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ONTOGENIC APPEARANCE OF Na^+ CHANNELS CHARACTERIZED AS HIGH AFFINITY BINDING SITES FOR TETRODOTOXIN DURING DEVELOPMENT OF THE RAT NERVOUS AND SKELETAL MUSCLE SYSTEMS

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SUMMARY : The appearance of the voltage-dependant Na^+ channel during the fetal and post-natal development of rat brain, cerebellum and skeletal muscle has been followed using a highly radiolabelled derivative of tetrodotoxin. The number of Na^+ channels is low at the fetal stage and increases drastically during post-natal development. The time-course of this increase is different in brain, cerebellum and skeletal muscle. Changes in affinity of the Na^+ channel for tetrodotoxin occur during brain and cerebellum development. The results are discussed in relation with the maturation of the three types of excitable tissues.

INTRODUCTION : The voltage-dependant Na^+ channel is a very important structural element of the surface membrane of nerve and muscle cells. It is essential for the generation of action potentials in these excitable tissues.

Several types of natural toxins bind with a high affinity to the Na^+ channel (1, 2) and they have been used to analyze the structural, functional and developmental properties of the Na^+ channel (1, 2, 3, 4, 5). Probably the most widely used radiolabeled toxins for studying the biochemistry of this ionic channel are tritiated derivatives of tetrodotoxin (TTX) and saxitoxin (6, 7).

The purpose of this work is to study with a highly radiolabeled TTX derivative the ontogenesis of the Na^+ channel in brain, cerebellum and skeletal muscle during the fetal and the post-natal development of the rat.

MATERIALS AND METHODS : Embryos and post-natal Wistar albino rats were used throughout this work. Midnight was considered as the time of mating and the day following mating was designated as day 0.5 of gestation. Pregnant rats were sacrificed at various times from 15 to 20 days of gestation by decapitation and fetal rats taken out. Postnatal rats were raised in litters of 5.

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On each animal, brain without cerebellum, cerebellum, and leg skeletal muscles were quickly taken out, weighted and rinced into ice-cold 20 mM Tris-HCl buffer containing 0.25 M sucrose and 1 mM EDTA at pH 7.5. For skeletal muscles, homogenization was performed in 10-volumes of the same ice-cold buffer using a polytron apparatus PT10S (Brinckman instruments) at setting 5 with three 5-sec bursts separated by 30-sec pauses. For brains and cerebella homogenization required a teflon Potter-Elvenjem C using 10 strokes in 10-vol of the same buffer. In all cases homogenates were filtered through four layers of cheese cloth and used for [^{3}H]en-TTX binding assays. Protein content was determined by the method of Hartree using bovine serum albumin as standard. For skeletal muscle binding assays were carried out at 4°C as previously described (6). For brains and cerebella 0.5 - 1.0 mg of protein of the respective homogenates were equilibrated at 4°C in 1 ml of the incubation medium (20 mM Tris-HCl, 50 mM choline chloride, pH 7.5) with increasing amounts of [^{3}H]en-TTX in the absence (total binding) or in the presence (non-specific binding) of 5 µM TTX. After 20 min, each incubation was stopped by filtering in duplicate two aliquots of 0.4 ml through prewetted GF/B glass fiber filters (Whatman) positioned over vacum. Filters were rinced twice with 5 ml of an ice-cold 20 mM Tris-HCl buffer containing 200 mM choline chloride at pH 7.5. Aliquots of 0.1 ml were taken in parallel to determine the total radioligand concentration. Filters were counted with Biofluor (N.E.N.) as scintillator on a Packard 2450 apparatus. Specific [^{3}H]en-TTX binding was determined from the difference between the radioactivity measured in the absence and in the presence of native TTX. [^{3}H]en-TTX was synthesized according to Chicheportiche et al. (8). The preparation had a specific radioactivity of 26 Ci/mmol and a radiochemical purity of over 90 %.

RESULTS : A study of the emergence of [^{3}H]en-TTX binding sites in rat brain is presented in Fig. 1. Typical binding data are shown in panel A using an homogenate of 31-day-old brain. These data show the respective proportions of specific and non-specific [^{3}H]en-TTX binding components. Scatchard representations of the specific binding component are linear at all stages of development (inset of panel A) indicating the presence of a single family of binding sites. Dissociation constants (K_d) of the [^{3}H]en-TTX - receptor complex and maximum binding capacities (B_{max}) were 1.3 nM and 0.4 pmol/mg of protein, 4.2 nM and 0.9 pmol/mg of protein and 7.6 nM and 1.8 pmol/mg of protein in brain homogenates from 3, 10 and 31-day-old rats. Panel B presents variations of K_d and B_{max} values during development from fetal to adult stage. K_d remained constant at a value near 1.0 $\pm$ 0.2 nM during the fetal stage till 4 days post-natal (PN). A marked increase in K_d was then observed between 5 and 20 days PN. A plateau was then attained at a K_d value of 7.9 $\pm$ 0.8 nM that remained invariant even in the adult stage. The maximal number of [^{3}H]en-TTX binding sites, B_{max}, regularly increased during development from 17 days post-coitum (PC) (0.08 pmol/mg of protein) to 23-26 days PN (1.9 pmol/mg of protein). B_{max} remained constant between 23 and 45 days PN and then slightly decreased

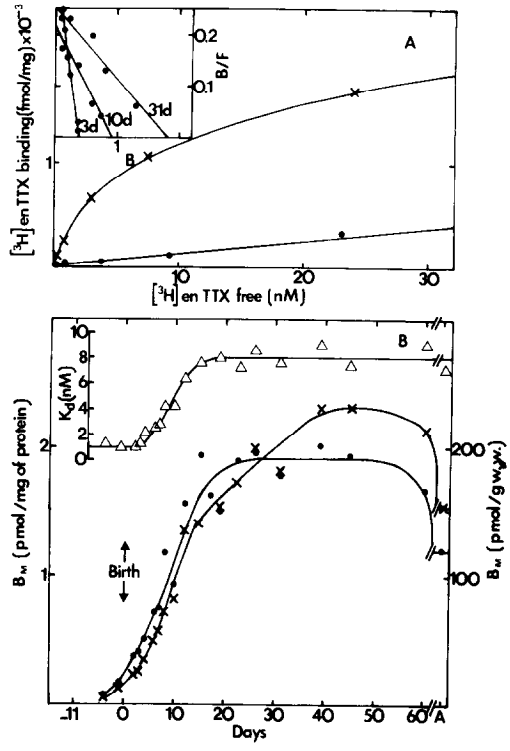

Fig. 1. Ontogenesis of TTX-binding components in rat brain. Panel A : saturation curve of [^{3}H]en-TTX binding to the Na^+ channel in a 31-day-old brain homogenate (without cerebellum). Total binding (X), non-specific binding (●) measured in the presence of 5 µM of unlabelled TTX. Inset panel A : Scatchard's representation of [^{3}H]en-TTX binding on homogenates of 3-10 and 31-day-old brain. Abscissa, B is bound [^{3}H]en-TTX expressed in fmol/mg of protein ; ordinate B/F expressed in ml/g of protein (F is free [^{3}H]en-TTX). Panel B : evolution of the maximum [^{3}H]en-TTX binding capacity as a function of time in days. K_d values (△) were plotted versus age. Maximum binding capacities (B_{max}) expressed in fmol/mg of protein (●) and in pmol/g of wet weight tissue (X) were obtained at each stages of development from Scatchard's analysis.

to adult stage to 1.2 pmol/mg of protein. B_{max} values have also been expressed in pmol/g of wet weight of tissue ; they have the same evolution in time as values expressed in pmol/mg of protein.

The ontogenesis of the TTX binding component of the Na^+ channel in rat cerebella is shown in Fig. 2. Typical binding data, presented in panel A for 31-day-old cerebella, show that the specific binding component is high. Scatchard representations of the specific binding data are presented in the inset. They are linear at all stages of development and correpond to the following K_d and B_{max} values : 1.1 nM and 0.3 pmol/mg of protein, 2.0 nM and 0.3 pmol/mg of protein, 3.2 nM and 1.8 pmol/mg of protein for 3-, 13- and 31-day-old cerebella. Variations of B_{max} and K_d during cerebellum development are presen-

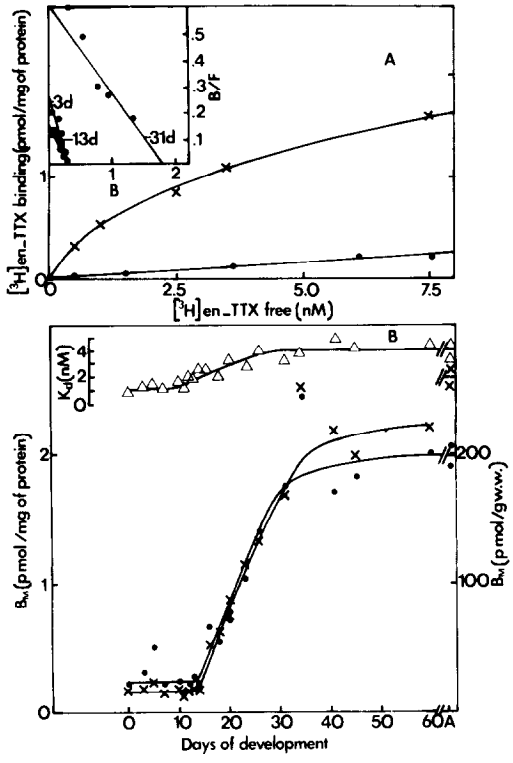

Fig. 2. Ontogenesis of TTX-binding components in rat cerebellum. Panel A and B show the same type of representation as those shown in Fig. 1 for brain.

ted in panel B. K_d remained constant between 0 and about 10 days at a value of 1.1 nM. Then K_d increased gradually from 10 to 30 days PN to reach a plateau value arround 4 nM that was maintained up to the adult stage. The maximal number of [^{3}H]en-TTX binding sites remained stable at 0.22 pmol/mg of protein between 0 and 14 days. Then a marked increase in TTX sites number occured that lasted up till 30-35 days PN when B_{max} reached a plateau value at 1.8-2.0 pmol/mg of protein that was maintained even in the adult stage. As previously observed with rat brain, variations of B_{max} expressed in pmol/mg of protein or in pmol/mg of wet weight tissues are very similar.

The same type of data are presented for skeletal muscle in Fig. 3. Direct binding data and Scatchard plots are shown in panel A, and variation of B_{max} and K_d values during muscle development are presented in panel B. Panel A shows that the specific [^{3}H]en-TTX binding component is high and that Scatchard plots of the data are linear. K_d and B_{max} values are 0.8 nM and 33 fmol/mg of protein, 1.2 nM and 109 fmol/mg of protein and 1.3 nM and 166 fmol/mg of

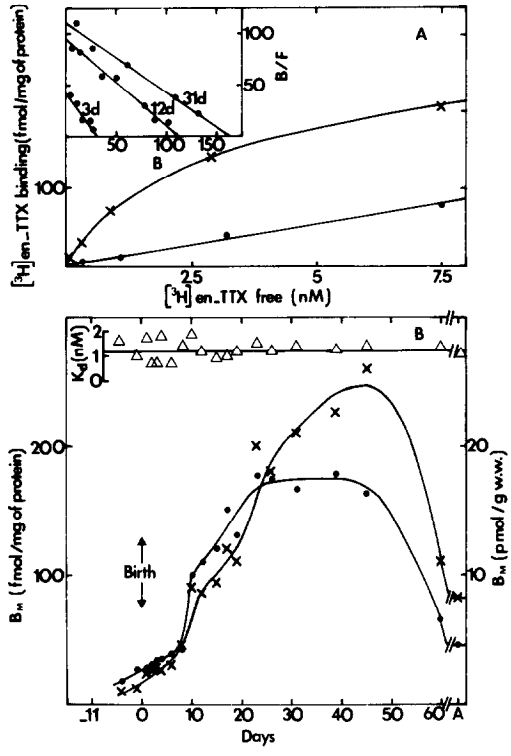

Fig. 3. Ontogenesis of TTX-binding components in rat skeletal muscle. Panels A and B show the same type of representations as those shown in Fig. 1 and 2 for brain and cerebellum.

protein at 3, 12 and 31 day PN. The affinity of [^{3}H]en-TTX for its receptor remained essentially unchanged at all ages (Panel B). Only the maximal number of binding sites varied during development. A slow increase of B_{max} was first observed from 17 days P.C. (19 fmol/mg of protein) to 8 days P.N. (41 fmol/mg of protein). Then, an important increase of binding capacity was observed between 8 and 10 days P.N. (100 fmol/mg of protein) which was followed by an other less dramatic increase to reach a plateau value at 23-25 days P.N. (175 fmol/mg of protein). B_{max} remained constant between 25 and 45 day PN and then dramatically decreased at the adult stage to reach a value of 45 fmol/mg of protein. B_{max} expressed in pmol/g of wet weight has the same type of variation profile as B_{max} expressed in pmol/mg of protein.

DISCUSSION : [^{3}H]en-TTX binding results presented in this paper show that Na^+ channels are present only in low amounts in rat brain at the fetal stage. A net increase in Na^+ channel number appears from 17 days PC to birth. However at birth, there are still only 10-15 % of the Na^+ channels that are present in

3-weeks-old animals. A plateau level corresponding to the maximal number of Na^+ channels is reached 20-days after birth at a time when the neonate is able to assume independent existence. In the chick which is well developed and independent very soon after birth, the number of Na^+ channels is esentially maximum about 1 or 2 days after hatching (9). At about 50 days after birth the maximal number of Na^+ channels decreases to reach a new plateau level at stages older than 80 days. The large variation in number of Na^+ channels which occurs during the 20 days that follow birth are accompanied by a gradual change in affinity of TTX for its receptor (Fig. 1). The dissociation constant K_d gradually varies from a first plateau level of 1 nM to another plateau level at 8 nM after about 3 weeks of development. Numerous anatomical and biochemical changes occur in the cerebral cortex during the 3-weeks that follow birth. They are believed to accompany the differentiation of neuroblasts into neurones with rapid dendritic growth. The post-natal maturation of the Na^+ channel is roughly parallel to that of several neurotransmitter receptors like the muscarinic receptor (10), the dopamine receptor (11), the opiate receptor (12), the GABA-receptor (13), the benzodiazepine receptor (14) and the histamine H_1 receptor (15). The change in maximal number of Na^+ channels and the changes in the TTX-receptor affinity also seem to follow myelinisation of the central nervous system. Central nervous system myelinisation starts at 3-4 days post-natally and is terminated at 30-60 days (16) depending on the brain regions. Myelinisation could be the factor responsible for the change in the affinity of TTX for its receptor.

Fig. 2 shows that the ontogenesis of the Na^+ channel in the cerebellum is different from that observed in the brain. In contrast with the brain for which there is a large increase in Na^+ channel number during the first two weeks after birth, the number of Na^+ channels in the cerebellum remains low and constant during the same period of time. Then, between 2 weeks and 3-4 weeks of development, there is a 9-10 fold increase in the number of Na^+ channels. The plateau value which is reached between 30-40 days remains constant to adult age. There are five neuronal types in cerebella cortex : Purkinje,

basket, stellate, Golgi and granule cells. The post-natal development of Na^+ channels in the cerebellum seems to be parallel to the development and maturation of granule cells which starts 13-14 days after birth (17). Moreover the ontogenesis of TTX binding sites during cerebellum development closely follows the ontogenesis of high affinity GABA receptors which have also been associated with granule cells (18). Like TTX receptors, GABA receptors reach their maximum density 4-5 weeks after birth.

Similarly to the situation found during brain development, the affinity of the TTX-receptor interaction varies during cerebellum development. The variation of K_d follows the variation in the maximum number of sites. However differences between K_d values at early (first 10 days) and late (after 30-days) stages of development of the cerebellum correspond to a 4-fold decrease in affinity instead of an 8-fold decrease during brain development. Again this change of affinity could be associated to changes in myelinisation which starts at about 10 days after birth to be completed at 40-days.

Fig. 3 indicates a first phase of development of muscle Na^+ channels between 17 days at the fetal stage and 25 days after birth. This first phase is followed by an important increase in the number of TTX binding sites which starts at 8-10 days PN. The maximum number of binding sites for TTX in muscle is attained between 20 and 40 days i.e. in the same period of time as previously found for brain development. Then, and very rapidly, the number of Na^+ channels decreases drastically from 170 fmol/mg of protein to a new plateau value at 45 fmol/mg of protein at the adult stage. The decrease of TTX receptors after 45 days to adult level could correspond either to a further maturation of Na^+ channels or to the indirect effect of the hypertrophic development of muscle fibers with an increase of the contractile protein content of muscle cells which would tend to decrease the B_{max} value expressed in fmol/mg of protein. However this latter explanation does not seem to be very likely since the number of nitrendipine-sensitive Ca^{2+} channels remains constant over the same period of time (unpublished observations). Unlike the situation found for the ontogenesis of the Na^+ channel in brain or cerebellum, the affinity of TTX for

its muscle receptor on the Na^+ channel (K_d) does not vary at all during muscle development.

Other results indicate that, immediately after birth most rat muscle fibers generate action potentials that are insensitive to TTX. TTX sensitivity starts to appear 10 days after birth and by 20 days of age TTX blocks action potential generation over the whole surface of the muscle fiber (19). This pattern of acquisition of TTX sensitivity is in good agreement with the gradual increase of TTX binding sites i.e. of Na^+ channels between birth and 20 days PN (Fig. 3). It is also of interest that the time at which the rat muscle fibers become totally sensitive to TTX is approximately the same time at which the normal adult pattern of end-plate innervation is established (19, 20, 21).

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