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Cannabinoid CB_1 receptor-mediated inhibition of hippocampal acetylcholine release is preserved in aged mice

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1 The cannabinoid CB_1 receptor inverse agonist/antagonist SR 141716 increases acetylcholine release in rodent hippocampus and improves memory in some experimental paradigms. Since drugs like SR 141716 may represent a novel class of cognition-enhancing drugs, we wanted to check whether the function of the CB_1 receptor is preserved during ageing.

2 Hippocampal and striatal slices from 2- to 3- and 24- to 28-month-old C57BL/6J mice were preincubated with [^{3}H]-choline or [^{3}H]-noradrenaline ([^{3}H]-NA) and superfused.

3 The cannabinoid receptor agonist WIN 55,212-2 inhibited, and SR 141716 facilitated, the electrically (3 Hz) evoked tritium overflow in hippocampal slices (preincubated with [^{3}H]-choline) from young and aged mice to the same extent. The evoked overflow *per se* was less by 33% in slices from aged animals.

4 WIN 55,212-2 and SR 141716 did not affect, but the muscarinic receptor agonist oxotremorine inhibited, the evoked (3 Hz) overflow in striatal slices (preincubated with [^{3}H]-choline) from young and aged mice to the same extent. The evoked overflow *per se* tended to be less in slices from aged animals.

5 The evoked (0.3 Hz) overflow in hippocampal slices (preincubated with [^{3}H]-NA) was not affected by WIN 55,212-2 and SR 141716, but was inhibited by histamine (via H_3 receptors) in slices from young mice and, to a somewhat less extent, in slices from aged mice. The evoked overflow *per se* did not differ between age groups.

6 In conclusion, the function of the CB_1 receptor involved in the tonic inhibition of hippocampal acetylcholine release is preserved in aged mice.

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Abbreviations: [^{3}H]-NA, [^{3}H]-noradrenaline; PSS, physiological salt solution

Introduction

Hashish/marijuana and agonists at cannabinoid CB_1 receptors impair learning and memory in humans and animals (for review, see Ameri, 1999; Sullivan, 2000). Two lines of evidence suggest that the endocannabinoid system is involved in cognitive processes. First, the CB_1 receptor inverse agonist/antagonist SR 141716 facilitates memory tasks in some behavioural paradigms (Terranova *et al*., 1996; Lichtman, 2000). Second, in each of the three CB_1 receptor knockout mice, marked alterations of learning were observed (Reibaud *et al*., 1999; Marsicano *et al*., 2002; Varvel & Lichtman, 2002).

CB_1 receptors are frequently located presynaptically on nerve endings, where their activation results in inhibition of release of the respective neurotransmitter (for review, see Schlicker & Kathmann, 2001; Howlett *et al*., 2002). CB_1 receptor activation also causes inhibition of acetylcholine release in the hippocampus of rodents, whereas SR 141716 or CB_1 receptor deficiency increases the release of this transmitter (for review, see Schlicker & Kathmann, 2001). Thus, the possibility has to be considered that the septohippocampal cholinergic system, the morphological and functional integrity of which is important for learning and memory (Dutar *et al*., 1995), is implicated in the detrimental effects of cannabinoids on cognitive processes. Even if this should not hold true, the fact that CB_1 receptor inverse agonists/antagonists (e.g. SR 141716), like the reversible cholinesterase inhibitors (e.g. donepezil; for review, see Fodero & Small, 2002), increase synaptic acetylcholine levels might mean that CB_1 receptor inverse agonists/antagonists might represent a new class of cognition-enhancing agents for use in humans. In this context, it is of interest that CB_1 receptor-mediated inhibition of acetylcholine release and its facilitation by SR 141716 have recently also been shown in superfused slices of the human brain (Steffens *et al*., 2002).

Since cognitive decline in humans most frequently occurs in aged individuals, the question has to be addressed whether the molecular substrates for potential cognition-enhancing drugs retain their function during the ageing process. With respect to CB_1 receptors, this aspect has so far been studied only rarely. An age-induced reduction of cannabinoid receptor binding and mRNA levels has been shown in the striatum and other extrapyramidal brain regions (Mailleux & Vanderhaeghen, 1992; Romero *et al*., 1998), but is less marked in other brain regions including the cerebral cortex or the hippocampus

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(Berrendero *et al*., 1998). In the study of Romero *et al.* (1998), a functional parameter, the (CB_1 receptor-mediated) stimulation of [^{35}S]-guanylyl-5′-*O*-(γ-thio)-triphosphate binding by WIN 55,212-2, was also studied in extrapyramidal areas and was found to be decreased in some brain regions.

The aim of the present study was to check whether the function of the CB_1 receptors involved in the inhibition of hippocampal acetylcholine release is preserved in aged mice of the C57BL/6 strain (frequently used for studies of the ageing brain; for review, see Jucker & Ingram, 1997). We examined the effects of cannabinoid receptor ligands on the electrically evoked tritium overflow from superfused hippocampal slices preincubated with [^{3}H]-choline. For the sake of comparison, the electrically evoked tritium overflow from striatal slices preincubated with [^{3}H]-choline and from hippocampal slices preincubated with [^{3}H]-noradrenaline and its modulation via presynaptic receptors were studied as well.

Methods

Superfusion studies

Hippocampal or striatal slices (0.3 mm thick, 2 mm diameter) were prepared from C57BL/6J mice of either sex (purchased from Jackson, Bar Harbor, ME, U.S.A. and further bred in our department). In each experiment, slices from a young adult (2 – 3 months) and aged mouse (24 – 28 months) were directly compared to each other. The slices were incubated for 30 min with physiological salt solution (PSS) at 37°C containing [^{3}H]-choline 100 nM or [^{3}H]-noradrenaline ([^{3}H]-NA) 25 nM. The PSS composition was as follows (mM): NaCl 118, KCl 4.8, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.3 (if not stated otherwise), glucose 10, ascorbic acid 0.06, Na_2EDTA 0.03; it was aerated with 95% O_2 and 5% CO_2.

After incubation, slices were superfused with PSS at 37°C for 110 min at a flow rate of 1 ml min^{-1}. The superfusate was collected in 5-min samples. Tritium overflow was evoked by two 2-min periods of electrical field stimulation 40 (S_1) and 90 min (S_2) after onset of superfusion. For hippocampal or striatal slices preincubated with [^{3}H]-choline, stimulation parameters were 3 Hz, 200 mA, 2 ms and the PSS used for superfusion contained $CaCl_2$ at a concentration of 3.25 mM and hemicholinium-3 at 10 μM. For hippocampal slices preincubated with [^{3}H]-NA, the stimulation parameters were 0.3 Hz, 50 mA, 2 ms and the PSS used for superfusion ($CaCl_2$ concentration 1.3 mM) contained desipramine 1 μM and rauwolscine 1 μM. WIN 55,212-2, SR 141716, oxotremorine or histamine was added to the PSS from 62 min of superfusion onward. At the end of superfusion, the radioactivities of the (solubilized) slices and the superfusate samples were determined by liquid scintillation counting.

Tritium overflow was calculated as the fraction of the tritium content in the slices at the beginning of the respective collection period (fractional rate of tritium efflux). Basal tritium efflux was quantified by calculating the ratio of the fractional rate in the 5-min period immediately before S_2 (i.e. from 85 to 90 min; t_2) over that in the collection period from 55 to 60 min (t_1). To determine the tritium content of the slice, for example, 85 min after onset of superfusion, the amounts of radioactivity in the slice at the end of superfusion and in the five 5-min superfusate samples collected from 85 to 110 min were added. The electrically evoked tritium overflow was calculated by subtraction of basal from total efflux during stimulation and the subsequent 13 min and expressed as per cent of the tritium present in the slice at the onset of stimulation (basal tritium efflux was assumed to decline linearly from the 5-min collection period before to that 15 – 20 min after onset of stimulation). To quantify the stimulated tritium overflow, the tritium overflow evoked by S_1 or the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined. As a measure for tritium accumulation, the mean of the tritium content of all (usually five) slices prepared from the same animal was determined at the end of superfusion.

Statistical analysis

Results are given as mean ± s.e.m. of n experiments. One value (t_1, S_1, tritium accumulation) or one to two values (t_2/t_1, S_2/S_1) were obtained from one animal. Student's t-test was used for comparison of mean values; if more than one experimental series was compared to the same control, the Bonferroni correction was applied.

Drugs used

[Methyl-^{3}H]-choline chloride (specific activity 75.0 – 86.0 Ci mmol^{-1}), (*R*)-(−)-[ring-2,5,6-^{3}H]-noradrenaline (specific activity 56.3 Ci mmol^{-1}) (NEN, Zaventem, Belgium); desipramine hydrochloride (Ciba-Geigy, Wehr, Germany); hemicholinium-3 (ChemCon, Freiburg, Germany); histamine dihydrochloride, oxotremorine, WIN 55,212-2 (*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-yl](1-naphthalenyl)methanone mesylate) (Sigma, München, Germany); rauwolscine hydrochloride (Roth, Karlsruhe, Germany); SR 141716 (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; Sanofi, Montpellier, France). Drugs were dissolved in DMSO (SR 141716, WIN 55,212-2), ascorbic acid (1 mg ml^{-1}; histamine) or water (other drugs) and diluted with PSS to obtain the concentration required. Diluted DMSO and ascorbic acid did not affect basal or evoked tritium overflow by themselves.

Results

Basal tritium efflux was expressed as t_1 or t_2/t_1. The t_1-values (min^{-1}) obtained for slices from young adult mice amounted to 0.0032 ± 0.0002 (hippocampal slices preincubated with [^{3}H]-choline; $n = 14$), 0.0049 ± 0.0004 (striatal slices preincubated with [^{3}H]-choline; $n = 15$) and 0.0044 ± 0.0002 (hippocampal slices preincubated with [^{3}H]-NA; $n = 10$). The corresponding t_2/t_1 values in these three experimental series were 0.73 ± 0.04, 0.83 ± 0.04 and 0.82 ± 0.05, respectively. The values obtained in slices from aged mice were identical or very similar to those from young adult mice (not shown). The drugs under study (WIN 55,212-2, SR 141716, oxotremorine, histamine) did not affect basal tritium efflux (t_2/t_1; not shown).

The evoked tritium overflow was expressed as S_1 or S_2/S_1. To study the effect of drugs on the evoked tritium overflow, the ratio of the overflow evoked by S_2 over that by S_1 was used (for S_2/S_1 values in controls, see legends to Figures 1-3). The cannabinoid receptor agonist WIN 55,212-2, the CB_1 receptor

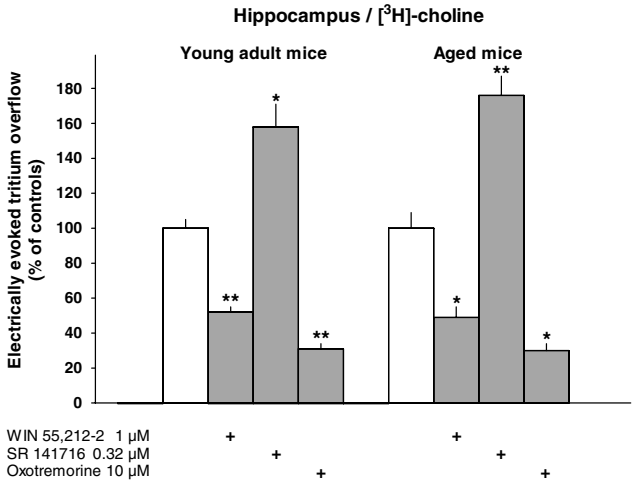

Figure 1 Influence of ageing on the effect of WIN 55,212-2, SR 141716 and oxotremorine on the electrically (3 Hz) evoked tritium overflow from superfused hippocampal slices preincubated with [^{3}H]-choline. Slices from young adult (2 – 3-months old) and aged (24 – 28-months old) C57BL/6J mice were superfused with PSS containing hemicholinium-3 10 μM. WIN 55,212-2, SR 141716 or oxotremorine was added to the medium from 62 min of superfusion onward. Tritium overflow was evoked twice, after 40 (S_1) and 90 min (S_2) of superfusion, and the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined. Tritium overflow is given as per cent of the S_2/S_1 value in the corresponding controls. The S_2/S_1 values in the control series were 1.30 ± 0.06 (young adult mice) and 1.24 ± 0.11 (aged mice). Means $\pm$ s.e.m. of four to 12 experiments. $^*P<0.05$, $^{**}P<0.001$, compared to the corresponding control (Student's *t*-test with Bonferroni correction). The effects of WIN 55,212-2, SR 141716 and oxotremorine did not differ between age groups ($P>0.05$; Student's *t*-test).

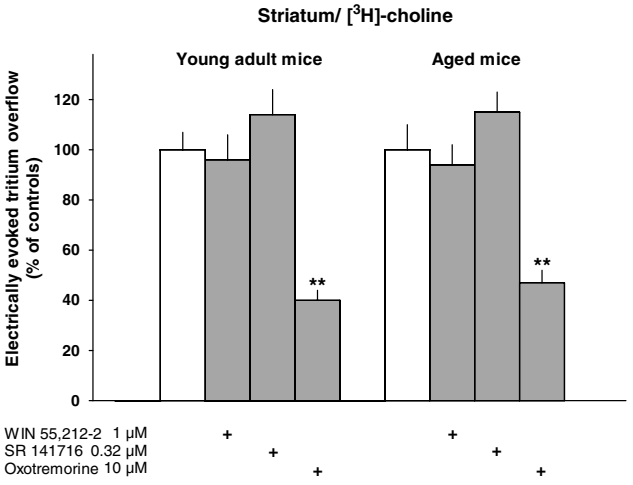

Figure 2 Influence of ageing on the effect of WIN 55,212-2, SR 141716 and oxotremorine on the electrically (3 Hz) evoked tritium overflow from superfused striatal slices preincubated with [^{3}H]-choline. Slices from young adult (2 – 3-months old) and aged (24 – 28-months old) C57BL/6J mice were superfused with PSS containing hemicholinium-3 10 μM. WIN 55,212-2, SR 141716 or oxotremorine was added to the medium from 62 min of superfusion onward. Tritium overflow was evoked twice, after 40 (S_1) and 90 min (S_2) of superfusion, and the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined. Tritium overflow is given as per cent of the S_2/S_1 value in the corresponding controls. The S_2/S_1 values in the control series were 0.96 ± 0.05 (young adult mice) and 1.06 ± 0.09 (aged mice). Means $\pm$ s.e.m. of seven to 20 experiments. $^{**}P<0.001$, compared to the corresponding control (Student's *t*-test with Bonferroni correction). The effect of oxotremorine did not differ between age groups ($P>0.05$; Student's *t*-test).

inverse agonist/antagonist SR 141716, the muscarinic receptor agonist oxotremorine or histamine was added to the medium before and during S_2. The evoked tritium overflow was inhibited by WIN 55,212-2 1 μM and facilitated by SR 141716 0.32 μM in hippocampal slices preincubated with [^{3}H]-choline without any significant difference between young adult and aged mice (Figure 1). WIN 55,212-2 and SR 141716 failed to affect the evoked tritium overflow from striatal slices preincubated with [^{3}H]-choline (Figure 2) and from hippocampal slices preincubated with [^{3}H]-NA (Figure 3) obtained from young and aged mice. There was also no difference between young and aged mice with respect to the inhibitory effect of oxotremorine 10 μM on the evoked tritium overflow from hippocampal slices preincubated with [^{3}H]-choline (Figure 1) and striatal slices preincubated with [^{3}H]-choline (Figure 2). In hippocampal slices preincubated with [^{3}H]-NA, the inhibitory effect of histamine 10 μM on the evoked overflow was reduced by about 20% ($P<0.02$) in slices from aged mice when compared to those from young animals (Figure 3).

To quantify the amount of tritium release in slices from young as opposed to aged mice, the tritium overflow evoked by S_1 (expressed as per cent of tissue tritium) was used. In hippocampal slices from aged mice preincubated with [^{3}H]-choline, the evoked tritium overflow was reduced significantly by 33% compared with young adult animals. In striatal slices preincubated with [^{3}H]-choline, the evoked tritium overflow from aged mice tended to be lower than that in slices from young animals, whereas in hippocampal slices preincubated with [^{3}H]-NA, it was identical (Figure 4).

To quantify tritium accumulation by slices from young adult vs aged mice, the tritium content remaining in the slices at the end of superfusion was determined. Table 1 shows that in none of the three experimental models the tritium content differed significantly between slices from 2- to 3- and 24- to 28-month-old mice although a tendency towards an age-dependent decrease occurred in hippocampal and striatal slices preincubated with [^{3}H]-choline.

Discussion

In the present study, we examined the effects of cannabinoid receptor ligands, oxotremorine and histamine on the electrically evoked tritium overflow from hippocampal and striatal slices from young adult and aged mice preincubated with [^{3}H]-choline or [^{3}H]-NA. Under the experimental conditions of the present study, the evoked tritium overflow is tetrodotoxin sensitive and Ca^{2+} dependent and can be assumed to represent quasiphysiological acetylcholine and noradrenaline release (Kathmann *et al*., 2001b). The cannabinoid receptor agonist WIN 55,212-2, the muscarinic receptor agonist oxotremorine and histamine were used at concentrations producing the maximum or near-maximum effect at the respective receptors (Schlicker *et al*., 1992 and unpublished; Kathmann *et al*., 2001a; Zhang *et al*., 2002). The CB_1 receptor inverse agonist/

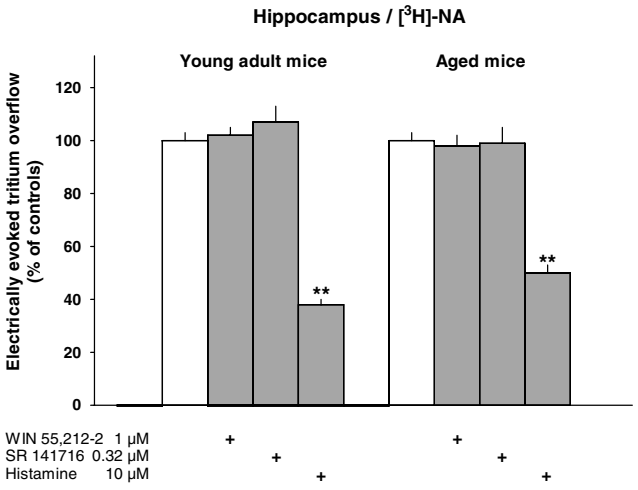

Figure 3 Influence of ageing on the effect of WIN 55,212-2, SR 141716 and histamine on the electrically (0.3 Hz) evoked tritium overflow from superfused hippocampal slices preincubated with [^{3}H]-NA. Slices from young adult (2 – 3 months old) and aged (24 – 28-months old) C57BL/6J mice were superfused with PSS containing desipramine 1 μM and rauwolscine 1 μM. WIN 55,212-2, SR 141716 or histamine was added to the medium from 62 min of superfusion onward. Tritium overflow was evoked twice, after 40 (S_1) and 90 min (S_2) of superfusion, and the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined. Tritium overflow is given as per cent of the S_2/S_1 value in the corresponding controls. The S_2/S_1 values in the control series were 1.03 ± 0.03 (young adult mice) and 1.00 ± 0.03 (aged mice). Means $\pm$ s.e.m. of eight to 13 experiments. $^{**}P < 0.001$, compared to the corresponding control (Student's *t*-test with Bonferroni correction). The effect of histamine was significantly less pronounced in slices from aged mice when compared to slices from young animals ($P < 0.02$; Student's *t*-test).

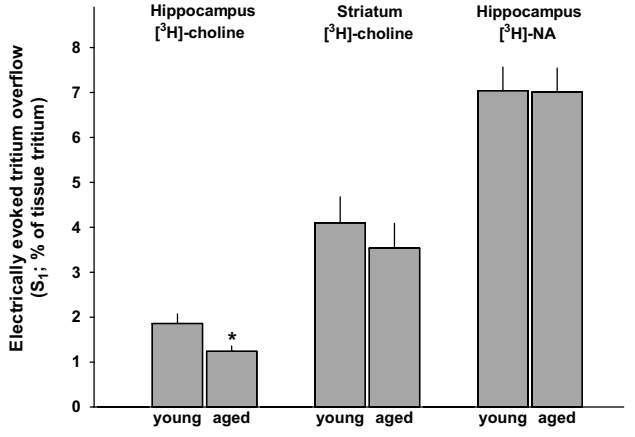

Figure 4 Influence of ageing on the electrically evoked tritium overflow from superfused hippocampal and striatal slices from young adult (2 – 3-months old) and aged (24 – 28-months old) C57BL/6J mice. The slices were superfused for 110 min with PSS containing hemicholinium-3 10 μM (hippocampal and striatal slices preincubated with [^{3}H]-choline) or containing desipramine 1 μM and rauwolscine 1 μM (hippocampal slices preincubated with [^{3}H]-NA). Tritium overflow was evoked after 40 min (S_1) of superfusion (and again after 90 min; not considered here); the stimulation frequency was 3 and 0.3 Hz for preparations preincubated with [^{3}H]-choline and [^{3}H]-NA, respectively. Means $\pm$ s.e.m. of 10 – 15 experiments. $^{*}P < 0.05$, compared to the evoked overflow of tritium in slices from young adult mice (Student's *t*-test).

antagonist SR 141716 was also considered in our study; unlike WIN 55,212-2, SR 141716 increases acetylcholine release in hippocampal slices, suggesting that the CB_1 receptors are subject to an endogenous tone in this tissue. It is not known so far for this experimental paradigm whether the tonic inhibition is because of accumulation of endocannabinoids in the biophase of the CB_1 receptors and/or because of precoupling of part of the CB_1 receptors to the transduction machinery.

In our investigation, the high-affinity choline uptake was inhibited with hemicholinium-3 in slices preincubated with [^{3}H]-choline. In slices preincubated with [^{3}H]-NA, desipramine and rauwolscine were used to block the neuronal noradrenaline transporter and the α_2-autoreceptor, respectively. The auxiliary drugs were used (i) to avoid possible interactions of the test drugs with uptake mechanisms and α_2-autoreceptors and (ii) to increase the amount of tritium overflow. Since in hippocampal slices preincubated with [^{3}H]-choline the amount of evoked tritium overflow was relatively low when compared to hippocampal slices preincubated with [^{3}H]-NA, a higher stimulation frequency, a higher current strength and a higher concentration of Ca^{2+} ions in the medium were used (also in striatal slices to have identical conditions). Nonetheless, the evoked tritium overflow in hippocampal slices preincubated with [^{3}H]-choline was relatively low and this is probably the reason why S_2/S_1 values in these experiments were higher than S_2/S_1 values in the other two paradigms (an inverse relation between extent of overflow and level of control S_2/S_1 values is not uncommon; see e.g. Göthert, 1980).

The major finding of our investigation is that there is no significant difference between young adult and aged mice with respect to the cannabinoid receptor-mediated modulation of acetylcholine release in the hippocampus, that is, the function of the CB_1 receptors is preserved in aged mice. These data fit well the results obtained on the rat brain, in which cannabinoid receptor binding does not differ between young adult and aged animals in most of the hippocampal subregions although it is changed in many other brain structures, particularly in the basal ganglia (Mailleux & Vanderhaeghen, 1992; Berrendero *et al*., 1998; Romero *et al*., 1998). The possibility had to be considered that, although the CB_1 receptor density does not change, the molecular events following the receptor level might undergo alteration. Furthermore, it would be very plausible that the presynaptic receptors involved in inhibition of acetylcholine release may behave differently from other sets of hippocampal CB_1 receptors.

Like in our previous study (Kathmann *et al*., 2001b), acetylcholine release from *striatal* slices and *noradrenaline* release from hippocampal slices were not affected by WIN 55,212-2 and SR 141716 in brain slices from young adult mice and, as shown here, were also not affected in brain slices from aged animals. The possibility that cannabinoid receptors, although absent in the brain of young adult animals, will be expressed later in life had to be taken into consideration since CB_1 receptor protein and mRNA levels increased in some brain regions of aged rats (Berrendero *et al*., 1998). In order to show that noradrenaline and acetylcholine release in these two brain regions can be modulated at all under the conditions of the present study, the effects of ligands at other presynaptic receptor systems were investigated. In striatal slices preincubated with [^{3}H]-choline, oxotremorine inhibited acetylcholine release, and in hippocampal slices preincubated with [^{3}H]-NA,

Table 1 Influence of ageing on the tritium content in hippocampal and striatal slices from young adult (2–3-months old) and aged (24–28-months old) C57BL/6J mice

Tissue	*Preincubation with*	*Age (months)*	*n*	*Tritium content (nCi)*
Hippocampus	[^{3}H]-choline	2–3	14	38.1±3.6
		24–28	14	33.3±2.5
Striatum	[^{3}H]-choline	2–3	15	60.4±5.4
		24–28	15	53.7±5.9
Hippocampus	[^{3}H]-noradrenaline	2–3	10	9.0±0.7
		24–28	10	8.4±0.7

The results represent means±s.e.m. of the tritium content present in the slices after completion of the experiments shown in Figures 1–3. Tritium content did not differ between age groups ($P > 0.05$; Student's *t*-test).

histamine inhibited noradrenaline release. Oxotremorine inhibits striatal acetylcholine release mainly via M_4 receptors (and hippocampal acetylcholine release, considered here as well, predominantly via M_2 receptors; Zhang *et al*., 2002). Histamine inhibits hippocampal noradrenaline release via H_3 receptors, since the selective H_3 receptor antagonist thioperamide antagonized the effect at an apparent pA_2 value of about 8 (unpublished results), similar to that found for other H_3 receptor models (Alexander *et al*., 2001).

It was beyond the scope of the present paper to examine in detail the impact of ageing (i) on the function of the latter receptors (muscarine and H_3) and (ii) on the release of acetylcholine and noradrenaline *per se*; therefore, the two age-dependent differences we found will be discussed only briefly. First, acetylcholine release was less by 33% in the hippocampus and tended to be less in the striatum. The S_1 values presented in Figure 4 are percentages of the tritium content in the slice. In order to determine whether there is an age-dependent alteration of release in absolute terms also, it is necessary to compare the tritium content in slices from young and aged mice. Since there was no difference, one can conclude that the reduction in hippocampal acetylcholine release in aged vs young adult mice also occurs in absolute terms. Taking into account that tritium accumulation tended to be reduced in hippocampal and striatal slices (preincubated with [^{3}H]-choline) from aged mice, one may even reach the conclusion that the release in absolute terms is reduced more markedly in hippocampal slices and also reduced to some extent in striatal slices. Our data fit well many previous studies on rodents showing that acetylcholine release in the brain declines with increasing age and that the extent of decrease differs among the various brain regions (for review, see Sherman & Friedman, 1990). An age-dependent decrease in acetylcholine release is also typical for the human cerebral cortex (for review, see Feuerstein & Seeger, 1997).

Second, the degree of H_3 receptor-mediated inhibition of hippocampal noradrenaline release was less by 20% in slices from aged mice, whereas in a previous study from our laboratory, in which the H_3 receptor-mediated inhibition of serotonin release in the cerebral cortex of rats was examined, a difference between young adult and aged animals did not occur (Schlicker *et al*., 1991). A more detailed analysis of H_3 receptor function in aged animals appears to be of interest since H_3 receptor antagonists have been proposed as potential drugs for the treatment of dementias (for review, see Leurs *et al*., 1998).

In conclusion, our study shows that the function of the CB_1 receptors involved in inhibition of acetylcholine release is preserved in the hippocampus from aged mice. This finding might have practical relevance since CB_1 receptor inverse agonists/antagonists might represent a new class of cognition-enhancing drugs.

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