CHAPTER 2

MATERIAL AND METHODS

Thirty seven male albino, Wistar strain, rats weighing 280-300 g, were used in the present study. After sodium pentobarbitone (Nembutal) anesthesia (45 mg/kg. i.p.), the rat was placed in a Takahashi stereotaxic instrument. Localization of the nucleus accumbens was based on stereotatic coordinates obtained from the stereotaxic atlas of the rat brain by Pellegrino, Pellegrino and Cushman (1979). After tissues overlying the frontal area were dissected and retracted away, a small burr hole was made in the skull by a dental drill for the insertion of the micropipette which was held by an the electrode carrier. The dura was then removed. The pipette was inserted into the brain and HRP was delivered by electrophoretic ejection.

2.1 Microelectrophoresis

HRP (Sigma type VI) was freshly prepared at a concentration of 10-30% in Tris-HCl buffer (pH 8.6) and then filled in the glass micropipette (tip diameter 20-60 μm). Positive square wave pulses (duration 500 ms, frequency 0.5 Hz, current 5-10 μA) were applied through the pipette for a period of 20-30 minutes using a Grass S 44 stimulator. The pipette was left in place for further 5-10 minutes after

termination of the current pulses. The micropipette was then withdrawn from the brain. During the withdrawal period a constant cathodal direct current of 0 15-0.30 uA was passed through the pipette to prevent diffusion of HRP. After removal of the pipette, the wound was closed by suture Following survival times of 24-48 hours the rats were deeply anesthetized with Nembutal perfused and subjected to histochemical techniques by either of the two procedures described below.

2.2 Histochemical Proceudre I

After cutting the jugular veins to provide drainage. the animal was perfused through the left ventricle with approximately 250 ml 0.05 M phosphate buffer (pH 7.4) and follow by a solution of fixative containing 1% paraformaldehyde, 1.5% glutaraldehyde, 4% sucrose in 0.05 M phosphate, buffer (pH 7.4). The brain was removed and stored overnight in 30% sucrose in 0.05 M phosphate buffer. Subsequently 50 μm frozen sections were cut in coronal plane. One from three serial sections were incubated according to the technique described by de Olmon. Hardy and Heimer (1978) using 3, 3′, 5, 5′ tetramethylbenzidine (TMB) as substrate. The sections were then picked up onto microscopic glass slides, blotted dry and were later stained lightly with 1% neutral red to facilitate color contrast between the red perikaryon and the blue TMB reaction-product. After the staining, the sections were dehydrated through graded series

of dehydrated ethanol and xylene, then mounted under cover-slip glass (for details of the procedure see appendix).

Examining under microscop. it was found that the presence of HRP labeled cells was interfered by a lot of precipitations and remaining of red blood cells in the blood vesseles. The HRP labeled cells eventhough, could be visualized but their processes were seldom seen. This might be due to either fixation or histochemical reaction or both. Therefore, another technique described below was used.

2.3 Histochemical Procedure II

The rat was perfused through left ventricle with normal saline (50 ml) and followed by a solution of fixative containing 1% of paraformaldehyde and 1.25% of glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Approximately 250 ml of fixative was used for each animal and then followed by 100 ml. of 10% sucrose in 0.1 M phosphate buffer. The brains were removed and subdivided into appropriate blocks. The right side of the brains were always marked with knife cut to facilitate identification of left-right position. The blocks were stored overnight at $4^{o}C$ in 0.1 M phosphate buffer containing 30% of source. The blocks were then cut in coronal plane into sections of 50 μm thickness on a freezing microtome. One from every three sections were collected in the phosphate buffer and processed for histochemical reaction according to method

described by Mesulam (1978), using 3, 3', 5, 5' tetramethylbenzidine (TMB) as substrate. The sections were picked up onto microscopic glass slide. blotted dry. stained and dehydrated as described in the proceudre I.

Comparing between the two procedures, the latter gave better results. In details, HRP-labeled cells obtained from the proceudre II were seen together with their processes. There were less precipitations and very few red blood cells were seen in blood vessels. In two cases that same amount of HRP were injected into the same area but the two brains were treated by different procedures (I or II); it was found that the number of HRP-labeled cells obtained from procedure II were more abundant than those obtained with procedure I

The serial coronal sections of the brain were projected by a microscopic projecting apparatus onto a large drawing paper and serial diagrams of brain sections were constructed in the similar way as shown in Figure 1 for identification of the brain areas. The sections were studied under light microscope for the presence of HRP labeled cells which were characterized by dark granules in the perikarya and dendritic processes. The location of each HRP labeled cell was then plotted as a dot onto the corresponding region in the serial diagrams (as shown in Figure 2-6).

Since it has been reported that under certain methodological conditions, endogenous peroxidase activities

in certain group of neurons can be demonstrated by the same histochemical reaction (Wong-Riley, 1976; Keefer and Christ, 1975). Several uninjected rats were perfused and processed by the same condition deseribed above and served as control. There were no endogenous labeled neurons found in brains treated by the methods and conditions used in our laboratory. In addition, the method produced by Mesulum (1978) using TMB as chromogen has been shown to give the most exogenous sensitivity while suppress endogenous peroxidase activity (Mesulam and Rosene. 1979).