MUSCARINIC BINDING SITES IN THE DEVELOPING RABBIT BRAIN

Regional distribution and ontogenesis in the prenatal and early neonatal cerebellum

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1. Introduction

Several studies are now available on the ontogenesis of the muscarinic binding sites in the developing chick [1] and rat brain [2] chick retina cells [3] and dissociated rat cerebral cell cultures [4]. The postnatal levels of the muscarinic receptor in several regions of the developing rat brain were investigated in more detail [2] and maximum binding activity by about 4 weeks postpartum was shown. A similar developmental profile has been recently reported for the muscarinic receptor in that rat hippocampus formation [5].

As part of our interest in studying the effects of vascular-induced intrauterine growth retardation on brain development [6] we have examined the ontogenesis of the muscarinic binding sites in the fetal rabbit brain.

Here we report on the concentration of the muscarinic receptor in several regions of the prenatal, newborn and adult rabbit brain. In addition we demonstrate that the maximal concentration of the receptor in the rabbit cerebellum is attained early in the neonatal stage.

2. Materials and methods

New Zealand white does raised at The Weizmann Institute Animal Colony were mated on appropriate dates and fetuses removed by cesarean section between 25–31 days gestation. Fetal brains were excised and a number of brain structures, easily identified as of day 25 gestation, were separated, weighed and stored at −70°C. When sufficient material had been collected, 50–100 mg tissue samples, freed of blood and meninges, were homogenized in 9 vol. ice cold 0.32 M sucrose using a Potter Elvehjem glass homogenizer fitted with a Teflon pestle. Protein content was assayed by the Lowry method [7] and DNA as in [8] after precipitating aliquots of the homogenate (0.1–0.2 ml) with 40 vol. chloroform/methanol 1/2 (v/v).

Determination and characterization of the muscarinic receptors was performed using [^{3}H]quinuclidinyl benzylate ([^{3}H]QNB), 29.4 Ci/mmol, obtained from New England Nuclear, Boston, Mass. Acetylcholine and scopolamine (chloride forms) and nicotine were from Sigma Chemical Co (St. Louis, MO), atropine sulfate and oxotremorine from Fluka AG (Bucks, Switzerland).

Incubation mixtures consisted of 50–150 μg protein homogenate, 60 mM NaCl/25 mM Tris·Cl buffer (pH 7.4) and 7 nM [^{3}H]QNB in final vol. 0.2 ml. Binding was carried out in triplicate for 45 min at 25°C and the reaction terminated by dilution with 2 ml incubation buffer, followed by vacuum filtration over glass fiber filter (GF/C, Tamar, Israel). The filter was rinsed 3 times with 3 ml portions of buffer, dried and the radioactivity determined in a Packard scintillation spectrometer at 38% efficiency using a scintillation mixture consisting of 33% (v/v) Triton X-100, 0.8% PPO and 0.01% POPOP in toluene.

3. Results and discussion

3.1. *Pharmacological properties of the QNB binding sites*

Specific binding of [^{3}H]QNB was defined as maximal binding less the binding in the presence of 10^{-5} M atropine. Specific binding was linearly proportional with protein concentration up to 0.2 mg protein and was saturable when either fetal cerebellum or adult cerebral cortex homogenates were assayed as illustrated in fig.1. The dissociation constant calculated from the double reciprocal plot was equal for both preparations, with an apparent K_d value of 1.5 nM, which is compatible with other values reported for the central nervous system [9].

The muscarinic properties of the QNB binding sites in both fetal cerebellum and adult cerebral cortex were investigated using a number of muscarinic antagonists and agonists. The inhibition constant K_1, for the [^{3}H]QNB binding sites was calculated from the equation

$$K_1 = ED_{50}/(1 + [L]/K_d)$$

where K_d = 1.5 nM, stands for the apparent dissociation constant of [^{3}H]QNB estimated from direct binding studies, $[L]$ = 7 nM is the molar concentration of [^{3}H]QNB in the assay mixture and ED_{50} is the concentration of the drug studied which displaces 50% of maximum bound radioactivity [10]

The muscarinic antagonists scopolamine and atropine displayed the highest affinity in protecting the binding sites, each with app. K_i 0.3×10^{-10} M and 1.3×10^{-9} M, respectively. The agonists oxotremorine and acetylcholine (the latter assayed in the presence of 5×10^{-6} M eserine) were less effective with inhibition constants of 1.1×10^{-6} M and 8×10^{-7} M, respectively. In contrast, the app. K_1 in the presence of nicotine was $> 5 \times 10^{-4}$ M. There were no marked differences between the inhibition constants obtained with either fetal cerebellar or adult cerebral cortex preparations. These results suggest that the [^{3}H]QNB binding sites in the fetal cerebellum display typical muscarinic characteristics.

3.2. *Regional distribution of muscarinic binding sites*

The distribution of muscarinic binding sites in various regions of the fetal, newborn and adult rabbit brain is shown in table 1. In all brain regions studied considerable levels of QNB binding sites are evident as of day 25 gestation. Significant differences in the regional distribution of the muscarinic receptor levels of the adult rabbit brain are encountered. When expressed per mg protein the highest level is seen in the caudate followed by the cerebral cortex and the hippocampus whereas the thalamus, hypothalamus and the midbrain structures exhibit intermediate

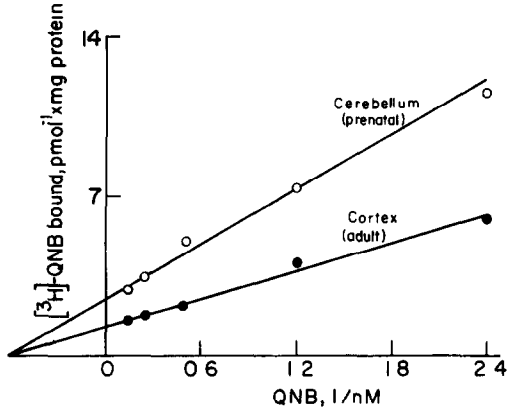

Fig 1 Double reciprocal plot of specific [^{3}H]QNB binding of adult cortex and fetal rabbit cerebellum (28 days gestation) For experimental conditions and specific binding definition see text

Table 1
Regional distribution of muscarinic binding sites of the prenatal (25 days gestation) newborn and mature rabbit brain

Brain region	Prenatal (fmol/mg protein)	Newborn	Adult
Cerebral cortex	72 ± 15	232 ± 25	723 ± 82
Hippocampus	44 ± 10	358 ± 30	560 ± 104
Caudate nucleus	–	410 ± 28	1232 ± 215
Thalamus	215 ± 20	360 ± 48	424 ± 52
Hypothalamus	154 ± 42	195 ± 35	305 ± 20
Midbrain	117 ± 23	393 ± 54	367 ± 54
Lower brain stem	194 ± 15	241 ± 35	143 ± 35
Cerebellum	203 ± 30	325 ± 28	54 ± 16

Specific [^{3}H]QNB binding was determined as described in the text. Values expressed as pmol/mg protein represent mean of 3–5 animals ± SEM

levels. In contrast a great reduction is observed within the lower brain stem and the cerebellar regions, in line with the values reported by others for rat and monkey brain [2,11].

3.3. *Ontogenesis of muscarinic receptor and other growth parameters in the developing cerebellum*

In almost every region studied, a progressive increase in receptor binding activity toward birth is apparent. Prompted by the observation that the concentration of the muscarinic receptor per mg protein in the cerebellum of newborn rabbits is relatively high, we investigated in more details the receptor development with respect to several growth parameters. As shown in fig.2, the specific [^{3}H]QNB binding rises from 0.2 pmol/mg protein at 25 days gestation to an ~0.4 pmol/mg protein peak at 2 days before parturition. This is followed by a significant reduction, when expressed per mg protein, which levels off at ~0.2 pmol/mg protein by 3 days postnatally. A further decline of about 4-fold is observed toward maturity. Similarly, when expressed per μg DNA (table 2), the levels of the QNB binding sites are maximal at 2–3 days prior to birth and decline by a factor of 7 in the adult state. This is evidently due to the fact that the protein/DNA ratios in the cerebellum are virtually the same at all ages studied. However, when expressed per whole cerebellum, the receptor density reaches an adult value by 10 days postnatally and remains constant. At that time the size of the cerebellum is only about 1/4 of the adult level. Furthermore, both the DNA and the protein content

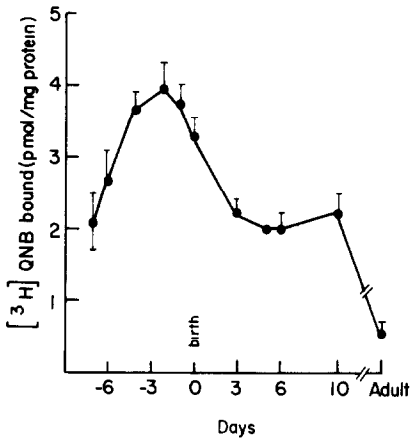

Fig 2 Development of [^{3}H]QNB binding sites in rabbit cerebellum Values are expressed in terms of mg protein and each point is a mean (± SEM) from at least 5 cerebella

per mg tissue are significantly elevated. These observations would suggest that the ontogenesis of the QNB binding sites is accomplished relatively early in development. As already pointed out the muscarinic receptor represents only one of several types of cholinergic receptors and its ontogenesis may not necessarily coincide with the acquisition of other cholinergic markers [2,12]. Furthermore one cannot rule out the possibility that its appearance is of a vestigious nature.

Whether its early onset is an indication for a highly specific cholinergic function related to early cerebellar

Table 2
Changes in the muscarinic binding sites and other growth parameters of rabbit cerebellum during development

Age (days from conception)		Cerebellar parameters: wet weight (mg)	Protein (mg/g brain)	DNA	^{3}H[QNB] binding: fmol/μg DNA	fmol/cerebellum
Prenatal	25	35 ± 8	57.6	2 8	4.3	412
	28	54 ± 5	57 8	2.6	8 1	1177
Newborn	31–32	80 ± 12	60.0	3 4	5 9	1564
Postnatal	42	390 ± 45	82 8	4.5	4.0	7040
Adult	>125	1510 ± 200	82 3	4.4	1.1	7134

For details see text

neurogenesis or if it is a phenomenon which is associated with functional cholinergic innervation remains to be seen. Preliminary autoradiography studies suggest QNB binding sites to be selectively localized in certain regions of the cerebellum.

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