5 Kubo, T., and Hashimoto, M., Archs int. Pharmacodyn. *232* (1978) 166.
6 Kuhn, D.M., Wolf, W.A., and Lovemberg, W., Hypertension *2* (1980) 243.
7 Buckingham, R.E., Hamilton, T.C., and Robson, D., Eur. J. Pharmac. *36* (1976) 431.
8 Dahlström, A., and Fuxe, K., Acta physiol. scand. *64* (1965) suppl. 247, 1.
9 Nygren, L.-G., and Olson, L., Brain Res. *132* (1977) 85.
10 Doba, N., and Reis, D.J., Circulation Res. *34* (1974) 293.
11 McCall, R.B., and Humphrey, S.J., J. Pharmac. exp. Ther. *222* (1982) 94.
12 Carruba, M.O., Keller, H.H., and Da Prada, M., Neurosci. Lett. *35* (1983) 173.
13 Da Prada, M., and Zürcher, G., Life Sci. *19* (1976) 1161.
14 Saavedra, J.M., Brownstein, M., and Axelrod, J., J. Pharmac. exp. Ther. *186* (1973) 508.
15 Carruba, M.O., Picotti, G.B., Miodini, P., Lotz, W., and Da Prada, M., J. pharmac. Meth. *5* (1981) 293.
16 Bühler, H.U., Da Prada, M., Haefely, W., and Picotti, G.B., J. Physiol., Lond. *276* (1978) 311.
17 Commissiong, J.W., and Neff, N.H., Biochem. Pharmac. *28* (1979) 1569.
18 Zivin, J.A., Reid, J.L., Saavedra, J.M., and Kopin, I.J., Brain Res. *99* (1975) 293.
19 Björklund, A., and Skagerberg, G., in: Brain Stem Control of Spinal Mechanisms, p. 55. Eds B. Sjölund and A. Björklund. Elsevier Biomedical Press, Amsterdam 1982.
20 Jonsson, G., Fuxe, K., Hökfelt, T., and Goldstein, M., Molec. Biol. *54* (1976) 421.
21 Ings, R., and Pappas, B., Eur. J. Pharmac. *70* (1981) 577.
22 Picotti, G.B., Carruba, M.O., Ravazzani, C., Bondiolotti, G.P., and Da Prada, M., Life Sci. *31* (1982) 2137.
23 Loewy, A.D., McKellar, S., Swensson, E.E., and Panneton, W.M., Brain Res. *185* (1980) 449.

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Neurotransmitter receptors as glycoproteins

A.J. Cross, T.J. Crow and J.A. Johnson

Division of Psychiatry. Clinical Research Centre. Watford Road. Harrow. Middlesex (England), November 8, 1982

Summary. Incubation of calf brain membrane preparations with the plant lectins, concanavalin A and wheat germ agglutinin did not inhibit neurotransmitter receptor binding sites directly. Plant lectins did however protect these sites against subsequent trypsin digestion suggesting that neurotransmitter binding sites may be associated with glycoprotein structures.

The structure of the neuronal cell surface plasma membrane determines not only the response of the neurone to transmitters and hormones but also their interaction with exogenous agents such as drugs, toxins, antibodies and viruses. In recent years glycoproteins have been shown to have a central role in interactions with such exogenous agents[1]. Glycoproteins are present in significant amounts on the cell surface[2,3], and may be concentrated in synaptic regions[4,5]. In brain it has been estimated that 80–90% of cell glycoproteins are membrane bound[4], but little is known of the specific functions of these membrane components. Several hormone receptors have been identified as glycoproteins[6], and the nicotinic acetylcholine receptor complex includes a number of glycoprotein subunits[7]. The present study was undertaken to determine if other neurotransmitter receptors include carbohydrate residues. A general rule that receptors are glycoproteins would suggest a wider involvement of transmitter receptors in cell recognition processes. Many of the properties of membrane bound glycoproteins have been elucidated by the use of plant lectins, the interaction with lectins involving the recognition of specific carbohydrate sequences[9]. We have therefore examined the direct and indirect interactions of plant lectins with a number of ligand binding sites in calf brain.

Materials and methods. Calf cerebral membranes were prepared as described previously[10], tissues were homogenized in 40 vol. of 50 mM tris/HCl buffer pH 7.4 and centrifuged at 50,000 × g for 20 min. The pellets were washed once and resuspended in the original volume of buffer to give a crude membrane preparation. Membranes were incubated in the presence or absence of plant lectin (see fig. 3) at 37 °C for 30 min. Trypsin was added at the appropriate concentration and the incubation continued for 15 min. Soyabean trypsin inhibitor was added and aliquots taken for the determination of ligand binding. Binding assays were performed using established techniques adapted for semi-automated analyses[11] using the following conditions: ^{3}H-N methyl scopolamine binding to muscarinic receptors[12] (^{3}H-NMS, ligand concentration 0.5 nm displaced by 10 μM atropine). ^{3}H-spiperone binding to dopamine D2 receptors[13] (^{3}H-SPIP, 0.5 nm displaced by 1 μM (+) butaclamol), ^{3}H-dihydroalprenolol binding to β-adrenoceptors[14] (^{3}H-DHA, 2.5 nM displaced by 1 μM propanolol), ^{3}H-WB4101 binding to α-adrenoceptors[15] (^{3}H-WB4101, 2 nM displaced by 1 μM aceperone), ^{3}H-pyrilamine binding to histamine H1 receptors[16] (^{3}H-PYR, 5 nm, displaced by 1 μM pyrilamine), ^{3}H-flunitrazepam binding to benzodiazepine receptors[17] (^{3}H-FNZ 1.5 nM displaced by 1 μM clonazepam). ^{3}H-etorphine binding to opiate receptors[18] (^{3}H-ETOR 2 nM displaced by 1 μM naloxone), and ^{3}H-muscimol binding to GABA receptors[19] (^{3}H-MUSC, 7 nM displaced by 10 μM GABA). All assays were performed with calf cortical membranes except ^{3}H-SPIP which used calf caudate membranes.

Results and discussion. Preliminary studies demonstrated that incubation of calf brain membrane preparations with the lectins given in figure 3 had no effect on the subsequent binding of any of the ligands used. When calf cerebral cortex membrane preparations were preincubated with trypsin, a rapid and almost complete loss of ^{3}H-N methyl scopolamine (^{3}H-NMS) binding to muscarinic cholinergic receptors was observed. In subsequent experiments a concentration of trypsin producing approximately 80% loss of ^{3}H-NMS binding was used (fig. 1). Incubation of membranes with the lectin concanavalin A (Con A), prior to trypsin treatment resulted in a significant protection of ^{3}H-NMS binding (fig. 1). When α-methyl mannoside (a hapten for Con A) was included in the Con A preincubation, the protective effect of Con A was no longer observed. α-Methylmannoside alone had no effect on the binding of ^{3}H-NMS, or on the reduction of ^{3}H-NMS binding by trypsin. Thus the action of Con A depends upon its affinity for a specific carbohydrate structure. Treatment of membranes with neuraminidase (an enzyme which removes

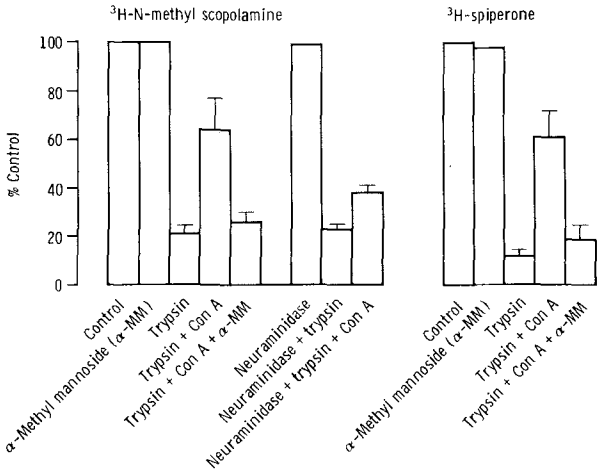

Figure 1. Interactions of concanavalin A and trypsin with ^{3}H-N methylscopolamine binding to calf cortical membranes (left) and ^{3}H-spiperone binding to calf caudate membranes (right). Results are expressed as a percentage of the appropriate controls, and are the means of at least 3 determinations, bars represent SEM.

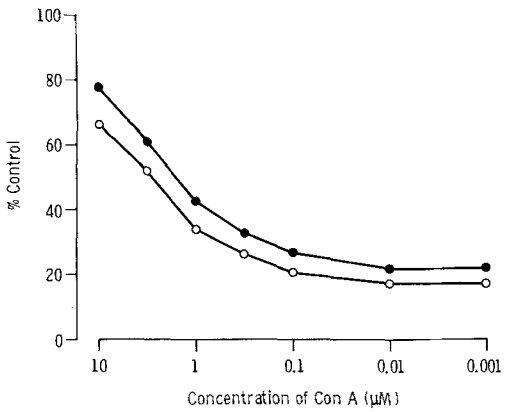

Figure 2. Dose-response curves for the protection of ^{3}H-NMS binding (●) and ^{3}H-SPIP binding (○) by Con A to proteolysis by trypsin. Results are the means of 2 or 3 determinations.

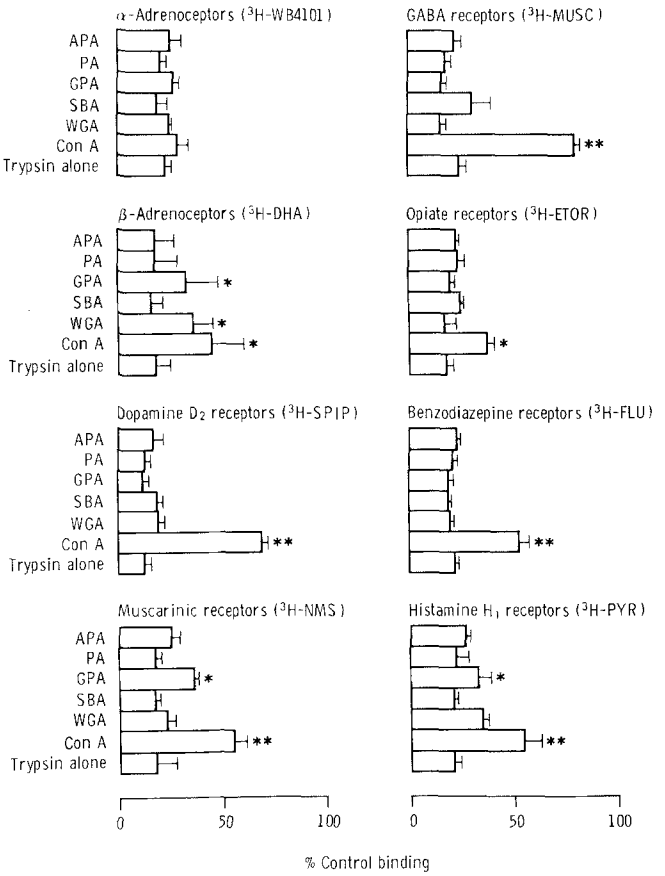

Figure 3. Protection of ligand binding sites against trypsin digestion by various plant lectins. Incubations were performed as described in figure 1. The lectins used were as follows: concanavalin A (Con A, 3 μM final concentration, active at mannose and glucose residues), wheat germ agglutinin (WGA, 3 μM, N-acetyl glucosamine), soya bean agglutinin (SBA, 1 μM, N-acetyl galactosamine), garden pea agglutinin (GPA, 2 μM, glucose and mannose), peanut agglutinin (PA, 1 μM, galactose), and asparagus pea agglutinin (APA, 2 μM, fucose). Results are expressed as a percentage of the appropriate control, bars represent SEM of 3-4 estimations. *$p < 0.05$, **$p < 0.01$ compared with trypsin treatment alone.

terminal sialic acid residues) had no effect on ^{3}H-NMS binding, however the protective effect of Con A was significantly reduced in neuraminidase pretreated membranes (fig. 1).

Similar results were obtained for the binding of the dopaminergic ligand ^{3}H-spiperone (^{3}H-SPIP) to calf caudate membranes. Thus trypsin was extremely potent in reducing ^{3}H-SPIP binding to caudate membranes, Con A protected ^{3}H-SPIP binding from trypsin digestion, and the effect of Con A was antagonized by α-methylmannoside.

The protection of both ^{3}H-NMS and ^{3}H-SPIP binding by Con A was dose-related (fig. 2). Con A having equal potency in protecting both binding sites. The potency of Con A in protecting ^{3}H-NMS and ^{3}H-SPIP binding was similar to that observed for the inhibition of ^{125}I-bungarotoxin binding to nicotinic acetylcholine receptors[20], however the binding of ^{125}I-Con A to isolated synaptic membranes appears to be of higher affinity[21].

The effects of various plant lectins in protecting a range of ligand binding sites are shown in figure 3. All the ligand binding sites studied were sensitive to trypsin treatment, with varying degrees of susceptibility. Con A was particularly active in protecting ligand binding sites, being most active in ^{3}H-muscimol binding (to GABA receptors) (75% protection) less active in ^{3}H-etorphine binding (to opiate receptors) and inactive in ^{3}H-WB4101 binding (to adrenergic receptors). Wheat germ agglutinin produced significant protection of ^{3}H-DHA (dihydroalprenolol) and ^{3}H-mepyramine binding (to β-adrenergic and histamine receptors respectively), and garden pea agglutinin protected ^{3}H-NMS, ^{3}H-mepyramine and ^{3}H-DHA binding. At the concentrations used in the present study (fig. 3) the other lectins tested were devoid of significant protecting effects in any of the binding sites. However, interaction may be revealed using higher lectin concentrations. Preliminary results suggest that Con A also protects ^{3}H-NMS binding sites in both rat and human cerebral cortex membranes.

The results of the present study are consistent with an association of oligosaccharide moieties with neurotransmitter receptor ligand binding sites. That the protection of binding sites against trypsin digestion is not due to nonspecific interactions is demonstrated by the selectivity of lectin effects to polysaccharides in general, and to specific polysaccharide components. Thus 3 μM Con A protected ^{3}H-muscimol binding sites by 75% whereas ^{3}H-WB4101 binding was not significantly protected. Moreover, the effects of Con A were antagonized by α-methylmannoside and prior neuraminidase treatment of the membrane preparation,

suggesting an interaction with specific carbohydrate structures. A nonspecific inhibition of trypsin by Con A would also appear unlikely as Con A is not a substrate for trypsin digestion[22].

The technique of protecting binding sites to trypsin digestion by ligands has been applied in several other systems[23,24], and recently Con A has been found to protect insulin receptors from trypsin digestion[24]. The present study provides no information on the mechanism of the protecting effect. As indicated by interactions with Con A and neuramindase both in the present and previous studies, carbohydrate structures are not directly involved in the binding of ligands to GABA and muscarinic receptors[25,26]. However, both receptors were protected against trypsin digestion by incubation with Con A. It would seem likely that although the oligosaccharide structure is not part of the active site of the receptor, it is closely associated with the receptor complex. Whereas Con A binding is unable to sterically inhibit the binding of small ligands, the interaction with the receptor of larger molecules such as trypsin is inhibited, presumably by steric factors. Although plant lectins are known to interact with glycolipids[9], the selectivity of Con A for some binding sites (e.g. ^{3}H-NMS binding) in preference to others (e.g. ^{3}H-WB4101 binding) suggests that such an interaction is unlikely. However, a selective association of some glycolipids with specific receptor binding proteins cannot be excluded.

A number of solubilized neurotransmitter receptors can be immobilized on Con A-linked stationary phases, including the benzodiazepine[27], GABA[27], nicotinic acetylcholine[28] and dopamine[29] receptors. That several peripheral hormone receptors (e.g. for insulin and epidermal growth factor) have a glycoprotein structure has been established[6]. The present findings suggest that many neurotransmitter receptors are associated with oligosaccharide structures. Furthermore, different receptors may be associated with different oligosaccharide moieties, as the pattern of lectin protection differs between ligand binding sites (fig. 3). Whilst differences in carbohydrate structure may exist between different neurotransmitter receptors, the present results suggest also that differences exist within receptor populations. Thus whilst Con A protected almost all ^{3}H-muscimol binding sites, a much lower proportion of binding sites for other ligands were protected. Previously it has been noted that Con A interacts only with a proportion of nicotinic[28] and insulin receptors[24], and not all solubilized brain nicotinic receptors bind to lectin columns[28]. Other glycoproteins such as the antigen Thy 1 contain similar protein primary structures, yet when present in different tissues may differ in oligosaccharide content[30]. The variety of carbohydrate structures associated with particular neurotransmitter receptors may be related to the presence of more than one cell type in the tissues used; alternatively it could be due to the activity of endogenous glycosidases remaining in the membrane preparations. Many of the ligands used in the present study bind to more than one sub-population of receptors[37]; it remains to be determined how these may relate to differences in carbohydrate structure.

The function of the carbohydrate component of neurotransmitter receptors is at present unknown. Topographical studies indicate that carbohydrate moieties of membrane-bound glycoproteins are generally located on the external surface of synaptic membranes[32,33]; thus they may be involved in stabilizing and orienting the receptor protein in the post-synaptic membrane and are exposed to the extracellular environment. Membrane glycoproteins are known to be involved in cell-cell recognition, intercellular adhesion and the development of regional brain differentiation[34]. In this respect it is interesting that neurotransmitter receptors may be involved not only in the recognition and transduction of specific neurochemical signals, but also in the development and stabilization of the presynaptic neurone in the synaptic junction[35]. The presence of two distinct receptor or recognition functions on one molecule or molecular complex has a number of implications. The carbohydrate moieties of membrane glycoproteins function as cell-surface antigenic determinants in many peripheral and central systems[1], and also act as receptors for the uptake of specific macromolecules[36]. Many neurotropic viruses are known to bind to the oligosaccharide components of membrane glycoproteins[37]. The potential of neurotransmitter receptors to act also as receptors for antibodies and viruses suggests a possible role in the pathogenesis of CNS disorders of viral or immunological[38] origin. The localization of neurotransmitter receptors on specific groups of neurones may thus account for the neuronal specificity exhibited by some neurotropic viruses[39]. For example, it has recently been demonstrated that the nicotinic acetylcholine receptor may function as a cellular receptor for rabies virus[40].

In conclusion, the present results suggest that some neurotransmitter receptors may be associated with oligosaccharide structures. Such an association may be involved in determining the neuronal specificity displayed by some immunological and virus-induced diseases of the CNS.

1 Margolis, R.U., and Margolis, R.K., Int. J. Biochem. *8* (1977) 85.
2 Matus, A.I., and Taff-Jones, D.H., Proc. R. Soc. Lond. B *203* (1978) 135.
3 Cotman, C.W., and Taylor, I, J. Cell Biol., *62* (1974) 236.
4 Gurd, J.W., and Mahler, H.R., Biochemistry *13* (1974) 5193.
5 Margolis, R.K., Margolis, R.U., Preti, C., and Lai, C.D., Biochemistry *14* (1975) 4797.
6 Cuatrecasas, P., A. Rev. Biochem. *43* (1974) 169.
7 Lindstrom, J., and Engel, A., in: Receptor regulation, p. 161. Ed. R.J. Lefkowitz. Chapman Hall, London 1981.
8 Sharon, N., and Lis, H., Science *177* (1972) 949.
9 Goldstein, I.J., and Hayes, C.E., Adv. Carbohydrate Chem. Biochem. *35* (1978) 127.
10 Owen, F., Cross, A.J., Crow, T.J., Longden, A., Poulter, M., and Riley, G.J., Lancet *2* (1978) 223.
11 Hall, H., and Thor, L., Life Sci. *24* (1979) 2293.
12 Hulme, E.C., Birdsall, N.J.H., Burgen, A.S.V., and Mehta, P., Moles Pharmac. *14* (1978) 737.
13 Fields, J.Z., Reisine, T.D., and Yamamura, H.I., Brain Res. *136* (1977) 578.
14 Bylund, D.B., and Snyder, S.H., Molec. Pharmac. *12* (1976) 568.
15 U'Pritchard, D.C., and Snyder, S.H., Life Sci., *24* (1979) 79.
16 Chang, R.S.L., Tran, V., and Snyder, S.H., J. Neurochem. *32* (1979) 1653.
17 Speth, R.C., Wastek, G.J., Johnson, P.C., and Yamamura, H.I., Life Sci. *22* (1978) 859.
18 Simon, E.J., Hiller, J.B., and Edelman, I., Proc. natl Acad. Sci. *70* (1973) 1947.
19 Beaumont, K., Chilton, W.S., Yamamura, H.I., and Enna, S.J., Brain Res. *148* (1978) 153.
20 Mittag, T.W., and Massa, T., J. Pharmac. exp. Ther. *218* (1981) 27.
21 Gurd, J.W., Can. J. Biochem. *58* (1980) 941.
22 Sumner, J.B., and Howell, S.F., J. Bact. *32* (1936) 227.
23 Kulmacz, R.J., and Lands, W.E.M., Biochem. biophys. Res. Commun. *104* (1982) 758.
24 Hill, R., and Helton, D.C., Biochim. biophys. Acta *720* (1982) 42.
25 Greenlee, D.V., Van Ness, P.C., and Olsen, R.W., J. Neurochem. *31* (1978) 933.
26 Enna, S.J., and Snyder, S.H., Moles Pharmac. *13* (1977) 442.
27 Lo, H.H.S., Strittmatter, S.M., and Snyder, S.H., Proc. natl Acad. Sci. *79* (1982) 680.
28 Salvaterra, P.M., Gurd, J.W., and Mahler, H.R., J. Neurochem. *29* (1977) 345.
29 Strange, P., personal communication.

30 Barclay, A.N., Letarte-Muirhead, M., Williams, A.F., and Faulkes, R.A., Nature *263* (1976) 563.
31 Snyder, S.H., and Goodman, R.R., J. Neurochem. *35* (1980) 5.
32 Wang, Y.S., and Mahler, H.R., J. Cell Biol. *71* (1976) 639.
33 Chiu, T.C., and Babitch, J.A., Biochim. biophys. Acta *510* (1978) 112.
34 Rees, R.P., Fedn Proc. *37* (1978) 2000.
35 Olden, K., Parent, J.B., and White, S.C., Biochim. biophys. Acta *65* (1982) 209.
36 Anderson, R.G.W., Vasite, E., Mello, R.J., Brown, M.S., and Goldstein, J.C., Cell *6* (1978) 919.
37 Lonberg-Holm, K., and Phillipson, L., eds. Virus Receptors. Chapman Hall, London 1981.
38 Carnegie, P.R., and Mackay, I.R., Lancet *2* (1975) 684.
39 Johnson, R.T., Brain *103* (1980) 447.
40 Lentz, T.L., Burrage, T.G., Smith, A., Crick, J., and Tignor, G.H., Science *215* (1982) 182.

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Monoaminergic neurons in the brain of goldfish as observed by immunohistochemical techniques

M. Yoshida, I. Nagatsu[1], Y. Kawakami-Kondo, N. Karasawa, M. Spatz and T. Nagatsu

Department of Anatomy, School of Medicine, Fujita-Gakuen University, Aichi 470-11 (Japan), Laboratory of Neuropathology and Neuroanatomical Sciences, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda (Maryland 20205, USA), and Laboratory of Cell Physiology, Department of Life Chemistry, Graduate School at Nagatsuta, Tokyo Institute of Technology, Yokohama 227 (Japan), March 4, 1983

Summary. With an immunofluorescent technique, catecholaminergic neurons were identified for the first time in the dorsal and medial thalamus and in the ventralis telencephali (the rostro-medial part of the lobus olfactorius) of the goldfish brain. Serotonin-containing neurons were found in the pretectal area.

The localization and distribution of monoaminergic neurons in the brains of lower vertebrates is important for the study of the phylogenetic development of such neuronal systems. Until now, most information about the central monoaminergic neurons of the vertebrates has been gathered by means of aldehyde-induced histofluorescent techniques[2-11]. These findings have been confirmed, mainly in higher vertebrates, by immunohistochemistry using antibodies against amine-synthesizing enzymes[12-14] and monoamines themselves[15]. Immunohistochemistry has revealed more extensive distribution of these monoaminergic neuron systems than was shown by histofluorescence. However, immunohistochemical information on monoaminergic neurons of the brains of lower vertebrates is almost lacking. Also, there are some discrepancies between the findings by the 2 methods in regard to monoamines contained in the neurons of the circumventricular organs of the frog[16,17].

In this paper, we report on the immunohistochemical localization of the monoaminergic neurons of the brain of the goldfish, especially of those neuron somata not hitherto detected in regions of the teleost brain, in comparison with the previously reported histofluorescent findings.

Materials and methods. Antiserum to bovine tyrosine hydroxylase (TH) was produced in rabbits and tested for its specificity as described previously[13]. Two kinds of rabbit antiserum to serotonin (5-HT) were used. One was purchased from Immuno Nuclear Corporation, Stillwater, Minn., USA, and the other was produced in our laboratory in rabbits using 5-HT coupled to bovine serum albumin (BSA) as antigen, according to the method of Steinbusch et al.[15,18]. The pre-immune serum and antiserum against BSA were used as controls. Control sera did not produce any specific staining in the brain sections of various animals. The 2 antisera to 5-HT produced specific 5-HT staining in brain sections of all the animals; similar results were obtained with the 2 antisera to 5-HT in dilutions of about 1:3000.

Brains of goldfish *(Carassius auratus)* were removed and fixed with Zamboni's solution (2% paraformaldehyde-0.2% picric acid in 0.1 M phosphate buffer. Subsequently they were processed for the indirect immunofluorescent staining. Details of the procedure were described earlier[12,16,17].

Results. At the level of the medulla oblongata, 2 distinct groups of TH-positive catecholaminergic neurons were observed in the reticular formation and in the post-obecular region. One cluster of catecholaminergic neurons extended bilaterally from the level of the rostral spinal region up to the middle portion of the expanded vagal lobe. The other group of catecholaminergic neurons was found to be located within the dorsal surface of the brain parenchyma of the post-obecular nucleus[9]. Processes of these neurons extended into the pia mater.

In the isthmic region of the upper rhombencephalon a small number of catecholaminergic neurons was found, bilaterally, at the ventral border of central gray. These neurons, situated dorso-laterally to the fasciculus longitudinalis medialis (FLM), were large in size but very few in numbers. At the same level of the isthmic tegmentum, 5-HT neurons were also seen in the midline portion between the FLM of both sides. This group of neurons, corresponding to the 5-HT neurons in brainstem raphé nuclei, could be followed up to the level of the caudal midbrain tegmentum. In contrast, no catecholaminergic neurons were found among the midbrain tegmentum.

In the diencephalon, a large number of monoaminergic neurons was found in close proximity to the ventricle. Hypothalamic 5-HT neurons, with processes reaching into the cerebro-spinal fluid (CSF) space, were clustered in the nucleus recessus posterioris (NRP) and the nucleus recessus lateralis (NRL) (fig. 1, b). TH-positive neurons were mostly situated within the brain parenchyma, and apparently had no contact with the CSF except for the rostro-medial portion of the NRL (the so-called paraventricular organ: PVO) (fig. 1, a). The most caudal catecholaminergic neurons of the hypothalamus were found in the nucleus posterior tuberis (NPT) lying rostromedially to the mammillary body. Some neurons were seen between the NRP and NRL in sagittal or horizontal sections (fig. 1, a). Their processes were not found to extend into the recessus posterioris and recessus lateralis. In contrast, the catecholaminergic neurons of the rostro-medial part of the NRL protrude with thick processes into the 3rd ventricle, together with the CSF-contacting 5-HT neurons of this nucleus (fig. 2, a and b). The NRP and the remaining part (ventro-