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Effects of Age on Selective Antagonist Binding to Muscarinic Receptors in Rat Striatum

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Abstract – The objective of the present study was to investigate the effect of senescence on the binding properties of muscarinic receptors in the neostriatum of young (3 months), middle-aged (18 months) and aged (33 months) male Fischer 344 x Brown Norway hybrid rats by employing direct binding of selective radiolabeled antagonists. Using the selective M_2 muscarinic receptor antagonist, [^{3}H]AF-DX 384, as the ligand, no significant difference in the maximal receptor density (Bmax) was observed in the neostriatum among any age-groups. In contrast, with the selective M_3 receptor antagonist, [^{3}H]4-DAMP, a significant increase in the number of muscarinic receptors was observed in neostriatal membrane fractions prepared from the aged animals relative to that observed in the young rats. For each ligand there was no age-related change in its affinity (Kd) for the muscarinic receptors. These results indicate that the observed age-related changes in the muscarinic receptor density may not be necessarily decremental and depend upon the muscarinic receptor subtype examined.

Keywords☐ muscarinic receptors, selective antagoninst, rat striatum, binding

Muscarinic cholinergic receptors have been characterized in experimental animals or human brain by pharmacological and molecular biological approaches (Hulme *et al.,* 1990). The muscarinic receptors have been divided into four major subtypes on the basis of radioligand binding and functional studies. These pharmacologically distinguishable subtypes of muscarinic receptors are present in varying degrees in different tissues, and have been designated as M_1, M_2, M_3 and M_4 by their differential affinities for selective ligands. Muscarinic antagonists, especially, used in this classification include pirenzepine (M_1-selective), AF-DX 116, methoctramine, himbacine (M_2-selective), and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), and hexahydrosiladifenidol (M_3-selective) (for reviews, see Mitchelson, 1988; Eglen & Watson, 1996). Although tropicamide and secoverine are reported to show higher affinities for M_4 than for other subtypes (Lazareno *et al.,* 1990), the pharmacological profile of the M_4 subtype remains to be further clarified. Genes for five subtypes of muscarinic receptor (m1~m5) have been cloned (Bonner *et al.,* 1987, 1988; Liao *et al.,* 1989), and pharmacological studies with these cloned subtypes individually expressed in various cell lines show that m1~m4 receptors correspond closely to the pharmacologically defined M_1~M_4 receptors (Bonner *et al.,* 1989; Buckley *et al.,* 1989; Dorje *et al.,* 1991; Guo *et al.,* 1995). No specific ligands are known for the M_5 (m5) subtype.

Radioligand binding studies have provided useful information about drug-receptor interaction. Muscarinic agonists with a high degree of selectivity for particular muscarinic receptor subtypes are not currently available, but selective muscarinic antagonists such as those described above have been useful tools for studying the binding characteristics of muscarinic receptors. These antagonists have also been utilized in investigations on age-related changes in muscarinic receptor binding. In these investigations in which the effect of aging on muscarinic receptors in the brain was assessed, only the total population of receptors was examined using non-selective muscarinic antagonists such as [^{3}H]quinuclidinyl benzilate (QNB) or [^{3}H]N-methyl scopolamine (NMS). Moreover, the majority of the studies were indirect binding experiments in which unlabeled agonists or antagonists

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were used to compete for [^{3}H]QNB. Recently, however, the muscarinic receptor subtype-selective radioligands such as the M_1-selective antagonist, [^{3}H]pirenzepine, or the M_2-selective antagonist, [^{3}H]AF-DX 116, were used to examine age-related changes in M_1 and M_2 muscarinic receptor subtypes (Watson *et al.*, 1988; Schwartz *et al.*, 1990; Araujo *et al.*, 1990). Whether with aging there are changes in the binding characteristics of M_3 receptor subtypes has not, to our knowledge, been reported. Reports on age-related alterations in the binding sites of muscarinic receptors in rodent brain are conflicting. In aged rats, decreases (Strong *et al.*, 1980; Lippa *et al.*, 1981; Pedigo *et al.*, 1984; London *et al.*, 1985; Norman *et al.*, 1986; Gurwitz *et al.*, 1987; Michalek *et al.*, 1989; Waller and London, 1989), and no changes (Strong *et al.*, 1980; Morin and Wasterlain, 1980; Consolo *et al.*, 1986; Surichamorn *et al.*, 1988; Schwartz *et al.*, 1990) in muscarinic binding sites have been observed in the hippocampus and cortex. In the striatum, large age-related reductions in the densities of muscarinic binding sites have been frequently reported for various strains (Pedigo *et al.*, 1984; Gurwitz *et al.*, 1987; Surichamorn *et al.*, 1988; Ogawa *et al.*, 1994; Narang, 1995), whereas no changes also have been shown (Schwartz *et al.*, 1990). Because of these conflicting results, it was necessary to examine whether age-related changes in muscarinic binding sites occurred in the population of rats used in our age-related studies.

In a previous study (Lee *et al.*, 1991), we reported an age-related decrease in the binding sites of M_1 muscarinic receptors, as revealed by [^{3}H]pirenzepine binding in rat neostriatum. In the present study, the binding properties of the remaining two pharmacologically defined muscarinic receptor subtypes were assessed. The M_2 and M_3 receptor populations in the neostriatum of young, middle-aged, and aged rats were assessed by direct binding with [^{3}H]AF-DX 384 and [^{3}H]4-DAMP, muscarinic antagonists which selectively label M_2 and M_3 muscarinic binding sites, respectively.

MATERIALS AND METHODS

Materials

AF-DX 384, [2,3-dipropylamino-^{3}H] (specific activity, 105.6 Ci/mmol) and 4-DAMP, [N-methyl-^{3}H] (specific activity, 84.3 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Atropine sulfate was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Animals and Membrane Preparation

Male Fischer 344 (F344)×Brown-Norway (BN) hybrid rats were obtained from the Aged Animal Colonies of the National Institute on Aging (NIA) through Harlan Sprague Dawley (Indianapolis, IN) or Charles River Breeding Laboratory (Stoneridge, NY). The F1 hybrids of the F344×BN crosses, aged 3 months (young), 18 months (middle-aged), and 33 months (aged) were used for the binding experiments. The F1 hybrids of the F344×BN crosses were selected as an alternative model to the F344 inbreds, as recommended by NIA. This hybrid appears to produce progeny with fewer pathologies and at later onset and has a longer life expectancy, so the 18- and 33-month hybrid rats were used as representatives of middle-aged and aged rats instead of the 10- and 28-month F344 rats usually used in our previous studies (Lee *et al.*, 1991; Weiler & Lee, 1992). The rats were individually housed in suspended stainless steel cages under a 12-hour light/dark cycle with food and water available ad libitum. The mean (±S.E.) body weights of the young, middle-aged and aged rats (hybrids) were 332±5 g, 546±6 g, 562±11 g, respectively.

Following sacrifice by decapitation, the brain was quickly removed and placed on ice. The whole neostriatum was then dissected out and weighed. In some experiments, striatal tissue blocks were quickly frozen in liquid nitrogen and then stored in -80℃ until assays were performed. Membrane fractions were prepared from the striata of seven to eight rats from each of the three age-groups. For the [^{3}H]4-DAMP binding assays, four separate experiments (for each with triplicate) were done for a total of 30 to 32 rats per age-group; in one experiment, [^{3}H]4-DAMP binding was done with hippocampal membrane prepared from each age-group. For the [^{3}H] AF-DX 384 binding assays, three separate experiments (for each with triplicate) were performed for a total 20 rats per age group. All membrane preparation procedures were conducted at 4℃ and with ice-cold buffers. The neostriatal tissues were homogenized with Tekmar polytron (setting 5 for 30 to 40 seconds) in 25 mM Tris-Krebs buffer which contains the following (in mM): Tris · HCl 25, NaCl 124, KCl 5.1, $MgSO_4$ 1.4, 1.22, $CaCl_2$ 2 and glucose 10.2 (pH 7.4). Homogenates were centrifuged for 10 min at 1,000×g, then the supernatant was re-centrifuged for 30 min at 30,000×g. The result-

ing pellet was resuspended in the same buffer (0.1 g/mL) and aliquots were stored at -80℃ until the binding assays were performed.

Receptor Binding Assay

For the [^{3}H]AF-DX 384 binding assays, membrane aliquots (50~100 g protein) were incubated with [^{3}H]AF-DX 384 (final concentrations, 0.5~100 nM) for 60 min at room temperature (0.5 mL total volume) in the absence and presence of 10 μM atropine sulfate. For the [^{3}H]4-DAMP binding assays, the membrane aliquots (20~40 g protein) were incubated with [^{3}H]4-DAMP at final concentrations between 0.05 and 20 nM (total volume 0.5 mL) for 45~60 min at room temperature in the absence and presence of 1 μM atropine sulfate.

The incubation was terminated by rapid filtration through Watman GF/B glass filters pretreated with 0.1% polyethyleneimine in a Brandel M-24 cell harvester. The filters were washed three times with 3 mL ice-cold buffer. All binding assays were carried out in triplicate. The radioactivity on the filters was extracted with 10 mL of ScintiSafe Econo2 (Fisher) and measured in a liquid scintillation counter (Beckman LS 6000TA) at about 38% counting efficiency.

The binding data for saturation experiments were analyzed by Scatchard plots and linear least-squares regression analysis (Scatchard, 1949) using a computer program called Pharmacological Calculating System, prepared by Thallarida (1987). The Hill coefficient (nH) values were calculated using a linear regression analysis and analyzed for statistically significant deviation from unity. Protein concentration was determined by the Bradford method (Bradford, 1976) with Bio-Rad dye reagent using bovine serum albumin as standard.

Analysis of variance and Student's t-test were employed to determine the statistical significance of the differences of means.

RESULTS

In kinetic experiments, the time course of specific binding of radiolabeled ligands was assessed by incubating crude membrane homogenate with 2.5 nM [^{3}H] AF-DX 384 or 0.1 nM [^{3}H]4-DAMP for the designated time periods (Fig. 1). Equilibrium binding of [^{3}H]AF-DX 384 to neostriatal membrane fractions at room temperature was achieved by 20 min and maintained for 90 min. The binding of [^{3}H]4-DAMP reached equilibrium within

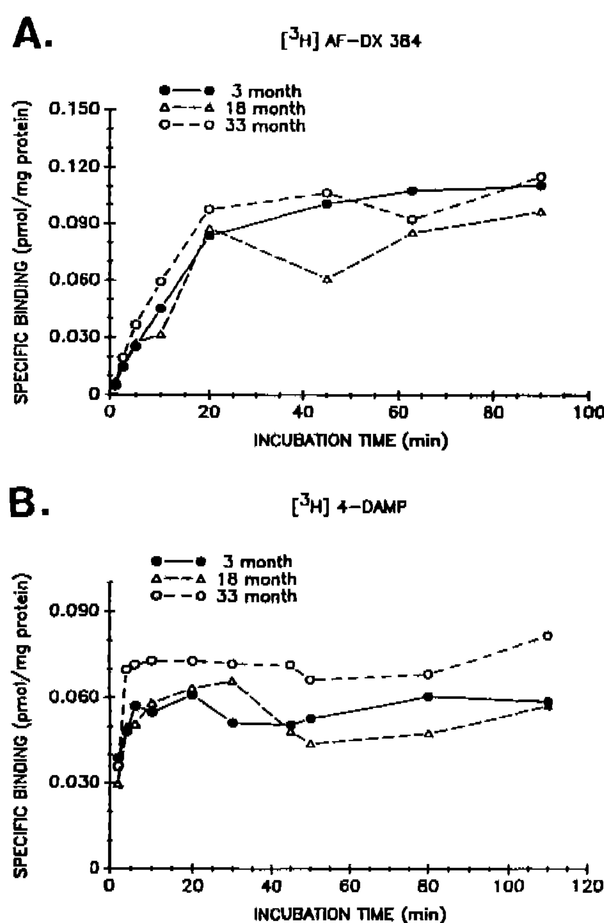

Fig. 1. Time course of specific binding of [^{3}H]AF-DX 384 (A) and [^{3}H]4-DAMP (B) in neostriatal membranes prepared from 3-, 18- and 33-month rats. Each point was determined from duplicate assay tubes. Nonspecific binding was determined in the presence of 10 μM (A) or 1 μM (B) atropine.

6 min and equilibrium binding was stable for 110 min. The pattern of the association curves for both radioligands was similar among the three age groups.

The binding of AF-DX 384 and 4-DAMP were determined directly using the radioligands [^{3}H]AF-DX 384 and [^{3}H]4-DAMP, and representative binding curves obtained with neostriatal membrane fractions prepared from three age-groups of the F344×BN hybrid rats (3, 18 and 33 months old) are shown in Fig. 2A. In all membrane fractions, specific binding was saturable and represented 70 to 95% of total binding over the concentration ranges tested. Scatchard plots (Scatchard, 1949) of both [^{3}H]AF-DX 384 and [^{3}H]4-DAMP binding in all three age-groups displayed straight lines indicating that [^{3}H]AF-DX 384 and [^{3}H]4-DAMP labeled a single population of receptors (Fig. 2B). A single binding site was confirmed

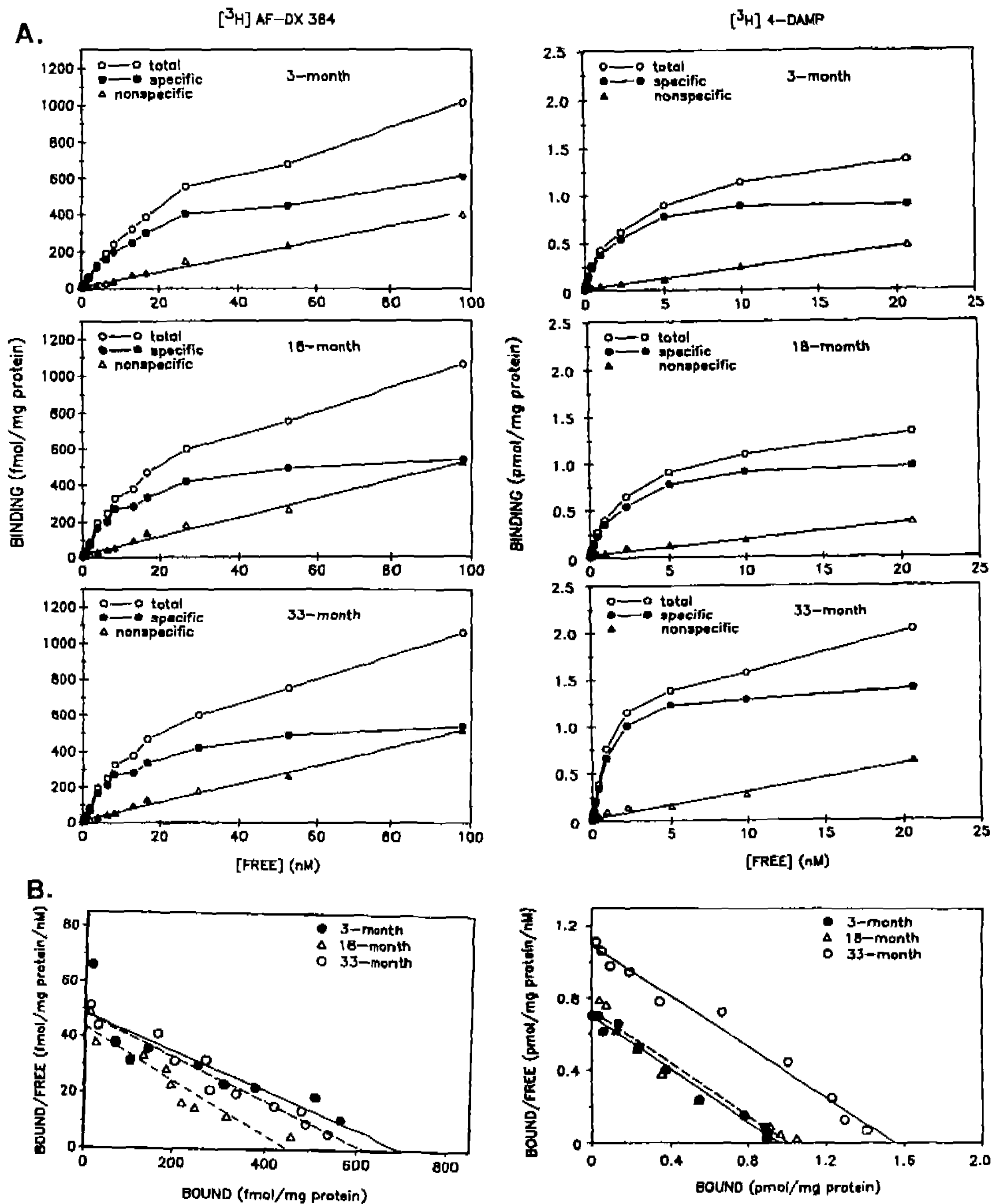

Fig. 2. Total, specific and nonspecific binding (A) and Scatchard plots of the specific binding (B) of [^{3}H]AF-DX 384 and [^{3}H]4-DAMP in neostriata from 3-, 18- and 33- month rats . Membrane fractions (0.02~0.1 mg of protein) were incubated in triplicate with increasing concentrations of the radioligands for 60 min at room temperature. Non-specific binding was obtained in the presence of either 10 μM or 1 μM atropine for [^{3}H]AF-DX 384 or [^{3}H]4-DAMP binding, respectively. Data shown are the results from a single representative experiment.

with a Hill analysis of the binding data. The binding data summarized in Table I for [^{3}H]AF-DX 384 shows no changes with aging in either the affinity or the number of binding sites for [^{3}H]AF-DX 384 in rat neostriatal membrane fractions. The data for [^{3}H]4-DAMP indicate that there is no age-related change in the affinity (Kd) of the muscarinic binding sites for [^{3}H]4-DAMP. Interestingly, however, there was an 26% increase ($p<0.05$) in the

Table I. Parameters of [^{3}H]AF-DX 384/M_2 and [^{3}H]4-DAMP/M_3 binding in neostriatum of three age-groups of rats

Age Group	Kd (nM)	Bmax (fmol/mg protein)	nH
[^{3}H]AF-DX			
3-month	13.17±2.985	772±211	1.03±0.015
18-month	10.00±2.870	549±125	0.93±0.020
33-month	13.31±3.405	775±86	0.98±0.020
[^{3}H]4-DAMP			
3-month	1.70±0.365	1105±64	1.02±0.029
18-month	1.64±0.326	1178±72	0.99±0.017
33-month	1.65±0.309	1396±55*	1.01±0.019

All binding data were derived from full saturation analysis. Striatal membranes were incubated in buffer containing various concentrations of the [^{3}H]AF-DX 384 (0.5~100 nM) or [^{3}H]4-DAMP (0.05~20 nM) for 60 min at room temperature. Determinations of equilibrium dissociation constants (Kd) and maximum binding capacities (Bmax) are based upon three to four separate experiments done with striatal membranes from a total of 20~32 rats per age-group. The Hill coefficient (nH) values analyzed were not different from unity. Values are mean ± SEM of 3~4 experiments, where each concentration was tested in tripicate. *$p<.05$ relative to the 3-month group.

number of [^{3}H]4-DAMP binding sites (Bmax) in neostriatal membranes prepared from the aged rats (Table I).

To determine whether similar age-related differences in [^{3}H]4-DAMP binding occurred in other brain regions, one [^{3}H]4-DAMP binding experiment was performed with hippocampal membrane fractions. No apparent age-related differences in the affinity and number of binding sites for 4-DAMP were found (data not shown). The binding parameters obtained in hippocampal membranes of the three age-groups, 3, 18 and 33 months, respectively, were as follows (the 95% of confidence intervals in parentheses): Kd for each age-group (in nM)=2.14 (1.74-2.76), 2.63 (1.82-4.77), 2.19 (1.83-2.73); Bmax (in fmol mg protein-1)=1089 (938-1240), 924 (625-1233), 1214 (1054-1373).

DISCUSSION

Three muscarinic receptor subtypes (M_1, M_2 and M_3) are commonly discriminated in binding and pharmacological studies with a use of antagonists selective for each of the subtypes (Hulme *et al.*, 1990). Although the existence of fourth class of muscarinic receptor, M_4, has been demonstrated in the rat neostriatum, rabbit lung, chick heart and NG 108-15 cell line (Waelbroek *et al.*, 1990; Lazareno *et al.*, 1990), muscarinic antagonists highly selective for this subtype are limited. Whether there are differential changes with aging in functional roles or binding characteristics of individual muscarinic receptor subtypes is not clear. Muscarinic antagonists, radiolabeled and highly selective for each subtype would be useful to assess the effect of aging on ligand binding properties of any particular muscarinic receptor subtype. Previously, a small, but significant loss of muscarinic-M_1 sites, which was determined by [^{3}H]pirenzepine binding, was observed in neostriatal tissues of aged F344 rats (Lee *et al.*, 1991). In the present study, the age-related changes occurring in the binding characteristics of muscarinic receptors were investigated by directly labeling M_2 and M_3 receptors in neostriatum with [^{3}H]AF-DX 384 and [^{3}H]4-DAMP, respectively. The results show that normal aging leads to changes in muscarinic binding sites depending on subtypes. The enhancement of M_3 binding sites and no alteration in M_2 binding sites in neostriatum of the aged rats were observed.

The M_2 muscarinic receptor subtypes are present in various brain regions of the rat, and the radioligand, [^{3}H]AF-DX 116 has been a useful selective ligand for muscarinic M_2 receptor sites in the rat brain (Wang *et al.*, 1987, 1988; Araujo *et al.*, 1989; Lapchak *et al.*, 1989; Regenold *et al.*, 1989). A derivative of AF-DX 116, AF-DX 384, has been reported to be more potent and selective (Ebeline *et al.*, 1989), and the radiolabeled ligand of the derivative has been used to study muscarinic M_2 receptors in rat brain tissues (Castoldi *et al.*, 1991). Therefore, [^{3}H]AF-DX 384 was used in the present study to examine whether there is an alteration with age in M_2 binding sites in the neostriatum. The direct binding data obtained from saturation analysis of [^{3}H]AF-DX 384 binding to rat neostriatal membrane fractions revealed binding of this radioligand to a single class of relatively high affinity muscarinic binding sites in all three age groups (3, 18 and 33 months old). A single class of M_2 binding sites for [^{3}H]AF-DX 384 was also shown in a binding study of various brain regions of the rats (Castoldi *et al.*, 1991), as had been seen in cardiac and ileal tissue (Entzeroth and Mayer, 1990, 1991). The binding parameters obtained in the present study were similar to those reported in other studies, even though the maximal density of [^{3}H]AF-DX 384 binding sites (772 fmol mg protein-1) in young (3 month old) rats in the present study was somewhat lower than the Bmax (1565 fmol

mg protein-1) in striatum reported by Castoldi *et al.* (1991). The dissociation constant (Kd) of [^{3}H]AF-DX 384 (10~14 nM) in neostriatum was about 2 fold higher than that previously reported in same brain area (5.28 nM; Castoldi *et al.*, 1991), and similar to that previously observed with this ligand in rat heart (8.7 nM) and ileum (9.2 nM). However, the Kd of [^{3}H]AF-DX 384 was several fold lower than that of the analog [^{3}H]AF-DX 116 in neostriatum (43.8~68.4 nM; Wang *et al.*, 1987), and it confirms a greater potency of AF-DX 384 over AF-DX 116.

No age-related alterations in binding properties of [^{3}H] AF-DX 384 to muscarinic M_2 binding sites, either Kd or Bmax, were observed in the present study. In contrast, Araujo and his colleagues (1990) have observed a significant age-related decline in muscarinic M_2 binding sites as identified by direct binding analysis of [^{3}H]AF-DX 116 in the brain regions of Long-Evans rats. These discrepancies, in part, may be due to differences in radioligand, the incubation parameters (e.g. incubation buffers and temperatures), and rat species used in studies.

The existence of the M_3 subtype of muscarinic receptors in rat brain has also been shown with the use of selective muscarinic antagonists (Waelbroeck *et al.*, 1987, 1990). Pharmacologically, 4-DAMP (Doods *et al.*, 1987; Michel and Whiting, 1988) and hexahydrosiladifenidol (Mutschler and Lambrecht, 1984) were found to be selective antagonists of M_3 muscarinic receptors in glandular tissues and ileal smooth muscle in both functional and ligand binding studies. In indirect replacement studies with [^{3}H]QNB or [^{3}H]NMS, the inhibition constant (Ki) of 4-DAMP ranged from 0.5 to 1.2 nM in rat brain (cortex, hippocampus and neostriatum) and glandular tissue, and ranged from 4.7 to 34 nM in heart (Doods *et al.*, 1987; Nilvebrant and Sparf, 1988; Waelbroeck *et al.*, 1990). In a binding study used CHO-K1 cell lines individually expressing the various muscarinic receptor subtypes, 4-DAMP exhibited high affinity for the m3 subtype, although it was almost equally potent in competing for [^{3}H]QNB binding to m1, m4 and m5 subtypes (Dorje *et al.*, 1991). Direct binding studies with [^{3}H]4-DAMP have shown that this radioligand selectively labels the M_3 (and possibly M_1) receptor subtypes as opposed to the M_2 receptors in rat cerebral cortex, rat submaxillary gland and ileal smooth muscle (Michel *et al.*, 1989; Michel and Whiting, 1990). It was observed that in rat cortex and gland, [^{3}H]4-DAMP bound to an heterogeneous population of binding sites, predominantly with high affinity (Kd=0.2~0.4 nM) and minimally with low affinity (Kd=4~17 nM), but whether the sites with low affinity represented a different subtype of a non-saturable component of [^{3}H]4-DAMP binding was not clear (Michel *et al.*, 1989). In the same study, the radioligand was observed to label the M_2 receptor with low affinity (Kd=4 nM). Another study which addressed a more detailed characterization of [^{3}H]4-DAMP binding (0.05~250 nM) in the rat brain, however, reported that it bound to a population of high-affinity sites (M_3, Kd<1 nM) and a population of low-affinity sites (M_1, Kd>50 nM) (Araujo *et al.*, 1991). In the present study, to assess the effects of aging on M_3 muscarinic binding parameters in neostriatum, direct binding of [^{3}H]4-DAMP in neostriatal membranes of three age-groups of rats was done. [^{3}H]4-DAMP, at concentrations between 0.05 and 20 nM, was observed to label a single population of binding sites with relatively high affinity (Kd=0.9~2.5 nM) in rat neostriatal membrane fractions of all age-groups tested, and the number of the maximal binding sites increased with senescence. The failure to find a second class of [^{3}H]4-DAMP binding sites can be partly explained by the fact that concentration range of the radioligand used in the present study did not include the higher concentrations at which lower affinity binding sites should have been detected.

When considered in the context of previous studies (Lee *et al.*, 1991; Weiler and Lee, 1992), the present study also indicates that there are age-related changes in certain muscarinic receptor subtypes. Potassium-stimulated acetylcholine release in the presence of muscarinic receptor subtype-selective antagonists was not consistent across all age-groups. The neostriatal acetylcholine release response in the presence of pirenzepine and 4-DAMP was greater in the 28-month rats than in the 3-month F344 rats. The release response in the presence of methoctramine was greater in the tissue from 3-month rats than in that from the 28-month rats. Likewise, the binding experiments indicate that shifts in the muscarinic receptor subpopulation are occurring during senescence. Some subpopulations are decreasing (M_1, as defined by [^{3}H]pirenzepine binding), others are increasing (M_3, as defined by [^{3}H]4-DAMP binding), while still others are relatively unchanged (M_2, as defined by [^{3}H]AF-DX 384 binding). Taken in the larger context of the previous release studies and present binding studies, it can be con-

cluded that in the rat neostriatum an age-related change in muscarinic receptor population is not consistent for all muscarinic receptor subtypes. Instead, the density of a given muscarinic receptor subtype might be increasing, decreasing, or remain unchanged relative to the other subtype represented in a given brain region.

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REFERENCES

Araujo, D. M., Lapchak, P. A., Meaney, M. J., Collier, B. and Quirion, R. (1990). Effects of aging on nicotinic and muscarinic autoreceptor function in the rat brain: Relationship to presynaptic cholinergic markers and binding sites. *J. Neurosci.* **10**, 3069-3078.

Araujo, D. M., Lapchak, P. A., Regenold, W. and Quirion, R. (1989). Characterization of [^{3}H] AF-DX 116 binding sites in the rat brain: Evidence for heterogeneity of muscarinic-M_2 receptor sites. *Synapse* **4**, 106-114.

Araujo, D. M., Lapchak, P. A. and Quirion, R. (1991). Heterogeneousbinding of [^{3}H]4-DAMP to muscarinic cholinergic sites in the rat brain: Evidence from membrane binding and autoradiographic studies. *Synapse* **9**, 165-176.

Bonner, T. I. (1989). New subtypes of muscarinic acetylcholine receptors. *Trends Pharmacol. Sci.* **10** (Suppl. Subtypes Muscarinic Recept. IV), 11-15.

Bonner, T. I., Buckley, N. J., Young, A. C. and Brann, M. R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. *Science* **237**, 527-532.

Bonner, T. I., Young, A. C., Brann, M. R. and Buckley, N. J. (1988). Cloning and expression of the an and rat muscarinic acetylcholine receptor genes. *Neuron* **1**, 403-410.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Buckley, N. J., Bonner, T. I., Buckley, C. M. and Brann, M. R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.* **35**, 69-476.

Castoldi, A. F., Fitzgerald, B., Manzo, L., Tonini, M. and Costa, L. G. (1991). Muscarinic M_2 receptors in rat brain labeled with [^{3}H]AF-DX 384.. *Res. Comm. Chem. Pathol. Pharmacol.* **74**, 371-374.

Consolo, S., Wang, J.-X., Fiorentini, F., Vezzani, A. and Ladinsky, H. (1986). In vivo and in vitro studies on the regulation of cholinergic neurotransmission in striatum, hippocampus and cortex of aged rats. *Brain Res.* **374**, 212-218.

Doods, H. N., Mathy, M.-J., Davidesko, D., Van Charldorp, K. J., De Jonge, A. and Van Zwieten, P. A. (1987). Selectivity of muscarinic antagonists in radioligand and in vivo experiments for the putative M_1, M_2 and M_3 receptors. *J. Pharmacol. Exp. Ther.* **242**, 257-262.

Dorje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschler, E. and Brann, M. R. (1991). Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.* **256**, 727-733.

Eberlein, W. G., Engel, W., Mihm, G., Rudolf, K., Wetzel, B., Entzeroth, M., Mayer, N. and Doods, H. N. (1989). Structure-activity relationships and pharmacological profile of selective tricyclic antimuscarinics. *Trends Pharmacol. Sci. Suppl.* **10**, 50-54.

Eglen, R. M. and Watson, N. (1996). Selective muscarinic receptor agonists and antagonists. *Pharmacol. Toxicol.* **78**, 59-68.

Entzeroth, M. and Mayer, N. (1990). Labeling of rat heart muscarinic receptor using the new M_2 selective antagonist [^{3}H]AF-DX 384. *Biochem. Pharmacol.* **40**, 1674-1676.

Entzeroth, M. and Mayer, N. (1991). The binding of [^{3}H]AF-DX 384 to rat ileal smooth muscle muscarinic receptors. *J. Receptor Res.* **11**, 141-152.

Guo, D. Z., Kameyama, K., Rinken. A. and Haga, T. (1995). Ligand binding properties of muscarinic acetylcholine receptor subtypes (m1-m5) expressed in baculovirus-infected insect cells. *J. Pharmacol. Exp. Ther.* **274**, 378-384.

Gurwitz, D., Egozi, Y., Henis, Y. I., Kloog, Y. and Sokolovsky, M. (1987). Agonist and antagonist binding to rat muscarinic receptors: influence of aging. *Neurobiol. Aging* **8**, 115-122.

Hulme, E. C., Birdsall, N. J. M. and Buckley, N. J. (1990). Muscarinic receptor subtypes. *Ann. Rev. Pharmacol. Toxicol.* **30**, 633-673.

Lapchak, P. A., Araujo, D. M., Quirion, R. and Collier, B. (1989). Binding sites for [^{3}H]AF-DX 116 and effect of AF-DX 116 on endogenous acetylcholine release from rat brain slices. *Brain Res.* **496**, 285-294.

Lazareno, S., Buckley, N. J. and Roberts, F. F. (1990). Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG108-15 cells. *Mol. Pharmacol.* **38**, 805-815

Lee, H.-J., Cozzi, N. V. and Weiler, M. H. (1991). Age-related differences in the effects of some muscarinic agents on acetylcholine release from rat neostriatal slices. *J. Pharmacol. Exp. Ther.* **258**, 496-501.

Liao, C. F., Themmen, A. P. N., Joho, R., Barberis, C., Birnbaumer, M. and Birnbaumer, L. (1989). Molecular cloning and expression of a fifth muscarinic acetylcholine receptor. *J. Biol. Chem.* **264**, 7328-7337.

Lippa, A. S., Critchett, D. J., Ehlert, F., Yamamura, H. I., Enna, S. J. and Bartus, R. T. (1981). Age-related alterations in neurotransmitter receptor: an electrophysiological and biochemical analysis. *Neurobiol. Aging* **2**, 3-8.

London, E. D., Waller, S. B., Ellis, A. T. and Ingram, D. K. (1985). Effects of intermittent feeding on neurochemical markers in aging rat brain. *Neurobiol. Aging* **6**, 100-104.

Michalek, H., Fortuna, S. and Pintor, A. (1989). Age-related differences in brain choline acetyltransferase, cholinesterase

and muscarinic receptor sites in two strains of rats. *Neurobiol. Aging* **10**, 143-148.

Michel, A. D., Stefanich, E. and Whiting, R. (1989). Direct labeling of rat M_3 receptors by [^{3}H]4DAMP. *Eur. J. Pharmacol.* **166**, 459-466.

Michel, A. D. and Whiting, R. (1990). The binding of [^{3}H]4-diphenylacetoxy-N-methylpiperidine methiodide to longitudinal ileal smooth muscle muscarinic receptors. *Eur. J. Pharmacol.* **176**, 197-205.

Michel, A. D. and Whiting, R. (1988). Methoctramine, a polymethylene tetra-amine, differentiates three subtypes of muscarinic receptor in direct binding studies. European. *J. Pharmacol.* **145**, 61-66.

Mitchelson, F. (1988). Muscarinic receptor differentiation. *Pharmac. Ther.* **37**, 357-423.

Morin, A. M. and Wasterlain, C. G. (1980). Aging and rat brain muscarinic receptors as measured by quinuclidinyl benzilate binding. *Neurochem. Res.* **5**, 301-308.

Mutschler, E. and Lambrecht, G. (1984). Selective muscarinic agonists and antagonists in functional tests. *Trends Pharmacol. Sci.* **Suppl. 5**, 39-43.

Narang, N. (1995). In situ determination of M_1 and M_2 muscarinic receptor binding sites and mRNAs in young and old rat brains. *Mech. Ageing Dev.* **78**, 221-239.

Nilvebracht, L. and Sparf, B. (1988). Receptor binding profiles of some selective muscarinic antagonists. *Eur. J. Pharmacol.* **151**, 83-96.

Norman, A. B., Blaker, S. N., Thal, L. and Creese, I. (1986). Effects of aging and cholinergic deafferentation on putative muscarinic cholinergic receptor subtypes in rat cerebral cortex. *Neurosci. Lett.* **70**, 289-294.

Ogawa, N., Asanuma, M., Kondo, Y., Nashibayashi, S. and Mori, A. (1994). Reduced choline acetyltransferase activity and muscarinic M_1 receptor levels in aged Fished 344 rat brains did not parallel there respective mRNA levels. *Brain Res.* **658**, 87-921.

Pedigo, N. W., Minor, L. D. and Krumrei, T. N. (1985). Cholinergic drug effects and brain muscarinic receptor binding in aged rats. *Neurobiol. Aging* **5**, 227-233.

Regenold, M., Araujo, D. M. and Quirion, R. (1989). Quantitative autoradiographic distribution of ^{3}H-AF-DX 116 muscarinic-M_2 receptor binding sites in rat brain. *Synapse* **4**, 115-125.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**, 660-672.

Schwartz, R. D., Bernabei, A. A., Spencer, C. J. and Pugsley, T. A. (1990). Loss of muscarinic M_1 receptors with aging in the cerebral cortex of Fischer 344 rats. *Pharmacol. Biochem. Behavior* **35**, 589-593.

Strong, R., Hicks, P., Bartus, R. T. and Enna, S. J. (1980). Age-related alterations in the rodent brain cholinergic system and behavior. *Neurobiol. Aging* **1**, 59-63.

Surichamorn, W., Kim, O. N., Lee, N. H., Lai, W. S. and Al-Fakahany, E. E. (1988). Effects of aging on the interaction of quinuclidinyl benzilate, N-methylscopolamine, pirenzepine, and gallamine with brain muscarinic receptors. *Neurochem. Res.* **13**, 1183-1191.

Thallarida, R. J. and Murray, R. B. (1987). Manual of pharmacological calculations with computer programs. Springer-Verlag, New York.

Waelbroeck, M., Gillard, M., Robberecht, P. and Christophe, J. (1987). Muscarinic receptor heterogeneity in rat central nervous system. I. Binding of four selective antagonists to three muscarinic receptor subclasses: a comparison with M_2 cardiac muscarinic receptors of the C type. *Mol. Pharmacol.* **32**, 91-99.

Waelbroeck, M., Tastenoy, M., Camus, J. and Christophe, J. (1990). Binding of selective antagonists to four muscarinic receptors (M_1 to M_4) in rat forebrain. *Mol. Pharmacol.* **38**, 267-273.

Waller, S. B. and London, E. D. (1989) Choline acetyltransferase activity and muscarinic binding in brain regions of aging Fischer-344 rats. *Neurochem. Int.* **14**, 483-490.

Wang, J. X., Roeske, W. R., Gulya, K., Wang, W. and Yamamura, H. I. (1987). [^{3}H]AF-DX 116 labels subsets of muscarinic cholinergic receptors in rat brain and heart. *Life Sci.* **41**, 1751-1760.

Wang, J. X., Roeske, W. R., Wang, W. and Yamamura, H. I. (1988). Himbacine recognizes a high affinity subtypes of M_2 muscarinic acetylcholine receptors in rat cerebral cortex. *Brain Res.* **446**, 155-158.

Watson, M., Roeske, W. R. and Yamamura, H. I. (1988). Decreased muscarinic receptor density in the aged rat brain. *Proc. West. Pharmacol. Soc.* **3**, 61-65.

Weiler, M. H. and Lee, H.-J. (1992). The muscarinic modulation of acetylcholine release from the rat neostriatum changes with senescence. In: Meyer, E. M., Simpkins, J. W., Yamamoto, J. and Crews, F. T. (eds) Treatment of Dementias: A New Generation of Progress. Plenum Press, New York, vol. **40**, pp. 35-42.