THE JOURNAL OF COMPARATIVE NEUROLOGY 194:291–301 (1980)

Neuronal Increase in Various Areas of the Nervous System of the Guppy, *Lebistes*

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ABSTRACT The numbers of 1) dorsal root ganglion cells in the 2nd spinal segment, 2) ventral horn cells in the 2nd spinal segment, 3) Purkinje cells of the cerebellum, and 4) neurons in the nucleus glomerulosus were counted and correlated with age and size in the guppy, *Lebistes*. The findings were that the neuronal numbers in all these areas increased throughout much of the life of the animal. These data, combined with the previously demonstrated increases in retinal neurons in goldfish and sensory and spinal neurons in stingrays, suggest that neurons are added to many areas of the nervous system of fish as the animal ages and grows. In this respect, the nervous systems of fish differ from the nervous systems of other vertebrates. We offer the suggestion that the comparatively greater ability of fish to regenerate their nervous system after injury may be related in part to their ability to add neurons to various parts of the nervous system throughout life.

It is at present generally accepted that neuronal numbers do not change during postnatal life in mammals (e.g. Konigsmark and Murphy, '72; Cowan, '73). This is not the case in fish or amphibia, however. In these animals, the eyes increase in size throughout life, and concomitant with this size increase is an increase in the number of retinal photoreceptors and ganglion cells (Straznicky and Gaze, '71; Easter et al., '77; Johns and Easter, '77; Beach and Jacobson, '79). In addition, the optic tecta of fish and amphibia contain dividing cells, and some of these cells may be neurons (Richter and Kranz, '77; Johns and Easter, '77; Meyer, '78; Stevenson and Yoon, '78; Stevenson and Yoon, '80).

The proliferation of neurons throughout the life of fish is not restricted to the optic system. Recently, for example, it was shown that the numbers of dorsal root ganglion cells and motor cells of the spinal cord increase throughout much of the life of the stingray, *Dasyatis sabina* (Leonard et al., '78). These findings might indicate that neurons in many parts of the nervous system of fish increase during the life of the animal. If this is so, this would seem to be a major difference between the nervous systems of fish and mammals.

The present study is designed to test the idea that the number of neurons in many parts of the nervous system of fish increases throughout most of the life of the animal. The guppy, *Lebistes*, was chosen because the fish is easily bred and grows rapidly. The areas of the brain that were counted are 1) neurons in the ventral horn of the spinal cord, 2) dorsal root ganglion cells, 3) Purkinje cells of the cerebellum, and 4) neurons in the nucleus glomerulosus. These are widely disparate regions, their boundaries are clear, and the neurons are relatively easily distinguished from glial cells. This work has been partially presented in an abstract (Coggeshall et al., '79).

In addition, we obtained an extremely large stingray, *Dasyatis sabina*, and the number of dorsal root ganglion cells and ventral and dorsal root axons were obtained to compare with our earlier data on the stingray (Leonard et al., '78).

MATERIALS AND METHODS

Guppies (Lebistes)

Pregnant female guppies (*Lebistes reticularis*) were isolated in single tanks. Directly after birth, the mothers were taken from the tanks and 4 broods from 4 different mothers gave the animals used in the present study. After appropriate intervals, fish of known age were anesthetized in 2% MS-222 (tricaine methanesulfonate, Finquel) and immersed in a standard formalin–acetic acid–alcohol fixative (80% ETOH 90 cc; glacial acetic acid 5 cc, 40%

0021-9967/80/1942-0291$02.30

formaldehyde solution 5 cc) for 2 days–6 months. This fixative was acidic enough to give a good decalcification for small animals, but for larger animals, 2.5 cc of concentrated hydrochloric acid was added to each 100 cc of 70% ETOH in the dehydration sequence, and the fish were left in this solution for 8–24 hours. This gave excellent decalcification of large animals. After fixation and dehydration, each fish was embedded in paraffin and serially sectioned at 10, 12, or 15 micra. The sections were mounted on glass slides and then stained in 1% cresylecht violet.

Neurons were counted in 4 regions: 1) the ventral horn of the second spinal segment, 2) the dorsal root ganglia of the second spinal segment, 3) the Purkinje cells of the cerebellum, and 4) the neurons in the nucleus glomerulosus at the diencephalic–mesencephalic junction. The numbers of neurons were determined by counting nucleoli. A standard correction factor to take into account split nucleoli (the same nucleolus counted in facing sections) was applied (Konigsmark, '72). This correction factor was appropriately modified to take into account the fact that the nucleoli enlarged somewhat as the animals aged.

Table 1 presents the number of animals, their ages, and the neuronal counts for the 4 areas mentioned above.

Stingray (Dasyatis)

By chance a very large Atlantic stingray (*Dasyatis sabina*) was taken from Galveston Bay. This animal could not be weighed because it was too heavy for available scales (it had to be lifted by a winch), but it weighed at least 90 kg, whereas our previous heaviest animal weighed 8.8 kg (Leonard et al., '78). It was a female and measured 56 inches across the pectoral fins. We obtained specimens of the proximal peripheral nerves at approximately the same segments examined in the earlier study (Leonard et al., '78). It was impossible to be absolutely certain of segmental level because there was 6–8 inches of tissue that had to be removed to get to the spinal cord, and we were unable to expose the entire spinal

TABLE 1. List of the guppies, by age, and the numbers of neurons in the parts of the nervous system examined in this study

Fish	Age	Spinal cord	Dorsal root ganglion L.	Dorsal root ganglion R.	Nucleolus glomerulosus L.	Nucleolus glomerulosus R.	Purkinje cells
1	1	924	151	161	2034	2178	1984
2	1	1014	213	209	2846	2586	1906
3	4	918	189	161	2312	2376	1906
4	4	947	218	203	3066	3064	2363
5	4	764	116	117	3308	3754	—
6	4	912	174	164	3610	3632	2381
7	8	1013	184	161	3396	3104	1913
8	8	1146	235	219	3646	3048	2248
9	10	867	208	727	2776	2754	2672
10	19	1165	—	—	3484	3194	2672
11	23	906	312	312	4808	4104	—
12	23	1318	478	470	5624	6116	—
13	24	933	203	203	3730	2748	2527
14	32	2146	482	497	4270	4984	3495
15	32	1178	386	346	5384	5042	3717
16	38	1022	222	180	4598	4254	2865
17	38	857	173	206	4880	4794	3288
18	61	1282	407	454	6672	6774	3864
19	61	1401	558	564	7260	7272	4856
20	61	1645	—	—	—	—	4036
21	61	—	371	391	—	—	—
22	61	—	—	—	5304	5586	2921
23	61	—	—	—	—	—	3042
24	81	1106	454	397	9436	8260	4812
25	81	1463	562	447	8654	8708	4286
26	82	1794	500	511	7954	8704	—
27	82	1775	317	—	—	6418	—
28	82	1392	438	429	5568	5460	—
29	82	1456	625	—	6090	6194	3393
30	101	1292	—	—	7576	9036	5140
31	199	1537	596	—	10854	10096	5161
32	199	1485	388	291	9438	8730	5523
33	199	1125	334	356	8046	8504	4332

cord to count the segments. Dorsal root ganglia were obtained from approximately the same segments from which we obtained the peripheral nerves. The tissues were fixed in a mixture of 3% formaldehyde, 3% glutaraldehyde and .01% picric acid. The nerves were post-fixed in buffered osmium tetroxide and embedded in plastic; the dorsal root ganglia were embedded in paraffin and serially sectioned. The axons in the peripheral nerve were counted in the light microscope (there are no unmyelinated axons in these spinal nerves, Coggeshall et al., '78), and the dorsal root ganglion cells were counted as in the guppy.

RESULTS

Spinal cord

The dorsal horn of the spinal cord of *Lebistes* is relatively reduced at the 2nd spinal segment, but the ventral horn is well outlined (Figs. 1, 2). All neurons were counted in the ventral horn of the 2nd spinal segment, and the segmental boundaries we used were from the middle of the 1st rib to the middle of the 2nd rib.

In young animals, the neurons of the ventral horn are similar in size and they have a distinct rim of cytoplasm surrounding the nucleus (Fig. 1). The nucleus is round and relatively pale, and the nucleolus has a diameter of approximately 1 micron. In older fish, the neurons are larger, and the basophilic cytoplasm is clearly drawn out into proximal dendrites (Fig. 2). Some of these neurons are very large and presumably correspond to anterior horn cells of other animals. Others are somewhat smaller and are probably interneurons. Glial cells are distinguished by the absence of cytoplasm in these stains and were not counted. Figure 3 presents the number of neurons in the ventral horn in relation to the age of the animal.

Dorsal root ganglion

The dorsal root ganglion in the guppy resembles that of other animals in that it is a cluster of large cells on the dorsal root (Figs. 4,5). The right and left ganglia of the 2nd spinal segment are found between the 1st and 2nd ribs. The ganglion cells are large and round and each contains a nucleus with a prominent nucleolus. In young animals the nucleolus is approximately 1 μm, and in older animals the nucleolar diameter increases to 1.5 μm. There was no difficulty in distinguishing neurons from supporting cells in these ganglia at any age. Figure 6 presents the number of dorsal ganglion cells in each spinal ganglion in relation to age.

Cerebellum

The Purkinje cells of the guppy are very large neurons located between the inner granule cell layer and the outer molecular layer of the cerebellum (Figs. 7,8). It is possible that some cerebellar efferent neurons are included in these counts because they may not be clearly separated from typical Purkinje cells (e.g. Finger, '78), but for convenience, these neurons will be referred to simply as Purkinje cells. The nuclei of the Purkinje cells are large, the nucleoli prominent, and in older animals, the size difference between Purkinje cells and other cells is even more apparent. The nucleolar diameter in small fish was approximately 1 μm, and it increases to 1.5 μm in larger animals. The number of Purkinje cells plotted as a function of age is shown in Figure 9.

Nucleus glomerulosus

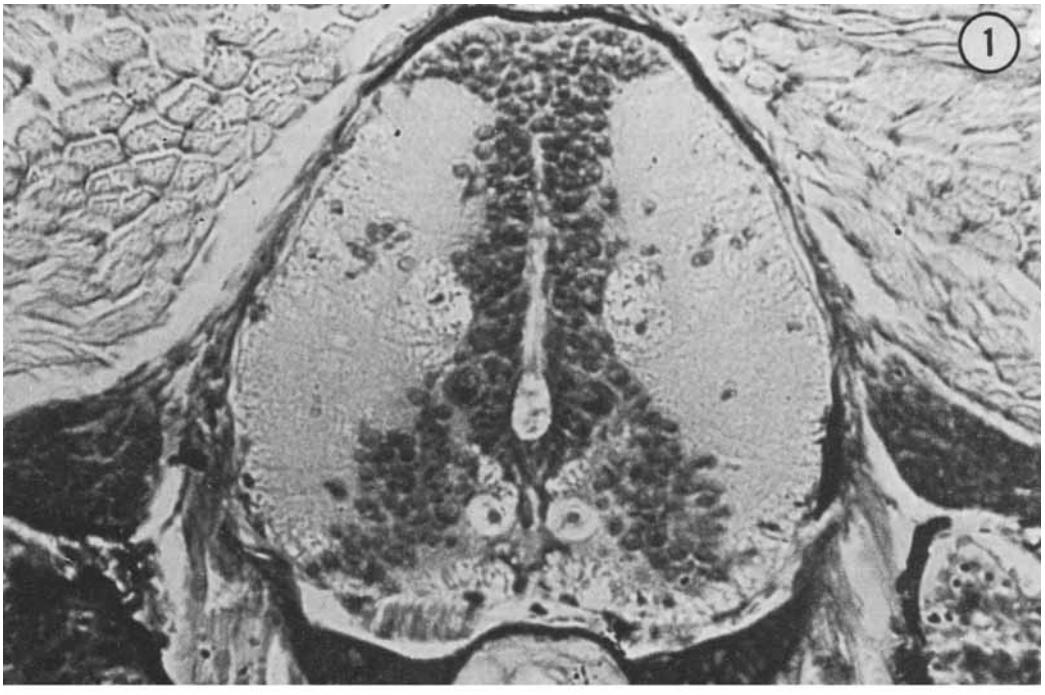
The nucleus glomerulosus is located at the junction of the diencephalon and mesencephalon in fish. This nucleus is clearly outlined as a well-defined sphere of neuronal cell bodies surrounding a relatively acellular core (Figs. 10, 11). Counting was stopped caudally at the point where the spherical shape of the nucleus ended. The nucleolar diameter in small fish was approximately 1 μm, and it increases to 1.5 μm in larger animals. The number of neurons in the nucleus glomerulosus plotted as a function of age is shown in Figure 12.

Stingray

The dorsal root, ventral root and 2 dorsal root ganglia from the middle of the animal were counted. The number of axons in the ventral root was 900, and the number of axons in the dorsal root was 6273. The ganglion cells in 2 dorsal root ganglia in this area numbered 6454 and 5945, respectively.

DISCUSSION

Much work, described and summarized in many places (e.g. Prestige, '70; Cowan, '73; Jacobson, '78), shows that there is in early neural development a neuronal proliferation that often results in the production of considerably more neurons in a particular area than are present in the same area in the adult. Concomitant with or at the end of the neuronal proliferation, which is still early in development, there is a wave of neuronal death



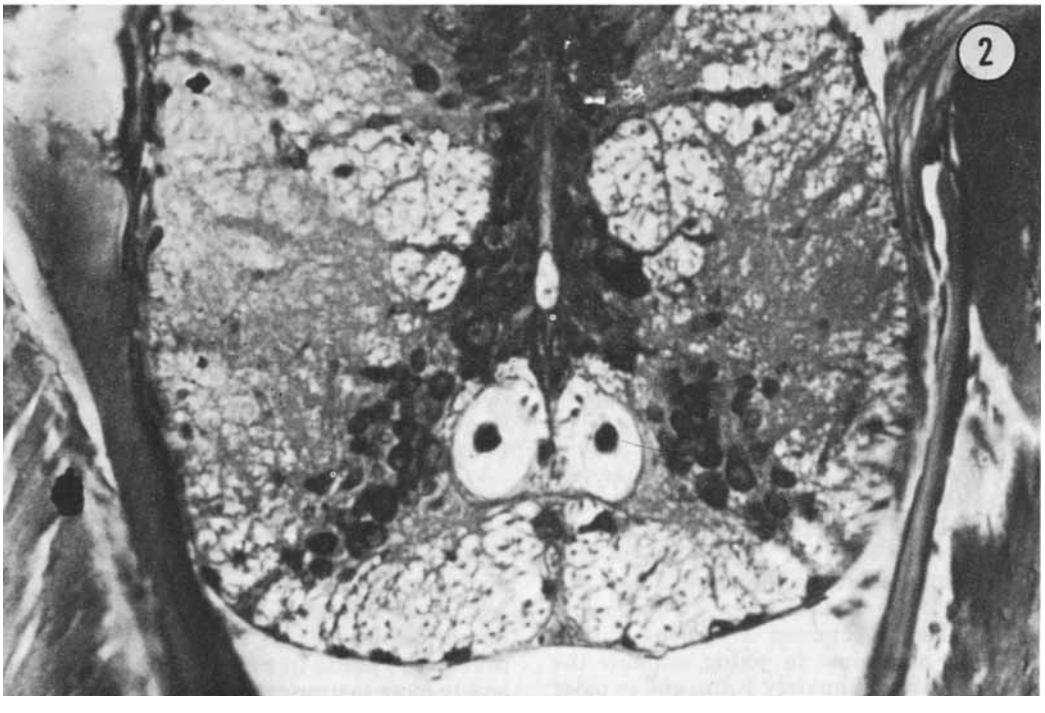


Fig. 1. A cross-section of the spinal cord in a 4-day-old guppy. The neurons of the ventral horn extend down on either side of the spinal cord from the central ependymal lining. The 2 large circular axons on either side of the ventral midline are the Mauthner cell axons. × 400.

Fig. 2. A cross-section of the ventral part of the spinal cord of an 81-day-old guppy. Note that the cells are somewhat larger and farther apart than in the 4-day-old guppy. Also the Mauthner axons are more prominent. × 400.

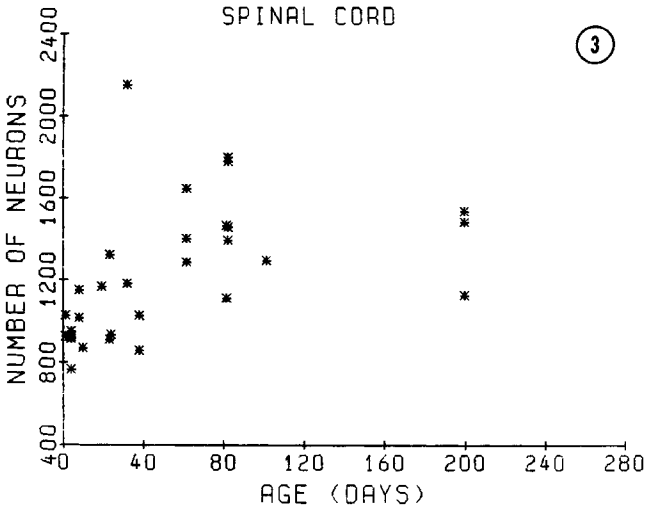


Fig. 3. A graph of the number of neurons in the ventral horn of the 2nd segment of the spinal cord plotted as a function of age. Note that the numbers of cells increase relatively steadily up to 80 days of age but that there is no apparent increase from 80 to 200 days.

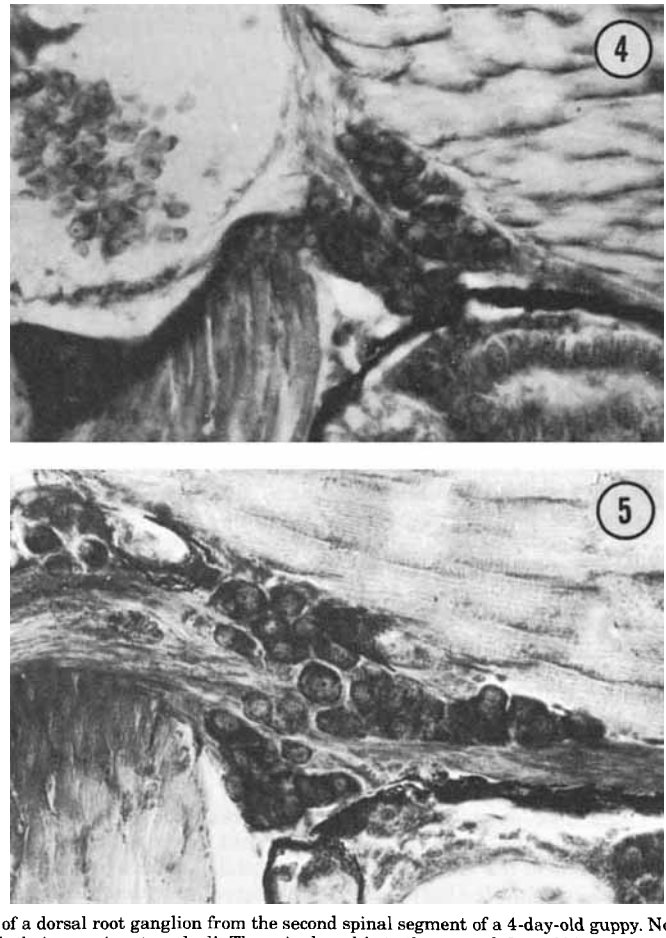


Fig. 4. A view of a dorsal root ganglion from the second spinal segment of a 4-day-old guppy. Note the large ganglion cells with their prominent nucleoli. The spinal cord is to the upper left. × 600.

Fig. 5. A view of a dorsal root ganglion from the second spinal segment of an 81-day-old guppy. Note that the dorsal root ganglion cells are larger than the dorsal root ganglion cells in the 4-day-old animal. × 600.

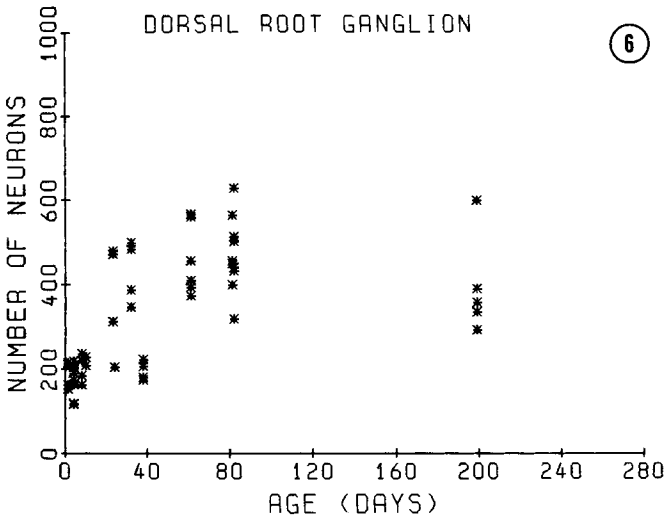


Fig. 6. A graph of the number of neurons in the dorsal root ganglion of the 2nd spinal segment plotted as a function of age. Note that the number increases steadily until 80 days but that there is no apparent increase from 80 to 200 days.

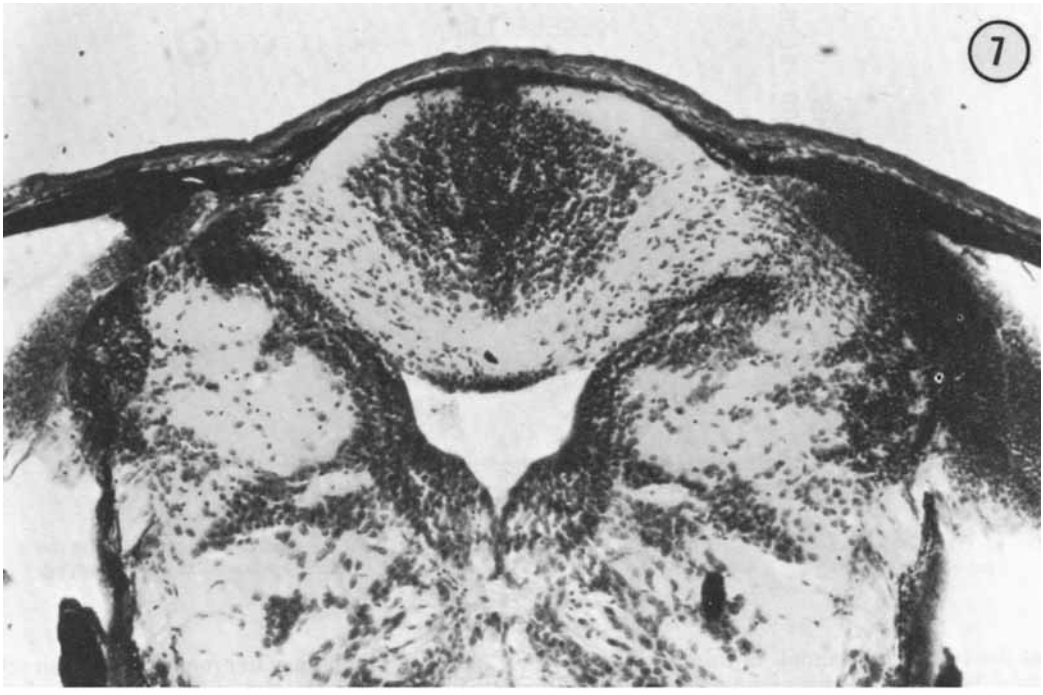
that reduces the number of neurons to the adult number. This sequence of events might be called the early phase of neural development, and it is well described in amphibia (e.g. Hughes, '61, '65; Prestige, '67, '70) and birds (e.g. Hamburger, '48, '75; Hamburger and Levi-Montalcini, '49; Rogers and Cowan, '73). It seems also to be true in mammals (Cowan, '73). It has not, to our knowledge, been examined in fish.

The next phase in neural development might be called the mid-life phase. This phase starts when early neuronal death is over and persists until old age, when there seems to be a drop in neuronal numbers. This is the phase that is characterized by a constant number of neurons (e.g. Konigsmark and Murphy, '72; Cowan, '73).

The final phase we can refer to as the late phase, and it is the phase in late life when neuronal numbers drop, either due to intrinsic causes such as genetic programming or extrinsic causes such as vascular insufficiency. Admittedly, in some cases a drop cannot be demonstrated, and in this case the mid-life phase would persist until the animal died.

The above formulation, if it is correct, implies that the number of neurons for any part of the nervous system does not change from late embryonic life until old age, and as Cowan ('73) points out, this is one of the "remarkable features of the nervous system." In this regard, it should be noted that there is a slow proliferation of neurons in the vertebrate forebrain in adolescent and adult life (Altman and Das, '66; Kaplan and Hinds, '77), but the number of proliferating neurons is so small compared to the total that this is not regarded as a serious contradiction to the generalization about the constancy of neuronal numbers. It is the purpose of the present paper to point out, however, that the idea of constancy of neuronal numbers in midlife clearly does not apply to fish.

The basic findings of the present study are that the numbers of neurons in the dorsal root ganglion and ventral horn increase at least until 80 days of age, and the numbers of Purkinje cells of the cerebellum and neurons of the nucleus glomerulosus are still increasing after 200 days of age. These increases extend well into the adult life of the guppy, because the gonads, by histological criteria, are mature at 30–40 days of age. Thus neuronal populations in the diencephalon, mesencephalon, cerebellum, spinal cord and peripheral nervous system increase throughout most of the life of the guppy. If we add the data on increasing cell populations in the retina of fish and amphibia and spinal nerves and dorsal root ganglion cells in the stingray, it would seem reasonable to speculate that neuronal numbers in many or all areas of the fish nervous system increase throughout much



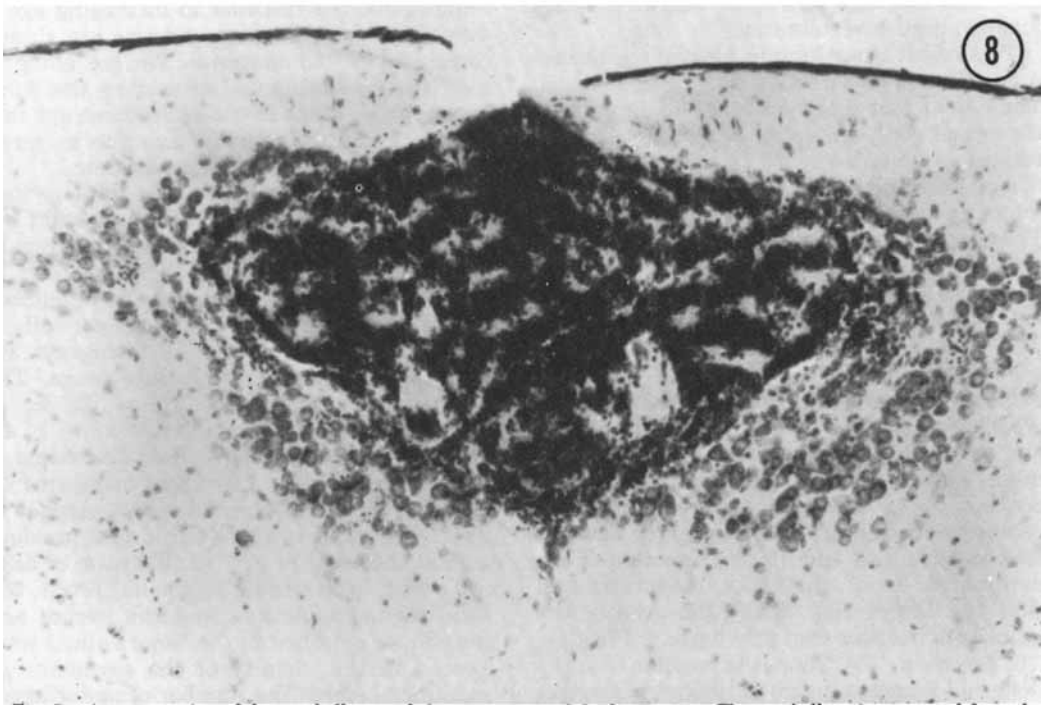


Fig. 7. A cross-section of the cerebellum and the upper part of the brain stem. The cerebellum is separated from the brain stem by the 4th ventricle. The Purkinje cells are not well seen as individuals at this magnification, but they are located in a layer outside of the tightly packed granule cells in the center of the cerebellum. × 250.

Fig. 8. A cross-section of cerebellum of a 199-day-old fish. Note that the cerebellum is much larger and that the large relatively solitary Purkinje cell bodies surround the more numerous, tightly packed, central granule cells. × 250.

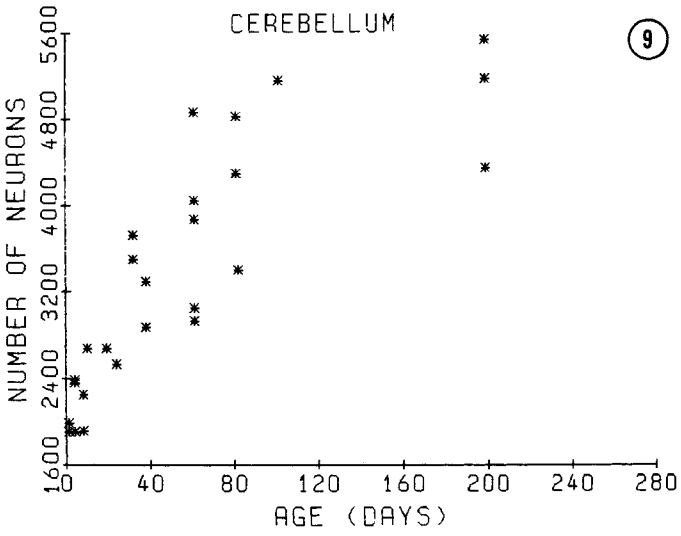


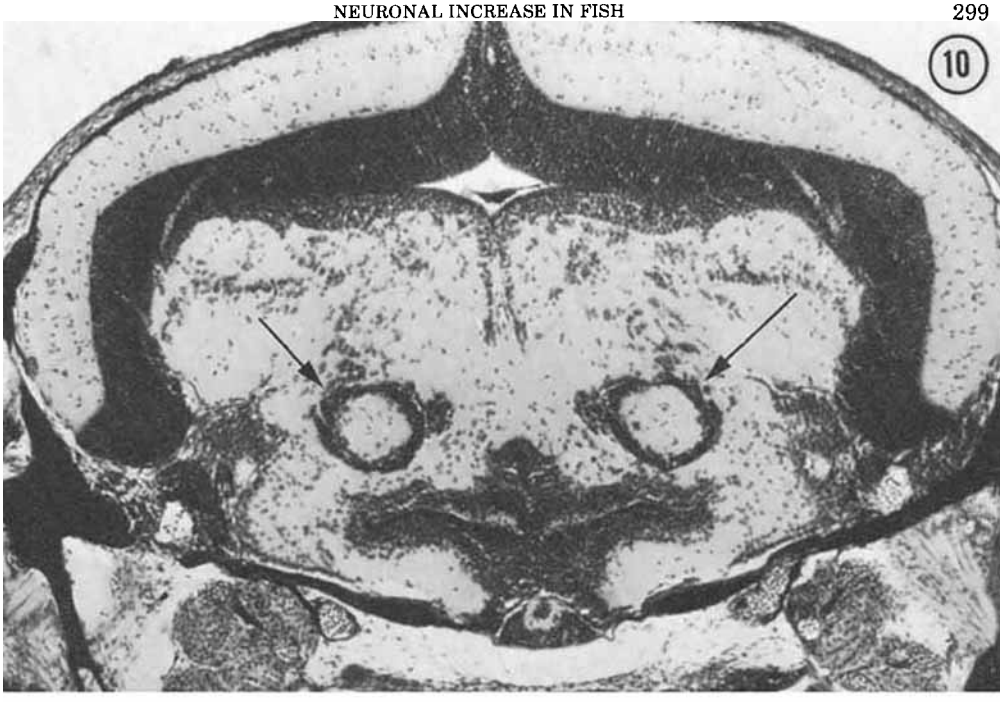
Fig. 9. A graph of the number of Purkinje cells of the cerebellum plotted as a function of age. Note that the rate of increase slows in older animals but even at 200 days the number of Purkinje cells is still increasing.

of the life of the animal. In this regard, therefore, fish neural development seems to differ in a fundamental way from neural development in higher vertebrates.

This result is striking in view of the particular cell populations that have been counted. The dorsal root ganglion cells, motor cells of the spinal cord, cerebellar Purkinje cells, and retinal ganglion cells are type I cells of Golgi (Cajal, '09), and dorsal root ganglion cells and motor cells are type I cells of Jacobson ('78). These populations of cells are widely regarded as being stable (e.g. Jacobson, '78), and these larger cells are often contrasted to small neurons which would be more likely to be modified in terms of numbers during the life of the animal.

If one speculates as to why there are increasing numbers of neurons in the fish nervous system during the mid-life period, one might consider the size of the periphery. A concept that has come into prominence in recent years is that the number of neurons that survive into mid-life is a function of the peripheral "field" that they innervate, and that the larger the "field," the greater the number of neurons that survive (e.g. Prestige, '70; Jacobson, '78). Thus it is possible that the neuronal increase in fish is related to the fact that many fish grow, albeit at a steadily decreasing rate, almost indefinitely, whereas the higher animals reach an upper size limit (if one neglects adipose tissue). Thus the increasing numbers of neurons in adult fish may be a response to the increasing size of the animal, and the response to increasing age is due to the fact that age and size are closely correlated in our material. We are going to test this possibility by measuring the same neural populations in fish of the same age that are markedly different in size due to variations in feeding, living space, sex, etc.

Another conclusion from our data is that the rate of neural addition is not constant but slows as the fish ages and grows. In this regard the stingray in this study is interesting. In our previous study we noted that the number of dorsal root ganglion cells and motor cells in the spinal cord increased as stingrays increased in size and, presumably, age. The animals ranged from 55 gm to 8.8 kg in weight, and the largest had a width of 23 inches across the pectoral fins. The stingray that was caught for this study measured 56 inches across the pectoral fins and weighed at least 10 times as much as our largest previous animal (Leonard et al., '78). Because of difficulty with determining segmental levels, our data are not definitive, but the nerves and ganglia we obtained in the large animal were located in the vicinity of the segments we examined before. The number of motor axons in the large animal is slightly less than the number in the largest of the smaller animals, but the number of sensory axons is somewhat greater. Thus there is a very definite slowing,



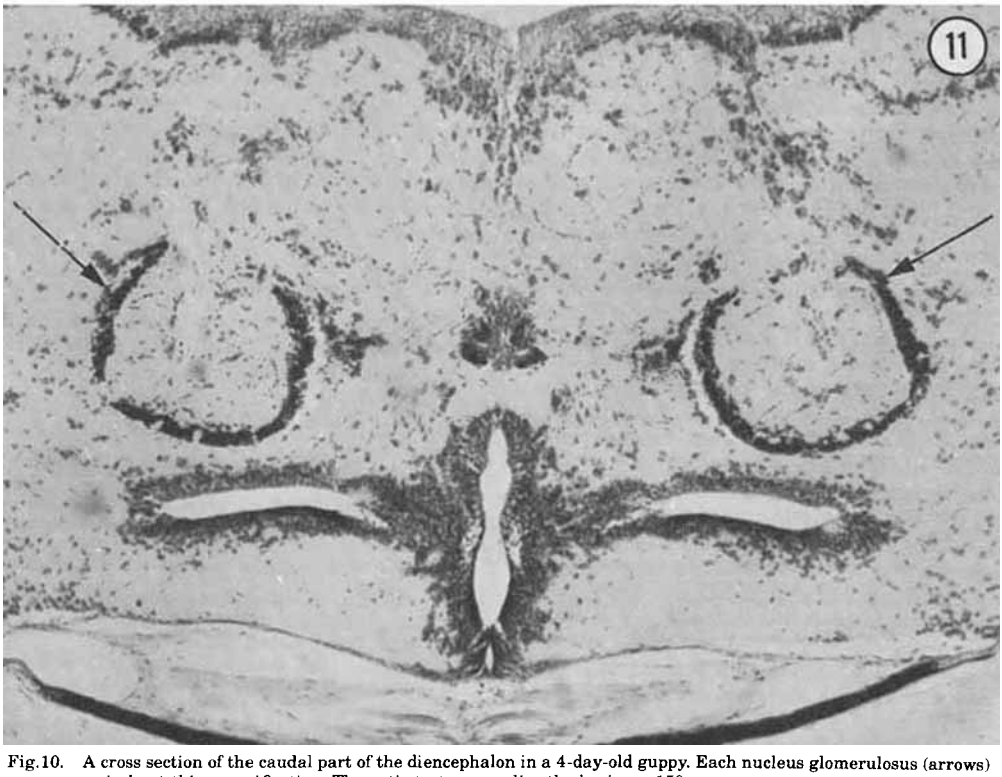


Fig.10. A cross section of the caudal part of the diencephalon in a 4-day-old guppy. Each nucleus glomerulosus (arrows) appears as a circle at this magnification. The optic tectum overlies the brain. × 150.

Fig. 11. A view of the nuclei glomerulosi (arrows) in an 81-day-old fish. Notice that the nuclei are larger and in this particular section, the nucleus is not a complete sphere but is open dorsally. × 150.

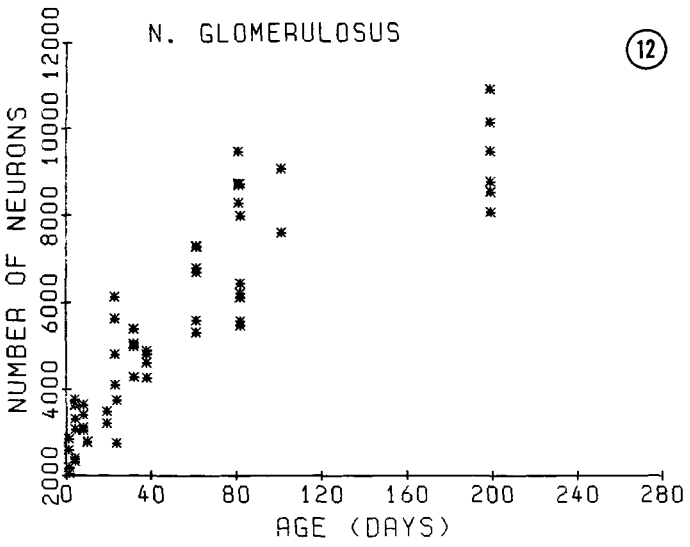


Fig. 12. A graph of the number of neurons in the nucleus glomerulosus plotted as a function of age. Note that the rate of increase slows in older animals but even at 200 days the number is still increasing.

if not a cessation, of neuronal addition in very large stingrays as compared to younger, smaller, but still adult animals. Further work in a more controlled situation will be necessary to determine whether there comes a period in old age in fishes when they no longer add neurons, but the preliminary indications suggest that in certain areas the rate of neuronal addition becomes so slow as to be negligible.

Our data also lead to the conclusion that the slowing of neuronal proliferation takes place at different rates in different areas of the brain. In our study, for example, the spinal neurons and dorsal root ganglion cells seem not to be adding new cells between 80 and 200 days after birth, whereas the neurons of the nucleus glomerulosus and cerebellum are. It is likely that different rates of neuronal increase characterize different regions of the fish brain. The trochlear nerve in goldfish is interesting in this regard, because it has recently been reported that the number of axons in this nerve does not increase with advancing age (Easter, '79). Also it might be pointed out that although amphibia add neurons to their retina until late in life, the numbers of ventral horn cells and dorsal root ganglion cells seem to be constant in midlife (Prestige, '70).

As a final point, it has been stated that fish have a much greater regenerative capacity following central nervous system injury than do mammals (Piatt, '55; Bernstein, '64). It has been suggested that this capacity for regeneration might be related to the fact that fish keep adding neurons in adult life, whereas higher vertebrates do not (e.g. Leonard et al., '78). It has also been shown that this capacity for regeneration decreases as fish age (Bernstein, '64). It is interesting, therefore, that the rate of addition of neurons that we describe here also seems to decrease as fish age and increase in size.

ACKNOWLEDGMENTS

This work is partially supported by grants NS 06039, NS 07377, and NS 11255 from the National Institutes of Health.

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