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Regional and temporal expression of sodium channel messenger RNAs in the rat brain during development*

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Summary. The distribution of mRNA expression for three types of voltage gated neuronal sodium-channels was studied in the rat brain at different developmental stages (embryonal day E18, postnatal day P5 and adult). With the in-situ hybridization technique, using synthetic DNA-oligomer probes, pronounced regional and temporal variations in the expression levels of the different channel subtypes could be detected. In comparison with types I and III, sodium channel II mRNA was the most abundant subtype at all developmental stages. Maximal expression of sodium channel II mRNA was seen at P5 in virtually all parts of the grey matter, except for the cerebellum. In adult rat brain in contrast, sodium channel II mRNA levels were maximal in the granular layer of the cerebellum, whereas in all other regions expression had decreased to roughly 50% of postnatal levels. Na channel I expression was virtually absent at E18 and showed highest levels at P5, with maxima in the caudate nucleus and hippocampus. In the adult brain, expression of Na-channel I was nearly absent in the neocortex, but well detectable in the cerebellum and, at lower levels in the striatum and thalamus. Sodium channel III was mainly expressed at the embryonal stage and showed a decrease to very low levels with little regional preferences in the adult.

Key words: Sodium channel – mRNA expression – Development – In situ-hybridization – Rat brain

Introduction

Sodium channels play a central role in signal transduction within the central nervous system.

These large transmembrane glycoproteins facilitate the voltage dependant ion flux through the cell membrane and thus the propagation of an action potential along a nerve fiber. In rat brain, they consist of an alpha subunit of 260 kDa (Agnew 1984; Caterall 1986) and two β-subunits of 36 kDa and 33 kDa (Caterall 1984, 1986; Hartshorne and Caterall 1984).

Three distinct sodium channel alpha-subunits, in the following termed NaCh I, II and III have so far been characterized in the rat brain through the application of molecular cloning techniques (Noda et al. 1986a; Kayano et al. 1988). A further type, which has been characterized recently and is almost completely homologous to type II has been denoted IIA (Auld et al. 1988).

The electrical properties of the different sodium channels have been studied by expression of the respective full-length mRNAs in Xenopus oocytes. Whole-cell patch-clamp recordings demonstrated almost identical electrical properties for channels II, IIA and III (Noda et al. 1986b, Suzuki et al. 1988). Sodium channel I has not been studied in this manner due to lack of sufficient expression in oocytes (Noda et al. 1986b).

The regional and temporal expression patterns of sodium channels within the central nervous system have been studied by protein-blot (Schmidt et al. 1985; Gordon et al. 1987) and RNA-blot techniques (Scheinmann et al. 1989; Beckh et al. 1989; Westenbroek et al. 1989). According to these findings, sodium channel III is expressed predominantly in the neo- and perinatal stages of CNS development (Beckh et al. 1989). Channel I is practically absent until birth, then exhibits a rapid increase in expression during the first postnatal week, dropping afterwards to adult levels, which are about 50% of maximum values (Gordon et al. 1987; Scheinman et al. 1989; Beckh et al. 1989). Sodium channel II is expressed throughout development in most regions of the central nervous system but expression also peaks around the first postnatal week (Gordon et al. 1987; Scheinman et al. 1989; Beckh et al. 1989).

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Investigations of regional differences in the distribution of sodium channels have shown, that type I is predominant in the more caudal regions of the CNS, such as the midbrain, medulla oblongata and spinal cord (Gordon et al. 1987; Beckh et al. 1989; Westenbroek et al. 1989). Channel II is present throughout the CNS but expression is markedly higher in the more rostral parts of the brain such as the cerebral cortex and the hippocampus (Gordon et al. 1987; Beckh et al. 1989). There seems to be some expression of channel III in the adult brain, excluding cerebellum and spinal cord, but expression is only a small fraction compared to channels I and II (Beckh 1989).

The question of cellular localization of sodium channel gene expression has not been studied on the histological level. In this study we have applied in-situ hybridization histochemistry using synthetic oligonucleotide probes to study the regional distribution of the expression of the different types of sodium channel mRNAs in the embryonal, postnatal and adult rat brain.

Material and methods

Preparation of tissues

Rats (postnatal day 5 and adult) were given lethal doses of Nembutal (90 mg/kg i.p.) and rapidly decapitated. The brains were dissected out quickly and frozen within a block of cryostat embedding medium (Tissue-Tek) in dry ice. Sections (12 µm) were cut on a cryostat and thaw-mounted onto gelatin/chrome alaum coated slides. Sections from at least three different animals were prepeared and hybridized for each developmental stage.

All solutions were prepared with glass-destilled, deionized water that had been treated with diethylpyrocarbonate (400 µl/l). Sections were fixed in 4% paraformaldehyde in 10 × PBS for 5 min, dipped twice in 1 × PBS and acetylated with 0.25% acetic anhydride/0.1% triethanolamine in 0.9% NaCl for 10 min, followed by dehydration in a graded series of alcohols, delipidated for 5 min in chloroform and stored either frozen dessicated at −70° C or submerged in 96% ethanol at 4° C.

Hybridization probes

Oligonucleotides were synthesized on an Applied Biosystems 381A DNA-Synthesizer using β-cyanoethyl phosphoroamidite chemistry. Probes were purified on a 20% polyacrylamide/8M urea gel and desalted on a Sephadex-G25 (Pharmacia) column.

All probes used in this study had a length of 30 bases. Two to three separate "antisense" probes from the coding region were used for the detection of each sodium-channel subtype. Probes used had the following sequences (numbers of bases of respective coding regions): R_{I-1}(850–879), R_{I-2}(2003–2032), R_{II-1}(903–932), R_{II-2}(5970–5999), R_{III-1}(846–875), R_{III-2}(1589–1618), R_{III-3}(2974–3003).

The "sense" counterparts of these probes were also synthesized and used as negative controls. All probes were 3'labeled with alpha-^{32}P-dATP, using terminal transferase (Eschenfeldt et al. 1987) to a specific activity of about 12000–15000 Ci/mmol. Probes were separated from unincorporated nucleotides by spun-column chromatography through Sephadex G-25(Pharmacia) (Blumberg 1987).

In-situ-hybridization

Sections were removed from the freezer or 96% ethanol and air-dried for 30 min. Each section was surrounded by a ring of rubber-cement and covered with 50–100 µl of prehybridization buffer (4 × SSC, 5 × Denharts, 50 mM Na-phosphate, pH 6.5, 1 mM Na–PP, 120 µg/ml heparin, 100 µg/ml sonicated salmon-sperm DNA, 100 µg/ml poly-[dA], 100 µg/ml yeast-tRNA, 50% deionized formamide). Prehybridization was for 2 h at 42° C in a humid box. Prehybridization buffer was blotted off and replaced by hybridization buffer (prehybridization buffer with addition of oligonucleotide probe, 3×10^6 CPM/100 µl). 50–100 µl of hybridization buffer were applied to each section. Hybridization was performed over-night at 30° C in a humid-box.

Sections were washed for 1h in 1 × SSC/0.05% Na–PP/0.05% β–ME at room temperature and 1h in 0.1 × SSC/0.05% Na–PP/0.05% β–ME at 38°–40° C with agitation. Slides were dehydrated briefly in a series of graded ethanols containing 300 mM ammonia acetate. Autoradiography was performed by direct exposure to autoradiography film (Hyperfilm MP, Amersham) for 2–4 weeks or by dipping in autoradiographic emulsion (LM1, Amersham) and exposure for 3–4 weeks. Dipped slides were developed in Kodak D–19 developer and fixed in Kodak Unifix. All slides were counterstained with cresyl-violet.

Quantification of hybridization signals

The intensity of the hybridization signal was assessed by measuring the optical density of autoradiographic films in corresponding circular areas of 0.3 mm diameter with an image-analysis system. The system consisted of a CCD b/w camera with a resolution of 256 grey levels, connected via a frame grabber (DT2255, Data Translation, Marlboro, MA, USA) to an Apple Macintosh II personal computer. Software used was the public domain program Image 1.26 (NIH, Bethesda, MD, USA). The results of the measurements were corrected for differences in the specific activities of the particular hybridization probes.

Solutions and abbreviations

1 × PBS: 2.7 mM KCl, 137 mM NaCl, 81 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.0; 1 × SSC: 150 mM NaCl, 15 mM Nacitrate, pH 3.5; 1 × Denhardt's (0.02% of each Ficoll, bovine serum albumin and polyvinylpyrrolidon); Na–PP: sodium pyrophosphate; β–ME: β-mercaptoethanol.

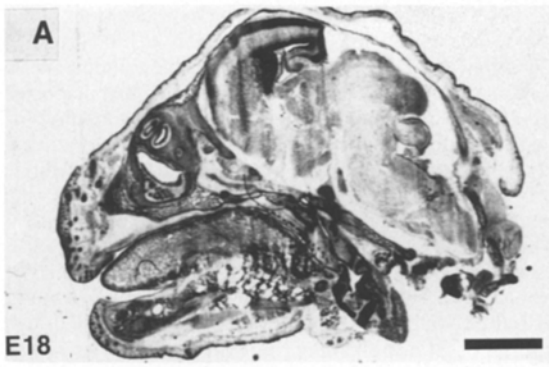
Results

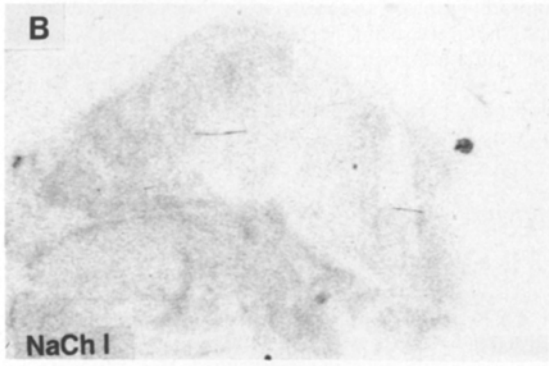
Sodium channel I

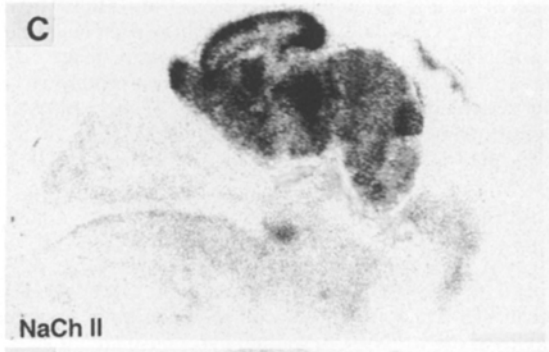
In-situ-hybridizations with NaCh-I probes showed virtually no signal in embryonal rat-brain (day E 18, Fig. 1B). At postnatal day 5, NaCh I is expressed at moderate levels in the cerebral and cerebellar cortex, the thalamus, midbrain and brainstem (Fig. 2B). Expression levels in the cerebellar cortex are comparable to those in the cortex. A comparison with hybridization signals, obtained with a probe against β-actin (data not shown) revealed that the seemingly higher signal intensity in the hippocampus is mainly due to the higher neuron density.

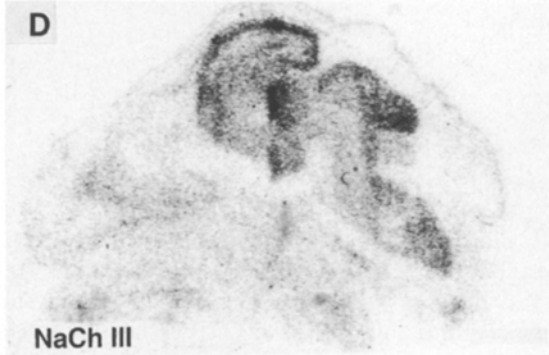
The amount of NaCh I mRNA expressed in the caudate-putamen is clearly above the cortical levels and is especially high in the germination zone of these structures (compare Figs. 2A, B, arrows).

In adult rat brain, NaCh I mRNA expression is very low in the cerebral cortex (Fig. 3B). Increasingly higher levels are detected in the putamen, thalamus and cerebel-


A
E18


B
NaCh I


C
NaCh II


D
NaCh III

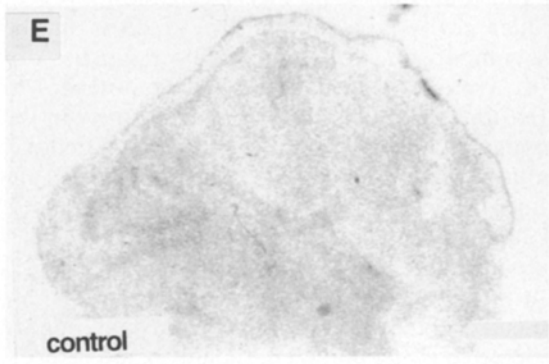
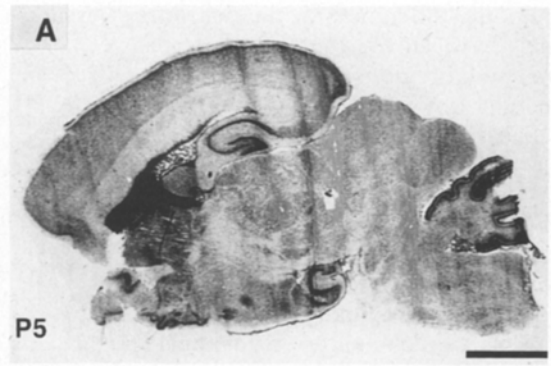
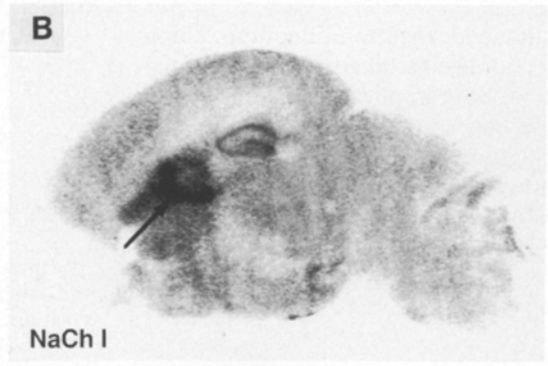

E
control

Fig. 1


A
P5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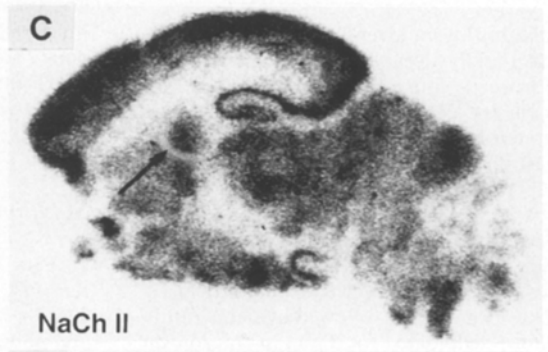
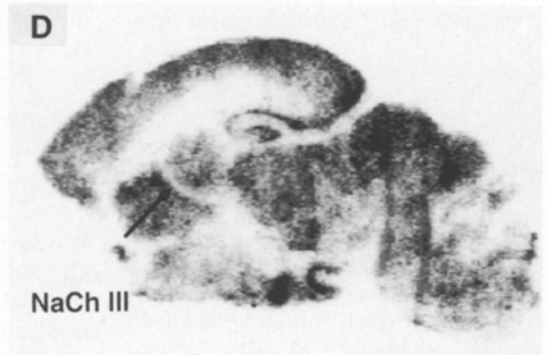
NaCh I


C
NaCh II


D
NaCh III

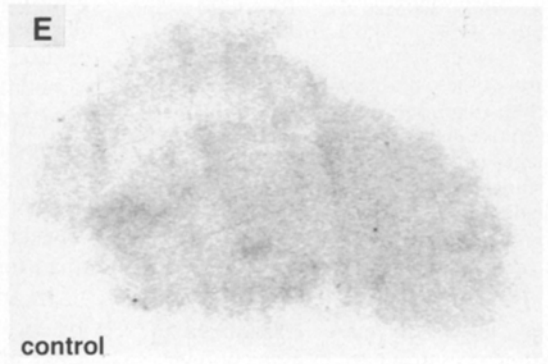

E
control

Fig. 2

lar cortex. Expression of NaCh I in the hippocampus, though clearly detectable at postnatal day 5, was never seen in adult rat brain. This is in striking contrast to the other two channel types, which are always seen in the postnatal and adult hippocampus (compare regions pointed out by arrows in Figs. 3A–D).

Sodium channel II

In embryonal rat-brain (E18), NaCh-II probes hybridized in the cerebral cortex, the thalamus and somewhat less in the inferior colliculus (Fig. 1C). Signal intensities in the cortex at this stage were comparable to those seen with NaCh-III probes, indicating that both channel types are co-expressed at approximately equal levels (compare Figs. 1C and 1D).

At postnatal day 5 (P5), expression of NaCh II in the cerebral cortex, hippocampus, thalamus, hypothalamus, and colliculi had increased to the highest level of any of the three subtypes seen at any stage (Fig. 2C). Expression levels in the caudate-putamen at this stage were somewhat lower than in the cerebral cortex and interestingly, the germination zone of these nuclei, which expresses NaCh I maximally at postnatal day 5, is virtually spared by NaCh II expression (compare regions pointed out by arrows in Figs. 2B, C). In the developing cerebellum, hybridization signals at day 5 are considerably lower than in the cerebral cortex (Fig. 2C). In adult brain, the overall expression of NaCh II is lower than in the postnatal brain and the spatial distribution now shows a gradient with hybridization signals being highest in the cerebral cortex and the hippocampus and progressively lower in the caudate-putamen, the thalamus, the midbrain and brainstem. Signal intensities in the latter two were approaching background levels. A region standing out with a signal intensity clearly above that in the cerebral cortex, is the granular layer of the adult cerebellum. NaCh II expression has increased here to very high levels, comparable to those seen in the cerebral cortex at postnatal day 5. This is in strict contrast to the low signal

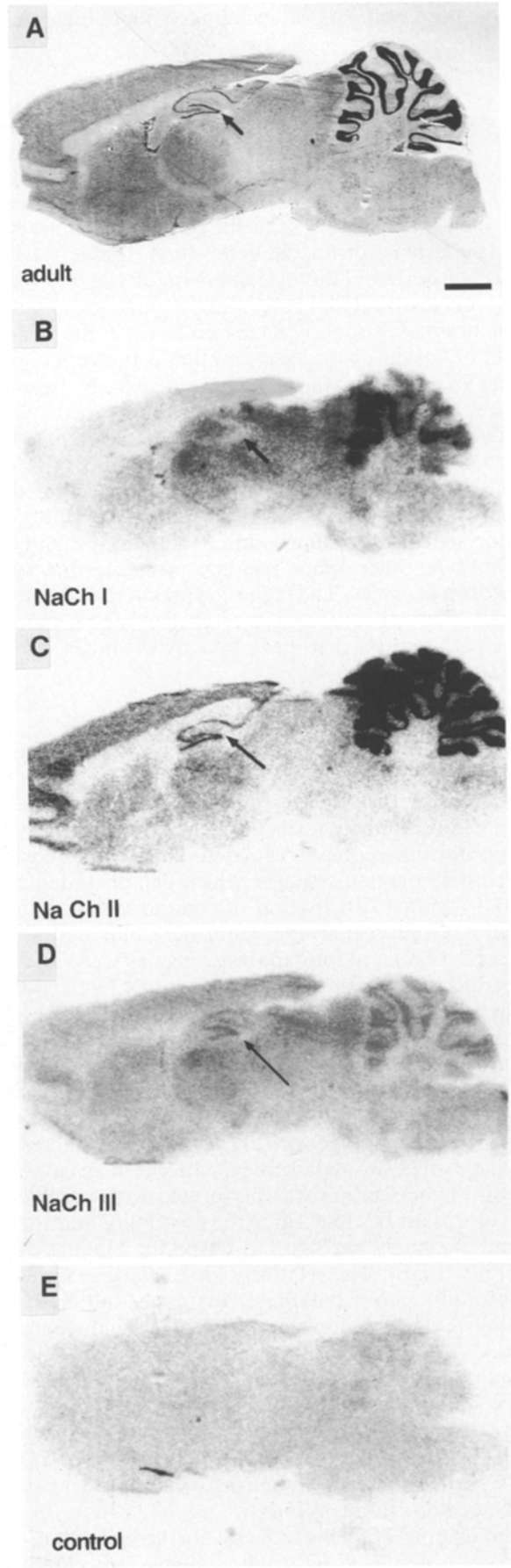


Fig. 1A–E. Sagittal section through the head of an embryonal rat at day E18. **A** Photomicrograph of cresyl-violet stain; **B–E** in-situ hybridization signals of (**B**) NaCh I mRNA, (**C**) NaCh II mRNA, (**D**) NaCh III mRNA and (**E**) hybridization with a sense-control probe, which was complementary to the NaChII probe. Bar = 2 mm

Fig. 2A–E. Sagittal section through rat brain at postnatal day P5. **A** Photomicrograph of cresyl-violet stain; **B–E** in-situ hybridization signals of (**B**) NaCh I mRNA, (**C**) NaCh II mRNA, (**D**) NaCh III mRNA and (**E**) hybridization with the sense-control probe. Bar = 2 mm. Arrows point to the germination zone of the corpus striatum

Fig. 3A–E. Sagittal section through adult rat brain. **A** Photomicrograph of cresyl-violet stain; **B–E** in-situ hybridization signals of (**B**) NaCh I mRNA, (**C**) NaCh II mRNA, (**D**) NaCh III mRNA and (**E**) hybridization signal with the sensecontrol probe. Bar = 2 mm. Arrows point to the granule-cells of the dentate gyrus

intensity in the developing cerebellum (compare Figs. 1C, 2C and 3C).

Sodium channel III

Expression of NaCh III at embryonal day 18 is mainly localized in the cerebral cortex, the thalamus, the colliculi and at somewhat lower levels in the brainstem. There is virtually no expression in the cerebellum at this stage (Fig. 1D). At postnatal day 5, expression of NaCh III is almost evenly distributed throughout the entire grey matter of the brain with slightly increased levels in the midbrain and brainstem. Notable exemption is the germination zone of the caudate-putamen, where only background levels could be detected (arrow in Fig. 2D).

In adult rat brain, NaCh III expression is again roughly equal in cerebral cortex, midbrain and brainstem. The overall expression level however has decreased somewhat from the levels seen at postnatal day 5. Hybridization signals in the hippocampus stand out slightly above those in other brain regions, probably due to higher neuron densities. The highest expression levels are seen in the granular layer of the cerebellar cortex, even when the high neuron density in this area is taken into consideration.

Discussion

Our results show that the expression of three types of voltage gated sodium channels in rat brain is regionally and temporally differentially regulated. These results correlate well with previous studies, which demonstrated a differential regional distribution of sodium channels in protein-blot (Gordon et al. 1987), northern-blot analysis (Beckh et al. 1989) and immunohistochemistry (Auld et al. 1988). Specificity of our results was assured by the use of synthetic oligonucleotides as hybridization probes. Each probe was selected in a way that it showed little or no sequence homology to the other types of sodium channels or any other sequence in the EMBL or GeneBank data-bases (Devereux et al. 1984). Hybridizations for each channel-type with at least two independent and distinct probes showed identical patterns in all cases. Each in-situ-hybridization was performed with parallel "sense" control probes to exclude the possibility that the hybridization signals were due to unspecific binding of probes (Figs. 1–3E). The relatively long exposure times of the autoradiographs (compare "materials and methods" section) point to a low abundance of sodium-channel mRNA's in the CNS. This is indicative of a very low turnover-rate for these proteins, considering the high density of sodium channels in the brain. Such low turnover-rates are typical for proteins wich have a relatively long half-life and are not involved in short-term responses to cellular stimuli by means of variing density within the cell or cell-membrane.

Sodium-channel II seems to be by far the most abundant subtype in the CNS (Gordon et al. 1987; Beckh et al. 1989). Its overall distribution correlates well with the distribution of tetrodotoxin binding-sites in the CNS (Mourre et al. 1988). Thus there seems to be a gradient in NaCh II expression with higher levels in the cerebral cortex and the caudate-putamen and decreasing levels towards midbrain and brainstem (Beckh et al. 1989). Two areas however stand out with exceptionally high expression levels: the hippocampus and the cerebellum. Both areas also show high levels of TTX-binding (Mourre et al. 1988). In the hippocampus, TTX-binding is restricted to the white matter, whereas NaCh II mRNA expression is confined to the dentate gyrus and areas CA1–CA4. This indicates that synthesis of these channel proteins is restricted to the soma while the functioning channels are mostly located along the neurites. A similar distribution pattern is seen in the cerebellum, where the granular layer shows the most intense in-situ-hybridization signal whereas TTX-binding is confined to the molecular layer (Mourre et al. 1988). The high level of NaCh II expression in hippocampus and cerebellum, which has also been detected by northern- and western-blots (Beckh et al. 1989; Gordon et al. 1987) may to some extent be due to high cell densities in this area since in-situ-hybridizations with a probe against β-actin (data not shown) also showed the highest expression in the hippocampus and the granular layer of the cerebellum. NaCh II expression, particularily in the granular layer of the adult cerebellum was however, much higher than neuron density alone would predict, indicating a genuinely high cellular expression of this channel subtype in cerebellar granular neurons.

The time course of NaCh II expression during development shows low levels at day E18, an increase to roughly five-fold levels at postnatal day 5 and then an approximately 50% decrease in adult rat brain. This suggests, that NaCh II is most strongly expressed, during neurite outgrowth to facilitate the biosynthesis of sodium-channels for the vast amount of new membrane surface which is generated during this process. This explanation does not apply to the adult cerebellum, however. One possible reason for the high expression of NaCh II in this area might be a high degree of ongoing plasticity, with new neuritic processes constantly being formed.

The distribution of sodium channel I expression in adult rat brain shows some marked differences as compared to NaCh II. There seems to be an increase of expression from rostral to caudal with almost background levels in cerebral cortex a slight increase in expression levels towards the striatum, thalamus and cerebellum. This is in good accordance with data obtained with northern blots (Beckh et al. 1989) and immunoprecipitation (Gordon et al. 1987). Interestingly, the hippocampus shows no signal at all, despite high cell densities in the dentate gyrus and areas CA1–CA3. The most interesting aspect of NaCh I expression is its developmental regulation, which shows a marked expression only during the postnatal stage. Furthermore this expression is highest in regions where cells are deviding or just becoming postmitotic, as in the germination zone around the caudate nucleus (Fig. 2C). Data from recent immunohistochemical studies showed that NaCh

I is the predominant channel-type of the soma-membrane (Westenbroek et al. 1989) and therefore a high expression would be needed where new somata are developing.

Westenbroek et al. (1989) have shown NaCh I immunoreactivity in the pyramidal layer of the adult hippocampus. This is somewhat contradictory to our observation, that there is no NaCh I mRNA expression in this region (see Fig. 3B). One possible explanation could be, that this channel protein has a very prolonged half-life, so that the amounts of mRNA needed for its replacement are below our detection level. Another explanation would be the existence of yet another type of sodium channel in this area, which cross-reacts with the anti NaCh I-antibody used by Westenbroek et al., but is not detected by the highly specific oligonucleotide-probes used in our experiments.

Sodium channel III seems to be mostly an embryonally and perinatally expressed type. Substantial expression levels are only seen at day E18 (Fig. 1D). Its more or less even distribution throughout all areas of grey matter and its electrophysiologic similarity to NaCh II (Suzuki et al. 1988) give little clue as to its specific function during development. The developmental regulation of sodium-channel mRNA levels in the rat brain suggests, along with previous data (Beckh et al. 1989; Westenbroek et al. 1989), that NaCh I and NaCh II expression is mainly regulated by the new formation of excitable membranes around somata and fibers of neurons. This would imply that NaCh I expression is elevated just during development, when neuronal stem cells become postmitotic, and returns to maintenance levels in the adult. NaCh II expression is accordingly increased during development in areas, where neurons are making new connections and in the adult brain in areas of high plasticity, as in the cerebral cortex, the hippocampus and the cerebellum.

References

Agnew WS (1984) Voltage regulated sodium channel molecules. Ann Rev Physiol 46:517–530

Auld VJ, Goldin AL, Krafte DS, Marshall J, Dunn JM, Catterall WA, Lester HA, Davidson N, Dunn RJ (1988) A rat brain Na^+ channel subunit with novel gating properties. Neuron 1:449–461

Beckh S, Noda M, Lübbert H, Numa S (1989) Differential regulation of three sodium channel messenger RNAs in the rat central nervous system during development. EMBO J 8 (N12):3611–3616

Blumberg DD (1987) Equipping the laboratory. Meth Enzymol 152:3–20

Catterall WA (1984) The molecular basis of neuronal excitability. Science 223:653–661

Catterall WA (1986) Molecular properties of voltage-sensitive sodium channels. Ann Rev Biochem 55:953–985

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucl Acids Res 12:387–395

Eschenfeldt WH, Puskas RS, Berger SL (1987) Homopolymeric tailing. Meth Enzymol 152:337–342

Gordon D, Merrick D, Auld V, Dunn R, Goldin AL, Davidson N, Catterall WA (1987) Tissue-specific expression of the R_I and R_{II} sodium channel subtypes. Proc Natl Acad Sci USA 84:8682–8686

Hartshorne RP, Catterall WA (1984) The sodium channel from rat brain: purification and subunit composition. J Biol Chem 259:1667–1675

Kayano T, Noda M, Flockerzi V, Takahashi H, Numa S (1988) Primary structure of rat brain sodium channel III deduced from the cDNA sequence. FEBS Lett 228:187–194

Mourre C, Moll C, Lombet A, Lazdunski M (1988) Distribution of voltage-dependent Na^+ channels identified by high-affinity receptors for tetrodotoxin and saxitoxin in rat and human brains: quantitative autoradiographic analysis. Brain Res 448:128–139

Noda M, Ikeda T, Kayano T, Suzuki H, Takeshima H, Kurasaki M, Takahashi H, Numa S (1986a) Existence of distinct sodium channel messenger RNAs in rat brain. Nature 320:188–192

Noda M, Ikeda T, Suzuki H, Takeshima H, Takahashi T, Kuno M, Numa S (1986b) Expression of functional sodium channels from cloned cDNA. Nature 322:826–828

Schmidt J, Rossie S, Catterall WA (1985) A large intracellular pool of inactive Na channel subunits in developing rat brain. Proc Natl Acad Sci USA 82:4847–4851

Scheinman RI, Auld VJ, Goldin AL, Davidson N, Dunn RJ, Catterall WA (1989) Developmental regulation of sodium channel expression in the rat forebrain. J Biol Chem 264 (N18):10660–10666

Suzuki H, Beckh S, Kubo H, Yahagi N, Ishida H, Kayano T, Noda M, Numa S (1988) Functional expression of cloned cDNA encoding sodium channel III. FEBS Lett 228:195–200

Westenbroek RE, Merrick DK, Catterall WA (1989) Differential subcellular localization of the R_I and R_{II} Na^+ channel subtypes in central neurons. Neuron 3:695–704