EXPERIMENT K-6-18

STUDY OF MUSCARINIC AND GABA (BENZODIAZEPINE) RECEPTORS IN THE SENSORY-MOTOR CORTEX, HIPPOCAMPUS AND SPINAL CORD

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INTRODUCTION

Frontal lobe samples of rat brains flown aboard Cosmos 1887 were processed for the study of muscarinic (cholinergic) and GABA (benzodiazepine) receptors and for immunocytochemical localization of the neurotransmitter gamma-aminobutyric acid (GABA) and glial fibrillary acidic protein (GFAP).

MATERIAL AND METHODS

Receptor binding studies

a) Tissue preparation

Brain fragments were placed in a microtome chuck in a cryostat kept at -15 deg. C and surrounded with phosphate buffer 0.1M. Sections were cut at 20 µm, placed on gelatinized slides and thawed by warming the back of the slides with palm of hand. The slides were then transferred to slide boxes and kept at 0 deg. C for at least two hours to allow them to dry.

b) Radioligand binding experiment

The slides were incubated with ^{3}H-ligand, followed by washes. To allow for the determination of specific binding a slide was incubated with a displacing amount of drug ("blank" slide). The incubation mixture consisted of 40 ml buffer, 5 ml ^{3}H-ligand, and 5 ml buffer (for total binding) or drug (for "blank"). The concentrations of ^{3}H-ligand and drug are given below since they vary according to the receptor to be studied.

The incubation procedure was performed in Coplin jars (50 ml capacity) and the washes in staining trays (250 ml capacity) with slides transferred in glass racks. After the last wash the slides were dipped in distilled water and then air dried. They were subsequently stored in a slide box with dessicant at 4 deg. C for 24 hours prior to autoradiographic exposure.

Receptor localization studies were as follows:

(1) Muscarinic (cholinergic)

Incubation: 1nM [^{3}H]-1-quinuclidinyl benzilate ([^{3}H]-QNB) for 2 hours, room temperature.

Blank: 1 µM atrophine

Washes: two 5-minute washes in ice cold buffer.

Buffer: phosphate buffer saline (PBS) at pH 7.4

Exposure: 1 month at 4 deg. C (see below for details)

(2) GABA (benzodiazepine)

Incubation: 1nM [^{3}H]-flunitrazepam for 40 minutes in ice cold buffer

Blank: 1 µM clonazepam

Washes: 10 minutes in ice cold buffer.

Buffer: 0.17M Tris, pH 7.4

Exposure: 1 month at 4 deg. C (see below for details)

c) Autoradiography

The dried labelled slides were arranged on a thin sheet of cardboard and fixed to the surface with double-stick paper. They were placed in x-ray cassette, tritium sensitive film (^{3}H-Ultrafilm LKB, Gaithersburg, M.D.) was placed over slides (emulsion side facing slides) and the cassette closed. This procedure was done in total darkness.

After the period of exposure the film was developed with Kodak D-19 developer under sodium safe-light for 5 minutes at 20 deg. C and fixed with full strength rapid fix.

Immunocytochemistry of gamma-aminobutyric acid (GABA) and glial fibrillary acidic protein (GFAP).

1. GABA

a) Tissue preparation

Tissue sections slide-mounted adjacent to those used for receptor binding studies were fixed for two hours in 5% glutaraldehyde in 0.1M phosphate buffer at 4 deg. C. After fixation the slides were thoroughly rinsed in cold phosphate buffer saline (PBS).

b) Immunocytochemical procedure

The ABC method was used. The slides were incubated as follows:

(1) 2% normal goat serum in PBS for one hour, room temperature

(2) GABA antiserum conjugated to bovine serum albumin (Immunonuclear Corp.) diluted 1/4000 in PBS, overnight at 4 deg. C.

(3) Biotinylated goat anti-rabbit IgG (Vector Labs.) for one hour, room temperature

(4) Vectastain reagent (Vector Labs.) for one hour, room temperature.

(5) Reaction at room temperature, with 25 mg. diaminobenzidine tetrahydrochloride in 50 ml tris buffer saline (TBS)+5 µl 30% hydrogen peroxide 5-15 minutes, to develop reaction product.

(6) Final rinse of the sections in TBS, two changes x 10 minutes, room temperature.

Between steps the sections were rinsed with cold PBS, three changes x 10 minutes.

Some sections were dehydrated and mounted and others were counterstained with Cresyl violet, dehydrated and mounted. Omission of the primary antibody was employed as control.

2. GFAP

a) Tissue preparation

The slide-mounted sections wer fixed for 15 minutes in 4% paraformaldehyde in 0.1M phosphate buffer. After fixation the slides were rinsed in cold PBS for 30 minutes.

b) Immunocytochemical procedure.

The peroxidase-antiperoxidase (PAP) method was employed. Incubation proceeded as follows:

(1) 10% normal swine serum in PBS, 30 minutes, room temperature.

(2) GFAP antiserum (Dr. Lawrence Eng, Veterans Administration, Palo Alto) diluted 1/100 in PBS, one hour, room temperature.

(3) Swine anti-rabbit IgG diluted 1/100 in PBS, 30 minutes, room temperature.

(4) PAP diluted 1/200 in PBS, 30 minutes, room temperature.

(5) Sections reacted with 12.5 mg diaminobenzidine tetrahydrochloride in 50 ml tris buffer saline (TBS) + 5µl 30% hydrogen peroxide, 5-15 minutes.

(6) Rinse of the sections in TBS, two changes x 10 minutes, room temperature.

Between steps the slides were rinsed with cold PBS, three changes x 10 minutes.

The sections were subsequently counterstained with hematoxiline for 2 minutes, dehydrated and mounted. Normal rabbit serum was employed as control instead of the primary antibody.

RESULTS

Although radioactive labeling of both muscarinic cholinergic and GABA (benzodiazepine) receptors proved to be successful with the techniques employed, distinct receptor localization of individual laminae of the frontal neocortex was not possible since the sampling of the area was different in the various groups of animals (Fig.1). In spite of efforts made for proper orientation and regional identification of laminae it was found that a densitometric (quantitation of autoradiograms) analysis of the tissue did not contribute to the final interpretation of the effects of weightlessness on these receptors.

As to the immunocytochemical studies the use of both markers, GFAP and GABA antiserum, confirmed the suitability of the techniques (Fig.2) for use in frozen material. However, similar problems to those encountered in the receptor studies prevented an adequate interpretation of the effects of micro-G exposure on the localization and distribution of GABA and GFAP.

This study did, however, confirm the feasibility of investigating neurotransmitters and their receptors in future space flight experiments.

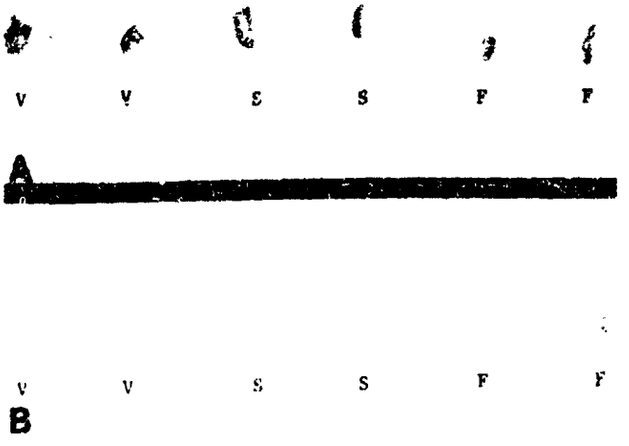

Figure 1: Autoradiograms for muscarinic cholinergic ([^{3}H]-QNB) (A) and GABA (benzodiazepine) (B) receptors of frontal cerebral cortex. Notice the difference in size and orientation of samples. V, vivarium; S, synchronous, F, flight.

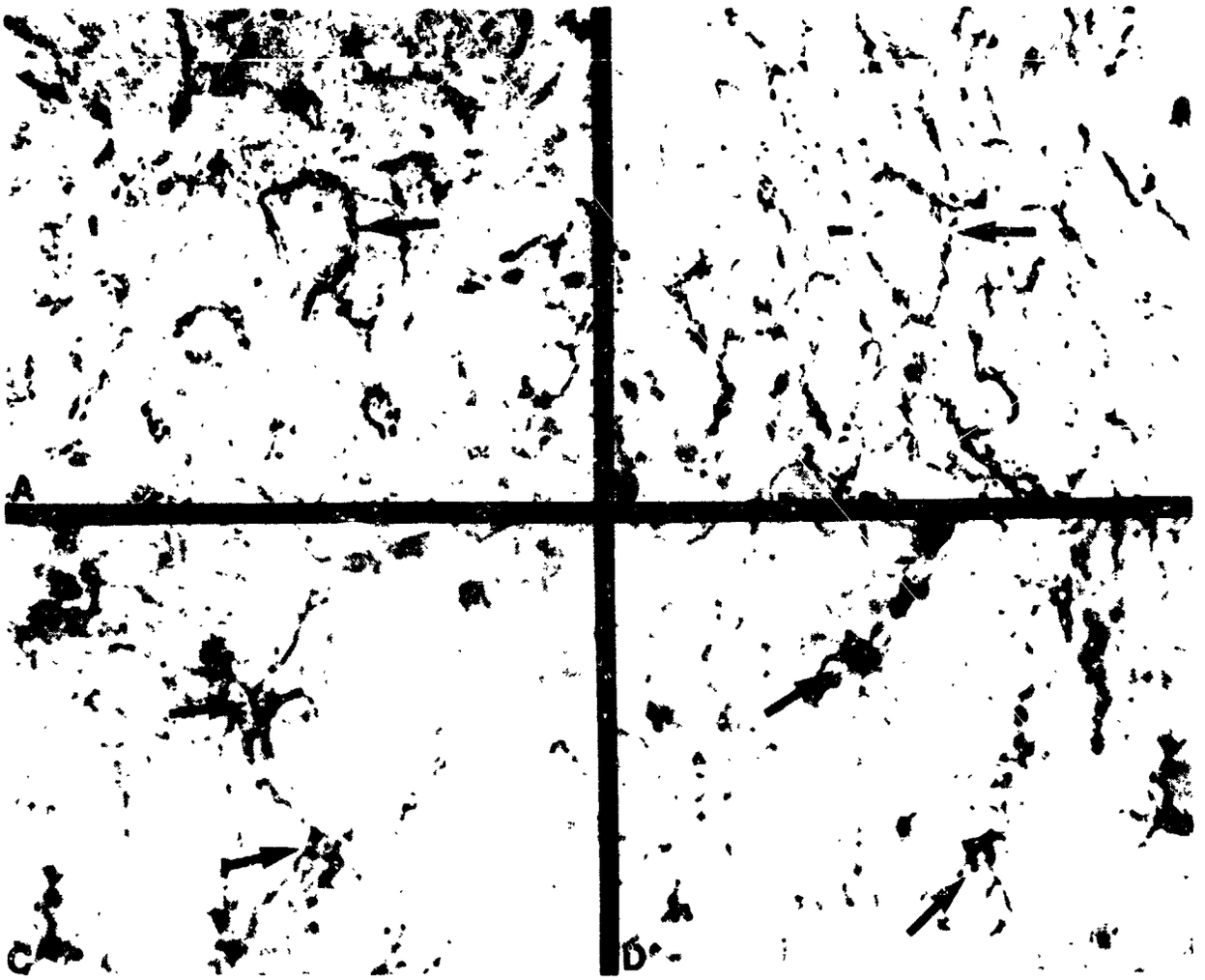

Figure 2: In (A) and (B) GABAergic boutons are seen surrounding pyramidal cells (arrows). The tissue shows some artifactual modifications due to the freezing process. (A), fligh. animal; (B), synchronous control. In (C) and (D) GFAP immunoreactive astrocytes (arrows) are seen in the boundaries between white and grey matter of the frontal cerebral cortex. (C), flight animal; (B), vivarium control.