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Cerebellar Monoamine Nerve Terminals, a new Type of Afferent Fibers to the Cortex Cerebelli

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Summary. The monoamine innervation of the cerebellum of the rat has been studied by both *in vivo* and *in vitro* techniques using the histochemical fluorescence method for the demonstration of catecholamines (CA) and certain tryptamines. By way of a pharmacological approach using *inter alia* protriptyline, which acts mainly by blocking the membrane pump of the noradrenaline (NA) neurons, evidence was obtained that CA nerve terminals in the cerebellum mainly represent NA nerve terminals. These were found to innervate practically all parts of the cerebellar cortex with a patchy innervation pattern and with an innervation of especially the anterior and posterior lobes. The terminals mainly seem to make axodendritic contacts in the molecular and granular layers without any strict localization of the terminal plexus to any special plane of the cerebellar folia. The fibers enter the cerebellum via the inferior cerebellar peduncle and run in the white matter of the cortex cerebelli. Incubation studies with 6-hydroxytryptamine indicate that there exists also a 5-hydroxytryptamine (5-HT) innervation of the cortex cerebelli, although not as pronounced as the NA innervation. The 5-HT nerve terminals are very fine, varicose fibers and innervate mainly the molecular layer, especially of the anterior lobe. The terminals run mainly in the transverse plane of the folium parallel to the surface. Thus, the pattern of innervation of these 5-HT afferents is different from that of the NA nerve terminals. In the uvula, structures which may represent the "rosettes" of the mossy fibers or golgi axon terminals in the granular layer take up and accumulate monoamines after incubation with amine *in vitro*. The exact nature of these structures remains to be elucidated. — The cerebellar nuclei receive a very low to low degree of innervation of NA and 5-HT nerve terminals.

Key Words: Noradrenaline and 5-hydroxytryptamine nerve terminals — Cortex cerebelli — Rat

Introduction

It is generally accepted that there exist two types of afferent nerve terminals to the cerebellar cortex, the mossy fibers and the climbing fibers (ECCLES, ITO and SZENTÁGOTHAI 1967; BELL and DOW 1967; FOX and SNIDER 1967). Most of the fiber systems to the cerebellar cortex end as mossy fibers, including the vestibular, spinal, pontine and reticular projections to the cerebellar cortex. The origin of the climbing fibers is believed by many workers to be the inferior olivary complex (SZENTÁGOTHAI and RAJKOVITS 1959). However, this is probably not the only major source of climbing fibers.

Previous studies using the histochemical fluorescence method for demonstration of catecholamines (CA) and 5-hydroxytryptamine (5-HT) (FALCK, HILLARP, THIEME and TORP 1962; HILLARP, FUXE and DAHLSTRÖM 1966; CORRODI and JONSSON 1967) have revealed the existence of small numbers of noradrenaline (NA) nerve terminals in the cerebellar cortex of the mouse and rat (see FUXE 1965). However, with certain modifications of the histochemical fluorescence method (FUXE and JONSSON 1967; FUXE, HÖKFELT and HAMBERGER 1968) and the use of *in vitro* techniques involving *inter alia* incubation with 6-hydroxytryptamine (6-HT), which has recently been shown to be an important tool for visualizing central monoamine, especially 5-HT nerve terminals (JONSSON, FUXE, HAMBERGER and HÖKFELT 1969), it has now been possible to perform a detailed mapping of the monoamine and especially the NA innervation of the cerebellar cortex and the cerebellar nuclei.

Material and Methods

About 50 male Sprague-Dawley rats have been used (180—200 g body wt.). In the *in vivo* experiments both untreated rats (7) and rats pretreated with nialamide (8) were used. Immediately before killing most of the rats were infused under Nembutal anaesthesia (40 mg/kg, i.p.) with 10 ml of a strongly hypertonic salt solution (NaCl 7.6 g, KCl 0.42 g, $CaCl_2$ 0.12 g, NaH_2PO_4 0.186 g, $NaHCO_3$ 2.1 g, Dextros 2.0 g, Sucros 4.5 g, aq. redest ad 250 ml) based on MCEWEN's (1956) buffer solution and saturated with 93.5% CO_2 and 6.5% O_2, since this procedure has been found to facilitate the visualization of the central monoamine nerve terminals (FUXE, HAMBERGER and HÖKFELT 1968).

To obtain evidence whether the CA nerve terminals observed in the cerebellum represented dopamine (DA) or NA nerve terminals, 10 rats were injected at a 2 h interval with two doses of 4-*a*-dimethyl-m-tyramine (H 77/77) (12.5 mg/kg, i.p.) which mainly depletes amine stores in the NA neurons in the brain (CARLSSON, CORRODI, FUXE and HÖKFELT 1969) and were killed 2 h after the last injection. Four of the rats were injected i.p. with protriptyline, which probably acts by blocking the membrane pump in the central NA neurons (see CARLSSON, FUXE, HAMBERGER and LINDQVIST 1966) 30 min before each injection of H 77/77. Protriptyline (25 mg/kg) was given before the first injection of H 77/77 and a second dose (12.5 mg/kg) was given before the last injection. This dose of protriptyline is known to block almost completely the depletion of NA induced by H 77/77 in the central NA nerve terminals (CARLSSON, CORRODI, FUXE and HÖKFELT 1969).

In the experiments *in vitro* serial sagittal and transversal slices were made from the cerebellum, after which the slices were incubated at 37° C for 30 min in Tyrode's solution (with

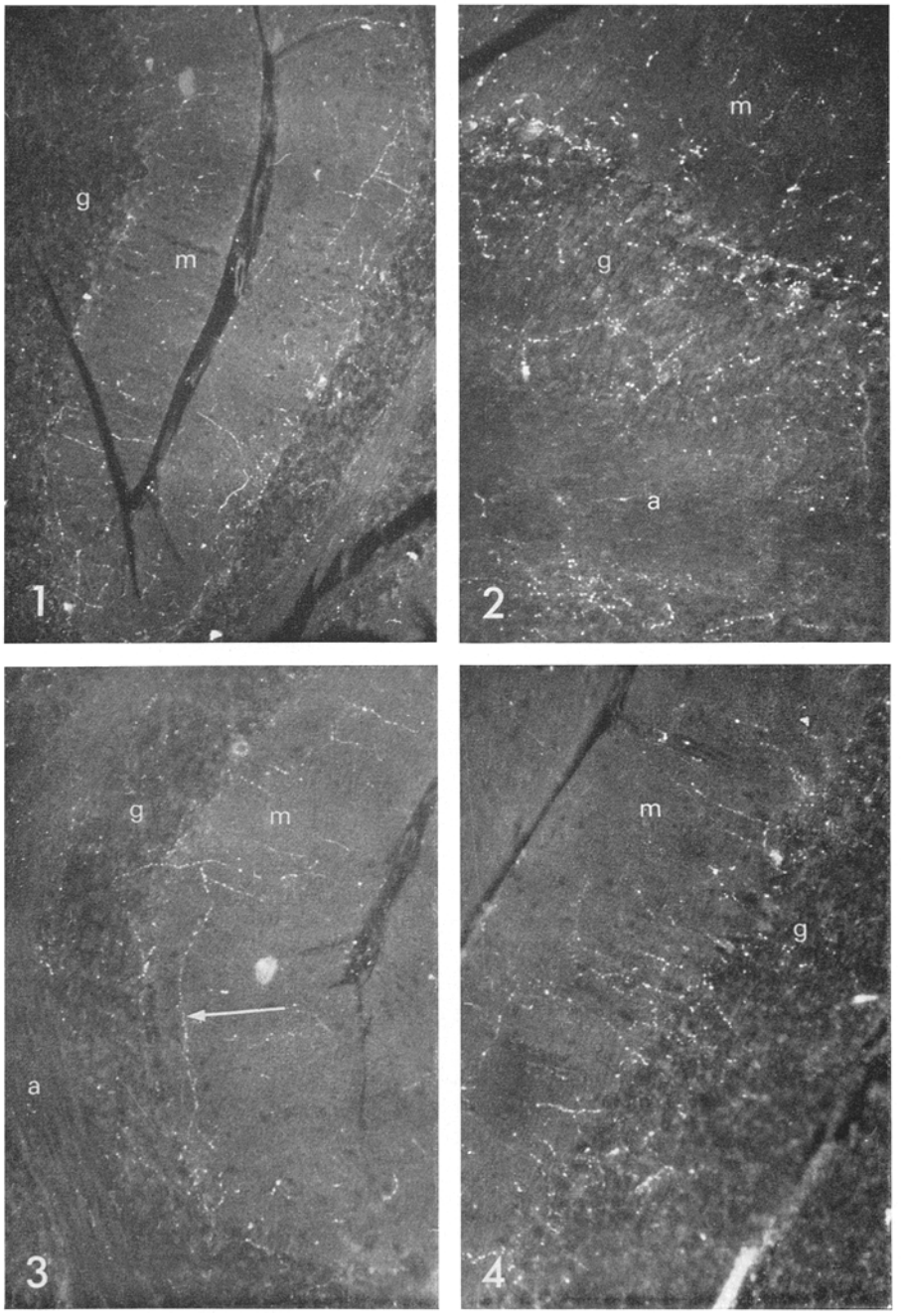
Fig. 1. *Section of a sagittal slice from the cortex cerebelli of a normal rat, providing a transverse section of the folium.* Fissura prima with culmen posterior to the left and the lobulus simplex to the right. Slice was incubated for 30 min with 6-HT (1 μg/ml). A diffusely oriented plexus of NA nerve terminals of a low to medium density is observed, mainly in the molecular layer (m), but also in the granular layer (g). × 80

Fig. 2. *Section of a sagittal slice from the cortex cerebelli (lobulus simplex) of a normal rat.* Slice was incubated for 30 min with 6-HT (1 μg/ml). A diffusely oriented plexus of NA nerve terminals of low to medium density is observed, mainly in the granular layer (g), but also in the molecular layer (m). a = white matter. × 120

Fig. 3. *Section of a sagittal slice from the cortex cerebelli (culmen posterior) of a normal rat.* Slice incubated for 30 min with 6-HT (1 μg/ml). One NA nerve terminal (↗) runs parallel to the cortical surface in the molecular layer (m). For other abbreviations, see text to Fig. 2. × 120

Fig. 4. *Section of a sagittal slice from the cortex cerebelli (culmen anterior) of a normal rat.* Slice was incubated for 30 min with 6-HT (1 μg/ml). NA nerve terminals in the molecular layer (m) are mainly oriented perpendicular to the cortical surface. For other abbreviations, see text to Fig. 2. × 120

added glucose 0.5 g/l and sucrose 1 g/l) containing NA, α-methyl-NA or 6-HT ($5 \cdot 10^{-7}$ to $5 \cdot 10^{-5}$ M) (HAMBERGER 1967; HÖKFELT 1968). Eight rats had been pretreated with reserpine (10 mg/kg, i.p., 22 h before killing) and nialamide (500 mg/kg, i.p., 30 min before killing). Before



Figs. 1, 2, 3 and 4

incubation with NA and 6-HT the cerebellar slices from 3 of these rats were preincubated for 15 min with protriptyline (10^{-5} and 10^{-4} M). For details on the incubation and preparation procedure see HAMBERGER (1967) and HÖKFELT (1968).

All the samples from the cerebellum were taken for fluorescence histochemistry to demonstrate catecholamines and certain tryptamines (DAHLSTRÖM and FUXE 1964; FUXE and JONSSON 1967; HAMBERGER 1967; JONSSON and SANDLER 1969; JONSSON, FUXE, HAMBERGER and HÖKFELT 1969).

Results

The same principal results were obtained in the *in vivo* and the *in vitro* experiments. However, because the monoamine nerve terminals appeared very distinct after incubation of cerebellar slices with 6-HT, most of the pictures in the present paper represent photographs of slices incubated in this way.

Experiments in Vivo

In horizontal, sagittal and transversal sections of the *cerebellar cortex*, the CA nerve terminals formed a sparse plexus of very fine, green-fluorescent, varicose nerve terminals throughout the molecular and granular layers (Figs. 1—4). The varicosities had a diameter of about 0.4—1.0 μ as measured in fluorescence microscopic pictures. This plexus was found in the medial, intermediate and lateral parts of the anterior, posterior and flocculo-nodular lobes, but the density of the CA nerve terminals in the latter lobe was less than in the other two lobes (Fig. 5). The density in the uvula (Fig. 6) was similar to that in the flocculo-nodular lobe. There was a patchy innervation of each folium of the anterior and posterior lobes, with some areas of medium density and others of very low density (Figs. 1 and 7). The plexus of CA nerve terminals was similar in morphological appearance and in topographical localization throughout the cortex cerebelli (Figs. 8—10). The plexus was not orientated in a special plane as is observed with the climbing fibers but was randomly distributed in all the planes. Some terminals ran at right angles to the surface (Fig. 8), others ran parallel to the surface (Fig. 3) in the sagittal or the transversal plane of the folium.

In the molecular layer the CA nerve terminals may establish mainly axo-dendritic contacts, since this layer is poor in cell-bodies and the fluorescent nerve terminals do not seem to be in close contact with the cell-bodies in this layer. In the Purkinje cell layer only a few CA nerve terminals are observed and these do not lie close to the cell-bodies. In the granular layer the CA nerve terminals again seem to make mainly axo-dendritic contacts between the cell-bodies of the granular cells. There seems to be a higher density of CA nerve terminals in the parts of the granular and molecular layers lying close to the Purkinje cell layer (Fig. 10) than elsewhere in these two layers.

Treatment with H 77/77 caused a marked disappearance of the fluorescence in the CA nerve terminals so that practically no fluorescent nerve terminals remained in the cerebellar cortex after this treatment. This effect of H 77/77 could be blocked, however, by pretreatment with protriptyline. These results strongly indicate that the green-fluorescent, varicose nerve terminals observed in the cerebellar cortex represented NA nerve terminals.

In the *cerebellar nuclei* (N. fastigii, interpositus and lateralis) there was a very low to low density of green-fluorescent, varicose nerve terminals with morphologic characteristics similar to those of the cortex cerebelli (Fig. 11). These terminals

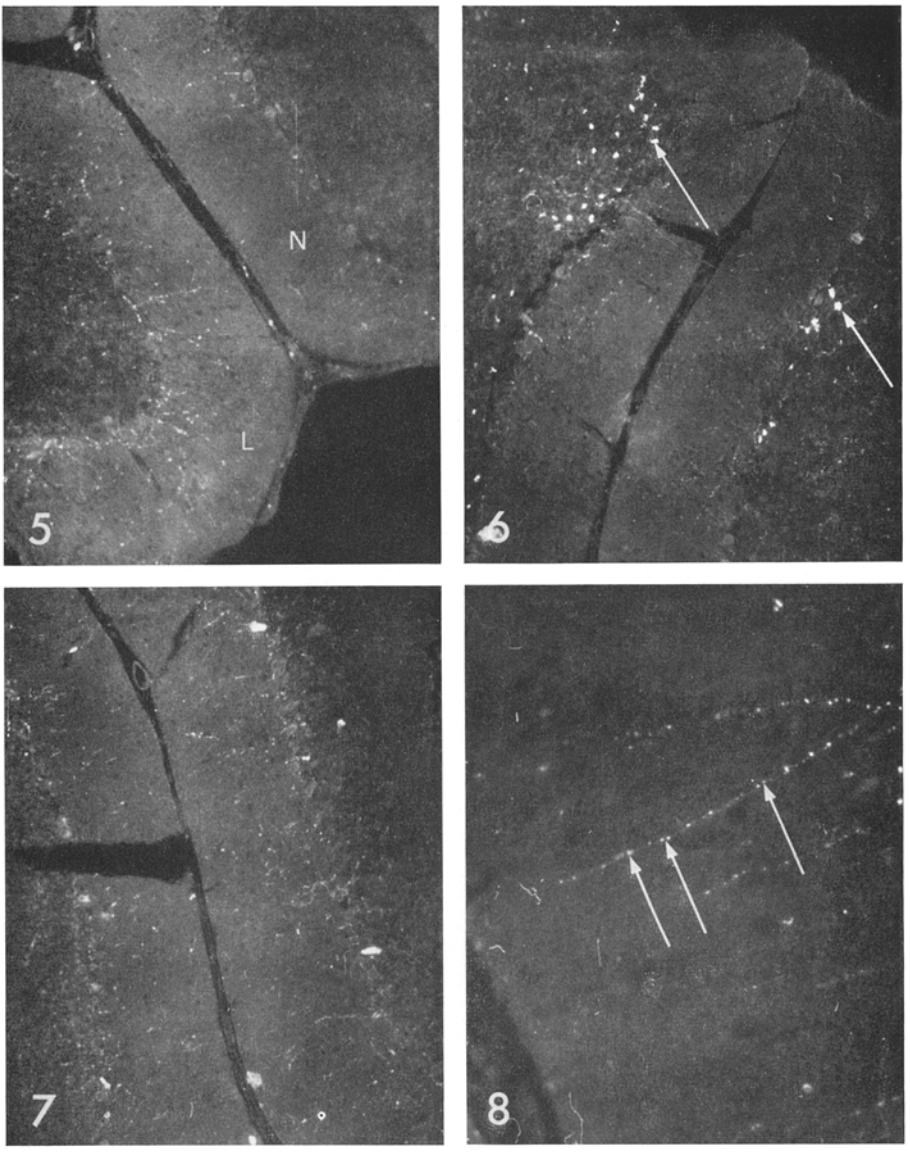


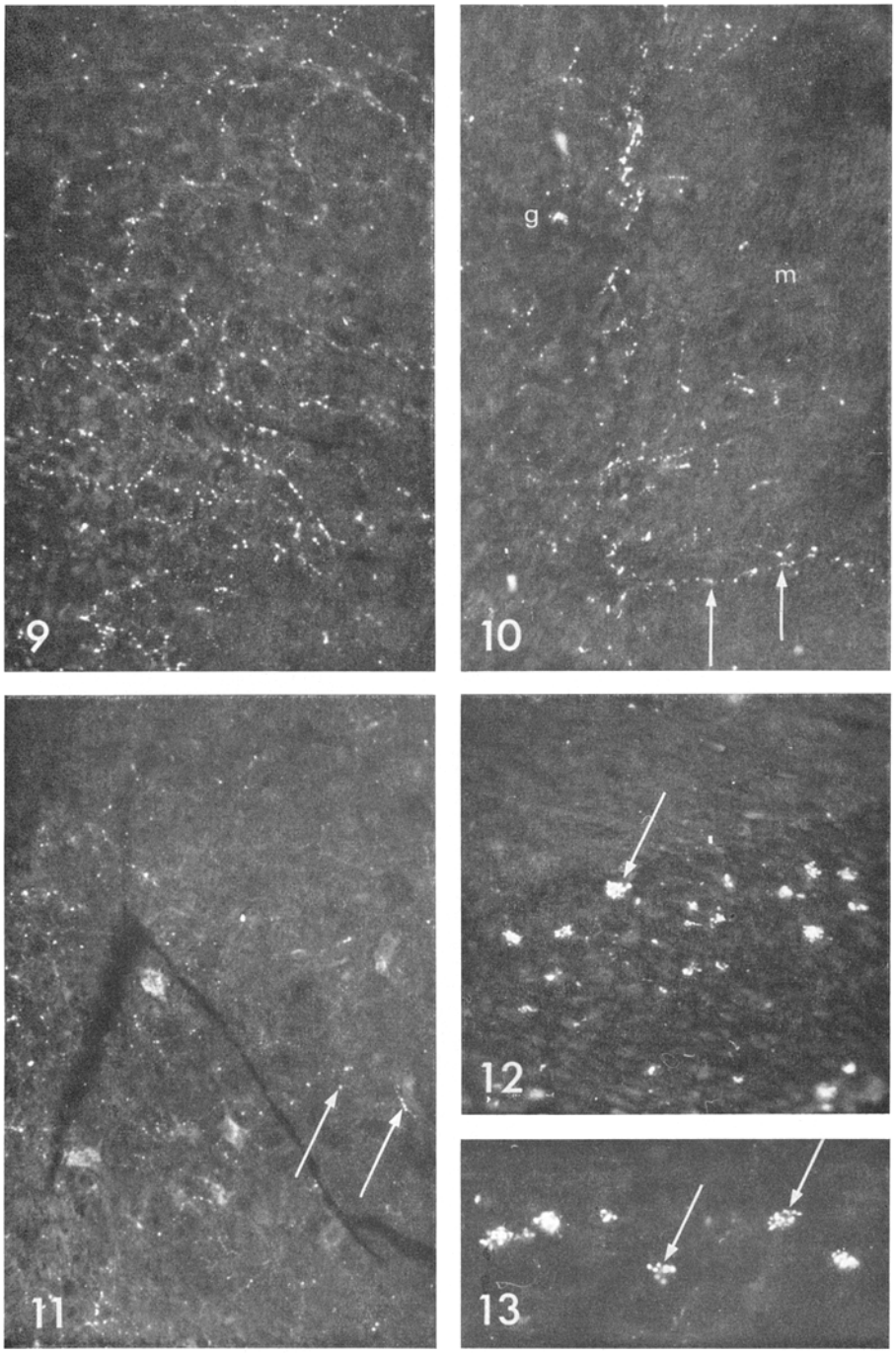
Fig. 5. *Section of a sagittal slice from the cortex cerebelli of a normal rat:* Lingula (L) to the left and nodulus (N) to the right. Slice was incubated for 30 min with 6-HT (1 μg/ml). A low density of NA nerve terminals is observed in the molecular layer of the lingula whereas practically no terminals are observed in the nodulus. × 80

Fig. 6. *Section of a sagittal slice from the cortex cerebelli (uvula) of a normal rat.* Slice was incubated for 30 min with 6-HT (1 μg/ml). Few NA nerve terminals are observed, but a strong yellow-green fluorescence appears in "dotted" structures (↗) in the granular layer close to the Purkinje cell layer, probably due to an accumulation of 6-HT. These structures do not seem to contain CA or 5-HT normally. × 80

Fig. 7. *Section of a sagittal slice from the cortex cerebelli (folium vermis) of a normal rat.* Slice was incubated for 30 min with 6-HT (1 μg/ml). A very low density of NA nerve terminals is observed in the granular layers. × 80

Fig. 8. *Section of a sagittal slice from the cortex cerebelli (lobulus centralis) of a normal rat.* Slice was incubated for 30 min with 6-HT (1 μg/ml). A very fine, varicose NA nerve terminal (↗) is oriented perpendicularly to the cortical surface. × 300

were also depleted by H 77/77, and this depletion could be prevented by treatment with protriptyline. Therefore these CA nerve terminals probably also are identical



Figs. 9, 10, 11, 12 and 13

with NA nerve terminals. In the rats treated with nialamide there were a certain number of yellow-fluorescent, very fine, varicose nerve terminals, which probably represented 5-HT nerve terminals. No yellow-fluorescent nerve terminals have so far been observed in the cortex cerebelli after this treatment.

Experiments in Vitro

Incubation with NA: Essentially the same results were obtained as in the experiments *in vivo*, although the number and intensity of the CA nerve terminals were somewhat increased. Furthermore, non-terminal, green-fluorescent axons were found in the subcortical white matter of the cerebellum and observed to give off collaterals to the granular layer. Such fibers were also found in the inferior cerebellar peduncle. If the slices had been preincubated with protriptyline very few green-fluorescent nerve terminals appeared in the cerebellum after incubation with NA, an observation which further supports the view that the CA nerve terminals represent NA nerve terminals. In the granular layer of the uvula, especially its ventral part and corresponding hemispheric areas, a strong green fluorescence appeared after incubation with NA in a large number of structures which may represent the "rosettes" of the mossy fibers or Golgi axon terminals (Figs. 6; 12—13). These were not observed *in vivo* in the normal rat cerebellum, but appeared only after incubation with amine.

Incubation with 6-HT: This procedure demonstrated the NA nerve terminals in the cerebellum more distinctly than any of the other techniques used. Furthermore, a new type of very fine, varicose fiber was observed in the molecular layer, especially in its superficial part. These fibers ran parallel to the surface of the cerebellar cortex in the transverse plane of the folium (Figs. 14—15). These terminals have been observed mainly in the anterior lobe, especially the lingula and lobus centralis and corresponding hemispherical parts. Since it is known that 6-HT is accumulated particularly well in the 5-HT nerve terminals (JONSSON, FUXE, HAMBERGER and HÖKFELT 1969), these terminals probably represent 5-HT nerve terminals in the cerebellar cortex. Previous failure to observe them is probably due to the fact that the histochemical fluorescence method is not as sensitive for 5-HT as for CA (see FUXE and JONSSON 1967). These fluorescent terminals were

Fig. 9. *Cortex cerebelli (paramedian lobule) of normal rat after i.v. perfusion with a strongly hypertonic buffer solution* immediately before killing. Section oriented transversely to the brain stem. The Purkinje cell layer here illustrated is cut obliquely. A low density of NA nerve terminals lies between the cell-bodies. × 200

Fig. 10. *Cortex cerebelli (paramedian lobule) of normal rat after an i.v. perfusion with a hypertonic buffer solution* immediately before killing. Section oriented transversely to the brain stem. The NA nerve terminals (↗) are localized mainly in the parts of the molecular (m) and granular (g) layers lying close to the Purkinje cell layer. × 200

Fig. 11. *Transverse section of the cerebellar nuclei of a rat* pretreated with reserpine plus nialamide (see text) before incubation with 6-HT (1 μg/ml) for 30 min. A very low density of very fine NA (↗) and 5-HT nerve terminals is shown. × 120

Fig. 12. *Section of a sagittal slice from the cortex cerebelli (uvula) of a normal rat* after incubation with α-methyl-NA (1 μg/ml). A strong fluorescence appears in the granular layer in structures, which may represent "rosettes" of mossy fibers or Golgi nerve terminals (↗). × 200

Fig. 13. *Higher magnification* of an area adjacent to that seen in Fig. 12. The fluorescent dots (↗) appear very distinct. × 300

not observed after incubation with NA. A strong yellow-green fluorescence appeared similar to that observed after incubation with NA in a large number of rosette-like structures in the granular layer of the uvula.

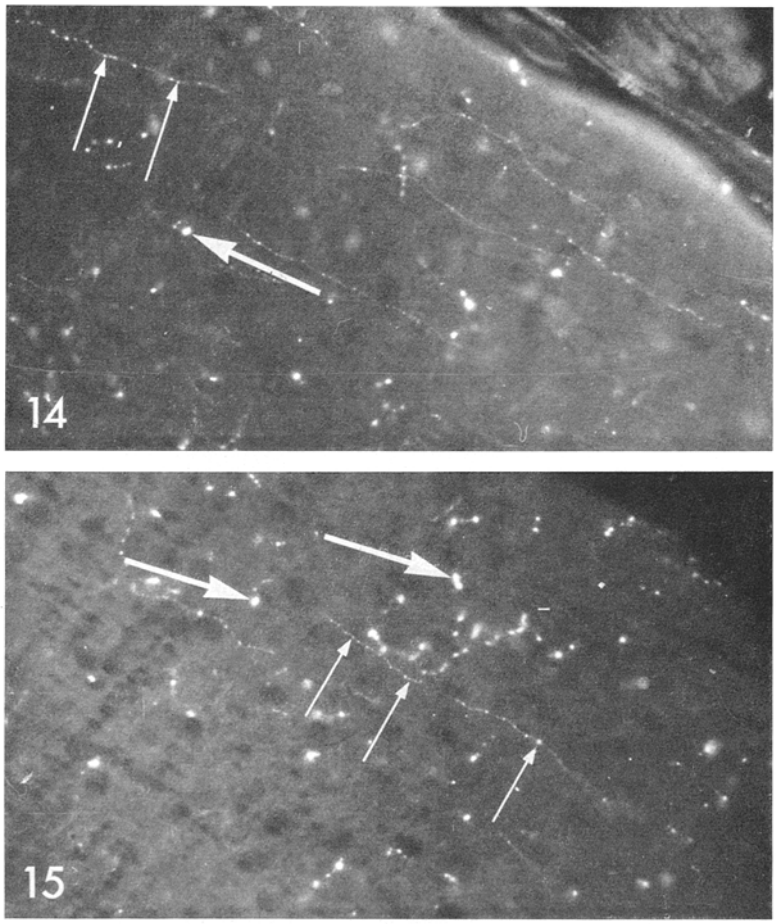


Fig. 14 and 15. *Section of a sagittal slice from the cortex cerebelli (lobulus centralis) of a normal rat.* The slice has been incubated with 6-HT (1 μg/ml) for 30 min. Besides varicose NA nerve terminals (thick arrow), very fine, fluorescent, varicose nerve terminals (thin arrow) are observed running parallel to the cortical surface in the transverse plane of the folium in the molecular layer. These fluorescent terminals are not observed without amine incubation but they may still represent 5-HT nerve terminals, since their morphological characteristics are similar to those found in 5-HT nerve terminals in the prosencephalon, and since 5-HT nerve terminals are known to take up and accumulate 6-HT to a marked extent. × 300

Discussion

A sparse plexus of CA nerve terminals has been found throughout the cerebellar cortex and nuclei. Previous biochemical determinations (see ANDÉN, FUXE and UNGERSTEDT 1967) and the present results with protriptyline (see CARLSSON, FUXE, HAMBERGER and LINDQVIST 1966; HAMBERGER 1967; CARLSSON, CORRODI,

FUXE and HÖKFELT 1969) strongly suggest that the predominant CA nerve terminals are identical with NA nerve terminals.

The NA nerve terminals are spread throughout the cerebellar cortex in a way similar to that observed in the cortex cerebri (FUXE, HAMBERGER and HÖKFELT 1967). The NA nerve terminals are characterized by their lack of stratification or delineation of innervated structures and their diffuseness of innervation. Only the uvula, especially the flocculo-nodular lobe, receive less NA innervation than the other parts, and there is a patchy innervation of the folia of the cortex cerebelli.

With the present technique no CA cell-bodies are found in the cerebellum of the normal rat, nor do any green-fluorescent CA cell-bodies show up after incubation with NA or 6-HT. Therefore, the NA nerve terminals probably arise from NA cell-bodies lying in the lower brain stem, where practically all the NA cell-bodies are found (DAHLSTRÖM and FUXE 1964). A previous investigation has revealed that certain NA cell-bodies mainly in the area of the N. reticularis lateralis increase markedly in intensity of fluorescence after removal of the cerebellum (ANDÉN, FUXE and UNGERSTEDT 1967). Hence at least some of the NA nerve terminals in the cerebellum may arise from these NA cell-bodies, and these NA nerve terminals may represent a special type of reticular cerebellar afferent, which probably ascend in the inferior cerebellar peduncle to the cortex cerebelli. Besides innervating the cerebellum some of these NA neurons also may innervate by way of an extensive collateral network a large number of other parts of the brain such as the spinal cord and the diencephalon (ANDÉN, FUXE and LARSSON 1966; ANDÉN, FUXE and UNGERSTEDT 1967).

After incubation with 6-HT additional numbers of varicose fluorescent nerve terminals appeared in the molecular layer running in the transverse plane of the folium parallel to the surface. These terminals may represent 5-HT nerve terminals, since 6-HT is known to accumulate to a great extent in this type of terminals (JONSSON, FUXE, HAMBERGER and HÖKFELT 1969) and since these nerve terminals resemble 5-HT nerve terminals found in other brain regions (FUXE 1965). On the other hand, it cannot be excluded that these terminals normally do not contain 5-HT but instead have the ability to take up and accumulate exogenous 6-HT. However, probable 5-HT nerve terminals seem to be restricted mainly to the molecular layer and to have a well-defined orientation in a special plane, in contrast to the NA nerve terminals. Because of this localization the 5-HT terminals may make mainly axodendritic contacts with the Purkinje cell dendrites. It has not been possible to detect 5-HT cell-bodies in the cerebellum not even after treatment with nialamide or after incubation with 6-HT. Since most of the 5-HT cell-bodies are found in the raphe nuclei (DAHLSTRÖM and FUXE 1964) the 5-HT nerve terminals in the cerebellum probably arise from these nuclei in the lower brain stem.

The fluorescent "rosettes" found were not observed in the normal cerebellum, but only after incubation with amine. These structures probably do not normally contain monoamines, but they may be able to accumulate catecholamines and certain tryptamines. Such fibers may belong to a specific neuron system with a mechanism for uptake and concentration of *inter alia* monoamines. Similar structures either containing endogenous monoamines or having the ability to take up and accumulate exogenous monoamines have not been found in any other region of the brain.

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