0270-6474/85/0504-1083$02.00/0

The Journal of Neuroscience
Vol. 5, No. 4, pp. 1083-1089
April 1985

Characterization of Muscarinic Cholinergic Receptors in the Brains of Copper-deficient Rats[1]

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Abstract

In order to assess a possible role for copper as a regulator of muscarinic receptors *in vitro*, the receptor was characterized in rats made copper deficient by a dietary regimen. In forebrain regions there was a decrease in both the affinity of the receptors for [^{3}H]-1-quinuclidinyl benzilate and the density of receptors in the copper-deficient animals compared with control animals. Copper treatment *in vitro* of homogenates from deficient animals did not reverse the *in vivo* effects on antagonist binding but, rather, decreased receptor occupancy and ligand affinity in a manner similar to copper treatment of control homogenates. Minimally deficient rats displayed very similar changes in receptor properties compared with the more severely deficient animals. Minimal copper deficiency produced robust effects on the binding of agonists, increasing ID_{50} and derived dissociation constants. The addition of copper to the assay medium caused an apparent reversal of the *in vivo* effect of copper deficiency on agonist binding, decreasing ID_{50} and derived dissociation constants to values near those observed with homogenates from normal animals in the presence of copper. Since copper deficiency has dramatic effects on both receptor number and the binding of agonists to muscarinic receptors in the central nervous system, it is suggested that copper, because of its ability to form complexes with some proteins, may have an endogenous role in the regulation of the receptor.

Transition and heavy metals affect muscarinic receptors by inhibiting the binding of antagonists at higher concentrations (Aronstam et al., 1978). The effect on antagonist binding is apparently reversible and competitive, with Hg^{2+} having the greatest inhibitory potency ($ID_{50} = 10^{-7}$ M). At lower concentrations, metals such as Cu^{2+}, Cd^{2+}, Pb^{2+}, and Zn^{2+} increase agonist binding without affecting antagonist binding (Aronstam et al., 1978). Since there is no further metal-induced increase in agonist binding after pretreatment with N-ethylmaleimide, sulfhydryl groups are probably involved in the ability of lower concentrations of metals to increase agonist binding.

We have recently reported that the *in vitro* addition of low concentrations of Cu to regional homogenates of rat brain alters the binding of both agonists and antagonists to muscarinic cholinergic receptors (Farrar and Hoss, 1984). The major effects of the treatment are a decrease in apparent receptor number, an increase in affinity for quinuclidinyl benzilate (QNB), and an increase in agonist affinity. A Cu-induced shift in receptor subtypes toward higher affinity receptors is suggested. Cu treatment also differentiates between muscarinic receptors of brainstem and forebrain—regions of low and high Cu content, respectively (Farrar and Hoss, 1984). The concentration of Cu required to produce these effects is a small fraction of the total Cu normally present in brain.

To test more directly the hypothesis that Cu is an endogenous regulator of the muscarinic receptor, the receptor was characterized in the brains of Cu-deficient rats. We report here that Cu deficiency produces significant decreases in both the apparent number of receptors and the affinity of the receptors for agonists.

Materials and Methods

Materials

Male and female albino rats of the Sprague-Dawley strain were purchased from the Charles River Breeding Laboratories. Carbamylcholine (CCH) chloride, gallamine triethiodide, and cupric chloride were obtained from Aldrich Chemical Co., K & K Laboratories, and Mallinckrodt, Inc., respectively. Scintanalyzed grade toluene was acquired from Fisher Chemical Co. The nitric and perchloric acids used for the preparation of atomic absorption samples were ACS grade (Baker-analyzed) from VWR. The copper standard solution for atomic absorption spectrophotometry was purchased from Ventron (Alfa Division). [^{3}H]-Quinuclidinyl benzilate (QNB)-1-isomer, specific activity 32 to 44 Ci/mmol, was obtained from Amersham. Radiolabeled samples were counted with a Searle model 300 liquid scintillation counter at an efficiency of 30%.

Animals

Binding assays and metal determinations were performed on tissue from male Sprague-Dawley albino rats. All animals examined were sacrificed at 42 days. The following groups were bred and compared.

Normal

These animals were raised on standard laboratory rat chow containing approximately 44 to 52 μg of Cu/gm of diet.

Deficient

Females from the F1 generation of normal Sprague-Dawley females were weaned at 19 days and separated randomly into control and deficient groups. The deficient population was placed on copper test diet (US Biochemical Corp.) containing less than 0.2 μg of Cu/gm of diet. These females were mated to normal males over a 48-hr period at 8 to 9 weeks of age (minimum weight, 180 gm). During the mating and gestation period the animals were maintained on test diet supplemented with 2 μg of Cu/gm of diet. This level of copper was necessary to prevent resorption of the fetuses. The F2 generation was weaned at 19 days and maintained on copper test diet until sacrifice.

Received June 18, 1984; Revised August 31, 1984;
Accepted August 31, 1984

[1] We are indebted to Lynn Arenella for the histological preparation of the tissue. The work reported here was supported by National Institutes of Health Grant DA 01851, a grant from the Council for Tobacco Research, National Institutes of Health Trainee Grant GM 07141 (J. R. F.), and National Institutes of Health Research Scientist Career Development Award DA 00010 (W. H.).

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TABLE I

Copper levels of isolated brain regions of normal, control, deficient, and two populations of minimally deficient (MCF2, D14) rats

Values are expressed as micrograms of copper per gram of dry weight of the isolated brain area. Errors are reported as the standard error of the mean of two to four experiments in which each value was determined in quadruplicate.

	Normal 4[a]	Control 3	Deficient 3	Minimally Deficient Region	
				D14 3	MCF2 2
Cerebellum	13.3 ± 0.57	12.2 ± 0.53	4.5 ± 0.53	9.1 ± 0.78	9.9 ± 0.86
Midbrain	15.5 ± 1.10	16.5 ± 1.57	5.0 ± 0.02	14.2 ± 0.89	14.3 ± 1.77
Brainstem	14.1 ± 0.46	13.8 ± 0.91	3.9 ± 0.31	12.0 ± 1.04	13.8 ± 0.58
Hippocampus	34.0 ± 1.84	29.3 ± 2.32	10.7 ± 0.79	21.5 ± 1.76	19.0 ± 1.95
Striatum	34.5 ± 1.11	31.5 ± 1.83	9.6 ± 0.91	19.8 ± 1.99	21.4 ± 2.91
Cortex	16.5 ± 0.99	17.0 ± 0.94	5.6 ± 0.42	12.8 ± 0.95	11.5 ± 1.20

[a] Number of experiments (2 to 4).

TABLE II

The binding of [^{3}H]-1-QNB to regional homogenates prepared from the brains of normal, control, copper-deficient, and minimally deficient rats and incubated in the presence and absence of 3 μM copper

Data are presented for direct binding assays using [^{3}H]-1-QNB as described under "Materials and Methods." Errors are reported as the standard error of the mean of two to four experiments (*N*), in which each value was determined in triplicate. No error indicates that only one experiment was performed.

	N	Apparent Number of Binding Sites (pmol/mg of protein)	
		No Added Cu	+3 μM Cu
Normal			
Hippocampus	4	2.70 ± 0.01	1.40 ± 0.02
Striatum	4	3.60 ± 0.03	2.40 ± 0.01
Cortex	4	2.10 ± 0.02	1.05 ± 0.01
Forebrain	4	2.45 ± 0.01	1.30 ± 0.01
Control			
Hippocampus	3	2.73 ± 0.02	1.35 ± 0.03
Striatum	3	3.50 ± 0.02	2.45 ± 0.02
Cortex	3	2.14 ± 0.06	0.90 ± 0.01
Forebrain	2	2.26 ± 0.02	1.11 ± 0.03
Deficient			
Hippocampus	3	1.50 ± 0.02	0.80 ± 0.01
Striatum	3	1.50 ± 0.02	0.80 ± 0.00
Cortex	3	0.90 ± 0.02	0.45 ± 0.01
Forebrain	2	1.50 ± 0.05	0.75 ± 0.02
Minimally Deficient (D14) Forebrain	3	1.60 ± 0.00	1.00 ± 0.03
Minimally Deficient (D14)-Reversed Forebrain	1	2.25	1.50

TABLE III

The binding of [^{3}H]-1-QNB to regional homogenates prepared from the brains of normal, control, copper-deficient, and minimally deficient rats and incubated in the presence and absence of 3 μM copper

The values for K_d were determined using data obtained from direct binding assays with [^{3}H]-1-QNB as described under "Materials and Methods." Errors are reported as the standard error of the mean of two to four experiments (*N*), in which each value was determined in triplicate. No error indicates that only one experiment was performed.

	N	Dissociation Constant for QNB Binding ($K_d \times 10^{-10}$)	
		No Added Cu	+3 μM Cu
Normal			
Hippocampus	4	0.53 ± 0.04	0.44 ± 0.06
Striatum	4	0.53 ± 0.08	0.43 ± 0.05
Cortex	4	0.49 ± 0.07	0.45 ± 0.07
Forebrain	4	0.59 ± 0.08	0.37 ± 0.03
Control			
Hippocampus	3	0.54 ± 0.08	0.42 ± 0.04
Striatum	3	0.52 ± 0.05	0.43 ± 0.01
Cortex	3	0.49 ± 0.03	0.33 ± 0.07
Forebrain	2	0.62 ± 0.04	0.38 ± 0.04
Deficient			
Hippocampus	3	2.15 ± 0.25	1.63 ± 0.10
Striatum	3	1.82 ± 0.20	1.33 ± 0.30
Cortex	3	1.44 ± 0.30	1.32 ± 0.23
Forebrain	2	1.07 ± 0.12	0.56 ± 0.08
Minimally Deficient (D14) Forebrain	3	0.91 ± 0.14	0.34 ± 0.02
Minimally Deficient (D14)-Reversed Forebrain	1	0.60	0.36

Control

The control females from the F1 generation described above were placed on copper test diet supplemented with 46 to 48 μg of Cu/gm of diet to approximate the copper level of the standard laboratory rat chow. The mating parameters were the same as for the deficient populations. Pups were weaned at 19 days and maintained on the supplemented diet until sacrifice.

Minimally deficient

Two groups of minimally deficient animals were bred.

MCF2. These animals represented the F2 generation of females maintained on copper test diet supplemented with 16 to 18 μg of Cu/gm of diet. Pups were weaned at 19 days of age and kept on the minimally supplemented diet until use.

D14. This population was bred to determine the development of minimal deficiency within one generation. Normal pregnant Sprague-Dawley females were placed on the minimally supplemented copper test diet at day 14 of gestation. Pups weaned at 19 days of age were maintained on this diet until sacrifice at 42 days. Control animals were born to females placed on the control test diet (containing 46 to 48 μg of Cu/gm of diet) at day 14 of gestation. These animals were maintained on the control diet from weaning until use.

Brain metal content

Metal content was determined in six specific brain regions (cerebellum, brainstem, midbrain, cortex, hippocampus, and striatum) as well as forebrain. Forebrain encompasses tissue anterior to the superior colliculus. Brain tissue was prepared for analysis using the wet digestion technique of Harrison et al. (1968). Briefly, this involved drying the tissue at 110°C for 12 hr followed by digestion in 1:1 concentrated HNO_3:$HClO_4$ at 200°C. Digestion was allowed to proceed until the white fumes of $HClO_4$ were evolved, and the samples were water clear. Copper content was determined by atomic absorption spectrophotometry using a Perkin Elmer model 372 atomic absorption spectrophotometer at a wavelength of 324.8 nm and a slit width of 0.7 nm. Acetylene and air were used as fuel and oxidizer, respectively. Digested samples were diluted as necessary with deionized water and measured against a reagent black. Metal recovery, as measured by the addition of two different amounts of Cu to tissue digests, was virtually 100% for all brain areas.

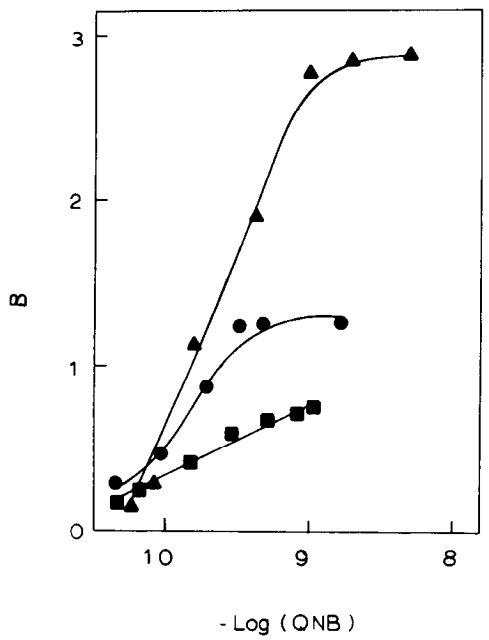

Figure 1. Log-dose curves for a typical experiment depicting the specific binding (*B*) of [^{3}H]QNB to crude synaptosomal membranes from copper-deficient cortex in the presence (■) and absence (●) of 3 μM copper *in vitro*. The binding curves are shown in comparison to the specific binding of [^{3}H] QNB in normal cortex in the absence of added medium copper (▲). Binding is expressed as picomoles per milligram of protein. The *curves* were drawn according to derived binding parameters.

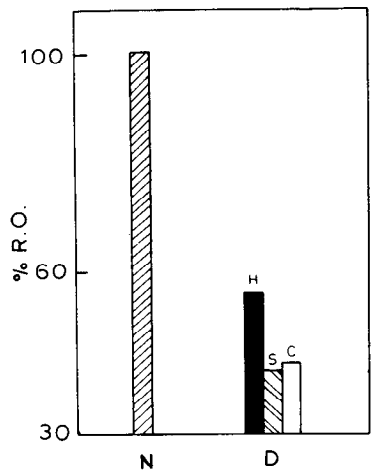

Figure 2. Receptor occupancy (*R.O.*) in hippocampal (*H*), striatal (*S*), and cortical (*C*) homogenates prepared from copper-deficient (*D*) animals expressed as a percentage of the specific QNB binding of normal and control (*N*) homogenates. The *bars* represent the composites of four experiments.

Neural membrane preparation

Crude membrane preparations from rat hippocampus, striatum, and cortex as well as from forebrain and brainstem were used in the various binding assays. The tissue was homogenized in 10 vol of 40 mM sodium/potassium phosphate buffer, pH 7.4, and centrifuged at 3,000 × *g* for 10 min. The supernatant was then centrifuged at 60,000 × *g* for 30 min. The resulting pellet was suspended in 40 mM sodium/potassium phosphate buffer, pH 7.4. Protein content was determined by a modification of the method of Lowry et al. (1951). Aliquots of the suspension were frozen rapidly in liquid nitrogen and stored at −20°C. Binding studies showed these samples to remain stable and comparable to fresh homogenates for at least 4 weeks.

Binding assays

The muscarinic binding assays utilized a modification of the filtration assay developed by Yamamura and Snyder (1974) which uses the binding of QNB,

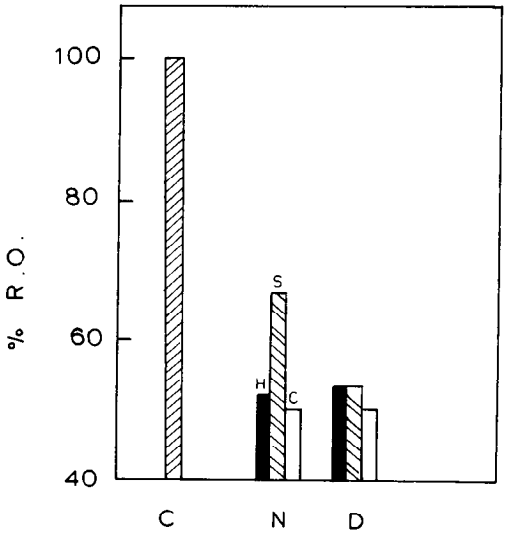

Figure 3. Receptor occupancy (*R.O.*) in the presence of 3 μM copper *in vitro* expressed as a percentage of the specific binding of [^{3}H]QNB in the absence of added medium copper. *Bars* show percentage of binding for homogenates prepared from normal (*N*) and copper-deficient (*D*) hippocampus (*H*), striatum (*S*), and cortex (*C*), and represent the mean of four experiments.

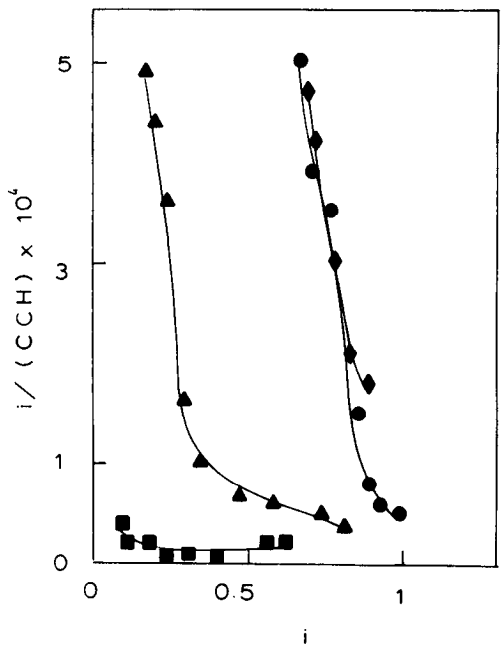

Figure 4. Scatchard plots of the binding of CCH to forebrain synaptosomal preparations from normal and minimally deficient (D14) rats. Curves are shown for CCH binding in the presence (●) and absence (▲) of 1 μM copper for normal animals and in the presence (◆) and absence (■) of copper for deficient animals. The data are representative of single experiments. Mean values of derived constants are presented in Table IV.

a specific and potent muscarinic antagonist. Suspensions containing 60 μg of protein/ml for forebrain and 30 μg of protein/ml for brainstem, 40 mM sodium/potassium phosphate buffer, pH 7.4, and various concentrations of [^{3}H]-I-QNB were incubated at room temperature for 1 hr. Control samples contained a 1000-fold excess of unlabeled QNB relative to the highest concentration of [^{3}H]-1-QNB. Samples were filtered with suction through Whatman GF/B glass fiber filters and were washed twice with 5 ml of cold phosphate buffer. The filters were placed in Nalge scintillation bags with 4 ml of Triton-toluene scintillation fluid. The bags were then placed in scintillation vials and held for at least 12 hr before counting. Specific binding was defined as the difference between the values obtained in the absence and presence of excess unlabeled QNB.

Indirect binding was determined by the ability of the ligand of choice to

TABLE IV

The binding of CCH to forebrain preparations from normal, control, and minimally deficient rats incubated in the presence and absence of 1 μM copper

Data are presented for indirect binding assays using the ability of CCH to displace [^{3}H]-1-QNB from the receptors as described under "Materials and Methods." K_H and K_L refer to high and low affinity constants, respectively; α denotes the proportion of high affinity receptors. Errors are reported as the standard error of the mean of two to four experiments (N), in which each value was determined in quadruplicate.

	N	ID_{50} (μM)	α	K_H (μM)	K_L (μM)
No added Cu					
CCH					
Normal	4	5.69 ± 0.21	0.42 ± 0.08	0.26 ± 0.02	40.14 ± 10.24
Control	2	5.44 ± 0.20	0.41 ± 0.01	0.22 ± 0.05	41.02 ± 6.91
Minimally Deficient					
D14	3	29.89 ± 3.04	0.41 ± 0.02	0.48 ± 0.02	118.50 ± 8.21
MCF2	3	29.42 ± 1.34	0.39 ± 0.01	0.32 ± 0.02	113.52 ± 7.18
+1 M Cu					
CCH					
Normal	4	1.00 ± 0.14	0.70 ± 0.06	0.28 ± 0.05	34.42 ± 5.82
Control	2	0.69 ± 0.05	0.67 ± 0.06	0.24 ± 0.03	25.31 ± 0.15
Minimally Deficient					
D14	3	0.51 ± 0.05	0.71 ± 0.01	0.34 ± 0.04	28.97 ± 5.33
MCF2	3	0.96 ± 0.02	0.61 ± 0.03	0.19 ± 0.03	35.77 ± 1.35

displace 50 pM [^{3}H]-1-QNB from the receptor. Experimental conditions were carefully determined and strictly maintained. Each sample contained approximately 10 pM receptors (4.0 μg/ml of protein for forebrain and 5.4 μg/ml of protein for brainstem) in a final volume of 20 ml. This receptor concentration had no significant effect on the ligand-induced inhibition of [^{3}H]QNB binding as determined experimentally. Addition of receptor homogenate to individual sample tubes was staggered such that the incubation time for each tube was precisely 90 min. Binding parameters for [^{3}H]QNB were determined directly by Scatchad analysis, and parameters for CCH were determined indirectly by competition with [^{3}H]QNB according to a two-site model as described elsewhere (Ellis and Hoss, 1980).

The effect of Cu on muscarinic binding properties *in vitro* was determined by including 3 μM $CuCl_2$ in the incubation medium for direct binding assays and 1 μM $CuCl_2$ in the medium for indirect studies. The concentration of Cu was unbuffered and thus represents total added Cu.

Nucleic acid analysis

The RNA and DNA content of whole brains from normal and copper-deficient rats was determined using the simple fluorometric assay developed by Prasad et al. (1972). This method exploits the increase in fluorescence of ethidium bromide (EBr) upon complexing with nucleic acids.

The brains were rinsed in deionized water, blotted, and weighed prior to homogenization in nucleic acid buffer (0.1 M Tris and 0.1 M Nacl), pH 7.4. Samples were diluted 1:100 with buffer. Sample tubes containing 1 ml of EBr (20 μg/ml), 0.9 ml of buffer, and 0.1 ml of homogenate were read against a reagent blank at fluorometer settings of 365-nm excitation and 590-nm emission wavelengths. Care was taken to permit as little time lapse as possible between homogenate preparation, sample addition, and measurement to prevent any degradative loss of RNA. Two sets of assay tubes were examined. The fluorescence of the first represented total nucleic acid content. Each tube of the second set received 0.02 ml of RNase (20 mg/ml; type IIIA from bovine pancreas, Sigma Chemical Co.), and the entire set was incubated at 50°C for 1 hr. This incubation ensured complete hydrolysis of the RNA. The fluorescence measurement, therefore, represented DNA content only. The RNA content of the tissue was determined by the difference of the two readings. Determinations of DNA and RNA were compared to standard curves prepared immediately preceding each experiment. The sensitivity of the method was checked periodically using recovery experiments with the addition of known concentrations of DNA and RNA to tissue homogenates. Recovery of added nucleic acids was virtually 100% for both DNA and RNA.

Histology

Two deficient rats were anesthetized with Chloropent and perfused with a 1% paraformaldehyde/2% glutaraldehyde/1% acrolein fixative in phosphate buffer. After fixation *in situ* for a minimum of 1 hr, the brains were removed and blocks of white matter were cut from the centrum semiovale. Following a 1-hr postfixation in 1% buffered osmium tetroxide, the tissue was stained en bloc with uranyl acetate in 50% ethanol, dehydrated, and embedded in Poly 812. Sections were cut using a diamond knife, retrieved on uncoated copper grids, and stained with lead citrate. Histological examination employed a Zeiss 10 electron microscope.

Results

Brain metal content. Metal levels in isolated brain regions of normal, control, and deficient animals (as described under "Materials and Methods") are shown in Table I. Control and normal animals were similar in general appearance, weight gain, and motor activity. Statistical analyses revealed no significant differences between the two groups for either brain metal content or mortality rate. Copper deprivation during development decreased brain copper levels by 65 to 75% in all regions examined. The deprived animals showed a number of observable abnormalities including subnormal weight, loss of hair, incoordination of the hindlimbs, and increased mortality.

Two populations of minimally deficient animals—MCF2 and D14—were also bred as described under "Materials and Methods." Brain copper content for both populations is included in Table I. The decrease in regional copper levels showed greater variation in the minimally deficient animals relative to the deficient rats. The copper content of the forebrain structures hippocampus, striatum, and cortex decreased by 27 to 37% in minimally deficient animals as compared to a 7 to 25% copper loss in non-forebrain regions. Animals of both MCF2 and D14 groups showed fewer coat problems, increased weight, and decreased mortality rate relative to deficient rats. Problems in coordination were diminished, but physical activity remained lower than for the normal group. Statistical analyses revealed no significant differences between MCF2 and D14 for either brain metal content or mortality rate.

Antagonist binding. The differential effect of minimal Cu deprivation on brain Cu content is interesting and in accord with the reported Cu sensitivity of forebrain. Studies performed in this laboratory have shown forebrain muscarinic receptors to be particularly susceptible to the effects of *in vitro* Cu addition (Farrar and Hoss, 1984). The binding data shown in Tables II and III indicate that forebrain muscarinic receptors are also sensitive to Cu deprivation *in vivo*. Cu-deficient preparations of hippocampus, striatum, cortex, and whole forebrain consistently showed significant decreases ($P < 0.001$) in ligand affinity and in the number of apparent binding sites relative to the normal or control groups. Binding curves for normal and deficient cortex are presented in Figure 1. These curves, as well as those in Figure 4, present data from single experiments delineating representative trends. The shift of the deficient plot down and to the right was evident in all preparations assayed.

The deficiency-induced decline in receptor number is clearly indicated in Figure 2, which presents receptor occupancy in deficient preparations as a percentage of QNB binding in the normal group. (Statistical analyses revealed no differences in the binding behavior of the normal and control groups.) Specific binding decreased by

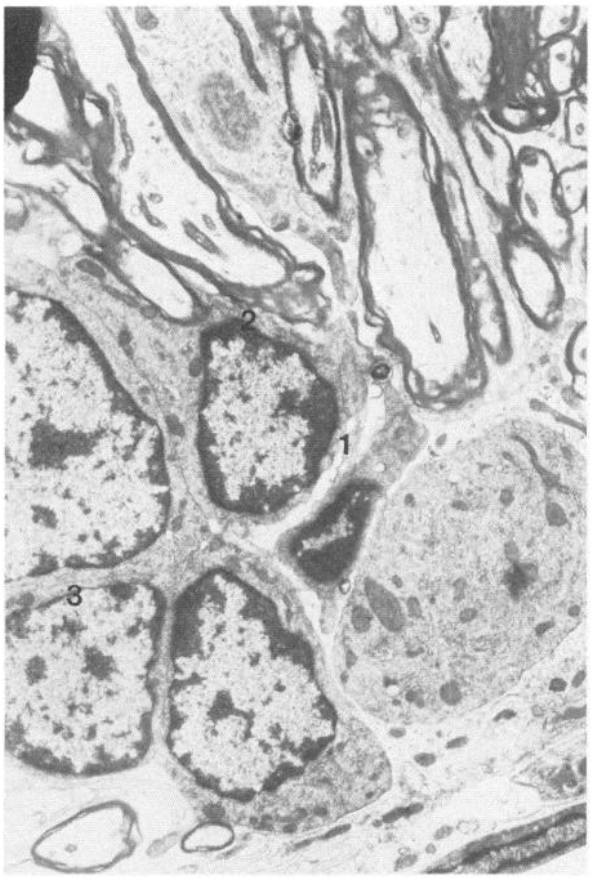

Figure 5. Abnormal white matter in copper-deficient rat brain, from the cerebellum of a 35-day-old rat. This field demonstrates the amorphous bubbling (*1*) and membrane fragmentation (*2*) affecting certain oligodendrocytes in copper-deficient brain. Normal-appearing oligodendrocytes (*3*) are also shown in this section. Magnification × 18,000.

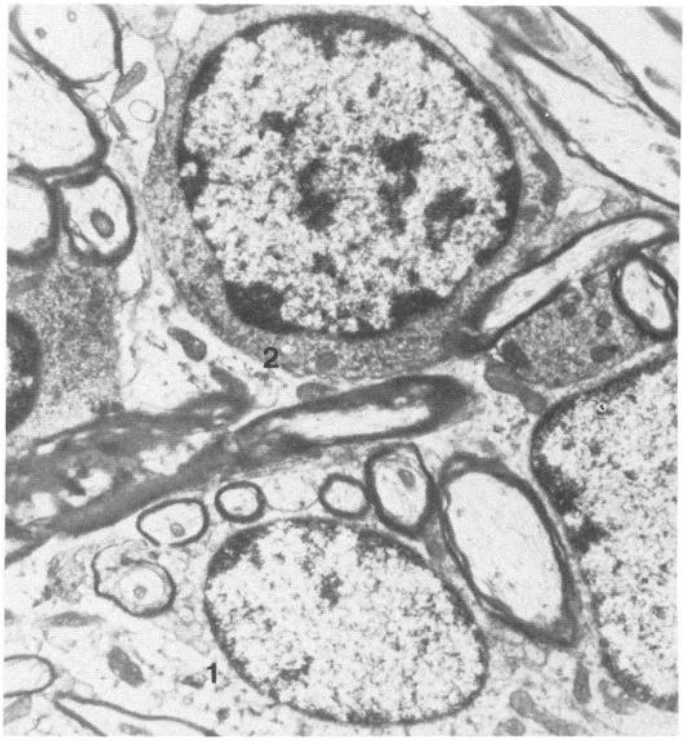

Figure 6. Abnormal white matter in copper-deficient rat brain, from the cerebellum of a 35-day-old rat. Astrocytic swelling (*1*) is evident in this section. The oligodendrocyte included in the field shows signs of increased membrane fragility (*2*). Magnification × 24,000.

44, 58, and 57% in the deficient hippocampal, striatal, and cortical homogenates, respectively. Specific binding in the deficient forebrain preparation decreased by 40%.

The effects of adding 3 μM Cu to a deficient preparation are included in Figure 1. It is apparent that the addition of Cu to the incubation medium did not reverse the effects of the deficiency but decreased receptor occupancy and ligand affinity in a manner similar to Cu treatment of normal and control homogenates (see Tables II and III for specific values). Similarities between the addition of 3 μM Cu to normal and deficient homogenates are obvious in Figure 3, which depicts receptor occupancy following Cu addition as a percentage of QNB binding in homogenates with no added Cu. The apparent number of binding sites decreased by 33% in normal striatal homogenates and by 45 to 50% in all other preparations. The decline in the number of QNB recognition sites was significant ($P < 0.001$) in all cases.

Use of minimally deficient animals for agonist assays necessitated examination of QNB binding in minimally deficient preparations relative to deficient homogenates. Although minimally deficient rats were similar to the control and normal animals in mortality rate and general appearance, the binding parameters of the minimally deficient group were comparable to those of the deficient preparations as shown in Tables II and III.

Nucleic acid content. Alterations in specific binding can occur from changes in cell number as well as from changes affecting the binding site itself. The possibility that the decline in apparent binding sites in deficient preparations was due to a decrease in the number of cells bearing QNB sites was addressed by nucleic acid analyses. The respective DNA and RNA contents expressed as micrograms per milligram of wet weight were 1.3 ± 0.2 and 1.2 ± 0.1 in normal homogenates and 1.2 ± 0.2 and 1.0 ± 0.2 in deficient preparations. These figures are in accord with those reported for rat forebrain by Prasad et al. (1972). The differences between the normal and deficient groups were not statistically significant.

Reversibility. The possibility of reversing the diet-induced developmental copper deficiency *in vivo* was of interest. One rat from the minimally deficient D14 group was placed on normal rat chow at 28 days of age. At 49 days, this animal showed normal weight, a normal-appearing coat, and increased physical activity. Results presented in Tables II and III allow comparison of the binding parameters for normal, minimally deficient, and deficiency-reversed forebrains in the presence and absence of added copper. However, these results should be treated with caution since they are derived from only one animal.

Agonist binding. Scatchard plots for the binding of the muscarinic agonist, CCH, to forebrain receptors from normal and minimally deficient D14 animals are presented in Figure 4. Binding parameters determined according to a two-site model are shown in Table IV. Forebrain homogenates prepared from the D14 animals showed significant ($P < 0.001$) increases in the values of ID_{50}, K_H, and K_L relative to the normal and control groups. The percentage of binding sites in the high affinity state (α) was unchanged by developmental Cu deprivation.

The addition of 1 μM Cu to the incubation medium appeared to reverse the effects of Cu deprivation on agonist binding, in contrast to the effects of Cu treatment on antagonist binding. However, in both cases, receptors from Cu-deprived animals actually responded to Cu treatment in a manner comparable to that of receptors from normal and control groups. In the presence of medium Cu, the Scatchard plot and the derived binding data for the minimally deficient forebrain homogenates approximated those of the normal and control preparations following *in vitro* Cu treatment. The change in all parameters upon the addition of Cu to the medium was significant ($P < 0.001$) for all experimental groups.

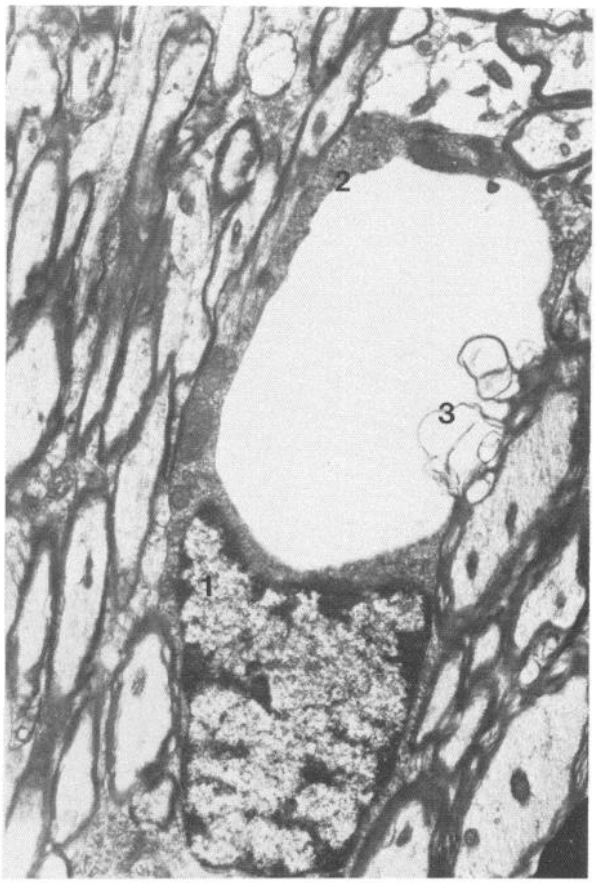

Figure 7. Abnormal capillary in copper-deficient rat brain, from the cerebellum of a 35-day-old rat. The endothelial cell appears thickened (*1*), and the capillary wall shows increased fragility (*2*). Myelin leakage into the capillary space is evident (*3*). Magnification × 16,000.

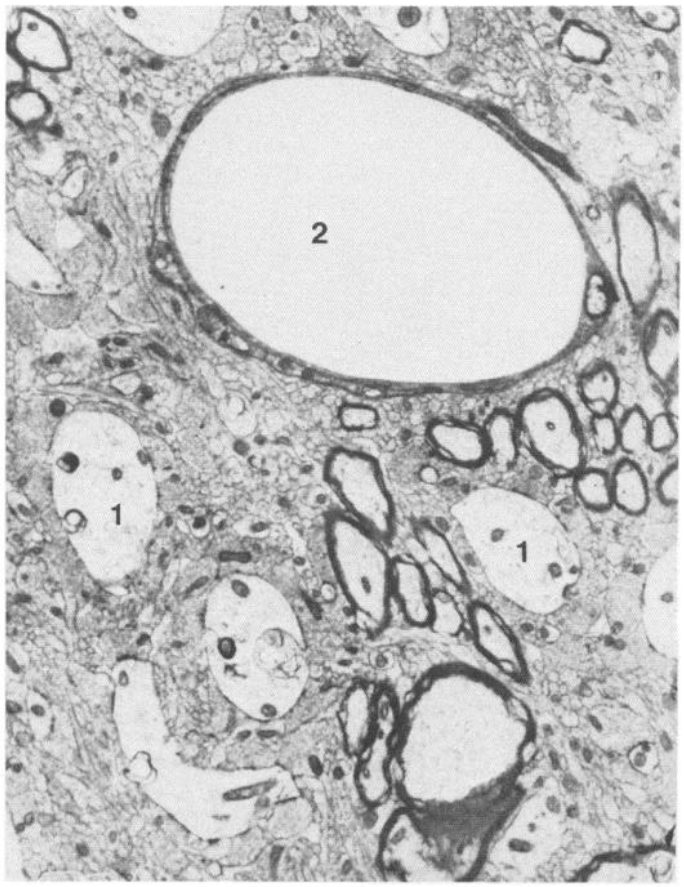

Figure 8. Neuropil of copper-deficient rat brain from the cortex of a 35-day-old rat showing swollen dendrites (*1*). The capillary (*2*) in this picture is normal in appearance. Magnification × 20,000.

Histology. Histological examination of the brain tissue of 35-day-old Cu-deficient rats found overt changes in white matter and indicators of vascular and dendritic disruption. Inspection of the perfused brain revealed increased translucence of myelin. Electron microscopic study of the tissue disclosed the formation of amorphous bubbles within oligodendrocytes and partial dissolution and swelling of astrocytes (Figs. 5 and 6, respectively). Figure 7 provides evidence of the capillary fragility of Cu-deficient brain. Rupture of the capillary with protrusion of myelin from the surrounding tissue into the capillary space suggests weakness of the capillary wall. We have never seen such rupture in normal animals. Electron microscopic examination of the neuropil of Cu-deficient rats also revealed dendritic effects including swelling and membrane fragmentation with formation of membranous whorls (Fig. 8).

Discussion

The experiments reported here used a nutritionally controlled animal model to examine a role for copper in the modulation of brain muscarinic binding parameters. Second generation animals born to females maintained on copper-restricted diets showed symptoms of severe copper deficiency. The behavioral and morphological anomalies noted for these animals are comparable to those reported for other cases of dietary or genetic copper deficiency (Everson et al. 1968; Carlton and Kelly, 1969; DiPaolo et al., 1974; Keen and Hurley, 1976; O'Dell et al., 1976; McMurray, 1980). Neurological abnormalities included ataxia, akinesia, tremor, seizure postures, and hyperirritability. Alterations in white and gray matter and evidence of vascular dysfunction were disclosed upon histological examination of the copper-deficient brain tissue. Histological examination of postmortem tissue from patients with Menke's syndrome, a human genetic disorder involving failure of intestinal absorption of copper, has also shown degenerative changes that affect the composition of white matter and neuropil (Danks et al., 1972). Vascular disorders have been described previously for these patients as well as for copper-deficient rats (DiPaolo et al., 1974). Lack of adequate copper intake is known to cause arterial defects due to diminished cross-linking of elastin and collagen. Lysyl-oxidase, a copper metalloenzyme, is the key enzyme in the cross-linking process.

Since histological examination of Cu-deficient rat brain revealed abnormalities of white matter and myelin, lipid analyses (Reese and Hoss, 1983) were performed in some animals. No significant differences in the phospholipid or cerebroside content or fatty acid composition due to Cu deficiency could be demonstrated. Although a decrease in phospholipid docosahexaenoic acid and a compensatory appearance of an unknown but highly unsaturated fatty acid were observed, these minor changes in unsaturated fatty acid distribution were also found in the brains of Cu-supplemented control animals (data not shown). Thus, these effects are due to dietary changes other than Cu deficiency and cannot account for the observed alterations in muscarinic receptors since the latter effects were not observed in control animals.

The biochemical effects of copper deficiency on several central neurotransmitter systems have been described by Feller et al. (1982). The authors noted a preferential reduction of muscarinic and dopaminergic receptors in forebrain regions. No change was reported for β-adrenergic receptor binding. The selective nature of the effect of copper deficiency suggests that the lesion is receptor specific. Receptor modifications secondary to lipid and/or vascular disruption presumably reflect widespread involvement of all neurotransmitter systems. Our results are compatible with the conclusions of Feller et al. (1982). We note specific effects on muscarinic systems,

including decreased receptor density, in forebrain areas, regions of relatively high copper content in normal rats. These regions appear to be more sensitive to copper deficiency than low copper areas such as brainstem. Results with marginally deficient animals indicated involvement of forebrain structures only. It is particularly interesting that binding parameters for the minimally deficient animals were comparable to data obtained with copper-deficient rats despite the lack of deficiency symptoms (e.g., hair loss, hindlimb incoordination, tail extension).

Consideration of the myriad effects of copper deficiency raises the possibility that receptor decline is secondary to changes in cellularity. Diffuse neuronal damage and gliosis have been associated with the genetic copper deficiency of Menke's syndrome (Danks et al., 1972). However, nucleic acid analyses performed on the copper-deficient brain tissue described here showed no change in the number of cells bearing QNB recognition sites. Neuron death was negated by the constancy of total nucleic acid content (DNA and RNA). A change in the proportion of neurons and glial cells (e.g., by gliosis) was contradicted by the lack of change in the RNA:DNA ratio. Glial cell proliferation with concomitant loss of RNA-rich neurons results characteristically in a reduction in the nucleic acid ratio (Rappoport et al., 1969). Examination of the neuropil of copper-deficient rats by electron microscopy indicated some dendritic disruption involving swelling and increased membrane fragility (Fig. 8). Such changes could account for the reduction of receptor sites in the presence of normal cell ratios. Light microscopic autoradiography with [^{3}H]QNB has indicated a preferential localization of muscarinic receptors on dendrites rather than cell bodies (Kuhar and Yamamura, 1975).

Developmental copper deprivation resulted in specific decreases in muscarinic ligand affinity in addition to the decrement in binding site number. Preparations from deficient animals showed marked decreases in receptor affinity toward the potent muscarinic antagonist, QNB, and agonist, CCH. The large increase in ID_{50} for CCH was correlated with significant increases in both K_H and K_L. These results are contrary to those of Feller et al. (1982), who noted no change in receptor affinity toward QNB or oxotremorine. (Agonist binding parameters, however, were not addressed.) It is likely that these differences reflect variations in the nutritional model and/or experimental conditions.

Two generalizations emerge from the studies of copper effects on muscarinic binding parameters: (*1*) copper deprivation during development induces an alteration in receptor density *in vivo* that is not correctable by the addition of copper *in vitro*, and (*2*) the receptors of copper-deficient homogenates are capable of responding to copper *in vitro* in normal manner.

It appears, moreover, that the decrease in agonist-binding affinity is due to the lack of Cu in the deficient animals. Cu^{2+} is a sulfhydryl ligand with a propensity for forming stable square planar complexes, allowing Cu^{2+} to bridge more than one protein. The data presented here are compatible with the notion that endogenous Cu may participate in the dimer-tetramer equilibrium corresponding to low and high agonist affinity states of the receptor, respectively, as suggested by Avissar et al. (1983). Thus, endogenous Cu may be involved in interactions between receptors or between a receptor and some other membrane protein.

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