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Regional localization and developmental profile of acetylcholinesterase-evoked increases in [^{3}H]-5-fluorowillardiine binding to AMPA receptors in rat brain

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1 In addition to its role in hydrolyzing the neurotransmitter acetylcholine, the synaptically enriched enzyme acetylcholinesterase (AChE) has been reported to play an important role in the development and remodelling of neural processes and synapses.

2 We have shown previously that AChE causes an increase in binding of the specific AMPA receptor ligand (S)-[^{3}H]-5-fluorowillardiine ([^{3}H]-FW) to rat brain membranes.

3 In this study we have used quantitative autoradiography to investigate the regional distribution and age-dependence of AChE-evoked increases in the binding of [^{3}H]-FW in rat brain.

4 Pretreatment of rat brain sections with AChE caused a marked enhancement of [^{3}H]-FW binding to many, but not all, brain areas. The increased [^{3}H]-FW binding was blocked by the specific AChE inhibitor BW 284c51.

5 The maximal potentiation of [^{3}H]-FW binding occurred at different developmental age-points in different regions with a profile consistent with the peak periods for synaptogenesis in any given region.

6 In addition to its effects on brain sections, AChE also strongly potentiated [^{3}H]-FW binding to detergent solubilized AMPA receptors suggesting a direct action on the receptors themselves rather than an indirect effect on the plasma membrane.

7 These findings suggest that modulation of AMPA receptors could provide one molecular mechanism for the previously reported effects of AChE on synapse formation, synaptic plasticity and neurodegeneration.

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Abbreviations: [^{3}H]-FW, [^{3}H]-5-fluorowillardiine; AChE, acetylcholinesterase; alv-or lim, limit between hippocampal alveus and stratum oriens; AMPA, α-Amino-3-hydroxy-5-methylisoxazolepropionate; Amy, amygdala; Arc, arcuatus nucleus; BW284c51, 1,5-bis(4-allyl-dimethylammoniumphenyl)pentan-3-one dibromide; BW284c51, 1,5-bis(4-allyl-dimethylammoniumphenyl)pentan-3-one dibromide; CA1, CA2, CA3, CA4, CA1-4 fields of hippocampus proper (Cornu Ammonis); Cpu, caudate putamen; DG, dentate gyrus; DHF, dorsal hippocampal formation; DIIIV, dorsal third ventricle; DTh, dorsal thalamus; GrDG, granular layer of dentate gyrus; H, hypothalamus; hf, hippocampal fissure; hil, hilus; IIV, third ventricle; lac-mol, stratum lacunosum-moleculare; molDG, molecular layer of dentate gyrus; mTh, medial thalamus; neoCx, neocortex; ocCx, occipital cortex; OG, octylglucoside; or, stratum oriens of hippocampus; pCx, parietal cortex; pirCx, piriform cortex; prhCx, perirhinal cortex; py (CA3), pyramidal cell layer of hippocampus (CA3 field); pyr, pyramidal cell layer of hippocampus; rad, stratum radiatum; rf, rhinal fissure; rsgCx, retrosplenial granular cortex; S, subiculum; SDS–PAGE, SDS polyacrylamide gel electrophoresis; temCx, temporal cortex; Th, thalamus; vlTh, ventrolateral thalamus; vmH, ventromedial hypothalamus; vmTh, ventromedial thalamus

Introduction

α-Amino-3-hydroxy-5-methylisoxazolepropionate (AMPA) receptors mediate most excitatory synaptic transmission in the mammalian CNS. They play a central role in synapse formation, stabilization and plasticity and their prolonged activation is potently neurotoxic (for a review see Dingledine *et al*., 1999). Developmental and activity-dependent changes in the functional expression of AMPA receptors at synapses are strictly regulated. Several proteins that interact with the intracellular C-termini of AMPA receptor subunits and which appear to be involved in receptor targeting and surface expression have been identified (for reviews see Dev & Henley, 1998; Braithwaite *et al*., 2000). Recently, another protein called NARP has been shown to interact with the extracellular N-terminal region of AMPA receptors and cause receptor clustering (O'brien *et al*., 1999).

AChE is a member of the serine esterase domain family of proteins (Ichtchenko *et al*., 1996) and it exhibits a number of morphogenetic activities in addition to its role in hydrolysing acetylcholine (Anderson & Key, 1999; Grifman *et al*., 1998;

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Holmes *et al*., 1997; Koenigsberger *et al*., 1997). AChE is concentrated at synapses and has a neuroligin-homology domain (Ichtchenko *et al*., 1995) and a carbohydrate HNK epitope, a hallmark of adhesion molecules (Koenigsberger *et al*., 1997). It is now widely accepted that AChE has actions which are relevant to the development and remodelling of neural processes and synapses (for reviews see Layer & Willbold, 1995; Grisaru *et al*., 1999). Some of these actions, such as effects on cell differentiation and promotion of neurite extension appear to be related to the homology of AChE to certain cell adhesion proteins including the neuroligins (Grifman *et al*., 1998; Ichtchenko *et al*., 1995; Koenigsberger *et al*., 1997). Significantly, most of the morphogenetic actions attributed to this enzyme do not involve a catalytic activity (for a review see Grisaru *et al*., 1999) whereas its synaptogenic activity appears to require a catalytic action, but not membrane association (Sternfeld *et al*., 1998). Thus, the synaptogenic properties of AChE are separable from its neuritogenic effects which do not require an hydrolytic capacity.

We have reported previously that AChE pretreatment of rat brain membranes caused an upregulation of agonist binding to AMPA receptors (Olivera *et al*., 1999). In that study we showed that AChE caused a concentration-dependent increase in the number of binding sites (B_{max}) for the AMPA receptor agonists (S)-[^{3}H]-5-fluorowillardiine ([^{3}H]-FW) and [^{3}H]-AMPA but had no effect on [^{3}H]-kainate binding to the related kainate-type glutamate receptors The actions of AChE were Ca^{2+}- and temperature-dependent, suggesting an enzymatic action for AChE in this system.

Here we have used quantitative autoradiography to investigate the regional and age-dependant effects of AChE pretreatment on [^{3}H]-FW binding to rat brain sections. In addition, we have investigated the action of AChE on [^{3}H]-FW binding to detergent solubilized AMPA receptors.

Methods

Rat tissue preparations

Whole brains were removed from halothane/O_2 anaesthetized Wistar rats, frozen in -40°C isopentane and stored at -80°C until use Cryostat sections (15 μm) were obtained in a OTF cryostat with a Bright 5030 microtome and mounted onto microscope slides with 0.1 mg/ml poly-L-lysine. Sections were dried and stored desiccated at -80°C for at least 24 h The age points studied were 8, 15, 21, 28 day-old (P8, P15, P21, P28), 6 and 10 weeks-old (PW6, PW10) rats.

AChE pre-treatment

Sections were slowly thawed (1–2 h) in air-tight containers and then immersed for 15 min in ice-cold 50 mM Tris-HCl buffer, pH 7.4 to remove debris due to mechanical damage during sectioning. A further 15 min wash in Tris-HCl buffer supplemented with 5 mM $CaCl_2$ ensured the complete removal of endogenous glutamate (Dev *et al*., 1995). Slices were preincubated with AChE (0.5–10 u ml^{-1}) in 50 mM Tris-HCl buffer plus 5 mM $CaCl_2$, for 30 min Control sections were incubated with buffer alone The AChE was removed with a 15 min wash in ice-cold Tris-HCl buffer Inhibition experiments were performed using the same protocol but in the presence of 1×10^{-5}–1×10^{-3} M 1,5-bis(4-allyl-dimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) as previously described (Olivera *et al*., 1999).

Quantitative autoradiography

Each slide, containing 3–4 brain sections, was incubated in a total volume of 1 ml Tris-HCl buffer containing [^{3}H]-FW (3–10 nM) or 100 nM [^{3}H]-kainate for 60 min on ice. Slides were washed six times (total wash time 10 s) in ice-cold buffer, rinsed in water and immersed for 1 s in acetone. Sections were then dried rapidly in a steam of cool air. Non-specific binding was defined by inclusion of 1 mM L-glutamate for [^{3}H]-FW or 100 μM kainate for [^{3}H]-kainate. The sections were then exposed to Amersham Hyperfilm-^{3}H alongside ^{3}H-microscales standards (Amersham). After 28 days, films were developed for 2 min in Kodak D19, fixed and dried. Anatomical coordinates, structure names and abbreviations, were as in Paxinos & Watson (1986).

Image acquisition and quantitative analysis

Pictures were digitized from developed ^{3}H-Hyperfilm autoradiograms using an EPSON Perfection 1200 U Scanner and Adobe Photoshop 4 Images were analysed with Scion Image Beta Release 3b, the NIH Image Software for Windows. Using the rectangular option of the selection tool we defined precise areas (6×6 pixels) from different brain regions (this area represents $<1\%$ of the entire image). The density values were calculated by the software from a grey scale calibrated in the range 0 (white)–256 (black) against ^{3}H-microscales standards (Amersham). An exponential non-linear fitting curve best fit the density of the microscales and was used in all subsequent experimental analyses. Corresponding areas from control and AChE treated sections exposed to the same sheet of ^{3}H-Hyperfilm were directly compared. Background from the film was determined by sampling three different areas surrounding the section, the mean value calculated and subtracted from each measurement. Data shown in Table 1 represent the ratio between the density of an area of brain in an AChE-treated slice and the corresponding brain area in an adjacent control slice.

In some experiments, to obtain quantitative analysis of the amount of radioligand binding, wet sections were wiped off the slide with Whatman GF/C glass microfibre filters. The filters were then transferred to scintillation vials containing 4 ml of Emulsifier-Safe scintillation fluid (Packard) and the [^{3}H]-label counted in a scintillation counter (Beckman LS6500).

Preparation of rat cortical membranes and solubilization of AMPA receptors

Cortex from adult male Wistar rats (150–250 g) was homogenized in a glass-Teflon homogenizer in 10 volumes of ice-cold EDTA buffer (50 mM Tris pH 7.4 at 0°C with HCl, 1 mM EDTA, 1 mM EGTA) supplemented with 0.32 mM sucrose. Following centrifugation at $1000\times g$ for 10 min the supernatant was centrifuged for 30 min at $40{,}000\times g$. The pellet (P2) was subjected to a freeze-thaw-wash protocol exactly as described previously (Dev *et al*., 1995). The final pellet was resuspended and used immediately Detergent extracts were prepared by incubating the mem-

Table 1 Quantification of AChE effects on AMPA receptors binding autoradiography

Animal age		*8 days*		*28 days*		*adult*			
AChE concentrations		*1 U ml^{-1}*	*5 U ml^{-1}*	*1 U ml^{-1}*	*5 U ml^{-1}*	*1 U ml^{-1}*	*2 U ml^{-1}*	*5 U ml^{-1}*	*10 U ml^{-1}*
CNS region	*Abbrev.*								
CA1	CA1	1.54±0.34	1.81±0.14	1.19±0.07	1.23±0.06	1.15±0.13	1.21±0.10	1.39±0.07	1.52±0.05
CA3	CA3	NSI	1.07±0.05	1.33±0.09	1.33±0.12	NSI	1.12±0.11	1.33±0.11	1.56±0.09
Dentate gyrus	DG	NSI	1.07±0.10	1.41±0.1	1.52±0.07	NSI	NSI	1.54±0.25	1.60±0.18
Occipital cortex, mediomedial	mmOc2	1.64±0.14	1.48±0.24	1.49±0.14	1.39±0.06	1.26±0.15	1.17±0.18	1.61±0.12	1.94±0.09
Occipital cortex, mediolateral	mlOc2	NSI	1.81±0.32	1.47±0.14	1.45±0.03	NSI	NSI	1.82±0.07	2.08±0.08
Retrosplenial granular cortex	rsgCx	NSI	NSI	1.58±0.10	1.38±0.09	NSI	1.19±0.20	1.24±0.07	1.51±0.12
Perirhinal cortex	prCx	1.98±0.61	2.17±0.40	1.43±0.17	1.57±0.13	NSI	NSI	1.31±0.30	1.74±0.19
Paraventricular posterior/ Parafasicular nucleus	pPV/PF	NSI	1.41±0.28	1.44±0.23	2.14±0.17	NSI	1.74±0.27	NSI	NSI
Arcuate nucleus	Arc	1.53±0.21	1.83±0.4	1.37±0.2	1.54±0.16	1.25±0.11	ND	1.35±0.26	1.71±0.11
Caudate putamen	CPu	ND	ND	1.66±0.18	1.85±0.12	ND	1.55±0.23	ND	1.72±0.25

AChe-evoked increases in [3H]-FW binding at specific CNS areas. Sections were incubated with 10 nM [3H]-FW following preincubation with the indicated concentrations of AChE. Control samples (without AChE) were simultaneously processed. All sections were opposed to [3H]-Hyperfilm (Amersham) for 30 days. Scion Image-Release Beta3b quantification analysis software was used to compare AChE-treated and contol areas at a resolution of (11×11 pixels) from digitized autoradiographs. After substraction of non-specific binding to each condition, increases were expressed as ratios between AChE-treated and control sections. Results are the mean±s.e.mean of 6–15 similar fields. ND, not determined; NSI, non significant increases.

branes at a ratio ~1:10 (w v^{-1}) with 1% octylglucoside (OG) in EDTA buffer containing the following protease inhibitors (final concentrations; EDTA, 2 mM; EGTA, 2 mM; Soybean Trypsin Inhibitor, 1 mg/100 ml^{-1}; Bacitracin, 20 mg 100 ml^{-1}, Benzamidine, 15 mg 100 ml^{-1}) overnight on a rotating wheel at 4°C The detergent-membrane mixture was then centrifuged for 1 h at 130,000×*g* and the supernatant containing soluble proteins collected and stored at −80°C. Where appropriate, 5 ml of the soluble extract was dialysed against EDTA buffer for 16 h with three changes of dialysis buffer [3H]-FW binding to membranes and detergent extracts was determined by filtration on Whatman GF/B filters soaked in 0.3% PEI (Henley & Barnard, 1989). Protein concentrations were assessed using the Pierce assay kit as described (Olivera *et al*., 1999).

SDS–PAGE and Western blots

SDS–PAGE and Western blots using pan-AMPA subunit antibodies (Archibald *et al*., 1998) were performed as described previously (Henley, 1993). Immunoreactivity was visualized using horseradish peroxidase and/or alkaline phosphatase-coupled anti-rabbit IgG secondary antibodies (Sigma) and the Amersham ECL kit.

Materials

[3H]-FW was from Tocris-Cookson and [3H]-kainate was from New England Nuclear Hyperfilm-[3H] and [3H]-standards were from Amersham International. All other ligands and reagents were from Tocris-Cookson, Sigma or BDH.

Results

AChE effects on [3H]-FW binding in different brain areas

In a previous study we have performed a senes of [3H]-FW saturation binding experiments to rat brain membranes (Olivera *et al*., 1999). We detected two classes of binding sites and showed that the increase in [3H]-FW binding following treatment with AChE is due predominantly to an increase in the number of receptors available for radioligand binding (B_{max}) rather than a conformational shift to a higher affinity state (K_D). The values for [3H]-FW binding in control experiments were: high affinity site–K_{D1}=62.2±6.5 nM, B_{max1} 1.56±0.12 pmol mg^{-1} protein; low affinity site–K_{D2}=1324±159 nM, B_{max2}=7.92±0.64. Following AChE preincubation the values for [3H]-FW binding were: high affinity site–K_{D1}=89.3±11.2 nM, B_{max1}=2.48±0.33 pmol mg^{-1} protein; low affinity site–K_{D2}=1039±130 nM, B_{max2}=13.12±0.64. In the present study [3H]-FW binding was assessed by quantitative autoradiography of coronal brain sections from immature (P8, P15, P21, P28, P42) and adult rats following preincubation with 1 u ml^{-1} or 5 u ml^{-1} AChE. The levels of [3H]-FW binding in distinct anatomical regions at P8, P28 and adult (P42), expressed as a ratio of non-AChE-treated controls are given in Table 1.

Consistent with the results from membrane binding experiments AChE pre-treatment augmented [3H]-FW binding to most areas of the adult brain (Table 1, adult) but regional differences in the degree of AChE-evoked enhancement were observed. For example, amongst nuclear structures, there was a clear difference in the AChE enhancement of [3H]-FW binding to the caudate-putamen compared to that in the globus pallidus. In addition, marked AChE-evoked increases in [3H]-FW binding were observed in certain, but not all, thalamic and hypothalamic nuclei of the adult brain (Table 1).

Age-dependence of AChE-evoked increases in [3H]-FW binding

At each of the age points investigated there was substantial enhancement of [3H]-FW, but not [3H]-kainate, binding in discrete areas following AChE pre-treatment (Figures 1 and 2). The maximal enhancement of [3H]-FW binding by AChE occurred at different times in different areas but in general it seemed that the phylogenetically more primitive a given area

(e.g., allocortex), the earlier in development it showed maximal AChE-enhanced [3H]-FW binding.

Developmental regulation of the AChE-evoked increase in [3H]-FW binding in the hippocampal formation

The maximal enhancement of [3H]-FW binding by AChE shifted during development, from a predominant subpyramidal (mainly at stratum onens) location at the P8 stage, to maximal enhancement in the adult, in stratum radiatum and lacunosum-moleculare of hippocampus and molecular layer of dentate gyrus. At P8, AChE potentiation of [3H]-FW binding was only detectable at CA1 as a thin, dense subpyramidal layer (Table 1) continuous with a wider layer of subpyramidal location at CA3 and hilus-CA4 fields (see Figures 1E and 3B; 1 u ml^{-1} AChE final concentration). At P15, this layer increased in intensity and widened to reach pyramidal cell layer, especially at CA3-CA4-hilus (Figure 3B). At P28, a striking AChE potentiation of [3H]-FW binding was seen at the hippocampal stratum radiatum and stratum lacunosum-moleculare, and also as a thin band beneath the granular layer of the dentate gyrus (roughly corresponding to stratum lucidum), and at the whole subiculum (2500–4000 Bq mg^{-1} with 5 u ml^{-1} AChE). At this stage of development AChE potentiation of [3H]-FW binding occurred within the two inner thirds (approximately

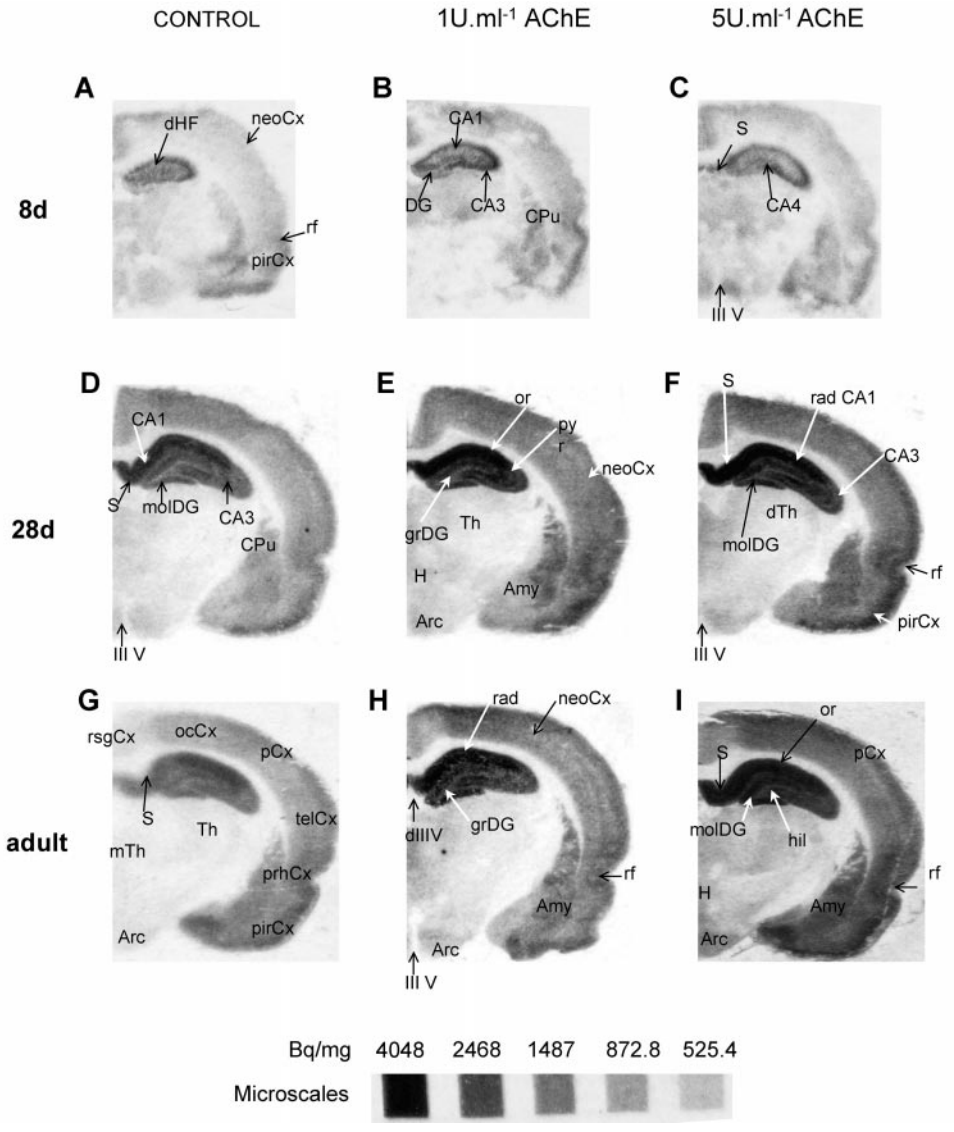

Figure 1 Regional- and age-dependence of AChE-enhancement of [3H]-FW binding to AMPA receptors in the rat brain. Autoradiographs of 15 μm coronal cryostat brain sections from 8-, 28-day-old and adult rats. Experiments were performed as detailed in the Methods. Quantitative analysis is given in Table 1. The abbreviations used are: Amy, amygdala; Arc, arcuatus nucleus; CA1, CA2, CA3, CA4, CA1–4 fields of hippocampus proper (Cornu Ammonis); CPu, caudate putamen; DG, dentate gyrus; DHF, dorsal hippocampal formation; dIIIV, dorsal third ventricle; dTh, dorsal thalamus; grDG, granular layer of dentate gyrus; H, hypothalamus; hf, hippocampal fissure; hil, hilus; IIIV, third ventricle; molDG, molecular layer of dentate gyrus; mTh, medial thalamus; neoCx, neocortex; ocCx, occipital cortex; or, stratum onens of hippocampus; pCx, parietal cortex; pirCx, piriform cortex; prhCX, penrhinal cortex; pyr, pyramidal cell layer of hippocampus; rf, rhinal fissure; rsgCx, retrosplenial granular cortex; S, subiculum; temCx, temporal cortex; Th, thalamus.

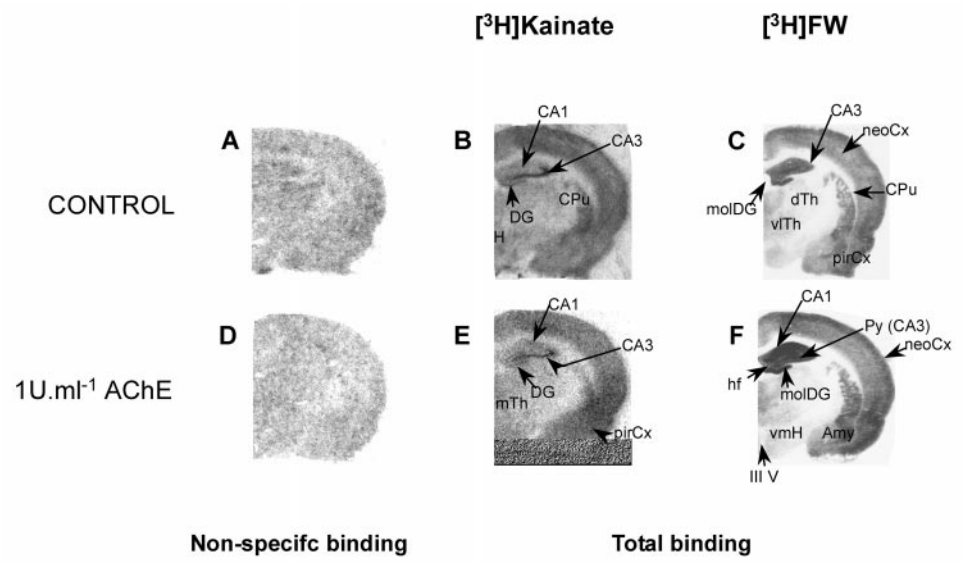

Figure 2 AChE enhances [3H]-FW but not [3H]-kainate binding to rat brain sections. Autoradiographs show [3H]-kainate (A,B,D,E) and [3H]-FW (C,F) binding to brain sections from adult rats 1 u ml^{-1} AChE evoked increased [3H]-FW binding but did not significantly affect [3H]-kainate binding. Note that whereas [3H]-FW non-specific binding was negligible (less than 3% of total, autoradiographs not shown), [3H]-kainate non-specific binding (A,D) represented ~50% of total binding. Independently from the agonist used, AChE alone (D) did not affect non-specific binding. Abbreviations, as in Figure 1; py(CA3), pyramidal cell layer of hippocampus (CA3 field); vlTh, ventrolateral thalamus; vmH, ventromedial hypothalamus; vmTh, ventromedial thalamus.

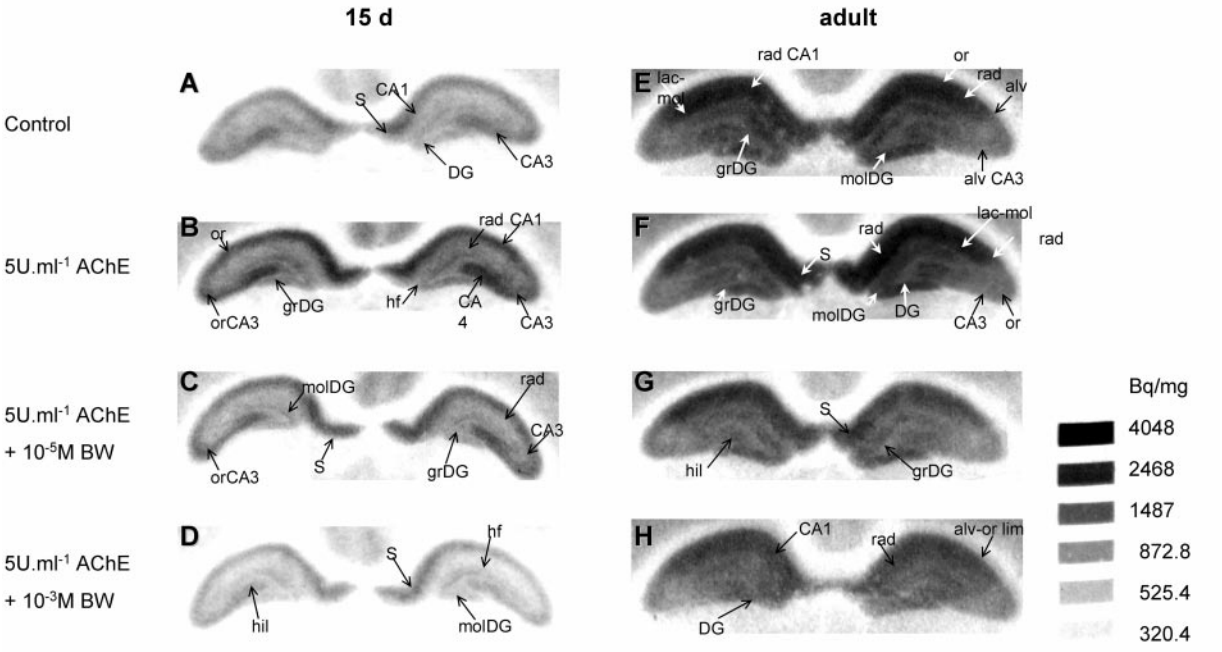

Figure 3 BW284c51 blocks the AChE-evoked potentiation of [3H]-FW binding to AMPA receptors. The autoradiographs show [3H]-FW binding to serial coronal sections of the hippocampal formation from a 15-day-old rat. Sections were preincubated under the conditions shown as described in the Methods. Abbreviations, as in Figure 2; alv-or lim, limit between hippocampal alveus and stratum oriens; lac-mol, stratum lacunosum-moleculare; rad, stratum radiatum.

50% increase as compared to P8, Table 1) and hilus of dentate gyrus. At later stages of maturation differences in the AChE potentiation of [3H]-FW binding amongst regions/layers were less pronounced and, unlike at P8-P15, at P28, 5 u ml^{-1} of AChE was required for marked potentiation in the subiculum-CA1 regions.

In parallel experiments using [3H]-kainate binding to adjacent sections from adult animals, no significant increases in binding were observed following AChE pretreatment (Figure 2). This was consistent with our previous observations using synaptic membrane preparations (Olivera *et al.*, 1999) and demonstrates that the effects of

AChE are selective for AMPA receptors over kainate receptors.

Effects of BW284c51 on AChE actions

Since dramatic changes were observed in the hippocampus we focused on this region in subsequent experiments to investigate the effects of the AChE inhibitor BW284c51 BW284c51 alone had no effect on [^{3}H]-FW binding but, as shown in Figure 3, BW284c51 completely prevented the increase in [^{3}H]-FW binding evoked by AChE.

AChE potently enhances [^{3}H]-FW binding to soluble AMPA receptors

We have shown previously that pre-treatment of brain cortical membranes with AChE increases the B_{max} for [^{3}H]-FW binding (Olivera *et al*., 1999). A similar increase in the B_{max} of [^{3}H]-AMPA binding has been shown following detergent solubilization of brain membranes (Hall *et al*., 1992; Henley & Barnard, 1989). We therefore investigated the effects of AChE on [^{3}H]-FW binding to octylglucoside-solubilized receptors. As shown in Figure 4A, AChE still caused a concentration-dependent increase in [^{3}H]-FW binding demonstrating that the two treatments are not mutually exclusive. Preincubation of solubilized receptors with AChE in the presence of the specific inhibitor BW284c51 (final concentrations used 1 μM – 1 mM) fully prevented the increase in [^{3}H]-FW binding. BW284c51 alone had no effect on binding. Moreover, immunoblots (Figure 4B) using a pan-AMPA specific antibody (Archibald *et al*., 1998) showed that AChE did not result in receptor degradation.

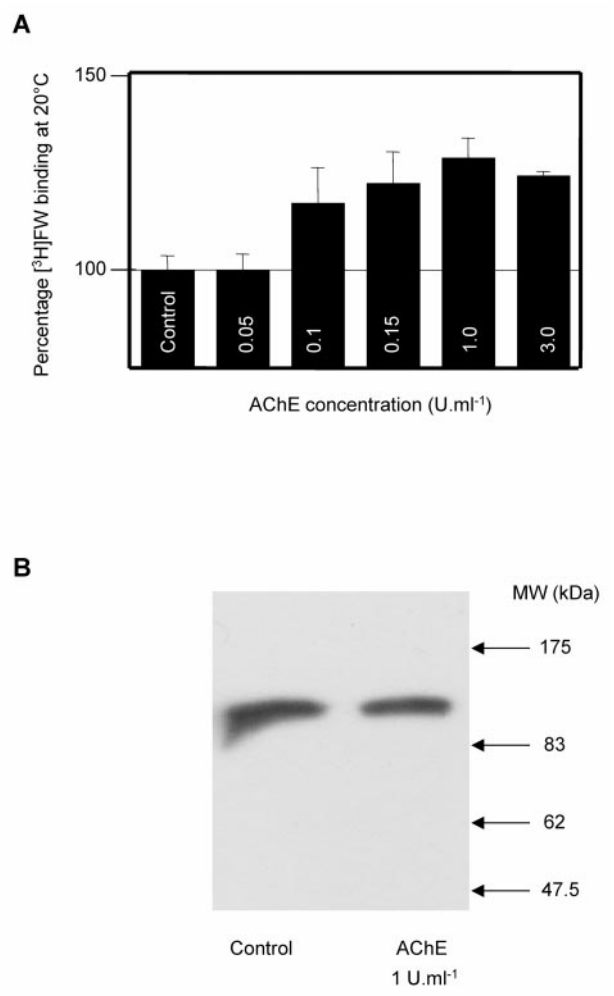

Figure 4 AChE enhances [^{3}H]-FW binding to solubilized AMPA receptors. (A) Histogram showing the concentration curve for AChE enhancement of [^{3}H]-FW binding to AMPA receptors solubilized with 1% octylglucopyranoside There was a significant increase ($P<0.01$, non paired Student's *t*-test) in binding to AChE-treated solubilized compared to solubilized control samples. (B) Western blot showing that AChE-treated solubilized AMPA receptors were not degraded.

Discussion

The autoradiographic data presented here demonstrate a substantial, anatomically-specific, and developmentally-modulated increase in [^{3}H]-FW, but not [^{3}H]-kainate, binding following pre-treatment with AChE. These data are consistent with our previous work showing that AChE causes an increase in the B_{max} of [^{3}H]-FW binding to rat brain membranes. The AChE potentiation of [^{3}H]-FW binding followed well defined anatomical patterns throughout the rat CNS, suggesting that the AChE-responsive sites may be associated with specific subsystems. In the adult brain, the distribution of AChE-enhanced [^{3}H]-FW binding sites does not parallel the localization of AChE (Matthews *et al*., 1974; Nadler *et al*., 1974).

There were developmental differences in sensitivity and in the chronology of the appearance of the AChE potentiation of [^{3}H]-FW binding and, in general, phylogenetically older structures appear more strongly responsive to AChE in the adult and show the earliest appearance of the enhancement of [^{3}H]-FW binding during development. For example, in the hippocampal formation the layer of enhanced binding occurs beneath CA1-CA3 pyramidal cell layer at P8 whereas at P15 it widens at CA3-CA4 to reach the pyramidal layer (Figure 1B,E). This pattern spatio-temporally parallels the localization of the septo-hippocampal afferent system that makes synapses with pyramidal cells at this time (Fiala *et al*., 1998; Matthews *et al*., 1974; Petralia *et al*., 1999).

The finding that there are developmental differences in the sensitivity to AChE agrees with our previous demonstration that the sensitivity of [^{3}H]-FW binding to AChE in cortical membranes prepared from 15-day-old rats is four orders of magnitude greater than that in membranes prepared from adult rats (EC_{50} value $= 4 \times 10^{-5}$ unit ml^{-1} corresponding to $\sim 4 \times 10^{-14}$ M) compared to adults (EC_{50} value $= 0.1$ unit ml^{-1}; Olivera *et al*., 1999). These observations are intriguing since AMPA receptors play an important role in synapse formation and the control of synaptic efficiency. Thus the fact that AMPA receptors are highly sensitive to AChE modulation and that the sensitivity is under developmental regulation is consistent with the hypothesis that AChE may be involved in neuronal development and synaptic stabilization.

Our results also demonstrate that AChE elicits a concentration-dependent increase in [^{3}H]-FW binding to detergent solubilized AMPA receptors. The effects of AChE were blocked by the specific inhibitor BW284C51 and by heat

treatment of the protein. These data suggest that the action of AChE on [3H]-FW binding is not due to any effect on the phospholipid membrane microenvironment surrounding the receptors as was previously suggested for AChE-evoked changes in Aplysia cholinergic receptors (Fossier *et al*., 1983). Furthermore, detergent solubilization of AMPA receptors has been shown to increase in apparent number of binding sites (B_{max}) (Hall *et al*., 1992; Henley & Barnard, 1989). The observation that prior solubilization does not occlude the AChE-mediated increase in binding indicates separate modes of action and implies that there is a direct modulation of AMPA receptors by AChE. However, the possibility remains that AChE could act on proteins tightly associated with AMPA receptors such as the proposed inhibitory modulatory component (Dev & Henley, 1998) which, in turn, regulates [3H]-FW binding to the receptor complex. If this were the case AChE could cause the dissociation of the inhibitory modulatory component from the receptor complex thereby causing an apparent increase in the number of [3H]-FW binding sites. There are a number of candidate proteins that are known to interact with AMPA receptors, mainly *via* the C-terminal domain of the GluR2 subunit (Braithwaite *et al*., 2000). There is also at least one protein, Narp (O'brien *et al*., 1999), that interacts with the extracellular N-terminal domain that is accessible to AChE. Narp possesses several properties that make it likely to play a key role in the genesis of excitatory synapses It is selectively enriched at excitatory synapses and its overexpression increases the number of excitatory but not inhibitory synapses in cultured spinal neurons. Narp-expressing HEK 293T cells can induce the aggregation of neuronal AMPA receptors suggesting that Narp can function as an extracellular clustering factor for AMPA receptors (O'brien *et al*., 1999). Future experiments will be required to determine the precise mechanisms by which AChE pretreatment alters the number of AMPA receptor agonist binding sites.

AChE contains an HNK-like carbohydrate domain and this motif is considered a hallmark of adhesion proteins (Koenigsberger *et al*., 1997). Its neurite-growth promoting activity (Holmes *et al*., 1997) has been attributed both to the E6-encoded C terminal domain of the protein and to AChE sequence homology to adhesion molecules such as laminin and neuroligins (Scheiffele *et al*., 2000). It is the extracellular AChE-homology domain of neuroligin that binds to the extracellular domain of β-neurexin and is proposed to act as a recognition mechanism for triggering synapse formation (Rao *et al*., 2000; Scheiffele *et al*., 2000). The correlation between the localization of AChE-enhanced [3H]-FW binding sites and layers undergoing intensive synapse formation and maturation suggests that this protein may be involved in one or more step of synaptogenesis AChE has been implicated in the control of neuronal growth and synaptogenesis, as well as in synaptic plasticity. It is now widely accepted that this protein causes extension of neurites (neuritogenic action) and can promote an increase in both the surface area and the structural organization of the postsynaptic membrane (Grifman *et al*., 1998; Sternfeld *et al*., 1998). These observations go some way towards explaining the fact that AChE is commonly expressed in developing neurones irrespective of the neurotransmitters they finally utilize (Layer & Willbold, 1995)

Our data are consistent with AChE potentiation of [3H]-FW binding occurring particularly in regions undergoing active synaptogenesis. The neuroligin-like domain of AChE may participate in recognition between pre- and postsynaptic membranes during synapse formation (Scheiffele *et al*., 2000; Rao *et al*., 2000), possibly competing with neurexins (Grifman *et al*., 1998). Whereas the catalytic domain of AChE can modify agonist binding to AMPA receptors AMPA receptor-mediated neuronal activity plays a crucial role in the establishment and stabilization of active synaptic connections (Wu *et al*., 1996).

AChE is released as a consequence of neuronal activation (Jones *et al*., 1994; Rodriguez-Ithurralde *et al*., 1997). Therefore, the synaptogenic action of AChE may, at least in part, be due to its effects on the molecular organization of postsynaptic membranes (Sternfeld *et al*., 1998), including an increase in available AMPA receptors. Thus activity-released AChE could provide a mechanism for transducing neuronal activity into structurally and functionally viable synapses. Further investigation into the molecular mechanisms underlying the AChE modulation of AMPA receptors should provide useful information about the roles of this enzyme in modulating synaptic plasticity and neuronal development and neurodegeneration.

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