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Estrogen effects on object memory and cholinergic receptors in young and old female mice

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Abstract

We investigated whether object recognition memory is modulated by estrogen in young (5 month) and aged (24 month) female C57Bl/6J mice, and if cholinergic muscarinic receptors might contribute to this response. Mice that were ovariectomized, or ovariectomized plus estradiol-treated three weeks before behavioral testing or quantitative autoradiography were compared to intact mice. Memory for a previously encountered object deteriorated significantly between 3 and 6h after initial exposure, regardless of animal age. In both young and aged mice, estradiol-treated mice showed significantly greater recall than did ovariectomized mice. In both age groups, the apparent number of [^{3}H]pirenzepine/M_1-like and [^{3}H]AFDX384/M_2-like muscarinic receptor binding sites was reduced in the basal forebrain as well as its projection areas following ovariectomy, but this decrease was not alleviated by estrogen. Aging poorly affected object memory, but reduced muscarinic binding in some cortical subregions and in the caudate nucleus. These findings suggest that estrogen effects on memory in C57Bl/6J mice are not due to changes in the number of muscarinic receptors.

Keywords: Acetylcholine; Aging; Alzheimer's disease; Basal forebrain; Cerebral cortex; Cholinergic; Cognitive function; Hippocampus; Muscarinic receptor; Object recognition; Working memory

1. Introduction

In a recent study, we reported that a 30 days of estradiol (E_2) treatment improved working memory in young as well as aged ovariectomized (OVX) C57Bl/6J mice in the spontaneous alternation T-maze test [29]. This effect was accompanied by an increase in the number of basal forebrain (BF) neurons immunostained for both choline acetyltransferase (ChAT) and estrogen α-receptor. These results were consistent with the idea that estrogen maintains normal brain functioning, especially in regard to cognitive performance, in humans [37] as well as in animals [19,27]. This hypothesis is based on the observed reduced cognitive abilities of subjects following menopause or ovariectomy [36, 42]. In animal models, gonadectomized rodents perform more poorly than E_2-treated gonadectomized rodents in various working memory paradigms. This is true for females [8,13,16,29] as well as males [24]. Working memory impairments are linked with cholinergic dysfunction and are ameliorated to some extent by restoring cholinergic activity [26,34]. Likewise, damage to cholinergic projections produces attention, learning and memory deficits [12,34]. Interestingly, cholinergic parameters such as ChAT mRNA levels [17], ChAT activity [25,39,43], acetylcholine release [21], and choline uptake [39] increased in BF-related structures after E_2 administration in OVX animals.

In spite of these data linking memory, estrogen and acetylcholine, there have been few studies exploring both

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memory and estrogen in relation to cholinergic function. In rats, estradiol microinjected into the hippocampus following a session of trials in a spatial water maze improves performance [32]. This effect is blocked by scopolamine, a muscarinic antagonist. Conversely, systemic injections of scopolamine disrupt the performance of ovariectomized rats in a T-maze alternation task but a three days pre-treatment with E_2 restores performance to normal [9,13]. The ability of estrogen to attenuate the effect of scopolamine has also been shown on acquisition of the multiple-trial passive avoidance paradigm [20].

The majority of the above studies were performed in young animals. The effects of E_2 on aged animals, which would represent a more appropriate model for changes occurring during menopause, have not been investigated. There have been several studies of muscarinic receptor number in the aging rat, suggesting that there may be an age-dependent decrease in muscarinic receptors [2,3,5,28,35]. However, there has been only one cholinergic receptor binding study performed in female mice [16], and none on the effects of estrogen. The aim of the present study, was 1) to evaluate the influence of estrogen status on memory performance, 2) to see if this influence was affected by the animal age, and 3) to establish if the effects observed in both cases could be related to a change in muscarinic receptor number in cerebral regions related to memory. We examined the effect of estrogen on a new non-spatial working memory paradigm, the object recognition test, which has already been shown to be sensitive to cholinergic treatments [11] in young (5 months old) and aged (22–24 months old) C57Bl/6J mice. We then quantified [^{3}H]pirenzepine/M_1-like and [^{3}H]AFDX384/M_2-like muscarinic receptor binding in different cerebral regions using quantitative autoradiography.

2. Materials and methods

2.1. Subjects

All protocols were approved by the McGill University and Royal Victoria Hospital Animal Care Utilization Committee and carried out in accordance with the requirements of the Canadian Council on Animal Care. Young (5 months) and old (22–24 months) female C57Bl/6J mice (Jackson Labs, Bar Harbor, Maine) were either gonadally-intact, OVX or OVX and E_2 (estratien-3,17-β-diol Sterloids Inc., Witten, NH, USA) treated (OVX/E_2). Mice were maintained in an isolated, pathogen-free colony with a 12-h light/dark photoperiod and food and water ad libitum. In normal animals, reproductive cyclicity patterns were established by daily vaginal smears. Young intact mice showed all stages of the reproductive cycle and old intact mice were in persistent diestrus. Surgery was performed during diestrus 21 days prior to behavioral testing and tissue collection as previously described [29]. Under anesthesia with ketamine (8.5 mg/100 *g*) and xylazine (0.3 mg/100 *g*) *i.m*, bilateral OVX surgery was performed using a posterior surgical approach and 5 mm silastic capsules containing E_2 crystals mixed in silastic adhesive were then implanted subcutaneously (s.c.) in the nape of the neck for OVX/E_2 animals. These capsules produce mean circulating levels of E_2 of 12 to 18 ng/ml, i.e. typical of diestrus animals, whereas levels of estradiol for OVX animals were undetectable [22]. The completeness of gonadectomy and efficiency of estrogen treatment was determined by the appearance of vaginal cytological smears exhibiting the absence or presence of mature epithelial cells, respectively. The young intact animals showing a cyclical pattern of estrus were tested in a randomized way for behavioral testing. They were sacrificed during diestrus for autoradiography experiments. Post-mortem uterus weight was taken as an index of the effect of estrogen deprivation (OVX) or supplementation (E_2 treatment) and was significantly reduced ($P < 0.05$) in OVX group (44 $\pm$ 1 mg, mean $\pm$ s.e.m) compared to intact (76 $\pm$ 2 mg) or OVX/E_2 mice (64 $\pm$ 3 mg).

2.2. Object recognition test

Six groups of female C57Bl/6J mice were tested for object recognition: young intact ($n = 12$), young OVX ($n = 12$), young OVX/E_2 ($n = 10$), old intact ($n = 25$), old OVX ($n = 16$) and old OVX/E_2 ($n = 13$). The object recognition test [10] was modified to make the test suitable for mice, and to allow two memory tests in each mouse. Mice were tested in clear plastic cages (36 cm L $\times$ 22 cm W $\times$ 25 cm H) placed on a tabletop in the mouse colony room. For each animal, two pairs of objects were selected at random from a set of four objects differing in shape, surface color, contrast and texture. These four objects had been selected from a larger pool of objects on the criterion that mice would spend approximately equal amounts of time exploring each of them. Mice were first habituated to the apparatus and room environment in 9 daily sessions of 15 min. For the first two sessions: animals were placed in the apparatus with their cage mates for 15 min. For the next 7 sessions: animals were placed singly in the apparatus.

On the test day, the 2 objects were placed on the centerline of the long axis of the floor, 5 cm from each end. Mice were allowed to become familiar with the first pair of objects for 10 min. After a delay of 3 h, mice were exposed to one of the original objects and one member of the second pair of objects for 5 min. The amount of time mice spent exploring each object was measured. Exploratory behavior was recorded if the animal touched the object with its forepaw or nose, or if the animal sniffed at the object within a distance of 1.5 cm. Longer time of exposure (i.e. 10 or 15 min) have been shown to provide same the same proportion of exploratory behavior for new or old objects (data not shown, pilot experiments). Six h after the initial exposure, mice were allowed to explore the other one of the original pair of objects and the remaining novel member of the second pair of objects for five min. The amount of time mice spent exploring each object was mea-

sured. After each exposure, the objects were wiped with 70% ethanol and the cage was sprayed and wiped with Cidex to eliminate odor cues. A Memory Index (MI) was calculated from the time (t) spent exploring each object where 'o'≈ represents an object seen in the original exposure and 'n' ≈ an object that is novel on the re-exposure.

$$MI = (t_n - t_o)/(t_n + t_o)$$

2.3. Autoradiographic visualization of cholinergic receptors

Muscarinic M_1-like and M_2-like receptor binding were investigated using quantitative receptor autoradiography with the ligand [^{3}H]pirenzepine (79.5 Ci/mmol) and [^{3}H]AF-DX 384 (106 Ci/mmol), respectively (New England Nuclear, Boston, MA, USA) as described in [4]. Mice (n = 4 for intact and OVX groups, n = 6 for OVX/E2 groups, distinct from the one used for behavioral experiments) were decapitated and their brains were rapidly removed, immediately frozen in 2-methylbutane at −40°C and stored at −80°C until further use. Coronal sections (20 μm thick) were cut at −18°C on a cryostat (H 500 M, Microm, Germany), thaw-mounted onto alternate gelatin coated slides, desiccated overnight at 4°C, and stored at −80°C until use. Brain sections were equilibrated at room temperature (5 min) before preincubation (15 min) in Krebs buffer (NaCl, 120 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; glucose, 5.6 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.5 mM; KCl, 4.7 mM, pH 7.4). Sections were then incubated (1 h, 22°C) in fresh Krebs buffer containing either 10 nM [^{3}H]pirenzepine or 2 nM [^{3}H]AF-DX 384. Alternate sections were incubated with 1 μM atropine (Sigma, St Louis, MO, USA), a non-selective muscarinic antagonist, to determine the level of specific binding. Slides were then transferred sequentially through three rinses (4 min each) in Tris HCl buffer (50 mM, pH 7.4) at 4°C followed by a rapid dip in cold distilled water. Sections were dried and juxtaposed to films alongside with ^{3}H-labeled standards (Amersham, Oakville, ON, Canada) for 15 days ([^{3}H]pirenzepine) or 3 weeks ([^{3}H]AF-DX 384). Specific receptor binding (expressed in fmol/mg tissue equivalent) was quantified from autoradiograms using computerized image analysis (MCID-M4 version 3.0, Mississauga, ON, Canada). Nine cerebral regions were selected according to their involvement in object memory processes (perirhinal and entorhinal cortices, hippocampus and basal forebrain) or as control structures (parentheses represent corresponding figures of the atlas of the mouse brain [15]): motor cortex (Fig. 22–26), somatosensory cortex (S_2, Fig. 33–43), perirhinal cortex (Fig. 46–50), visual cortex (Fig. 51–55), entorhinal cortex (Fig. 56–60), hippocampal formation (Fig. 46–50), caudate nucleus (Fig. 23–29), medial septum and diagonal band of Broca (Fig. 22–25).

2.4. Statistical analysis

A three way ANOVA was used to determine the significant interactions between the age, the estrogen status and the timing of the behavioral experiment. Subsequent two ways ANOVA followed by post-hoc multiple comparisons (Tukey HSD) was performed separately on the two age groups to determine the statistical significance of differences between estrogen status and timing. For the autoradiographic study, a two way ANOVA followed by Tukey test was used to evaluate the statistical significance ($P < 0.05$) of difference between treatment and age groups for each brain region.

3. Results

3.1. Object memory

Fig. 1 shows the histograms of memory performance of the young (Fig. 1A) and aged (Fig. 1B) mice for the different estrogen status groups at 3h and 6h after the initial exposure to the objects. Three way ANOVA analysis indicated that 1) memory for a previously encountered object deteriorated between the 3h and 6h after the initial exposure (main effect $F(1,82) = 9.904$, $P < 0.002$), and 2) memory performance was better in estrogen treated ovariectomized mice (main effect, $F(2,82) = 11.365$, $P < 0.001$). The age of the subjects did not affect the rate at which memory deteriorated between 3 and 6 h after the initial exposure (interaction $F(2,82) = 0.263$, ns). Because the intact groups are not identical in the young (normally cycling) and aged (in persistent diestrus) mice, we also analyzed the effect of estrogen status on memory for each group separately. Young OVX mice treated with estrogen showed greater recall than the estrogen-deficient OVX mice did ($\alpha = 0.05$ HSD). The scores of young intact mice fell in between, and neither OVX group differed significantly from young intact mice. Old OVX mice treated with estrogen showed greater recall than old estrogen-deficient OVX mice and old intact mice with non-functional ovaries ($\alpha = 0.05$ HSD). Estrogen treatment also affected the overall exploratory activity in both age groups, but in a way that was not related to the effect on object memory. Exploratory activity was almost the same at 3 h and 6 h after the original exposure ($F(1,82) = 0.441$, ns). Accordingly, these data were collapsed for further analysis. The total activity was not affected by age ($F(1,82) = 0.173$, ns) but OVX mice without estrogen showed more exploratory behavior than intact mice or OVX/E_2 mice ($F(2,82) = 3.726$, $P < 0.03$).

3.2. [^{3}H]-pirenzepine/muscarinic/M_1-like binding sites

The regional distribution of [^{3}H]pirenzepine/M_1-like binding sites in the intact C57Bl/6J female mice was similar to that already reported in the male rat [4], i.e. the main

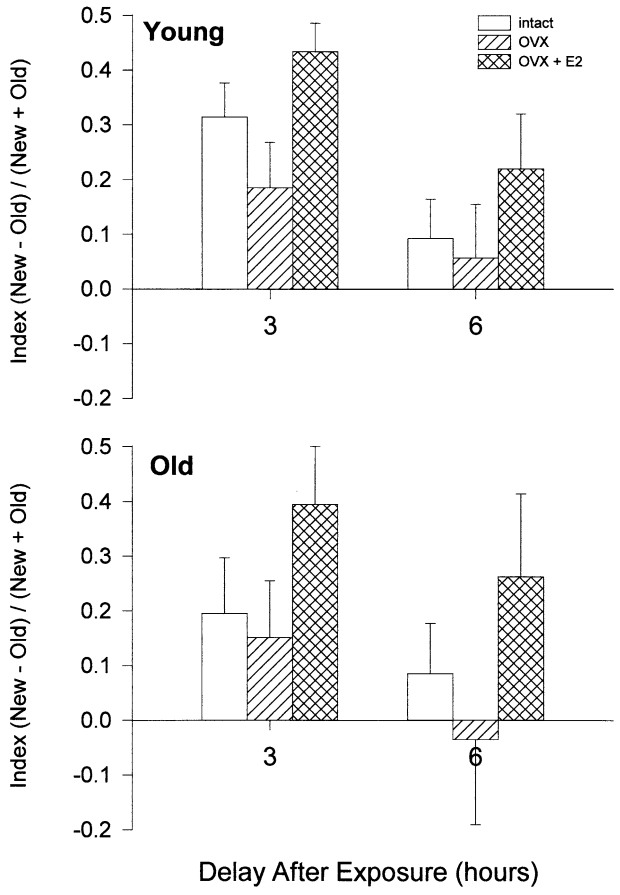

Fig. 1. Mean object memory index scores at 3 and 6 h after learning for young (5 months) and old (22–24 months) C57BL/6J female mice that were intact, ovariectomized or ovariectomized and estrogen treated.

regions showing significant binding level (ranging from 80 to 180 fmol/mg tissue equivalent) were the neocortex, hippocampal formation and caudate nucleus (Fig. 2, Table 1). Moreover, we report here that in the neocortex, layer I showed the greatest level of [^{3}H]pirenzepine/M_1-like binding sites compared to the other layers. Other regions, such as the thalamus or basal forebrain, demonstrated only negligible levels of [^{3}H]pirenzepine/M_1-like binding sites.

Ovariectomy affected [^{3}H]pirenzepine/M_1-like binding in the cerebral cortex of both young (Fig. 2) and aged animals. Except for layer I of the perirhinal cortex which was not affected by ovariectomy, [^{3}H]pirenzepine/M_1-like binding was significantly decreased (up to −30%, $P < 0.05$) in OVX animals compared to the intact groups. E_2 treatment alleviated the decrease in layer I and layer IV-V of the somatosensory cortex, but not in other regions. Ovariectomy produced no significant changes in the caudate nucleus or the CA1 and CA3 subfield of the hippocampal formation.

Aging induced a decrease in [^{3}H]pirenzepine/M_1-like binding sites ($P < 0.05$) in caudate nucleus, and in motor and somatosensory cortices (Fig. 3). These changes were not seen in visual, perirhinal, and entorhinal cortices or in the hippocampus. There was a trend toward estrogen's ability to prevent the decrease in [^{3}H]pirenzepine/M_1-like binding sites after ovariectomy in young animals but not old animals. However, the interaction failed to reach significance.

3.3. [^{3}H]-AFDX 384/muscarinic M_2-like binding sites

The regional distribution of [^{3}H]AFDX 384/M_2-like binding in intact female mice was also similar to that reported in the male rat brain [4], and high levels of M_2-like binding sites were located in the neocortex, caudate nucleus and basal forebrain (septum and diagonal band of Broca). We showed here that in contrast to M_1-like binding sites, M_2-like binding was more densely located in layers II and IV-V of the neocortex.

OVX induced a significant ($P < 0.05$) decrease in [^{3}H]AFDX 384/M_2-like binding (up to −30%) in all the regions studied (Fig. 4, Table 2) including the neocortex, hippocampal formation and basal forebrain in both age groups. This decrease was not alleviated by E_2 treatment except in the deep layers of the motor and somatosensory cortices and in perirhinal cortex ($P < 0.05$). Overall, aged animals had fewer [^{3}H]AFDX-384/M_2-like binding sites ($P < 0.05$) in the caudate nucleus, basal forebrain and layer IV-V of the somatosensory and motor cortices (Fig. 3). However, as for [^{3}H]pirenzepine/M_1-like binding, no statistical interaction between treatment and age of the animals was detected.

4. Discussion

The present study was designed to examine the influence of estrogen status on non-spatial working memory in young and old female C57Bl/6J mice, and the possible contribution of muscarinic receptors to these changes. OVX mice showed slightly reduced performance in the object recognition task in both age groups. Regardless of age, OVX also produced a significant decrease in the level of [^{3}H]pirenzepine/M_1-like binding sites in the cortex and hippocampus, and a significant decrease in the amounts of [^{3}H]AFDX-384/M_2-like binding in all regions except the deep layers of the perirhinal cortex. E_2 treated mice showed better cognitive performance in both young and aged groups but this was not correlated with OVX or E_2 induced changes in muscarinic receptor levels. Aging had little effect on object recognition performance, although the levels of muscarinic receptors significantly decreased in many structures in 22–24 months old mice. The results suggest that alterations in muscarinic receptor number do not play a significant role in the facilitatory effects of E_2 on object recognition in aged animals.

4.1. Estrogen status and working memory

Our results show a trend in memory performance to be poorer after 21 days OVX compared to intact animals, but this did not reach statistical significance. The fact that intact mice were tested at random with respect to estrus cycle

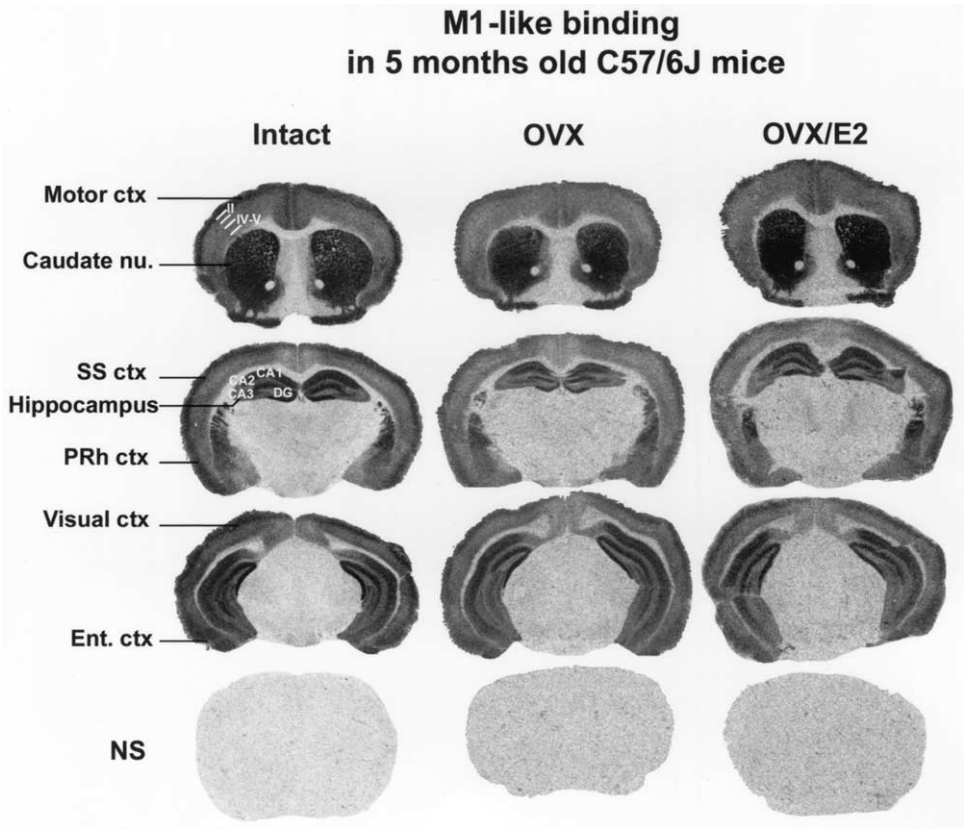

Fig. 2. Representative [^{3}H]pirenzepine/M_1-like receptor binding sites autoradiograms in intact, ovariectomized (OVX) and ovariectomized plus estrogen (OVX/E2) young C57/6J mice. 3 coronal sections at different antero-posterior levels are shown for each group to illustrate estrogen status effect on M_1-like binding in different brain structures related to memory. Bottom panel shows non-specific binding (NS). Ctx: cortex; SS, somatosensory; PRh, perirhinal; Ent., entorhinal.

might have increased the variability of memory scores in intact mice but it can be seen in Fig. 1 that this was not the case. On the other hand old intact mice or OVX mice, which had both had many months with low levels of estrogen, showed poor memory performance relative to young intact mice. It should be noted that OVX mice showed vigorous exploratory activity while intact and OVX/E_2 mice showed low levels of exploratory activity, as might be expected after 9 sessions of habituation in the apparatus. Although there was no correlation between the amount of exploratory activity and the memory index ($r = -0.08$, ns), the greater exploratory activity of E_2 deficient mice may reflect a failure to habituate to the apparatus.

We previously showed that working memory was affected by OVX in the spontaneous alternation task [29], and expected to obtain such a finding in the present behavioral experiment. However, other studies have also mentioned a lack of significant effects of OVX on memory tests in the relatively short-term after OVX, although effects were found after long-term OVX [39]. Thus, the effects of loss of ovarian hormones following OVX on cognition or neural systems may require some time to develop, and are only reliably found after long-term gonadectomy [39] and see [41]. In contrast, our results show that a facilitatory effect of estrogen on memory is present even before impairments due to estrogen deficiency are detectable. This suggests that estrogen may directly potentiate cognitive functioning rather than merely prevent cognitive decline due to estrogen deficiency. Likewise, it has been found that acute post-training injections of estrogen in the hippocampus can improve memory [32].

We have shown here that estrogen treated OVX mice had improved object memory as compared to OVX mouse in both young and old mice. Moreover, old E_2 treated OVX mice showed better object memory than old intact mice. Previous evidence for estrogen facilitation of memory in OVX animals has been found in tests of spatial working memory including spontaneous alternation [29], radial arm maze [24], delayed non matching to position [18], and Morris water maze. E_2 facilitation of memory has also been reported in reference memory tests such as active avoidance [39], and T-maze footshock avoidance [14]. Moreover, the object recognition test is based on the spontaneous, differential exploration of familiar and novel objects [10]. Since performance depends on non-spatial information that varies from trial to trial it would be classified as a test of non-spatial working memory. The object memory test involves different neuronal circuits than tests based on spatial recognition such as the majority of the maze tests [40]. It has been proposed that perirhinal, entorhinal and medial prefrontal cortical areas

Table 1
Levels of specific [^{3}H]-pirenzepine/M_1-like binding in intact, OVX and OVX/E2 mice

		5 months old intact	OVX	OVX/E2	24 months old intact	OVX	OVX/E2
Cerebral cortex							
Motor	Layer I	131 ± 4	106 ± 1*	116 ± 8*	121 ± 10†	99 ± 5*†	88 ± 5*†
	Layer II	91 ± 3	72 ± 1*	82 ± 4*	86 ± 6†	70 ± 4*†	63 ± 4*†
	Layer III	81 ± 2	64 ± 1*	73 ± 3*	74 ± 5†	62 ± 4*†	56 ± 4*†
	Layer IV-V	81 ± 2	64 ± 2*	75 ± 3*	70 ± 3†	57 ± 4*†	53 ± 4*†
Somatosensory	Layer I	150 ± 4	104 ± 2*	122 ± 5	144 ± 10	110 ± 5*	95 ± 3†
	Layer II	94 ± 1	66 ± 4*	71 ± 2*	86 ± 6†	65 ± 4*†	58 ± 3*†
	Layer III	86 ± 2	61 ± 2*	68 ± 3*	79 ± 5†	61 ± 4*†	56 ± 3*†
	Layer IV-V	92 ± 2	71 ± 2*	80 ± 2	82 ± 5	69 ± 4*	62 ± 4†
Perirhinal	Layer I	113 ± 2	104 ± 3	108 ± 3	115 ± 6	109 ± 3	103 ± 6
	Layer II	98 ± 2	80 ± 2*	84 ± 3*	93 ± 3	82 ± 2*	80 ± 5*
	Layer III	83 ± 2	67 ± 2*	75 ± 3*	83 ± 4	68 ± 2*	66 ± 3*
	Layer IV-V	81 ± 1	69 ± 2*	76 ± 3*	82 ± 6	66 ± 2*	64 ± 3*
Visual	Layer I	144 ± 1	107 ± 7*	102 ± 8*	142 ± 4	100 ± 6*	98 ± 4*
	Layer II	105 ± 6	73 ± 5*	75 ± 7*	98 ± 2	75 ± 5*	70 ± 3*
	Layer III	86 ± 3	60 ± 3*	61 ± 5*	83 ± 2	60 ± 3*	58 ± 2*
	Layer IV-V	96 ± 2	69 ± 6*	70 ± 6*	87 ± 2	64 ± 2*	65 ± 2*
Entorhinal		104 ± 3	80 ± 5*	76 ± 4*	104 ± 5	78 ± 3*	80 ± 7*
Subcortical structures							
Hippocampus	CA1	176 ± 5	151 ± 6	157 ± 10	165 ± 7	166 ± 12	144 ± 7
	CA2	173 ± 4	102 ± 4*	127 ± 8*	173 ± 12	124 ± 9*	115 ± 6*
	CA3	102 ± 3	70 ± 4	86 ± 4	101 ± 9	76 ± 4	73 ± 3
	dentate	178 ± 5	144 ± 7	158 ± 10	165 ± 13	150 ± 6	136 ± 6
Caudate nu.		164 ± 8	184 ± 4	181 ± 10	137 ± 13†	159 ± 6†	146 ± 7†

Values represent means ± sem of [^{3}H]-pirenzepine/M_1-like binding expressed in fmole/mg tissue equivalent in intact (n = 4), OVX (n = 4) and OVX/E2 (n = 6) young (5 months) and aged (24 months) C576J/Blmice.

Significant diference: *, vs. intact group ($p < 0.05$, two-way ANOVA); †, old vs. young equivalent ($p < 0.05$, two-way ANOVA)

are crucial for object memory, whereas spatial recognition involves hippocampal pathways in rats (see [40]). Thus, our results, together with those cited above, agree with recent human studies which suggest that estrogen is beneficial to a wide range of cognitive functions (see [37,38]). This suggests that the cognitive effects of estrogen are not limited to, or specific to, hippocampal pathways.

4.2. Estrogen and muscarinic receptor level

The muscarinic binding experiments were performed to see if these putative memory-related brain structures were sensitive to estrogen manipulation. Ovariectomy reduced muscarinic M_1-like and M_2-like binding sites in most regions examined, and the magnitude of this effect was similar in young and old female mice. Since a significant proportion of M_2 receptors are located on presynaptic nerve terminals of cholinergic and non-cholinergic neurons [31] the reduction of M_2 receptors is consistent with other evidence that cholinergic cells shrink after ovariectomy [29]. The loss of M_1-like binding sites may also be due to loss of post-synaptic elements such as dendrites and cell bodies [7,27]. However, except for one region, estrogen treatment failed to prevent either M_1-like or M_2-like binding decrease, while it apparently does prevent loss of cholinergic cells [29] and neurites [30]. This suggests that the change in cholinergic receptors is not simply due to atrophy of cholinergic cells, nor is it directly due to the loss of estrogen. Another possibility is that ovarian testosterone plays some role. Ovariectomy and menopause lead to a loss of testosterone as well as estrogen, and there is evidence that testosterone may be important in the maintenance of cognitive function and mood after menopause [6]. The possibility that effects of testosterone on cholinergic receptors may play a role in these effects needs to be investigated further.

4.3. Aging

Aging in animal and human females is accompanied by a loss of ovarian function, even if the deficit in estrogen is not as dramatic in rodents as in humans [23]. As the majority of the estrogen administration experiments are performed in young animals, one of our goals was to determine if estrogen might have differential effects on neuronal function depending on the age of the animal, especially in

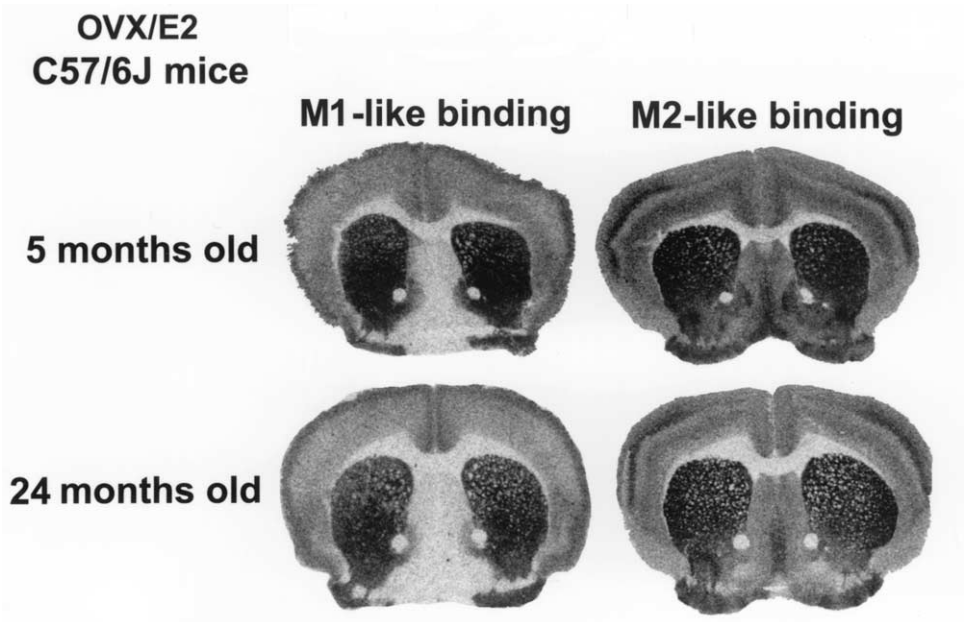

Fig. 3. Representative [^{3}H]pirenzepine/M_1-like (left) and [^{3}H]AF-DX384/M_2-like (right) receptor binding sites autoradiograms in ovariectomized plus estrogen (OVX/E2) young (upper panel) and old (bottom panel) C57/6J mice. One coronal section at the level of motor cortex and basal forebrain is shown as an example of age effect on muscarinic receptor binding.

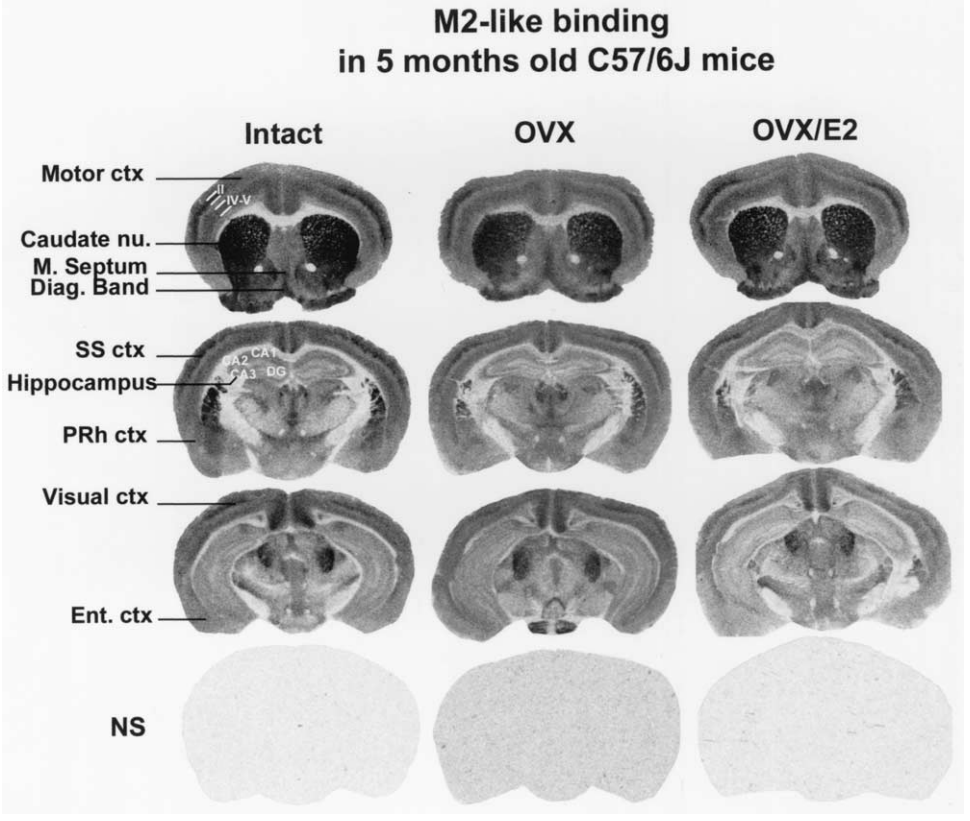

Fig. 4. Representative [^{3}H]AF-DX384/M_2-like receptor binding sites autoradiograms in intact, ovariectomized (OVX) and ovariectomized plus estrogen (OVX/E2) young C57/6J mice. 3 coronal sections at different antero-posterior levels are shown for each group to illustrate estrogen status effect on M_2-like binding in different brain structures related to memory. Bottom panel shows non-specific binding (NS). Ctx: cortex; SS, somatosensory; PRh, perirhinal; Ent., entorhinal; M. Septum, medial septum; Diag. Band, diagonal band of Broca.

Table 2
Levels of specific [^{3}H]-AFDX-384/M_2-like binding in intact, OVX and OVX/E2 mice

		5 months old intact	OVX	OVX/E2	24 months old intact	OVX	OVX/E2
Cerebral cortex							
Motor	Layer I	76.4 ± 4	57 ± 1*	65 ± 3*	85 ± 4	57 ± 1*	67 ± 5*
	Layer II	92 ± 3	68 ± 1*	78 ± 4*	106 ± 7	67 ± 2*	78 ± 4*
	Layer III	82 ± 4	61 ± 1*	69 ± 3*†	82 ± 2	58 ± 2*	67 ± 4*†
	Layer IV-V	104 ± 3	81 ± 1*	87 ± 4*	93 ± 2†	68 ± 3*†	75 ± 3*†
Somatosensory	Layer I	83 ± 2	65 ± 2*	75 ± 5*	93 ± 2	67 ± 2*	74 ± 4*
	Layer II	117 ± 4	94 ± 4*	110 ± 9*	130 ± 4	91 ± 3*	102 ± 5*
	Layer III	83 ± 3	61 ± 2*	67 ± 3*	81 ± 3	59 ± 3*	64 ± 3*
	Layer IV-V	104 ± 3	79 ± 2*	87 ± 4*	96 ± 2†	69 ± 4*†	76 ± 4*†
Perirhinal	Layer I	53 ± 1	42 ± 1*	49 ± 4	54 ± 1	45 ± 2*	50 ± 2
	Layer II	59 ± 2	46 ± 1*	55 ± 5	59 ± 1	50± 2*	55 ± 2
	Layer III	61 ± 1	49 ± 1*	58 ± 5	60 ± 2	53 ± 2*	56 ± 2
	Layer IV-V	78 ± 1	64 ± 2	72 ± 6	72 ± 1	64 ± 3	69 ± 2
Visual	Layer I	83 ± 3	60 ± 3*	67 ± 6*	85 ± 2	67 ± 1*	73 ± 5*
	Layer II	128 ± 4	82 ± 3*	98 ± 10*	123 ± 3	95 ± 2*	105 ± 10*
	Layer III	89 ± 4	61 ± 3*	69 ± 4*	87 ± 2	65 ± 1*	70 ± 5*
	Layer IV-V	94 ± 2	70 ± 3*	78 ± 5*	86 ± 1	69 ± 1*	77 ± 6*
Entorhinal		70 ± 4	44 ± 3*	50 ± 4*	63 ± 1	47 ± 2*	52 ± 4*
Subcortical structures							
Hippocampus	CA1	66 ± 3	43 ± 2*	53 ± 4*	61 ± 1	45 ± 3*	51 ± 4*
	CA2	67 ± 6	43 ± 2*	48 ± 3*	62 ± 2	46 ± 3*	45 ± 2*
	CA3	58 ± 2	39 ± 2*	44 ± 2*	53 ± 1	39 ± 4*	40 ± 2*
	dentate	49 ± 2	32 ± 2*	37 ± 3*	46 ± 1	35 ± 2*	34 ± 3*
Caudate nu.		258 ± 9	170 ± 7*	162 ± 8*	241 ± 18†	128 ± 5*†	151 ± 16*†
Medial septum		132 ± 7	112 ± 2*	113 ± 6*	113 ± 6†	90 ± 3*†	97 ± 5*†
Diagonal Band		138 ± 6	111 ± 4*	121 ± 3*	116 ± 6†	93 ± 10*†	103 ± 5*†

Values represent means ± sem of [^{3}H]-AFDX384/M_2-like binding expressed in fmole/mg tissue equivalent in intact (n = 4), OVX (n = 4) and OVX/E2 (n = 6) young (5 months) and aged (24 months) C576J/BI mice.

Significant difference: *, vs. intact group ($p < 0.05$, two-way ANOVA); †, old vs. young equivalent ($p < 0.05$, two-way ANOVA); ‡, OVX/E2 vs. OVX ($p < 0.05$, two-way ANOVA)

models of menopause. None of the experiments showed statistical interaction between age and estrogen treatment. Moreover, our findings showed that aging did not drastically decrease memory performance of the mice tested for object recognition, and affected muscarinic receptor binding levels in only or few brain regions, including motor and somatosensory cortex, caudate nucleus for M_1-like, and basal forebrain and caudate nucleus for M_2-like receptor binding. Consistent with our behavioral data for C57Bl/6J mice, object memory was not reduced in old male rats [1] or old primates [33]. Moreover, our muscarinic receptor binding data are consistent with previous studies which suggest an age-dependent decrease in muscarinic receptors in male rats [3,28] as well as female mice [16]. Taken together, these results suggest that aging, per se, does not affect the response to estrogen stimulation in regard to object recognition or to the number of muscarinic receptors.

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