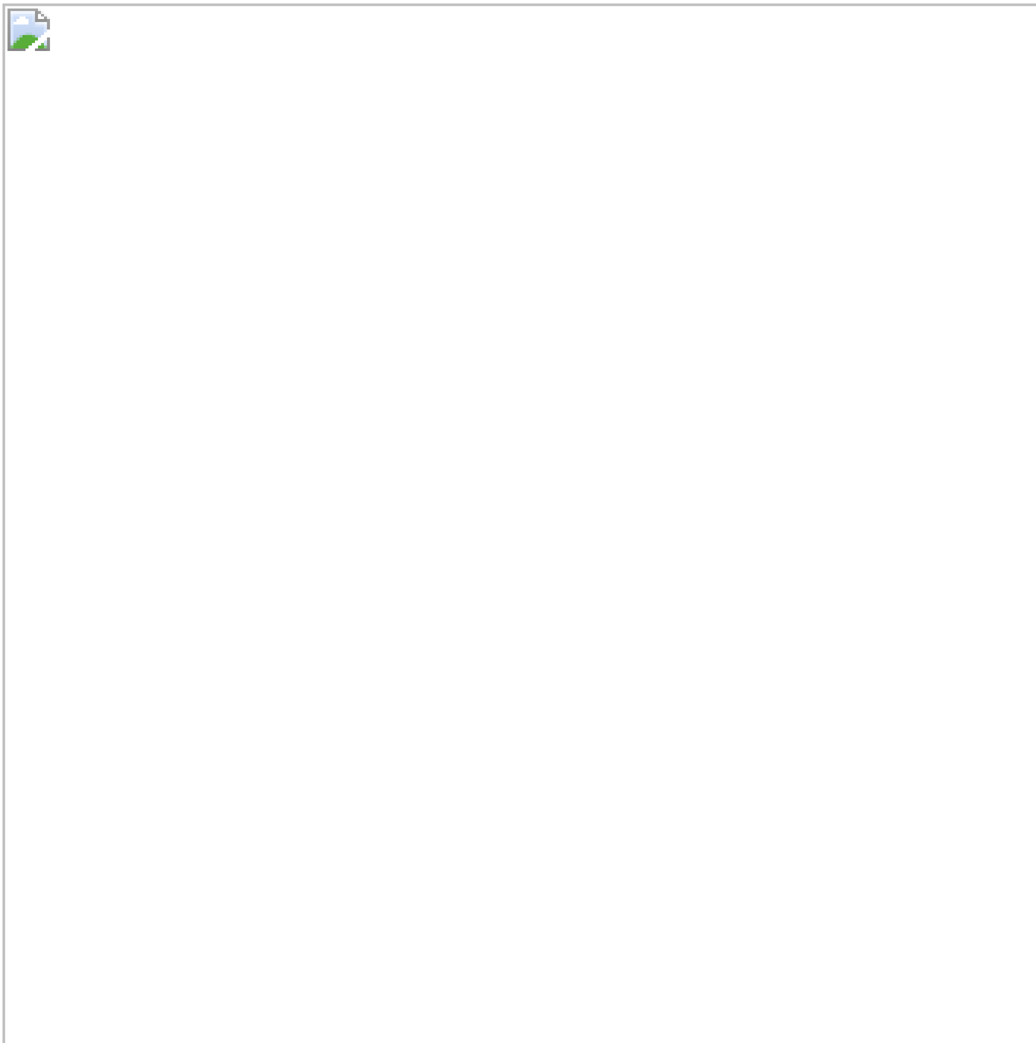

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Expression of GAP-43 mRNA in the Adult Carp Central Nervous System

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ABSTRACT—The distribution of neurons which express the gene for the growth-associated protein, GAP-43, in the adult carp central nervous system (CNS) was studied by *in situ* hybridization using newly formed RNA probes for carp GAP-43 mRNA. A great number of neurons heavily labeled by the ^{35}S-labeled antisense probe were found in the telencephalon, diencephalon, mesencephalon, optic tectum, pontine area, medulla oblongata and spinal cord. Motoneurons of the cranial nerves, i.e., the oculomotor, trochlear, trigeminal and spinal motor nerves, also strongly expressed GAP-43 mRNA, in contrast to the low level of GAP-43 signals in the motoneurons in the adult mammalian CNS. These results suggested that synaptogenesis and continuous synaptic reorganization might normally occur in the adult carp nervous system, since GAP-43 protein is generally accepted to be essential for the dynamic growth of axonal processes which leads to synaptogenesis.

In the mature skeletal muscle of the adult carp, a number of small-sized neuromuscular junctions (NMJs), which were visualized with acetylcholinesterase (AchE) histochemistry, were detected on each muscle fiber. This polyinnervation pattern was similar to that of the immature muscle of mammalian embryos. These findings indicate that, unlike mammalian muscles, maturation of carp muscles is not accompanied by the synapse elimination which is thought to be coupled with the down-regulation of motoneuron GAP-43. NMJs of the adult carp muscle are supposed to be continuously reorganized, keeping the motoneurons expressing GAP-43.

The expression of GAP-43 under physiological conditions in the adult carp CNS may facilitate axonal regeneration in various kinds of carp CNS neurons.

INTRODUCTION

The growth-associated protein, GAP-43, is a neuron-specific and membrane-associated phosphoprotein. GAP-43 is highly conserved in its amino acid sequences among vertebrates including mammals (Skene, 1989). This protein is rapidly transported to distal axonal segments and is localized in the growth cone membrane (Benowitz *et al.*,1989; Skene, 1989). The expression of GAP-43 is known to be closely correlated with axonal growth under developmental or regenerative conditions in mammals, amphibians and fishes (Benowitz *et al.*, 1981, 1983; Skene and Willard, 1981; Jacobson *et al.*, 1986; Basi *et al.*, 1987; Moya *et al.*, 1989; Skene, 1989; Dani *et al.*, 1991; Tetzlaff *et al.*, 1991; Chong *et al.*, 1992; Lindå *et al.*, 1992; Reh *et al.*, 1993; Palacios *et al.*, 1994).

In the central nervous system (CNS) of mammals, the expression of this protein and mRNA declines markedly to undetectable levels in most neurons after their synapse connections have been completed at the end stage of the development (Moya *et al.*,1989; Caroni and Becker, 1992). Exceptionally, in some brain regions of adult mammals such as the pyramidal layer of the hippocampus and monoaminagic neurons, GAP-43 expression continues throughout their lives. This persistence of GAP-43 mRNA synthesis in the adult brain has been thought to play a significant role in physiological synaptic plasticity and remodeling (Meberg and Routtenberg, 1991; Kruger *et al.*,1992,1993). In the case of axonal injuries, GAP-43 mRNA

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Abbreviations: CC, corpus cerebelli; DH, dorsal horn; E, ependymal cell; EG, granular eminence; FL, facial lobe; G, granular layer; GL, glossopharyngeal lobe; IL, inferior lobe; IO, inferior olivary nucleus; IS, nucleus isthmi; M, molecular layer; MB, mammillary body; NC, nucleus centralis; NCH, nucleus cerebellosus hypothalami; NDL, dorsolateral thalamic nucleus; NDM, dorsomedial thalamic nucleus; NFLL, nucleus of the lateral longitudinal fascicle; NLT, nucleus lateralis tuberis; NLV, nucleus lateralis valvulae; NMT, motor nucleus of the trigeminal nerve; NRL, nucleus recessus lateralis; NTP, posterior thalamic nucleus; NVL, ventrolateral thalamic nucleus; NVM, ventromedial thalamic nucleus; NIII, nucleus of the oculomotor nerve; NIV, nucleus of the trochlear nerve; NXM, motor nucleus of the vagus nerve; P, purkinje layer; PGN, nucleus preglomerulosus pars lateralis; RF, reticular formation; SAC, stratum album centrale; SFGC stratum fibrosum et griseum superficiale; SGC, stratum griseum centrale; SGN, secondary gustatory nucleus; SM, stratum marginale; SO, stratum opticum; SPV, stratum periventriculare; SRF, superium reticular formation; TGN, tertiary gustatory nucleus; TL, torus longitudinalis; TO, tectum opticum; TS, torus semicircularis; V, ventricle; VH, ventral horn; VL, lateral lobe of the valvula cerebelli.

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expression is up-regulated in the damaged neurons of both the peripheral nervous system (PNS) and CNS (Redshaw and Bisby, 1984; Hoffman, 1989; Verge *et al.*,1990). Certain CNS neurons such as cortical pyramidal neurons do not exhibit up-regulation of GAP-43 following axotomy and axonal regeneration does not occur (Elliott *et al.*,1997). Thus, the reactive re-expression of GAP-43 may be important for successful axonal regeneration (Tetzlaff *et al.*,1991; Elliott *et al.*,1997).

In fish also, the synthesis of GAP-43 has been reported to be induced in the retinal ganglion cells during optic nerve regeneration (Benowitz *et al.*,1981,1983; Benowitz and Schmidt, 1987; Perry *et al.*,1987). However, the distribution of the neurons which express GAP-43 in the normal brain of adult fish has not been fully elucidated.

In the present study, we demonstrate by *in situ* hybridization that many neurons of the brain and spinal cord of the adult carp express GAP-43 mRNA and we discuss the possible association between the expression of GAP-43 in motoneurons and the construction of neuromuscular junctions (NMJs) in adult carp.

MATERIALS AND METHODS

Tissue preparation

Adult carp (*Cyprirus carpio*), 21–23 cm in body length, which were capable of reproduction, were kept at 21–22°C in 70-litter aquarium. Fish were used for *in situ* hybridization and acetylcholinesterase (AchE) staining. Adult male Sprague-Dawley rats (10 weeks old) were used for AchE staining.

For *in situ* hybridization, carp were anesthetized with 0.02% tricaine methanesulfonate, and their brains were removed and frozen in OCT compound (Miles Inc.) on the dry ice.

For AchE staining, carp were anesthetized with the same anesthetic as above, and rats were anesthetized with pentobarbital sodium (i.p., 60 mg/kg body weight). They were then fixed by transcardinal perfusion with 2% paraformaldehyde and 2.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Skeletal muscles were removed from the dorsal part of carp or from the femoral region of rats and were further immersed in the same fixatives for 4 hr at 4°C.

Synthesis of carp cDNA probes

Total cellular RNA was extracted from the carp whole brain as described elsewhere (Chomczynski and Sacchi, 1987). A cDNA library was constructed using Superscript cDNA synthesis Kit (GIBCO-BRL, Gainthersberg, MD, USA). To isolate carp cDNAs encording GAP-43, PCR was carried out with oligomers F and R (5'-ATGCTGTGCTGTATCAGGAG-3' and 5'-TTAAACATTCTGGTCTTTGC-3') using part of the cDNA library as a template. The oligomers were designed from the previously reported nucleotide sequence of goldfish GAP-43 (Michael and Skene, 1989). PCR was performed according to the instruction of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) using a thermal cycler 480 (Perkin Elmer Cetus) under the following thermal cycling conditions: denaturation, 94°C for 30 sec; 55°C for 30 sec; extension, 72°C for 60 sec; the last extension, 72°C for 5 min; cycle number, 30. PCR products were ligated into the EcoRV site of P-Bluescript KS-vector (Stratagene, La Jolla, CA, USA), where a dideoxythymidine monophosphate had been added. Carp GAP-43 cDNA was thus cloned and sequenced, and it showed 90% homology with goldfish GAP-43 cDNA.

Northern blot analysis

Twenty μg of RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde. The RNA was transferred to nylon membrane (Pall Biosupport, NY, USA) and was UV cross-linked. The membrane was probed with carp GAP-43 cDNA which was labeled with ^{32}P-dCTP (6000 Ci/mmol) using a labeling kit Prime I t-II (Stratagene). Hybridization was performed in hybridization buffer (0.5 M sodium hydrogenphosphate, 1% bovine serum albmin (BSA), 1 mM sodium dodecyl sulfate (SDS)) containing the labeled probe (1×10^6 cpm/ml) at 65°C overnight. The membrane was washed with 0.1 × SSC, 0.1% SDS at 65°C for 20 mim and exposed to X-ray film.

In situ hybridization

Antisense RNA probe was synthesized with T3 or T7 RNA polymerase (GIBCO-BRL) in the presence of ^{35}S-UTP (800 Ci/mmol) using selected carp GAP-43 cDNAs as templates. Sense probe was synthesized in a similar manner and used for the control study.

Coronal, frozen sections of fresh carp brains were cut at 6 μm with a cryostat, mounted onto glass-slides coated with Vectabond reagent (Vector Lab., Inc., USA) and stored at –80°C until use. After being dried in air, sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min, and rinsed in 2 × SSC (0.3 M NaCl, 0.03 M Na citrate, pH 7.0). They were placed in acetic anhydride solution (0.25% acetic anhydride, 0.1 M triethanolamine HCl, pH 8.0) for 10 min, and then rinsed in 2 × SSC. Finally, sections were dehydrated through a graded ethanol series and air dried. RNA probes were heated to 60°C in hybridization buffer (50% formamide, 0.5 M NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 × Denhardt's solution, 0.1% SDS, 10% dextran sulfate, 20 mM DTT, 500 μg/ml yeast tRNA, 5 ng/μl UTPαs) just prior to hybridization. The probe concentration was 1×10^6 cpm/ml. Covered with silicone-coated coverslips, sections were incubated with RNA probes overnight at 55°C in a humidified chamber. After incubation, coverslips were removed in 4 × SSC containing 10 mM DTT, and sections were washed in the same solution for 15 min at 40°C. They were then incubated at 37°C for 30 min in RNaseA (Sigma, St. Louis, MO, USA) (20 μg/ml) dissolved in RNase buffer (0.5 M NaCl, 10 mM Tris-HCl pH 8.0), and washed in 0.1 × SSC containing 20 mM DTT at 55°C for 30 min. After wash in 0.1 × SSC at room temperature, sections were dehydrated in ethanol and air-dried. They were then dipped in NTB-2 emulsion (Eastman Kodak, USA) and exposed for 3 to 4 days at 4°C. After development with D-19 (Eastman Kodak), sections were counterstained with hematoxylin and eosin and were observed under a microscope (Zeiss Axiophoto, Karl-Zeiss, Germany).

AchE staining procedure

AchE histochemistry was carried out according to the method of Tago *et al.* (1986). Twenty to thirty μm-thick sections of sketetal muscles were cut with a cryostat and air-dried. They were washed in 0.1 M maleate buffer (pH 6.0) and were incubated in the reaction medium for AchE containing 1.8 mM acetylthiocholine iodide, 0.5 mM $K_3Fe(CN)_4$, 3 mM $CuSO_4$ and 5 mM sodium citrate in 65 mM maleate buffer (pH 6.0) for 1 hr at room temperature. After washing in 50 mM Tris-HCl (pH 7.6) several times, sections were incubated in a solution containing 0.04% 3,3'-diaminobenzidine tetrahydrocholide and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl (pH 7.6) for 5 mim. Subsequently, 0.003% H_2O_2 was added to the solution and sections were further incubated for 5 min. Sections were observed under a light microscope (Optiphoto, Nikon, Tokyo, Japan).

RESULTS

By *in situ* hybridization, we could detect the signals for GAP-43 mRNA on the perikaryal cytoplasm of nerve cells in various regions of the carp CNS. Control sections to which the sense probe had been hybridized showed no significant signals. The findings are described in detail below.

Identification and nomenclature of the brain nuclei were

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according to the goldfish brain atlas (Northcutt and Davis, 1983, Peter and Gill, 1975) and the zebrafish atlas (Wullimann *et al.*,1996).

Fig. 1. Northern blot analysis of carp brain mRNA probed with the carp GAP-43 cDNA.

Northern blot analysis of the carp brain mRNA

We used the carp GAP-43 cDNA to probe the RNA derived from the adult carp hole brain. The specific single band of carp GAP-43 mRNA was found at approximately 1.35 kb long (Fig. 1).

Telencephalon and preoptic area

In the telencephalon which consists of the area ventralis and the area dorsalis, strong signals for GAP-43 mRNA were found in almost all nerve cells (data not shown).

The preoptic area extends from the caudal end of the telencephalon to the rostral of the nucleus habenularis (NH) in the diencephalon. In the nucleus preopticus periventricularis (NPP), which is located around the third ventricle, many neurons displayed GAP-43 mRNA signals. The nucleus preopticus (NPO) existing around the third ventricle ventral to NPP was also positive for GAP-43 mRNA (data not shown).

Diencephalon

The diencephalon is composed of the epithalamus, dorsal thalamus, ventral thalamus and hypothalamus.

The epithalamus consists of only the nucleus habenularis (NH), which showed a very low level of mRNA signals for GAP-43 on small neurons (data not shown). The dorsal thalamus is divided into two thalamic nuclei, i.e., dorsomedial thalamic

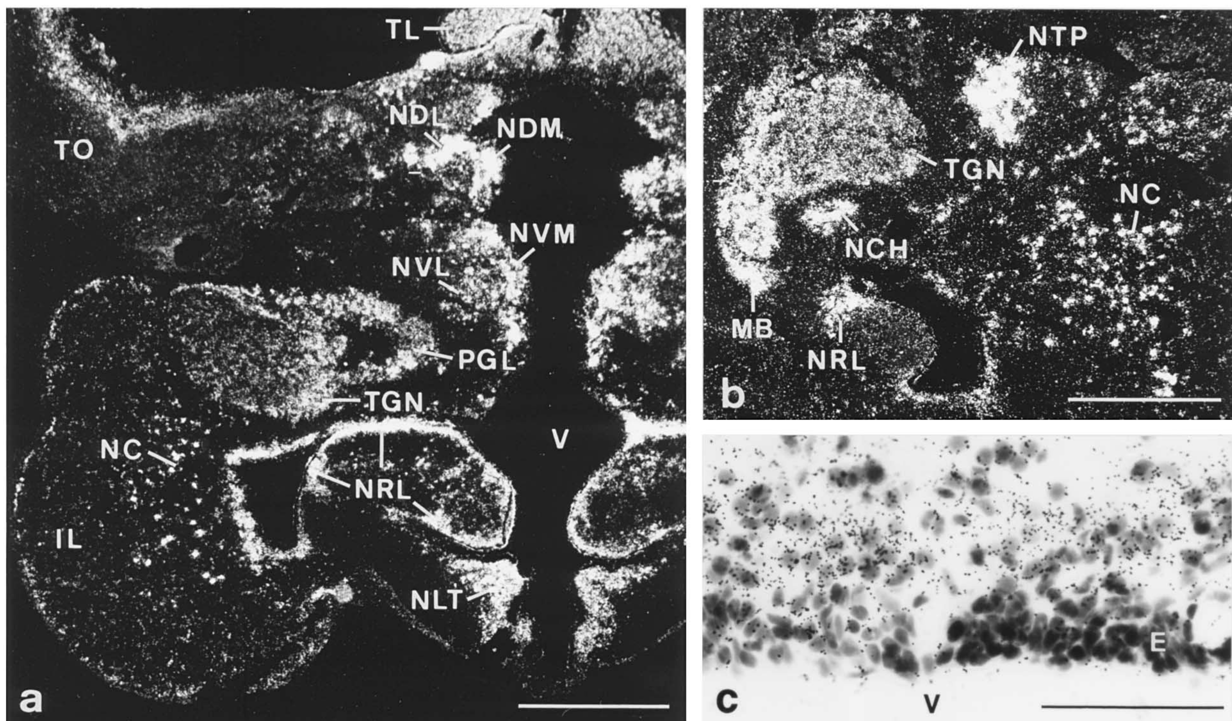

Fig. 2. Light microscopic autoradiography of *in situ* hybridization using the RNA probe for carp GAP-43 mRNA in the diencephalon. (**a**) Strong signals are seen in the dorsal and ventral thalamus, i.e., dorsomedial thalamic nucleus (NDM), dorsolateral thalamic nucleus (NDL), ventromedial thalamic nucleus (NVM) and ventrolateral thalamic nucleus (NVL), as well as in the hypothalamus, i.e., nucleus preglomerulosus pars lateralis (PGL), tertiary gustatory nucleus (TGN), nucleus recessus lateralis (NRL), nucleus lateralis tuberis (NLT) and nucleus centralis (NC). IL: inferior lobe. TL: torus longitudinalis. TO: tectum opticum. V: ventricle. Bar = 500 μm. (**b**) Intense signals are also seen in the other hypothalamic nuclei, i.e., posterior thalamic nucleus (NTP), nucleus cerebellosus hypothalami (NCH) and mammillary body (MB). Bar = 500 μm. (**c**) Bright-field photograph of a part of NRL. Hybridization signals are not found upon the ependymal cells (E). V: ventricle. Bar = 50 μm.

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nucleus (NDM) facing the third ventricle and dorsolateral thalamic nucleus (NDL) located lateral to NDM. These dorsal thalamic nuclei displayed strong mRNA signals for GAP-43 (Fig. 2a). The ventral thalamus is also separated into two thalamic nuclei: the ventromedial thalamic nucleus (NVM) and the ventrolateral thalamic nucleus (NVL). These ventral thalamic nuclei showed mRNA signals at the same levels as NDM and NDL (Fig. 2a). In the hypothalamus, some nuclei are located around the third ventricle and others in the medial-ventral part or the inferior lobe (LI). Strong mRNA signals for GAP-43 were found in almost all hypothalamic nuclei, i.e., nucleus anterior tuberis (NAT) (data not shown), nucleus lateralis tuberis (NLT), nucleus recessus lateralis (NRL), nucleus posterior tuberis (NPT) (data not shown) and nucleus centralis (NC) (Fig. 2a). In NAT, NLT, NRL and NPT located around the ventricle, the cells heavily labeled with the GAP-43 mRNA probe were not ependymal cells but nerve cells (Fig. 2c).

The diencephalic nuclei other than those described above also showed mRNA signals for GAP-43. In the medial and lateral part of the hypothalamus, strong signals were found in the mammillary body (MB), tertiary gustatory nucleus (TGN), nucleus preglomerulosus par lateralis (PGL), nucleus cerebellosus hypothalami (NCH) and posterior thalamic nucleus (NTP) (Fig. 2a,b).

Mesencephalon

The torus semicircularis (TS) is a pair of longitudinal ridges and forms the wall of the mesencephalic ventricle, and the nucleus isthmi (IS) lies more ventricle to the TS. Both TS and IS were found to show strong signals (Fig. 3a). The nucleus lateralis valvulae (NLV) is located medial to TS and ventral to the valvula cerebelli, and extending widely in a rostrocaudal direction. The central part of NLV showed the mRNA signals for GAP-43 at the same levels as TS and IS (Fig. 3a, b). The superium reticular formation (SRF), located medial to the central NLV, also showed strong signals (Fig. 3a). Torus longitudinalis (TL) is another pair of longitudinal ridges and extends from the medial border of the optic tectum into the mesencephalic ventricle. In TL, neurons in the dorsal part are larger in size than in the ventral part. The dorsal neurons had stronger mRNA signals for GAP-43 than the ventral neurons (Fig. 3b). Nuclei of the cranial nerves, i.e., nuclei of the oculomotor nerve (NIII) and trochlear nerve (NIV), are located in the mesencephalon. Although it is difficult to distinguish the NIII nucleus from the NIV nucleus, many neurons of this area were found to have strong signals for GAP-43 (Fig. 3a, c).

Optic tectum

The optic tectum is composed of six layers. Among them, the stratum fibrosum et griseum superficiale (SFGC) and stratum griseum centrale (SGC) contained many large-sized neurons which were found to show GAP-43 mRNA signals. In the stratum griseum periventriculare (SPV), strong signals were found in small-sized neurons (Fig. 4).

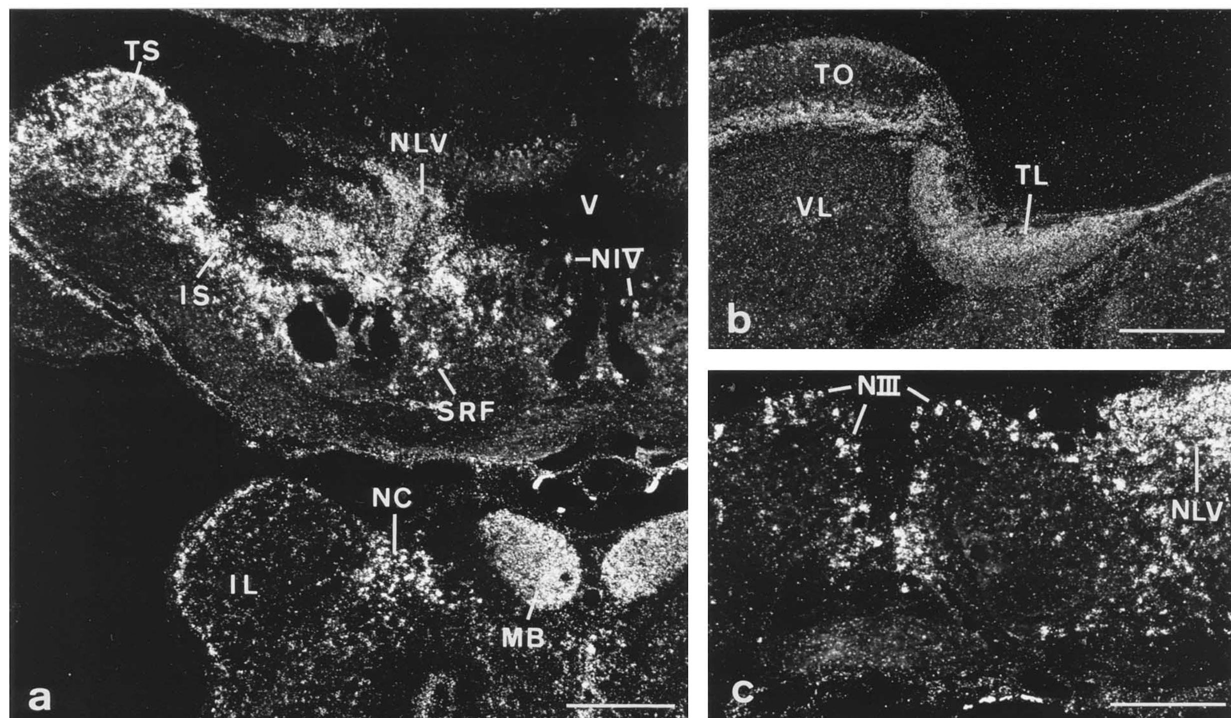

Fig. 3. Light microscopic autoradiography of *in situ* hybridization using the RNA probe for carp GAP-43 mRNA in the mesencephalon. (**a**) Strong signals are seen in the torus semicircularis (TS), nucleus lateralis valvulae (NLV), nucleus isthmi (IS) and superium reticular formation (SRF). The motoneurons of the trochlear nerve (NIV) are also labeled by the probe. MB, IL, NC, V: see legend of Fig. 2. Bar = 500 µm. (**b**) Strong signals are seen in the dorsal part of the torus longitudinalis (TL). TO: tectum opticum. VL: lateral lobe of the valvula cerebelli. Bar = 500 µm. (**c**) Strong signals are seen in the motoneurons of the oculomotor nerve (NIII). NLV: nucleus lateralis valvulae. Bar = 500 µm.

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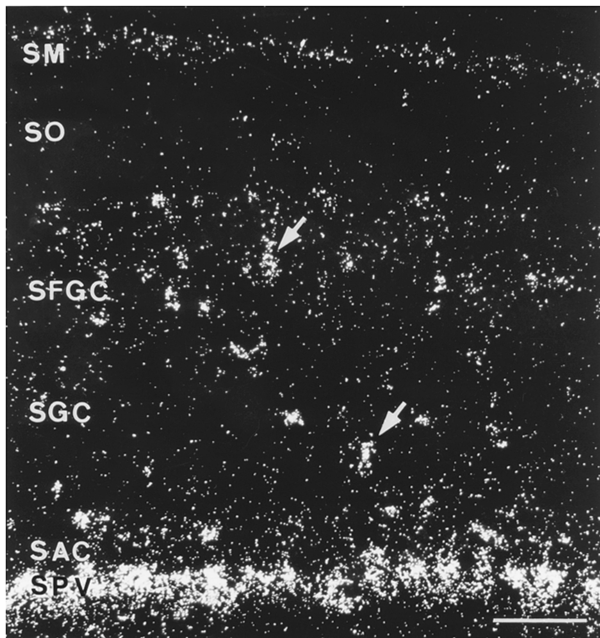

Fig. 4. Light microscopic autoradiography of *in situ* hybridization using the RNA probe for carp GAP-43 mRNA in the optic tectum. Strong signals are seen in the large-sized neurons (arrows) of the stratum fibrosum et griseum superficiale (SFGC) and stratum griseum centrale (SGC), and in the small-sized neurons in the stratum periventriculare (SPV). SM: stratum marginale. SO: stratum opticum. SAC: stratum album centrale. Bar = 100 μm.

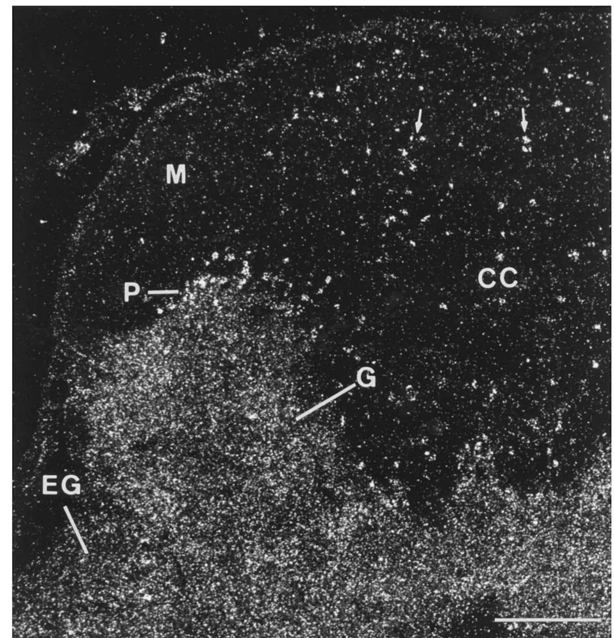

Fig. 5. Light microscopic autoradiography of *in situ* hybridization using the RNA probe for carp GAP-43 mRNA in the cerebellum. Strong signals are seen in the purkinje cell layer (P) and satellite cells (arrows) of the molecular layer (M). Weak signals are seen in the granular cell layer (G) and in the granular eminence (EG). CC: corpus cerebelli. Bar = 500 μm.

Cerebellum

Granular cells (G) located in the valvula cerebelli and the corpus cerebelli showed weak signals for GAP-43 mRNA. The granular eminence (EG) also displyed weak signals. In the purkinje cell layer (P), purkinje cells and eurodendroid cells, which are difficult to distinguish from each other, were posi-

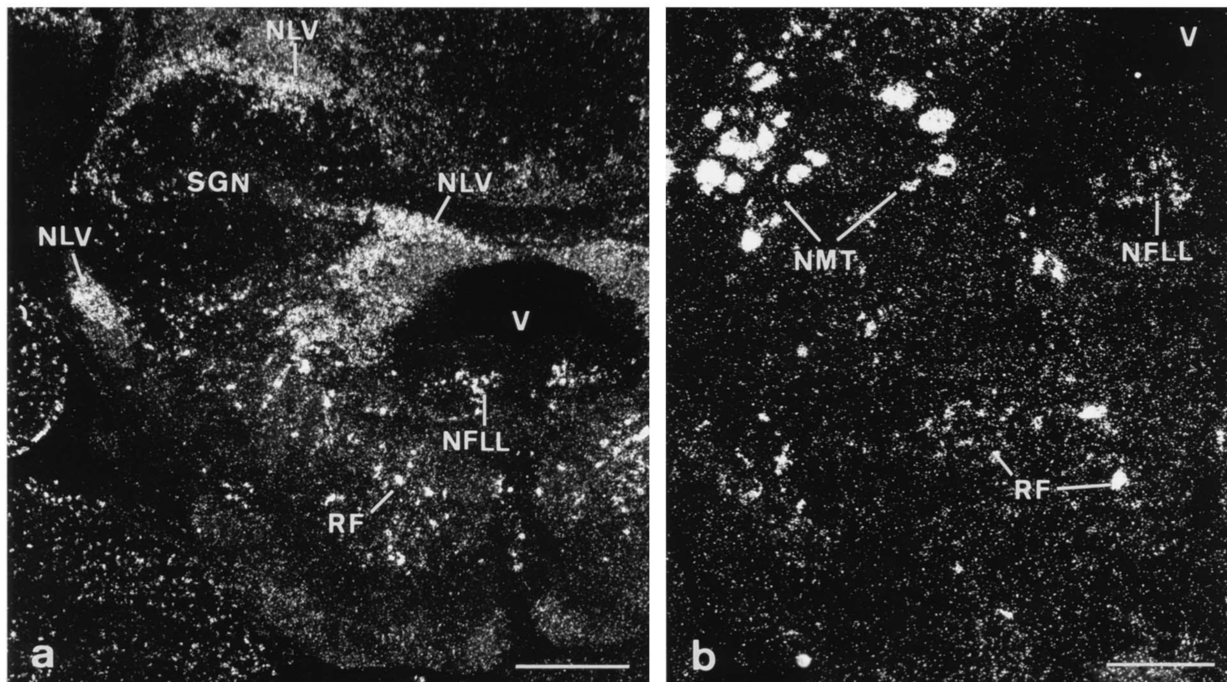

Fig. 6. Light microscopic autoradiography of *in situ* hybridization using the RNA probe for carp GAP-43 mRNA in the pontine area. (**a**) Strong signals are seen in the nucleus lateralis valvulae (NLV), secondary gustatory nucleus (SGN), nucleus of the lateral longitudinal fascicle (NFLL) and reticular formation (RF). V: ventricle. Bar = 500 μm. (**b**) Intense signals are seen in the motor nuclei of the trigeminal nerve (NMT). NFLL, RF, V: see above. Bar = 250 μm.

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tive for GAP-43 mRNA signals. In the molecular layer (M), many neurons thought to be satellite cells were positive for GAP-43 mRNA (Fig. 5).

Pontine area

In the secondary gustatory nucleus (SGN), the nucleus of the lateral longitudinal fascicle (NFLL) and the reticular formation (RF), many neurons showed mRNA signals for GAP-43. In the pontine area, NLV is situated medial and lateral to the SGN. Almost all the neurons in NLV were labeled clearly by the GAP-43 mRNA probe (Fig. 6a). Both the sensory and motor nuclei of the trigeminal nerve (NMT) and Mauthner cells (data not shown) showed strong signals (Fig. 6b).

Medulla oblongata

In carp, the dorsal medulla is composed of three lobes, i.e., the facial, glossopharyngeal and vagal lobes. These lobes showed weak GAP-43 mRNA signals. In the ventral medulla oblongata, the ventral motoneuron group of the vagus nerve (NXM) showed strong GAP-43 mRNA signals. In the most ventral part of the brain stem, the inferior olivary nucleus (IO) also showed strong signals (Fig. 7).

Spinal cord

GAP-43 mRNA signals were found in the gray matter of the spinal cord at the anterior and middle levels of the dorsal fin. In the dorsal horn (DH), most of the neurons appeared to have clear signals. In the ventral horn (VH) on the other hand,

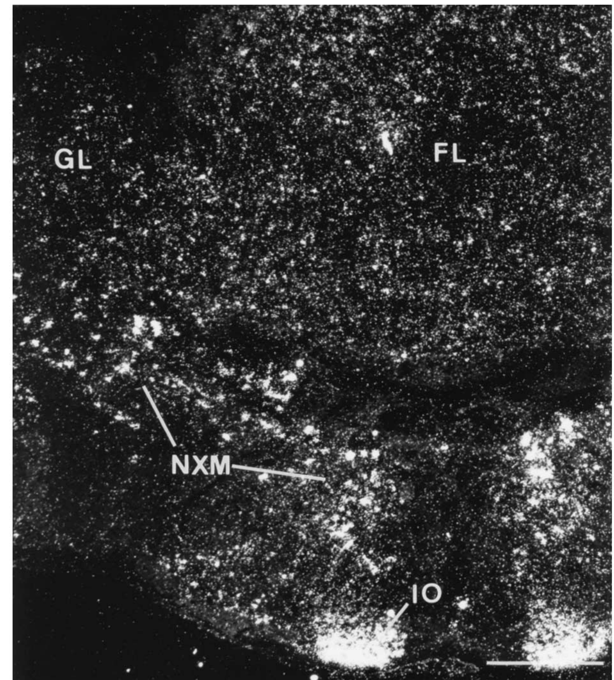

Fig. 7. Light microscopic autoradiography of *in situ* hybridization using the RNA probe for carp GAP-43 mRNA in the medulla oblongata. Strong signals are seen in the ventral motoneuron group of the vagus nerve (NXM) and nerve cells of the inferior olivary nucleus (IO). Weak signals are seen in the facial lobe (FL) and the glossopharyngeal lobe (GL). Bar = 500 μm.

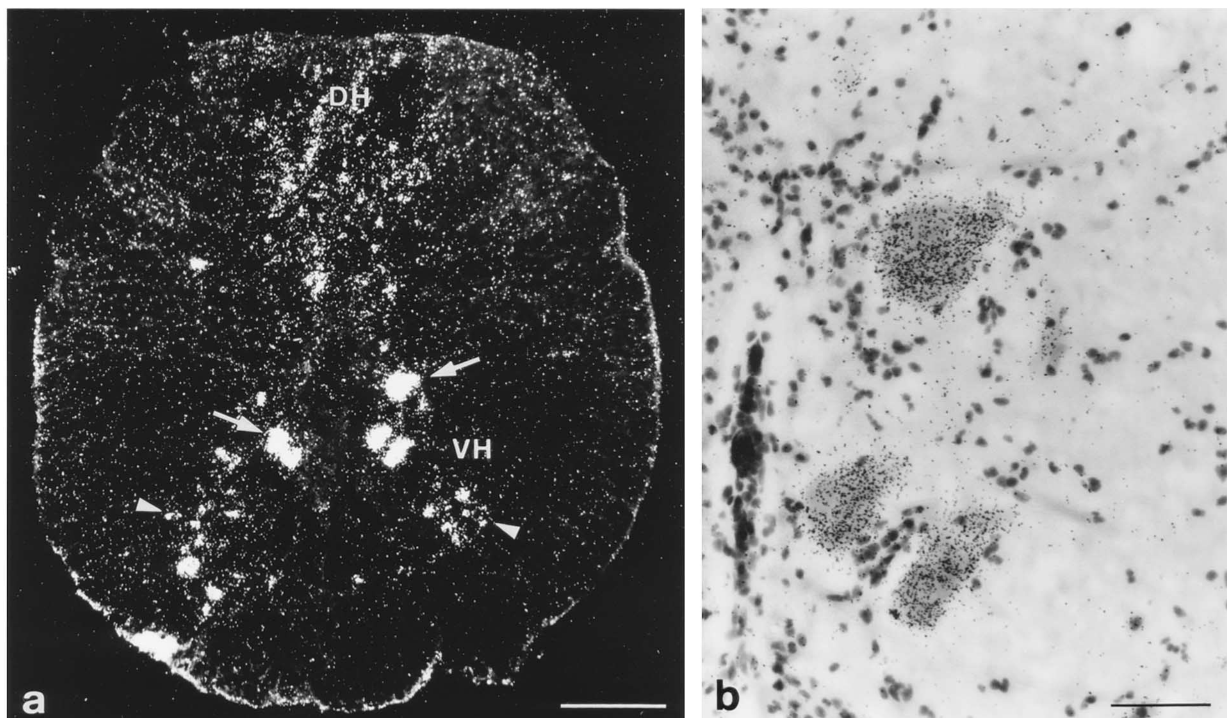

Fig. 8. Light microscopic autoradiography of *in situ* hybridization using the RNA probe for carp GAP-43 mRNA in the spinal cord. (**a**) Strong signals are seen in the motoneurons (arrows) and the small-sized neurons which are considered to be interneurons (arrowheads). DH: dorsal horn. VH: ventral horn. Bar = 250 μm. (**b**) Bright-field photograph of the motoneurons heavily labeled by the probe. Silver grains are seen on the cell bodies of the motoneurons. Bar = 50 μm.

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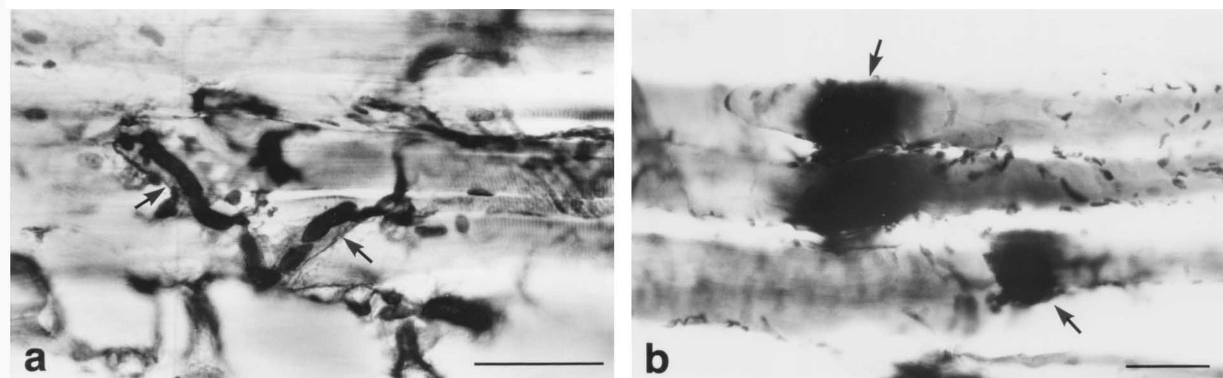

Fig. 9. Acetylcholinesterase histochemistry of the skeletal muscle of carp (**a**) and rat (**b**). (**a**) A number of long extended endplates (arrows) are seen on each muscle fiber. Bar = 50 µm. (**b**) A single oval-shaped endplate (arrows) is seen on each muscle fiber. Bar = 50 µm.

strong signals were detected in the motoneurons, and the small neurons which were thought to be interneurons were also labeled by the probe (Fig. 8a, b).

Neuromuscular junctions (NMJs) of the skeletal muscle

This study disclosed that the adult carp motoneurons show strong GAP-43 mRNA signals. On the other hand, it has been reported that, in the cranial and spinal motoneurons of adult rats, expression of GAP-43 mRNA and its protein was very low or undetectable. To explain this species difference, we investigated the morphology of NMJs in the carp and rat skeletal muscles with acetylcholinesterase histochemistry to compare the innervation pattern of skeletal muscles on these animals.

In the white skeletal muscle at the dorsal part of adult carp, which should be innervated by spinal motor neurons, a number of small round or oval-shaped junctional plaques were found over the entire length of the individual muscle fibers. Many long extended endplates were also detected around the muscle fibers (Fig. 9a).

On the other hand, in the skeletal muscle of the femoral region of adult rat, the endplates were oval-shaped and larger in size in comparison to those of the carp. Only a single endplate was found on each muscle fiber (Fig. 9b).

DISCUSSION

GAP-43 is one of the major components of growth cones (Skene, 1989; Van Hoff *et al*., 1989; Strittmatter *et al*., 1990), and the level of its mRNA expression increases during axonal elongation in the developmental stage (Skene and Willard, 1981; Jacobson *et al*., 1986; Skene and Virag, 1989). After development, in the mammalian CNS, most neurons down-regulate the GAP-43 gene to undetectable levels (Oestreicher and Gispen, 1986; Benowitz *et al*., 1988, 1989; Mc Guire *et al*., 1988; Neve *et al*., 1988; De la Monte *et al*., 1989; Dani *et al*., 1991; Meberg and Routtenberg, 1991). However, in some regions of the adult rat brain, such as the pars conpacta of substantia nigra, the locus coeruleus and raphé nuclei, which have long and extensively dispersed axons and many synaptic connections, neurons continue to express GAP-43 mRNA and its protein (Kruger *et al*.,1993). Those neurons which continue to express GAP-43 mRNA and protein may possess a propensity for continuous axonal growth and remodeling of synaptic connections.

Neurogenesis continues in adulthood in the optic tectum of the adult goldfish (Raymond and Easter, 1983) and in the cerebellum of the adult gymnoiform fish (Zupanc *et al*.,1996). In the present study using *in situ* hybridization, we demonstrated that the brain and spinal cord of the adult carp contain many neurons which strongly express GAP-43 mRNA. This result may indicate that neurons of the adult carp CNS have a potential to continue axonal growth and synapse remodeling. On the other hand, granular cells of the cerebellum and granular eminence (EG) and small-sized neurons of the nucleus habenularis (NH) and torus longitudinalis (TL) showed only weak signals. These types of neurons might not perform active synaptogenesis and/or axonal elongation in the adult carp brain.

In the mature rat brain, all cranial motoneurons lack expression of GAP-43 mRNA (Kruger *et al*., 1993), and its protein is difficult to detect by immunohistochemistry (Hassan *et al*., 1994; Johnson *et al*., 1995). On the other hand, our present study revealed that the cranial and spinal motoneurons of adult carp which project to the skeletal muscle are strongly positive for GAP-43 mRNA. This species difference in the motoneuron GAP-43 expression may depend on the development and plasticity of neuromuscular junctions (NMJs) for the following reasons.

In the CNS of mammals including rats, the down-regulation of GAP-43 in the spinal motoneurons are associated with synaptic elimination at the skeletal muscle. In rat embryos, axonal processes of motoneurons reach the developing skeletal muscle in a manner of polyinnervation at E11 and multiple synapses are formed on the muscle fibers from E12. The increase in the number of muscle fibers stops shortly after birth, and hypertrophy is responsible for the postnatal growth of muscle (Goldspink, 1972, 1974; Campion, 1984). Synapse elimination of the skeletal muscle begins at P8 and is completed at P14, so that one NMJ can be formed upon each

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muscle fiber (Brown *et al.*,1976; Dennis, 1981; Bennet, 1983). Caroni and Becker (1992) reported that the level of GAP-43 mRNA expression declined rapidly in the spinal motoneurons of the postnatal rat at the time corresponding to the onset of synapse elimination. In contrast, in fish, the skeletal muscle remains polyneuronally innervated through adulthood and motor axons form multiple terminals on each muscle fiber (Akster, 1983; Eisen *et al.*,1986; Westerfield *et al.*,1986). We also observed by acetylcholinesterase staining that there are many small NMJs on each white muscle fiber of the dorsal part of adult carp. Thus, synapse elimination, which is closely associated with the down-regulating of motoneuron GAP-43, does not significantly occur in fish. In the carp, muscle fibers keep increasing in number throughout adulthood (Koumans *et al.*,1993). This hyperplasia of the skeletal muscle fibers should be physiologically accompanied by an increase in the number of NMJs so that the neuromuscular system can work well as a functional unit. The strong expression of GAP-43 mRNA in the adult carp spinal motoneurons as revealed in our study is likely to reflect the increase in number of NMJs, i.e., the physiological neo-formation of synaptic terminals during adulthood.

Under the pathological conditions caused by axotomy of the peripheral motor nerves of rats, GAP-43 mRNA increases in the damaged motoneurons (Chong *et al.*,1992; Lindå *et al.*,1992; Palacios *et al.*,1994; Kitamura *et al.*,1995), and seems to contribute to axonal regeneration. Similarly, following injury of the rat CNS, such as by transection of the rubrospinal tract, up-regulation of GAP-43 mRNA occurs in the damaged rubrospinal neurons (Tetzlaff *et al.*,1991). In the carp CNS, axonal regeneration of large neurons in the reticular formation takes place after complete spinal cord transection (Yamada *et al.*,1995). We investigated the expression GAP-43 mRNA in the brainstem of carp 7 days after transection of the spinal cord, but could not detect significant increase for the signals of GAP-43 mRNA on the level of microautoradiography (data not shown). Since a large number of neurons of the adult carp brain including those of the reticular formation normally express GAP-43 mRNA strongly, further up-regulation of GAP-43 may not be needed by the damaged neurons of the carp CNS.

Our *in situ* hybridization made it clear that many neurons in the normal carp brain and spinal cord strongly express GAP-43 mRNA. This might suggests that synaptogenesis and continuous synaptic reorganization normally occur in the adult fish CNS which is considered to be ever-growing throughout life.

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REFERENCES

Akster HA (1983) A comparative study of fiber type characteristics and terminal innervation in head and axial muscle of the carp (*Cyprinus carpio* L.): a histochemical and electron-microscopic study. Netherlands J Zool 33: 164–188

Basi GS, Jacobson RD, Virag I, Schilling J, Skene JHP (1987) Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. Cell 49: 785–791

Bennet MR (1983) Development of neuromuscular synapses. Physiol Rev 63: 915–1048

Benowitz LI, Shashoua VE, Yoon MG (1981) Specific changes in rapidly transported proteins during regeneration of the goldfish optic nerve. J Neurosci 1: 300–307

Benowitz LI, Yoon MG, Lewis ER (1983) Transported proteins in the regenerating optic nerve: Regulation by interactions with the optic tectum. Science 222: 185–188

Benowitz LI, Schmidt JT (1987) Activity-dependent sharpening of the regenrating retinotectal projection in goldfish: Relationship to the expression of growth-associated proteins. Brain Res 417: 118–126

Benowitz LI, Apostolides PJ, Perrone-Bizzozero NI, Finklestein SP, Zwiers H (1988) Anatomical distribution of the growth-associated protein GAP-43/B-50 in the adult rat brain. J Neurosci 8: 339–352

Benowitz LI, Perrone-Bizzozero NI, Finklestein SP, Bird ED (1989) Localization of the growth-associated phosphoprotein GAP-43 in the human cerebral cortex. J Neurosci 9: 990–995

Brown MC, Jansen JKS, Van Essen D (1976) Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation. J Physiol (Lond) 261: 387–422

Campion DR (1984) The muscle satellite cell; a review. Int Rev Cytol 87: 225–251

Caroni P, Becker M (1992) The downregulation of growth-associated proteins in motoneurons at the onset of synapse elimination is controlled by muscle activity and IGF1. J Neurosci 12: 3849–3861

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analyt Biochem 162: 156–159

Chong MS, Fitzgerald M, Winter J, Hu-Tsai M, Emson PC, Wiese U, Woolf CJ (1992) GAP-43 mRNA in rat spinal cord and dorsal root ganglia neurons: Developmental changes and re-expression following peripheral nerve injury. Eur J Neurosci 4: 883–895

Dani JW, Armstrong DM, Benowitz LI (1991) Mapping the development of the rat brain by GAP-43 immunocytochemistry. Neurosci 40: 277–287

De la Monte SM, Federoff HJ, Shi-Chung Ng, Grabczyk ED, Fishman MC (1989) GAP-43 gene expression during development: persistence in a distinctive set of neurons in the mature central nervous system. Dev Brain Res 46: 161–168

Dennis MJ (1981) Development of neuromuscular junction, inductive interaction between cells. Annu Rev Neurosci 4: 43–68

Eisen JS, Myers PZ, Westerfield M (1986) Pathway selection by growth cones of identified motoneurons in live zebra fish embryos. Nature 320: 269–271

Elliott EJ, Parks DA, Fishman PS (1997) Effect of proximal axotomy on GAP-43 expression in cortical neurons in the mouse. Brain Res 775: 221–228

Goldspink G (1972) Postembryonic growth and differentiation of striated muscle. In “The Structure and Function of Muscle, Vol 1, 2nd ed” Ed by GH Bourne, Academic Press, New York, pp 179

Goldspink G (1974) Development of muscle. In “Differentiation and Growth of Cells in Vertebrate Tissues” Ed by G Goldspink, Chapman and Hall, London

Hassan SM, Jennekens FGI, Veldman H, Oestreicher BA (1994) GAP-43 and p75[NGFR] immunoreactivity in presynaptic cells following neuromuscular blockade by botulinum toxin in rat. J Neurocytol 23: 354–363

Hoffman PN (1989) Expression of GAP-43, a rapidly transported

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growth-associated protein, and class II beta tublin, a slowly transported cytoskeletal protein, are coordinated in regenerating neurons. J Neurosci 9: 893–897
Jacobson RD, Virag I, Skene JHP (1986) A protein associated with axon growth, GAP-43, is widely distributed and developmentally regulated in rat CNS. J Neurosci 6: 1843–1855
Johnson H, Mossberg K, Arvidsson ULF, Piehl F, Hökfelt T, Ulfhake B (1995) Increase in α-CGRP and GAP-43 in aged motoneurons: a study of peptides, growth factors, and ChAT mRNA in lumbar spinal cord of senescent rats with symptoms of hindlimb incapacities. J Comp Neurol 359: 69–89
Kitamura T, Miyake T, Gahara Y, Uwabe K, Yamada H, Nakayama M (1995) Molecular biological analysis on CNS repair process after axotomy. Sinkei Kenkyu no Shinpo 39: 975–982
Koumans JTM, Akster HA, Booms GHR, Osse JWM (1993) Growth of carp (*Cyprinus carpio*) white axial muscle; hyperplasia and hypertrophy in relation to the myonucleus/sarcoplasm ratio and the occurrence of different subclasses of myogenic cells. J Fish Biol 43: 69–80
Kruger L, Bendotti C, Rivolta R, Samanin R (1992) GAP-43 mRNA localization in the rat hippocampus CA_3 field. Mol Brain Res 13: 267–272
Kruger L, Bendotti C, Rivolta R, Samanin R (1993) Distribution of GAP-43 mRNA in the adult rat brain. J Comp Neurol 333: 417–434
Lindå H, Piehl F, Dagerlind Å, Verge VMK, Arvidsson U, Cullheim S, Risling M, Ulfhake B, Hökfelt T (1992) Expression of GAP-43 mRNA in the adult mammalian spinal cord under normal conditions and after different types of lesions, with special reference to motoneurons. Exp Brain Res 91: 284–295
Mc Guire CB, Snipes GJ, Norden JJ (1988) Light-microscopic immunolocalization of the growth-associated protein GAP-43 in the developing brain. Dev Brain Res 41: 277–291
Meberg PJ, Routtenberg A (1991) Selective expression of protein F1/GAP-43 mRNA in pyramidal but not granule cells of the hippocampus. Neurosci 45: 721–733
Michael EL, Skene JHP (1989) Selective conservation of GAP-43 structure in vertebrate evolution. Neuron 3: 299–310
Moya KL, Jhaveri S, Schneider GE, Benowitz LI (1989) Immunohistochemical localization of GAP-43 in the developing hamster retinofugal pathway. J Comp Neurol 288: 51–58
Neve RL, Finch EA, Bird ED, Benowitz LI (1988) The growth-associated protein GAP-43(B-50,F1) is expressed selectively in associative regions of the adult human brain. Proc Natl Acad Sci USA 85: 3638–3642
Northcutt RG, Davis RE (1983) Fish Neurobiology, Vol 1 and 2. University of Michigan Press, Ann Arber
Oestreicher AB, Gispen WH (1986) Comparison of the immunocytochemical distribution of the phosphoprotein B-50 in the cerebellum and hippocampus of immature and adult rat brain. Brain Res 375: 267–279
Palacios G, Mengod G, Sarasa M, Baudier J, Palacios JM (1994) De novo synthesis of GAP-43: in situ hybridization histochemistry and light and electron microscopy immunocytochemical studies in regenerating motor neurons of cranial nerve nuclei in the rat brain. Mol Brain Res 24: 107–117
Perry GW, Burmeister DW, Grafstein B (1987) Fast axonally transported proteins in regenerating goldfish optic axons. J Neurosci 7: 792–806
Peter RE, Gill VE (1975) A stereotaxic atlas and technique for forbrain nuclei of golfish, *Carassius auratus*. J Comp Neurol 159: 69–102
Raymond PA, Easter SS Jr (1983) Postembryonic growth of the optic tectum in goldfish. J Neurosci 3: 1077–1091
Redshaw JD, Bisby MA (1984) Fast axonal transport in central nervous system and peripheral nervous system axons following axotomy. J Neurobiol 15: 109–117
Reh TA, Tetzlaff W, Ertlmaier A, Zwiers H (1993) Developmental study of the expression of B50/GAP-43 in rat retina. J Neurobiol 24: 949–958
Skene JHP (1989) Axonal growth-associated proteins. Annu Rev Neurosci 12: 127–156
Skene JHP, Willard M (1981) Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. J Cell Biol 89: 86–95
Skene JHP, Virag I (1989) Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. J Cell Biol 108: 613–624
Strittmatter SM, Valenzuela D, Kennedy TE, Neer EJ, Fishman MC (1990) G_0 is a major growth cone protein subject to regulation by GAP-43. Nature 344: 836–841
Tago H, Kimura H, Maeda T (1986) Visualization of detailed acetylcholinesterase fiber and neurons staining in rat brain by a sensitive histochemical procedure. J Histochem Cytochem 34: 1431–1438
Tetzlaff W, Alexander SW, Miller FD, Bisby MA (1991) Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. J Neurosci 11: 2528–2544
Van Hooff COM, Holthuis JCM, Oestreischer AB, Boonstra J, De Graan PNE, Gispen WH (1989) Nerve growth factor-induced changes in the intracellular localization of the protein kinase C substrate B-50 in pheochromocytoma PC12 cells. J Cell Biol 108: 1155–1125
Verge VMK, Tetzlaff W, Richardson PM, Bisby MA (1990) Correlation between GAP-43 and nerve growth factor receptors in rat sensory neurons. J Neurosci 10: 926–934
Westerfield M, McMurray JV, Eisen JS (1986) Identified motoneurons and their innervation of axial muscles in the zebrafish. J Neurosci 6: 2267–2277
Wullimann MF, Rupp B, Reichert H (1996) Neuroanatomy of the Zebrafish Brain. Birkhäuser Verlag, Basel
Yamada H, Miyake T, Kitamura T (1995) Regeneration of axons in transection of the carp spinal cord. Zool Sci 12: 325–332
Zupanc GKH, Horschke I, Ott R, Rascher GB (1996) Postembryonic development of the cerebellum in gymnotiform fish. J Comp Neurol 370: 443–464

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