of the cloned rat DAT were expressed as the fusion protein with glutathione-S-transferase (GST) (Kim *et al.*, 1995; Cheong *et al.*, 1996). Based on the amino acids sequence of the cloned dopamine transporter, the regions containing glycosylation sites were excluded. Using the primers shown in the results part, the second extracellular loop and the N-terminal tail of the DAT were amplified (94°C, 1 min; 55°C, 1 min; 72°C, 1 min), digested with *BamHI/EcoRI*, then subcloned into pGEX-2T (Pharmacia). The expression of the fusion protein was confirmed on the SDS polyacrylamide gel.

Fusion protein was purified in a large scale and used for the antigen. For this, saturated bacterial culture was

10 times diluted and grown for 1 h to reach the log phase. Proteins were induced by adding IPTG (Isopropyl β-D-Thiogalactopyranoside) in the log phase at the final concentration of 0.5 mM. Cells were harvested after additional two hour incubation, and the expressed protein was purified using glutathione beads (Pharmacia or Sigma).

### Immunization

Purified GST fusion protein of the N-terminal or the second extracellular loop of DAT was injected into rabbits. For initial immunization, 500×g of the purified proteins was mixed with the equal volume of complete adjuvant (Gibco or Sigma). For the subsequent boosterings, 200 μg of the purified protein was injected with the equal amount of incomplete adjuvant (Gibco or Sigma). These treatments were intramuscularly applied on the monthly basis for 4 months.

### Solublization of dopamine transporter

Rat brain tissues were solublized according to the previously reported methods (Patel *et al.*, 1993). The cortex and striatum were removed from the rat brain, and crude extract was prepared in 50 mM Tris HCl, pH 8.0, containing 1 mM EDTA, 1mM PMSF (Phenylmethylsulfonyl Fluoride), aprotinin (10 μg/ml), and leupeptin (10 μg/ml). After centrifuging at 48,000×g for 10 min at 4, the pellet was resuspended in the same buffer containing 1.0% (v/v) CHAPS (3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonate) and gently stirred on ice for 30 min. The extracts were centrifuged at 48,000×g for 20 min at 4°C. The supernatant was removed and filtered through 0.22 m filter and the filtrate was used for the immunoprecipitation. The concentration of protein was determined by Bradford method.

### Enzyme-linked immunosorbant assay (ELISA)

Microtitration plates were coated at 4°C overnight with 50 μl/well of 5 μg GST or GST-DAT. The plates were washed four times with T-PBS (1% Tween 20 in PBS). Nonspecific sites were blocked by 200 μl of PBS containing 0.1% (w/v) of bovine serum albumin for 2 h at 37°C. After 3 times washing, 50 μl of antiserum was added to each well and incubated for 2 h at 37°C.

The same amount of pre-immunization serum was added as a negative control. Either crude or GST-treated antibodies were used for the ELISA. Because the crude antibodies were supposed to detect both GST and DAT, but the GST-treated antibodies to detect only DAT, the titer of the antibodies specific for the DAT was calculated by measuring the bindings of two preparations of antibodies to GST-DAT. The antiserum was used at the dilution of 1 : 1,000, 1 : 10,000, 1 : 100,000, 1 : 200,000. The plates were washed and 50 μl of peroxidase-labeled anti-rabbit antibodies (diluted 1 : 5,000) were added for 1 h. Tetramethylbenzydine, a peroxidase substrate solution (Sigma), was used for the color development and the absorbance was measured with the ELISA reader at 450 nm.

### Immunoblotting

For the characterization of the antibodies by immunoblotting, we prepared the membrane proteins from the rat brain regions of interest or from the COS-7 cells transfected with DAT constructs. The COS-7 cells transiently transfected between 48 to 72 h were harvested in 50 mM Tris HCl, pH 8.0, containing 1 mM EDTA and protease inhibitors. The cells were homogenized and centrifuged at 48,000×g for 10 min at 4 °C. The same procedure was repeated, and the pellet was used for the immunoblotting. Exactly the same procedure was followed for the brain tissues.

Proteins were separated by 8% polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membrane filter. The nitrocellulose membrane filters were incubated overnight with blocking buffer (PBS+1% nonfat dry milk, 0.1% Tween 20) at 4°C. The antisera diluted 1 : 250 in blocking buffer were added and incubated for 2 h with gentle stirring at room temperature. The nitrocellulose membrane filters were washed, treated with anti-rabbit IgG alkaline phosphatase conjugate (1 : 10,000 diluted) for 1 h at room temperature, then developed with BCIP/NBT solution (Sigma).

### Immunoprecipitation

Protein A beads (50 μl, 100 mg/ml in PBS) were coupled to the DAT antiserum which was diluted 1 : 10 in PBS. This mixture was incubated for 1 h at 4°C with gentle stirring and added to the solublized proteins from the rat brain, which was diluted 10~20 times in 0.05% SDS-PBS. After overnight incubation at 4°C, the pellet was boiled in SDS sample buffer. Proteins were separated on the 8% polyacrylamide gel and used for immunoblotting.

## RESULTS AND DISCUSSION

### Bacterial expression of the second extracellular loop of dopamine transporter

The dopamine transporter has 12 putative transmembrane domains. The alignment of neurotransmitter transporter showed that they are highly conserved at the transmembrane domains (Shimada *et al.*, 1991; Kilty *et al.*, 1991). We chose the amino terminal and the second extracellular loop as the potential sites for antibody production because these regions are distinct from those

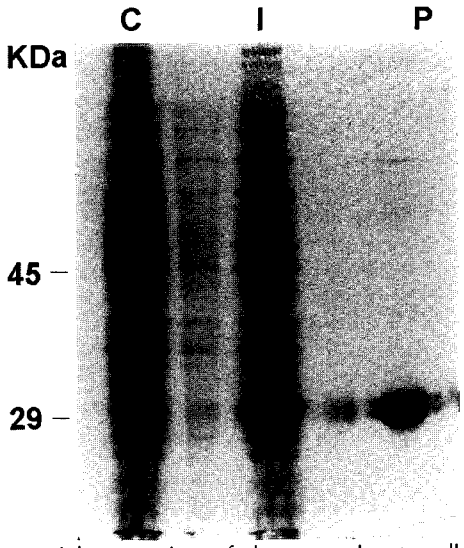


**Fig. 1.** Bacterial expression of the second extracellular loop of rat dopamine transporter. 'C' represents 'control', 'I' represents 'induced', and 'P' represents 'purified'. Protein expression was induced by IPTG at the final concentration of 0.5 mM. For the control and induced lanes, the whole bacterial protein was resuspended in SDS sample buffer and loaded on to the 9% polyacrylamide gel. The purified lane represents purified fusion protein through the glutathione-agarose affinity chromatography.

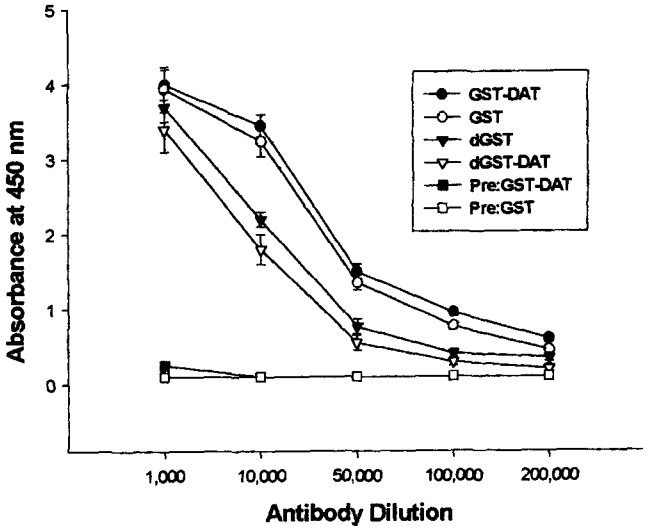


**Fig. 2.** ELISA test of the antibodies for dopamine transporter. GST alone or GST-DAT was used as coating antigen to differentiate the antibody specificities. Serum was used either intact or incubated with GST. Preimmune serum was used as negative control. Following designations were used to classify experimental groups. pre: pre-immunization serum, d: serum was treated with GST to remove the antibodies raised for GST; GST antibodies, gst: GST protein as a coating antigen, gst-dat: GST-DAT fusion protein as a coating antigen. Therefore, pre-GST means that preimmune serum was used as a primary antibody and coating protein was GST. d-GST-DAT means that the serum was cleared with GST and the coating antigen was GST-DAT.

bodies specific for the DAT or GST. For this, we introduced the changes either to the coating antigen or to the antibodies. For the coating antigen, GST was used for one group and GST-DAT for the other group. Meanwhile, the antibodies were used either as crude or as treated with GST. Pre-immune serum was used as a negative control.

As shown in Fig. 2, pre-immune serum recognized neither DAT nor GST. Since the antibodies were raised against both GST and DAT (refer to the method part, GST-DAT was used as the antigen), we tried to remove the antibodies raised for the GST. For this, the serum was incubated with GST overnight and centrifuged to remove precipitated antigen-antibody complex. When the serum was used without treatment with GST, only around 10% of antibodies specifically detected DAT (the difference of upper two graphs). When the serum was treated with GST, about 20% of the antibodies detected DAT. These results were expected because the size of GST was around 27.5 KD but that for DAT was only around 4 KD. These results also suggest that overnight treatment of antibodies with GST did not remove the substantial proportions of the antibodies raised for GST.

### Immunoblotting and immunoprecipitation from the rat brain tissues

As shown in Fig. 3, the antibodies recognized the in other transporter proteins, and long enough to produce antibodies.

To make antibodies specific for DAT, the 2nd extracellular domain of the DAT was amplified using the primers; 5'-ATATGGATCCACCACACCCGCTGCTG-3' vs 5'-ATCAGAATTCTCAGAGCTGCCACCGTGG-3', and subcloned into pGEX-2T vectors to produce fusion proteins (Kim *et al.*, 1995; Cheong *et al.*, 1996). This method was proven to be convenient and powerful in terms of time and effort. Milligrams of fusion proteins per liter of bacterial culture were obtained. Fig. 1 shows the expression of the protein, the 2nd extracellualar loop of rat DAT linked to GST, in the SDS polyacrylamide gel (the N-terminal part is not shown because this region failed to produce specific antibodies). The calculated molecular weight of the 2nd extracellular loop of DAT, we amplified, was about 4.1 kD, and the whole fusion protein produced was about 31.6 kD including 27.5 kD GST and the linker portion. Purified protein usually gave two bands in SDS gel, indicating that it was cleaved in the process of purification.

### Characterization of the antibodies using ELISA

Fig. 2 shows the ELISA tests of the anti-DAT serum. Since the whole fusion protein was used as the antigen (GST-DAT), we wanted to check the titer of the anti-

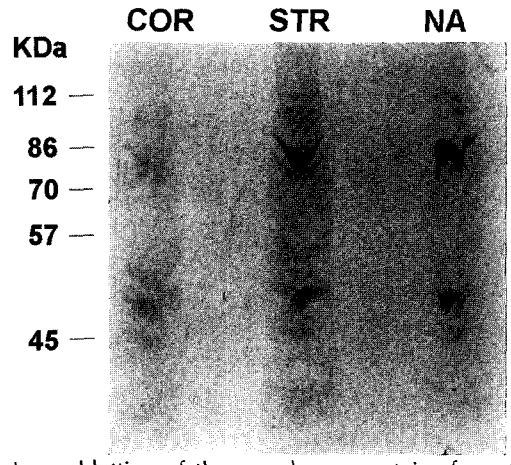


**Fig. 3.** Imunoblotting of the membrane proteins from the rat brain tissues. The membrane proteins were prepared from three brain regions and loaded onto 9% polyacrylamide gel, cerebral cortex (COR, 32 μg), striatum (STR, 24 μg), and nucleus accumbens (NA, 32 μg). Primary antibodies were diluted 500 times and anti-rabbit alkaline phosphatase conjugated secondary antibodies (Sigma) were 20,000 times diluted.

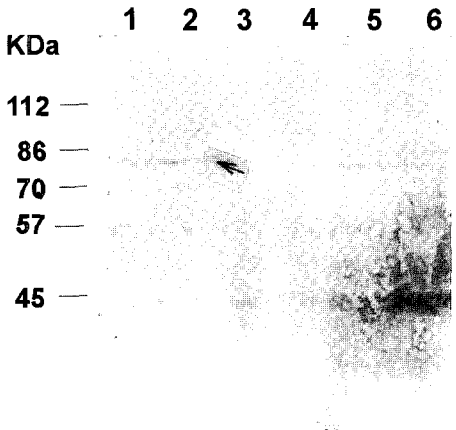


**Fig. 4.** Immunoprecipitation of the dopamine transporter from the brain tissues. The membrane proteins were prepared from the rat striatum and cortex, solublized in 1% (v/v) CHAPS for 30 min. The solublized membrane proteins were incubated with the protein A conjugate (Anti-DAT antibodies were precoupled with protein A beads for 2 h at 4°C) for 4 h at 4°C. The pellet (protein A beads) was incubated with SDS buffer for 30 min to elute bound proteins. Immunoblotting was conducted as in Fig. 3. The arrow shows the location of the rat dopamine transporter. 1-membrane proteins from cortex; 2-membrane proteins from striatum; 3-supernatant after precipitating the solublized membrane proteins from cortex with protein A-antiserum conjugate; 4-supernatant after precipitating the solublized membrane proteins from striatum with protein A-antiserum conjugate; 5-immunoprecipitates of cortex; 6-immunoprecipitates of striatum.

rat DAT in the cerebral cortex, striatum, and nucleus accumbens. The intensity of the protein bands recognized by DAT antiserum was the strongest in the striatum (24 μg membrane proteins were loaded) followed by nucleus accumbens (32 μg loaded) and very low at cerebral cortex (32 μg loaded) in accordance with the density of DAT expressed (Ciliax *et al.*, 1995; Madras *et al.*, 1998). The expression of DAT in the cerebellum was hardly detectable (data not shown). The calculated molecular weight of the cloned rat DAT is 69 KD (Giros *et al.*, 1991), however, the DAT in the brain tissues was recognized as a protein with the molecular weight around 80 KD. This discrepancy probably comes from the glycosylation of DAT (rat DAT has 4 potential glycosylation sites in the 2nd extracellular loop), and this value is in a good agreement with the previously reported results using photoaffinity binding technique (Patel *et al.*, 1993; Vaughan, 1995). These antibodies were also able to immunoprecipitate the DAT from the cortex and striatum of rat brain (Fig. 4).

### Cross-reactivity of the antibodies with human dopamine transporter

Immunoblotting was conducted using the membrane proteins prepared from the COS-7 cells transfected with the human DAT cDNA in pCMV5. For MOCK transfection, the COS-7 cells were transfected with the same amount of pCMV5 vector itself. As shown in Fig. 5, the antibodies raised against rat DAT clearly detected the human DAT. The human DAT was detected near 70 KD protein, but the lighter band was also detected around 100 KD, suggesting that some portion of DAT

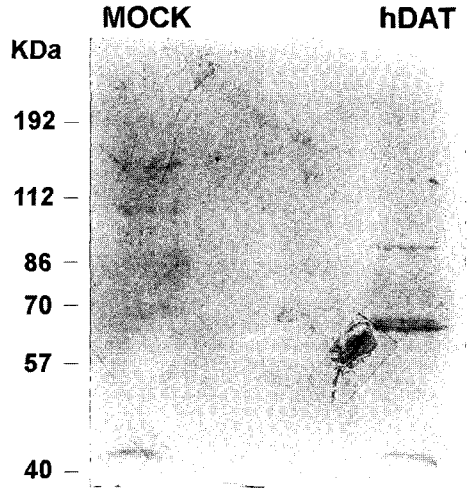


**Fig. 5.** Cross reactivity of the antibodies with human dopamine transporter. The membrane proteins was prepared from COS-7 cells transfected either with vacant vectors or with the human DAT constructs. The same procedure as in Fig. 3 was employed for the immunoblotting. The lane labelled 'MOCK' represent the membrane proteins prepared from the COS-7 cells in which pCMV5 DNA was transfected. 'H-DAT' represent the membrane protein prepared from the COS-7 cells in which the construct of human dopamine transporter in pCMV5 was transfected. Primary antibodies were diluted 500 times and anti-rabbit alkaline phosphatase conjugated secondary antibodies (Sigma) were 20,000 times diluted. The arrow represents shows the human dopamine transporter.

expressed in COS-7 cells are highly glycosylated (Patel *et al.*, 1991).

The cross-reactivity of the antibodies with human DAT was predictable because of the high homology in amino acids sequence between the rat and human in the 2nd extracellular loop of DAT, which was used as the antigen. Among 31 amino acids used for the antigen, only one amino acid was different from those of human DAT (arginine to histidine).

These antibodies detected both rat and human DAT in immunoblotting, and also demonstrated wide range of applications such as ELISA, immunoblotting, and immunoprecipitation. Therefore, these antibodies are expected to be quintessential to conduct more sophisticated studies for the pharmacological functions of dopamine transporter including drug addiction.

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