Acute β-Amyloid Administration Disrupts the Cholinergic Control of Dopamine Release in the Nucleus Accumbens

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The clinical presentation of Alzheimer's disease is characterized by memory deficits but it also involves the impairment of several cognitive functions. Some of these cognitive and executive functions are mediated by limbic areas and are regulated by dopaminergic neurotransmission. Furthermore, literature data suggest that β-amyloid (Aβ) can influence synaptic activity in absence of neurotoxicity and in particular can impair cholinergic modulation of other neurotransmitter actions. In the present study, we evaluated whether small concentrations of Aβ could disrupt cholinergic control of dopamine (DA) release in nucleus accumbens using *in vivo* (brain dialysis) and *in vitro* (isolated synaptosomes) models. The cholinergic agonist carbachol (CCh) greatly enhanced DA release from dopaminergic nerve endings in nucleus accumbens both *in vivo* and *in vitro*. This effect was mainly exerted on muscarinic receptors because it was inhibited by the muscarinic antagonist atropine and it was unaffected by the nicotinic antagonist mecamylamine. Also the nicotinic agonists epibatidine and nicotine evoked a dopaminergic outflow in nucleus accumbens, which, however, was lower. Aβ 1–40 in absence of neurotoxicity fully inhibited the DA release evoked by CCh and only marginally affected the DA release evoked by epibatidine. The PKC inhibitor GF109203X mimicked the effect of Aβ on DA release and, in turn, Aβ impaired PKC activation by CCh. We can suggest that, in nucleus accumbens, Aβ disrupted *in vivo* and *in vitro* cholinergic control of DA release by acting on muscarinic transduction machinery.
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INTRODUCTION

Two series of considerations, concerning the clinical presentation of Alzheimer's disease (AD) and the putative molecular mechanisms underlying this illness, prompted us to study the effect of β amyloid (Aβ) on classical neurotransmission in *in vivo* and *in vitro* experimental models.

The clinical presentation of AD involves the impairment of several cognitive functions including, but not limited, to memory. Thus complex symptoms of AD do not fit the single neurotransmitter deficit model even if there is no doubt that cholinergic transmission impairment plays an important role. Within this context some cognitive and executive functions are predominantly mediated by limbic areas that play a critical role in elaborating thoughts, behavior, and emotions using representational knowledge: operations often referred to as working memory. In

particular, studies have suggested a direct involvement of dopamine (DA) in the regulation of different aspects of cognitive and executive functions in limbic areas. DA acts as a key neurotransmitter in the brain and shows a regulatory role for limbic functions; it acts as a powerful regulator of different aspects of cognitive brain functions. The integrative properties of the dopaminergic system are probably associated with direct contribution to cognitive functions at the cortical level, namely in working memory and executive functions. Such functions are highly vulnerable to aging (Arnsten and Li, 2005; Nieoullon, 2002; Nieoullon and Coquerel, 2003) and profoundly affected by AD (Baudic *et al*, 2006; Germano and Kinsella, 2005; Waltz *et al*, 2004). Moreover, animal studies suggest that both AD and age-associated cognitive impairment reflect vulnerability of the same circuits (Hof and Morrison, 2004).

The second set of considerations concerns the recent observations made by various groups showing that Aβ may exert synaptic activities at levels that do not result in toxicity (Kamenetz *et al*, 2003; Zhong *et al*, 2003). Zhong *et al* (2003) suggested that Aβ, in conditions not resulting in neurotoxicity, impairs cholinergic responses mediated by cholinergic muscarinic receptors by acting on the transduction machinery, in particular on PKC. This last consideration fits several observations including ours (Govoni *et al*,

1993) on the impairment of the PKC transduction system in AD. Furthermore, in striatum and limbic areas, a cholinergic-DA link has been demonstrated showing that acetylcholine may promote the activation of DA terminals by regulating DA release (De Klippel et al, 1993; Giorquieff et al, 1977; Kitamura et al, 1999; Xu et al, 1989; Zhang et al, 2002). All this leads to the hypothetical scenario of an Aβ-driven early dysfunction of cholinergic responses occurring at relatively low concentrations of soluble Aβ, not giving origin to fibrils, that may disrupt cholinergic responses and consequently turn off DA terminals leading to an early impairment of executive functions.

This biological hypothesis is testable in animal models. In fact, it is possible to infuse Aβ into the brain of laboratory rodents and simultaneously monitor the extracellular release of neurotransmitters by the microdialysis technique. This was exactly our approach. Within this context we chose the nucleus accumbens as a test area because it is a region of convergence of limbic forebrain inputs and midbrain dopaminergic pathways, important for the control of various behaviors. In addition, there are studies that suggest the existence of a functional interaction among various neurotransmitters including acetylcholine and DA in this area of the brain. In particular, it has been shown that in nucleus accumbens the cholinergic receptor activation increases DA release (Kitamura et al, 1999). The choice of this brain area is further supported by the observation that in man the striatum (Brilliant et al, 1997; Mann and Iwatsubo, 1996) and in particular the ventral striatum, which includes the nucleus accumbens, is frequently affected by amyloid deposits and it is particularly vulnerable to AD (Suenaga et al, 1990). Moreover, taking advantage of previously developed (Marchi et al, 1985; Raiteri et al, 1984) in vitro models of synaptic neurotransmitter release, we investigated the direct effects of Aβ on isolated synaptosomes.

MATERIALS AND METHODS

In Vivo Experiments

Animals and microdialysis probe implantation. Male Wistar rats (275–300 g; Harlan, Udine Italy), housed in standard conditions (temperature $23 \pm 1^{\circ}C$; humidity 50%) with 12:12 light/dark cycles, water, and food *ad libitum*, were anesthetized with Equithesin 3 ml/kg (pentobarbital 9.7 g, chloral hydrate 42.5 g, MgSO$_4$ 21.3 g for 1L, 11% ethanol, 42% propylene glycole v/v) administered intraperitoneally and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). All experimental procedures were performed according to international regulations for the care and treatment of laboratory animals and in accordance with the Italian Act (DL n 116, GU, suppl 40, 18 February, 1992) and with EEC Council Directive (86/609, OJ L 358, 1, 12 December, 1987).

All *in vivo* experiments were performed using microdialysis probes, made in our laboratory according to the original method described by Di Chiara et al (1993) with cellulose (Emophan Bellco Artificial OR-internal diameter 200 μm, cutoff 40 KDa; Bellco, Mirandola, Italy), with a 2-mm nominal active length. The probe was implanted in the shell of nucleus accumbens (AP +1.6 mm, ML ±0.8 mm from bregma and DV −8.0 mm from dura) according to the Paxinos and Watson atlas (Paxinos and Watson, 1998).

Microdialysis samples collection. Twenty-four hours after the surgical procedure, the probe was perfused with artificial cerebrospinal fluid (aCSF: 145 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl$_2$, 1.2 mM MgCl$_2$, 1.4 mM, Na$_2$HPO$_4$ pH 7.2–7.4) at 2 μl/min using a 1000 μl syringe (Hamilton) and a microinjection pump (CMA/100, CMA/Microdialysis AB). Fractions were collected every 20 min in 100 μl Eppendorf tubes.

Chromatography. In vivo dialysate samples were analyzed for DA concentration using high-pressure liquid chromatography with electrochemical detection (ESA Coulochem II Bedford-MA) using a Supelcosil LC-18-DB Column 15 cm × 4.6 mm packed with a 5-μm C$_{18}$ stationary phase (Supelco) and a sample injection Rheodyne 7125 with 20 μl loop. The pH 5.5 mobile phase consisted of 0.71 g Na$_2$HPO$_4$, 6 g NaH$_2$PO$_4$, 15% methanol (v/v), 33.6 mg Na$_2$ethylenediaminetetraacetic acid (EDTA) and 116 mg octyl sodium sulfate dissolved in a final volume of 1L solution. The phase was degassed under vacuum and simultaneously filtered through a Millipore 0.2 μm pore membrane and was delivered with a Waters (Millipore, Mildford, MA, USA) Model 510 pump at flow rate of 1.3 ml/min. DA was detected at 2 nA with a Model 5011 dual-electrode analytical cell. The detector was operated at a nominal potential of +130 mV applied to the first electrode and a potential of −125 mV applied to the second electrode. Only the signal generated at the second electrode was routinely recorded. The analytical cell signal was recorded on a Houston Instruments (Gistel, Belgium) Omniscribe recorder. Dialysate samples (20 μl) were manually injected into the Rheodyne injector using a 50 μl Hamilton syringe. The sensitivity of the assay for DA was 3 fmol per sample.

Statistics. Values were expressed as percentage of basal values. The average concentration of four consecutive samples immediately preceding the drug dose (less than 10% variation) was considered as the basal value and was defined as 100%.

For statistical analysis the GraphPad Instat statistical package (version 3.05 GraphPad software, San Diego, CA, USA) was used. The data were analyzed by analysis of variance (ANOVA) followed when significant by an appropriate *post hoc* comparison test. When the Bartlett's test indicated nonhomogeneity of variance a nonparametric test, that is the Kruskal–Wallis ANOVA followed by Dunn's test, was applied. The reported data are expressed as means ± SEM.

Immunohistochemical analysis. Brain tissue samples were frozen and stored at −80°C. For immunodetection of infused Aβ peptide, 20 μm coronal sections were incubated with a primary monoclonal antibody recognizing Aβ protein (clone 4G8; Chemicon International). Sections were then incubated with a mouse anti-IgG antibody RPE conjugated (Dako). After the fluorescent labelling procedures, sections were finally counterstained for DNA with HOECHST 33342 and mounted in a drop of Mowiol (Calbiochem, Inalco SpA, Milan, Italy).

Ex Vivo Experiments

Western blot procedure. For detecting activated PKC, a phospho-PKC (pan) antibody that recognizes PKCα, βI, βII, ε, η, and δ isoforms only when phosphorylated at a carboxyl-terminal residue homologous to Ser660 of PKCβII was used in the Western blot analysis. At the end of Aβ infusion, the rats were killed and the cerebrum was extracted. Brain tissue corresponding to shell of accumbens was taken by punch and lysed by addition of ice-cold lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 50 mM EDTA, and 1% Triton X-100, 1 mM Na_3VO_4 and protease inhibitors). Proteins were subjected to SDS-PAGE (10%) and then transferred onto PVDF membrane (DuPont NEN, Boston, MA). The membrane was blocked for 1 h with 5% non fat dry milk in Tris-buffered saline containing 1% Tween 20. For the detection of PKC membranes were immunoblotted with the phospho-PKC (pan) antibody (Cell Signaling, 1:1000) overnight at 4°C. The detection was carried out by incubation with horseradish peroxidase conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburgh, MD, USA) for 1 h as secondary antibody. The blots were then washed extensively and phospho-PKC visualized using an enhanced chemiluminescent methods (Pierce, Rockford, IL, USA). Then the blots were stripped for 30 min at 50°C followed by saturation in 5% nonfat dry milk and incubated with a PKCalpha antibody (Transduction Laboratories, 1:1000).

Densitometry and statistics. Following acquisition of the Western blot image through an AGFA scanner and analysis by means of the Image 1.47 program (Wayne Rasband, NIH, Research Services Branch, NIMH, Bethesda, MD, USA), the relative densities of the bands were analyzed as described previously (Lanni et al, 2004). Data were analyzed using the analysis of variance test followed, when significant, by the Student's *t*-test. The data reported are expressed as mean ± SEM of at least three independent experiments.

In Vitro Experiments

Superfusion of isolated nerve endings. Crude synaptosomes were prepared as described previously and labelled with [³H]DA ([³H]DA; final concentration 0.05 μM) in the presence of 0.1 μM of the serotonin uptake blocker 6-nitroquipazine and 0.1 μM of the noradrenaline (NA) blocker nisoxetine (Marchi et al, 1985). After the labelling

period, identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel superfusion chambers thermostated at 37°C (Marchi et al, 1985; Raiteri et al, 1984).

Synaptosomes were superfused at 0.5 ml/min with standard physiological solution aerated with 95% O_2 and 5% CO_2, at 37°C.

The system was first equilibrated during 36 min of superfusion; subsequently, five consecutive 1-min fractions were collected. Synaptosomes were exposed to agonists at the end of the first fraction collected ($t = 37$ min) till the end of the superfusion, while antagonists were added 8 min before the agonists.

Fractions collected and superfused synaptosomes were counted for radioactivity.

The amount of radioactivity released into each superfusate fraction was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux). Drug effects were expressed as percent increase over basal release and were evaluated as the ratio between the percentage of tritium released into the fraction where the maximal releasing effect was observed and that in the first fraction collected; this ratio was compared with the corresponding ratio obtained under control conditions (no drug added).

The high K^+ evoked release was monitored according to Grilli et al (2005).

Direct comparison analyses were performed with the two-tailed Student's *t*-test.

RESULTS

In Vivo Experiments

To test the effect of Aβ infusion on DA release, we first studied whether Aβ (1 μM) administration through the dialysis probe allowed the delivery of the peptide to the tissue. Immediately after the perfusion of the peptide, Aβ immunoreactivity was detected within the brain area where the dialysis probe was located. At this time the peptide showed a range of diffusion comparable to the end of the tip (200 μM) (Figure 1a). Three hours after the interruption of 1 μM Aβ 1–40 infusion, the peptide was partially cleared from the area of interest (Figure 1b). The decreased content of Aβ 1–40, observed after 3 h from the injection of the peptide, suggested a putative clearance of the protein either
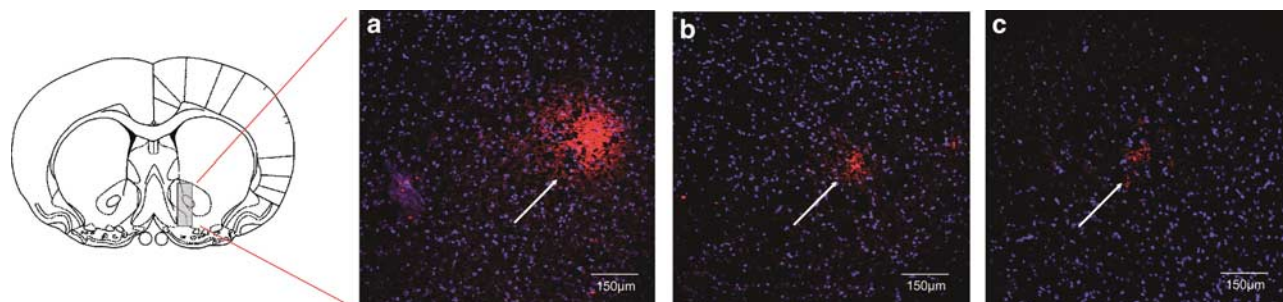

Figure 1 Coronal section indicating the area (nucleus accumbens) in which the dialysis probe was implanted and fluorescence micrographs showing the presence of human β-amyloid (Aβ) protein. (a) Aβ immunoreactivity (red-PE staining, white arrow) immediately after perfusion of 1 μM Aβ 1–40. (b) Aβ immunoreactivity 3 h following 1 μM Aβ 1–40 infusion. (c) Aβ immunoreactivity immediately after perfusion of 10 μM Aβ 1–42. Nuclear DNA was counterstained with Hoechst 33342 (blue staining). Magnification: × 20.

by the cerebral tissue or by the probe. We also perfused Aβ 1–42. At variance with Aβ 1–40, Aβ 1–42 had difficulty to be delivered to the tissue. Indeed even after the administration of 10 μM Aβ 1–42 the presence of the peptide within the nucleus accumbens of the perfused rats was at least one order of magnitude lower than that of Aβ 1–40, as estimated from the intensity of the staining (Figure 1c). Then we designed a pilot study in which we stimulated DA release in the nucleus accumbens by infusing carbachol (CCh). Carbachol (1–200 μM) elevated DA outflow in a concentration-dependent manner as shown in Figure 2a. The maximal effect (a stimulation of DA outflow up to 11 times the basal) was observed with 100 μM CCh. Increasing the concentration of CCh over 100 μM did not further stimulate DA outflow. A control experiment was performed to isolate presynaptic response, using the blocker of voltage-sensitive sodium channels tetrodotoxin (TTX). Therefore we coinfused through the dialysis probe 100 μM CCh with 1 μM TTX and we observed that TTX completely prevented the CCh-induced DA release (Figure 2a, inset).

The effect of CCh was at least partially mediated by muscarinic cholinergic receptors since it was inhibited (by 75%) by atropine (Atr 1 μM) administration but only marginally (by 14%) by the administration of mecamylamine (Mec 50 μM) (Figure 2b). The view that the effect of CCh on DA outflow was mainly mediated by muscarinic receptor activation was further supported by the observation that epibatidine (Epi, 1–125 μM), a nicotinic agonist,

was less effective than CCh in eliciting DA outflow from nucleus accumbens. The peak effect (4.2 times over basal) was observed with 100 μM Epi and was 37% of the effect observed with CCh (Figure 2a).

The effect of Epi was reduced by Mec by 64% (Figure 2b). Aβ 1–40 infusion, started 40 min before CCh, greatly impaired (72% inhibition) the ability of CCh (100 μM) to stimulate DA release at peptide doses as low as 1 μM. The concentration–response curve (0.1–10 μM) of the inhibition by Aβ of CCh-induced DA release was steep (Figure 2c, inset). Aβ 1–42 and the reverse peptide Aβ 40–1 (10 μM) were ineffective (Figure 2c). Aβ infusion did not inhibit the Epi-induced DA release as shown in Figure 2d. None of the tested concentrations of Aβ affected the basal level of extracellular DA in nucleus accumbens at any of the time points considered (from 20 min up to 6 h of infusion) (not shown).

To test the persistence of Aβ effect, we first examined the response to repeated CCh administrations. The effect of a single dose of CCh was maximal within the time frame of sample collection (20 min) and returned to prestimulus values 20 min after the infusion was stopped. Repeated administrations of CCh had the same effect on DA release (Figure 3a), indicating the absence of tachyphylaxis.

The effect of Aβ on CCh-elicited DA release persisted continuing the Aβ infusion as shown by the fact that a second and third stimulation with 100 μM CCh were ineffective, indicating that there was no tolerance to Aβ
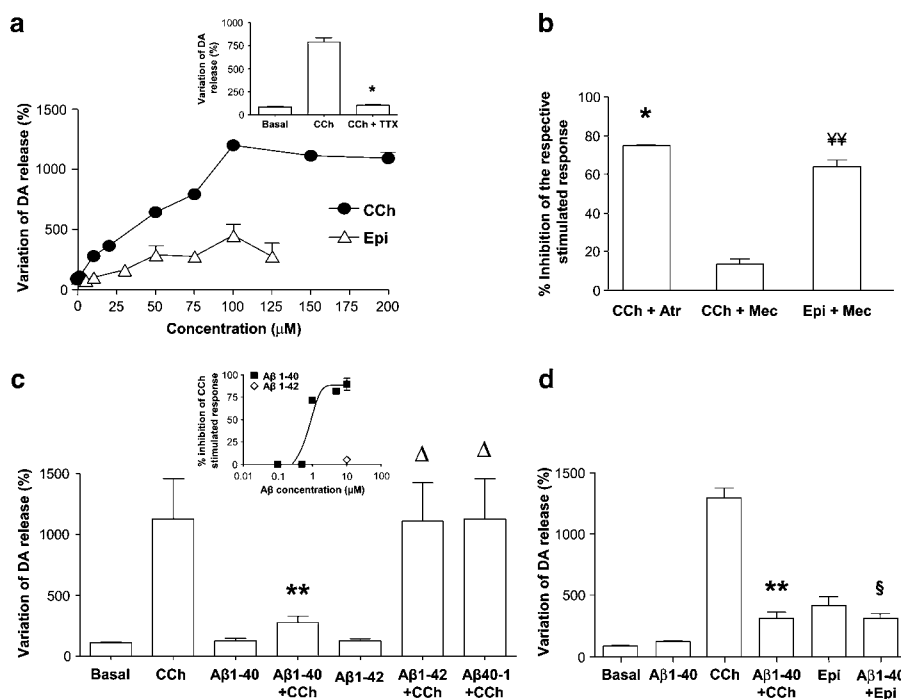

Figure 2 β-amyloid (Aβ) infusion inhibits carbachol-elicited DA outflow from nucleus accumbens by acting on cholinergic muscarinic receptors. (a) Carbachol (CCh) or epibatidine (Epi) were infused at various concentrations as indicated in the figure. Each sample was collected for 20 min in the presence of the respective carbachol concentration. The inset shows the effect of the coinfusion of 1 μM tetrodotoxin (TTX) with 100 μM CCh on DA release. *P < 0.05 (Dunn's test) compared to CCh. (b) Effect of a maximally effective CCh (100 μM) or Epi (100 μM) concentration on DA outflow and inhibition by atropine (Atr) (1 μM) or mecamylamine (Mec) 50 μM coinfused with CCh or Epi. *P < 0.05 vs CCh; ¥¥P = 0.001 vs Epi (Dunn's test). (c) Effect of the Aβ 1–40 (1 μM), Aβ 1–42 (10 μM), and the reverse peptide Aβ 40–1 (10 μM) on the CCh stimulated DA release. **P < 0.01 vs CCh; ΔP > 0.05 vs CCh (Dunn's test). Inset: the effect of various concentration of Aβ (0.1–10 μM) and, for comparison, of Aβ 1–42 (10 μM) on the CCh-stimulated DA release. (d) Effect of Aβ (1 μM) on Epi (100 μM) stimulated DA release. The effect of Aβ on CCh (100 μM) was repeated for control in this set of experiments. **P < 0.01 vs CCh; §P > 0.05 vs Epi (Dunn's test). Data are expressed as mean ± SEM of 5–8 individual rats for each experimental group in each panel.
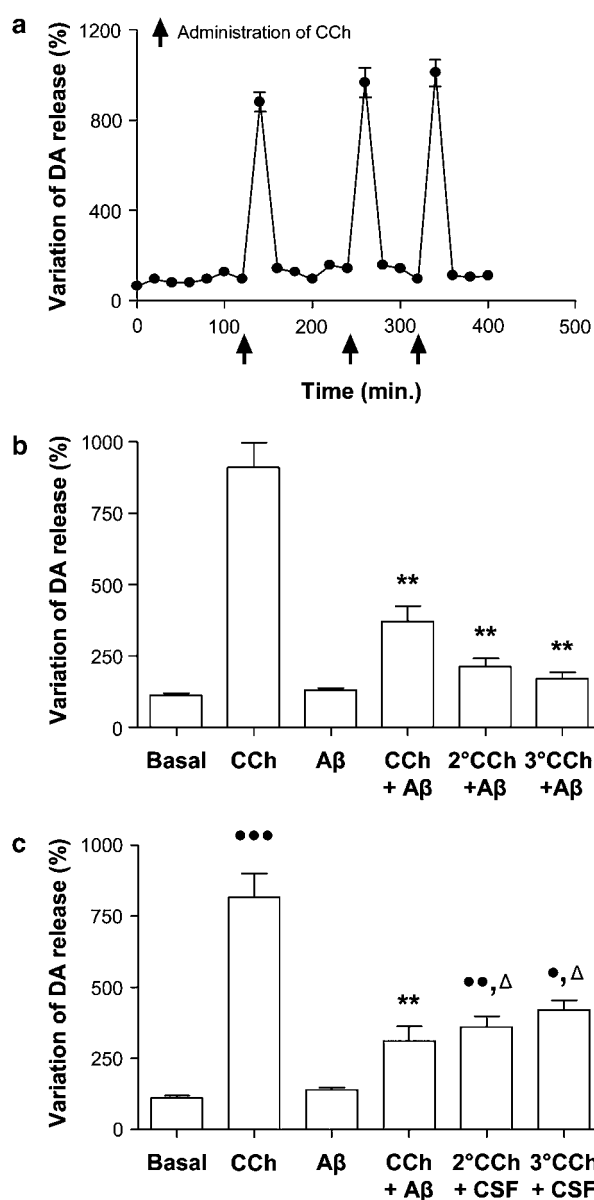
Figure 3 Effect of β-amyloid (Aβ) on carbachol-induced dopamine (DA) release after continuous infusion and after stopping the treatment. (a) Time of onset and offset of the action of carbachol (CCh) and the effect of repeated CCh administrations. The arrows indicate the time of CCh administration which ended after 20 min. (b) Effect of the continuous infusion of Aβ 1–40 on repeated CCh stimuli. (c) Effect of repeated CCh stimuli on DA release after ending the Aβ 1–40 infusion. **$P < 0.01$ vs CCh; •$P < 0.05$ vs basal; ••$P < 0.01$ vs basal; •••$P < 0.001$ vs basal; $^\Delta P > 0.05$ vs CCh (Dunn's test). Data are expressed as mean $\pm$ SEM of 5–8 individual rats for each experimental group in each panel.

inhibition (Figure 3b). The action of Aβ, however, was reversible as shown by the fact that after discontinuing the infusion of Aβ a second and third stimulation with 100 μM CCh led to a significant increase of DA outflow over basal even if the mean values were lower than those observed with CCh before Aβ infusion (Figure 3c). The administration of GF 109203X (25 μM, 100 min), a protein kinase C inhibitor, inhibited the effect of CCh on DA release to an extent (72%) similar to that observed by blocking muscarinic receptors or by infusing Aβ (1 μM) (Figure 4a).
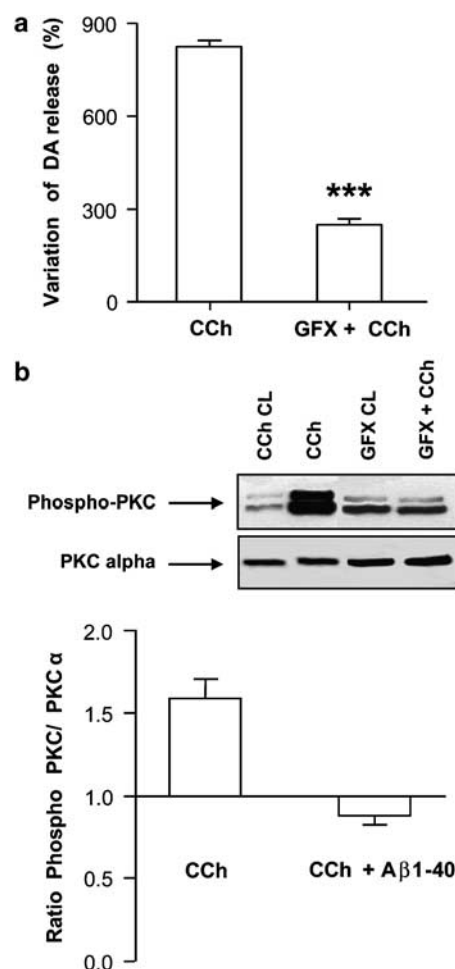

Figure 4 Protein kinase C (PKC) inhibition prevents the carbachol-elicited dopamine (DA) outflow from nucleus accumbens and β-amyloid (Aβ) impairs PKC activation by carbachol (CCh). (a) Inhibition of CCh effect on DA release by pretreatment with the PKC inhibitor GF109203X (25 μM). ***$P < 0.0001$ vs CCh (since the variance was homogeneous, a two-tailed Student's t-test was used to evaluate the significance of the difference). (b) Phospho PKC/PKCα ratio following PKC activation by CCh and inhibition by Aβ 1–40. CL: control side. Data are expressed as mean $\pm$ SEM of 5–7 individual rats for each experimental group in each panel.

Ex Vivo Experiments

To obtain information regarding the effect of Aβ on PKC machinery, we studied the PKC activation, by evaluating the ratio between phospho-PCK and PKCα, in CCh-stimulated rats. CCh-activated PKC as indicated by the increased ratio of phospho PKC versus PKCα, when comparing the injected versus the controlateral (CL) side (see inset, Figure 4b). The CCh effect was fully prevented by pretreatment with the PKC inhibitor GF109203X (see inset, Figure 4b) or Aβ, thus suggesting that the effect of Aβ on carbachol response was mediated by PKC activation (Figure 4b).

In Vitro Experiments

CCh and nicotine (Nic) were able to enhance significantly the basal release of [3H]DA from accumbal nerve endings. The dose–response curves of CCh and Nic reported in
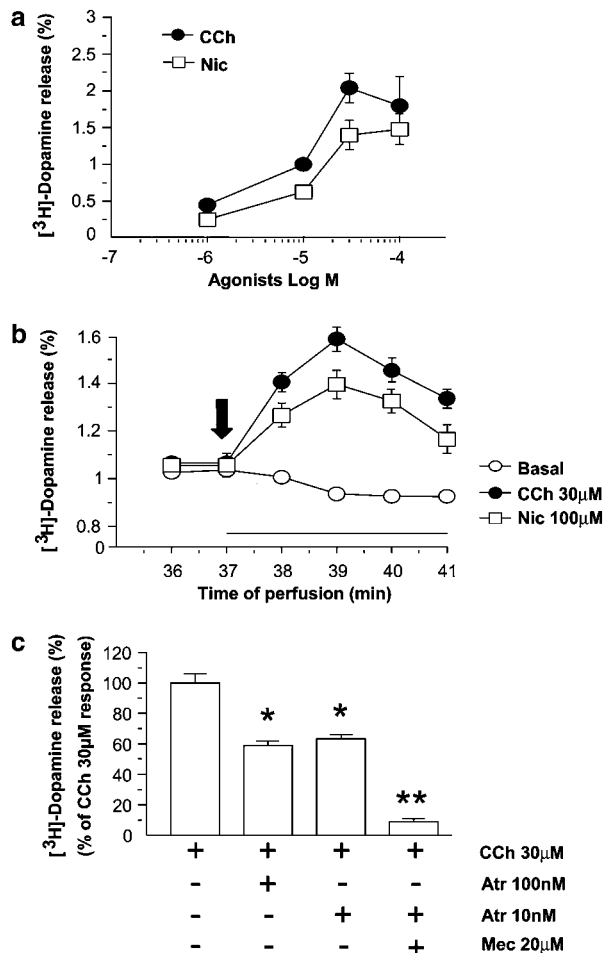
Figure 5 Characterization of carbachol (CCh) and nicotine (Nic) evoked [³H]dopamine (DA) release from rat accumbal synaptosomes. (a) Concentration–response curves of [³H]DA overflow by CCh and Nic. (b) Time courses for CCh and Nic (black arrow indicates the time of agonists administration and black line indicates permanent stimulation). (c) Effect of a maximally effective CCh concentration on DA release and inhibition by atropine (Atr) and mecamylamine (Mec). Data are means ± SEM of nine experiments run in triplicate. **$P < 0.01$; *$P < 0.05$ vs 30 μM CCh (two-tailed Student's t-test).

Figure 5a show that the maximum effect was reached at 30 μM for CCh and at 100 μM for Nic with an EC_{50} respectively of $6.76 \pm 2.01 \times 10^{-6}$ and $1.13 \pm 0.08 \times 10^{-5}$.

The time courses of [³H]DA release evoked by CCh and Nic are illustrated in Figure 5b. The peak effect was observed both for CCh and Nic at min 39 of superfusion.

Figure 5c shows that the release of [³H]DA elicited by 30 μM CCh was partially antagonized by atropine (Atr, 10 and 100 nM) and almost abolished by 10 nM Atr plus 20 μM of Mec.

The [³H]DA release evoked by CCh (30 μM) in accumbal isolated nerve endings was significantly reduced by 100 nM Aβ, while 10 nM Aβ was ineffective (Figure 6a). The CCh-induced release remaining after Aβ 1–40 was completely blocked by Mec. Also Aβ 1–42 (100 nM) significantly reduced the DA release evoked by CCh to a similar extent.

To shed light on the selectivity of the effect of Aβ on the muscarinic and nicotinic receptors (nAChRs), we performed experiments in which the KCl evoked release of
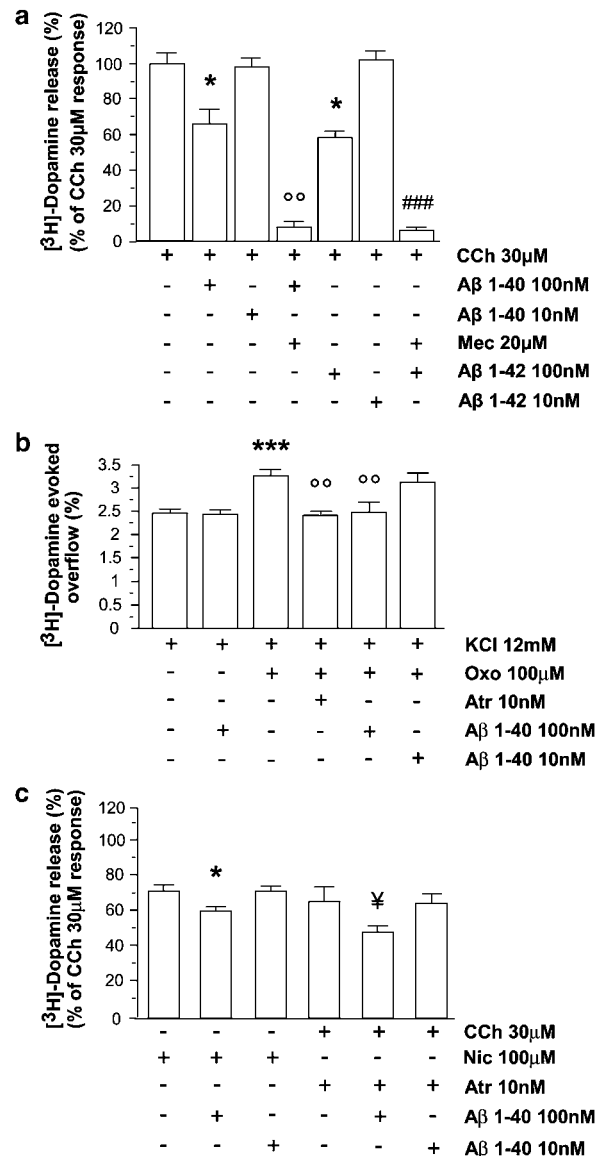

Figure 6 Effect of β-amyloid (Aβ) on the carbachol (CCh) and nicotine-stimulated [³H]dopamine (DA) release in accumbal synaptosomes. (a) Effect of Aβ 1–40 and Aβ 1–42 infusions on [³H]-DA release from accumbal synaptosomes. Aβ 1–40 (100 nM) and Aβ 1–42 (100 nM) are unable to modify basal release (data not shown). *$P < 0.05$ vs 30 μM CCh; °°$P < 0.01$ vs 30 μM CCh + 100 nM Aβ 1–40; ###$P < 0.001$ vs 30 μM CCh + 100 nM Aβ 1–42 (two-tailed Student's t-test). (b) Effect of Aβ 1–40 on [³H]DA overflow evoked by KCl 12 mM alone or in combination with oxotremorine (Oxo). ***$P < 0.001$ vs 12 mM KCl; °°$P < 0.01$ vs 12 mM KCl + 100 μM Oxo (two-tailed Student's t-test). (c) Nicotinic component of [³H]DA release is partially affected by Aβ 1–40 infusion. *$P < 0.05$ vs 100 μM Nic; ¥$P < 0.05$ vs 30 μM CCh + 10 nM Atr (two-tailed Student's t-test). Data are means ± SEM of 6–10 experiments run in triplicate.

[³H]DA was further enhanced by oxotremorine (Oxo) through the activation of muscarinic receptors as previously shown (Marchi et al, 1985).

Figure 6b shows that 100 nM Aβ, which did not modify the KCl evoked release of [³H]DA, completely counteracted the potentiating effect of 100 μM Oxo which was also abolished by Atr. Aβ (10 nM) was completely ineffective.

The effect of Aβ on the nicotinic stimulation of [³H]DA release is reported in Figure 6c.
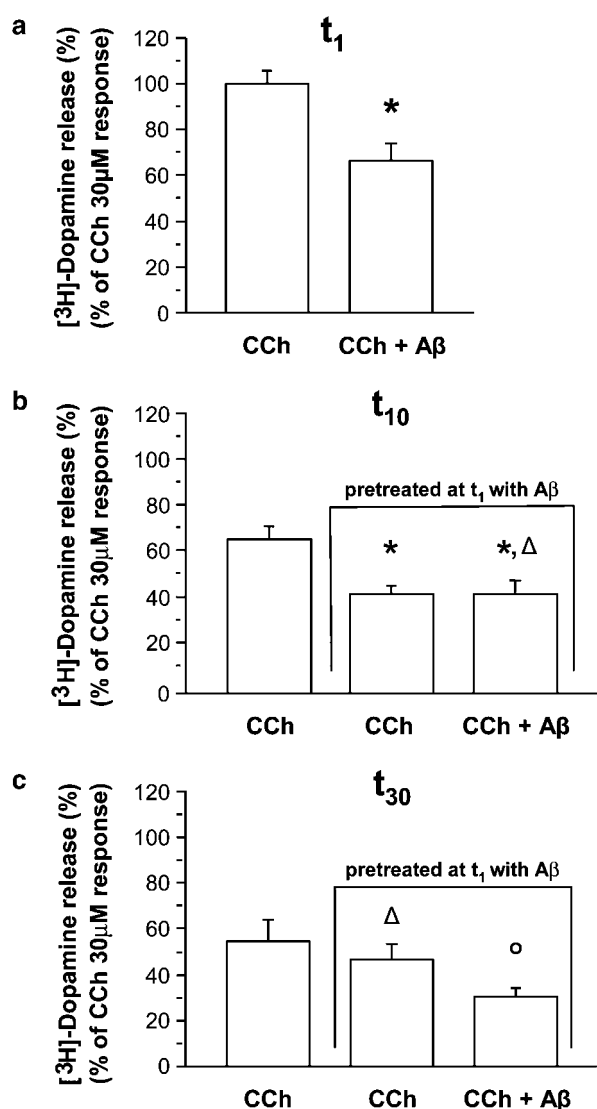
Figure 7 Effect of β-amyloid 1–40 (Aβ) on CCh-evoked DA release in nucleus accumbens synaptosomes following washing with physiological medium. (a) Effect of Aβ on CCh induced DA release. $*P<0.05$ vs 30 μM CCh (two-tailed Student's t-test). (b) Effect of Aβ on CCh-induced DA release following 10 min continuous washing with physiological medium of Aβ pretreated synaptosomes $*P<0.05$ vs 30 μM CCh, not pretreated with Aβ; $^{\Delta}P>0.05$ vs CCh, Aβ pretreated. (c) Effect of Aβ on CCh-induced DA release following 30 min continuous washing. $^{\circ}P<0.05$ vs 30 μM CCh, Aβ pretreated; $^{\Delta}P>0.05$ vs CCh, not pretreated with Aβ (two-tailed Student's t-test). Data are means ± SEM of 6–10 experiments run in triplicate.

The inhibitory effect of 100 nM Aβ on the nic evoked [³H]DA release was modest and similar to that produced by Aβ on the [³H]DA release evoked by CCh in presence of Atr.

Figure 7 shows the effect of Aβ 1–40 in control (panel a) and in Aβ 1–40 pretreated synaptosomes after 10 and 30 min washing with physiological medium (panel b and c, last two columns). Note that in each panel the control response to CCh is reported, which showed a time-dependent decrease (at t_{10} and t_{30} compared to t_1). After 10 min washing the release of [³H]DA elicited by CCh from nerve endings pretreated with Aβ was still inhibited. Moreover, further addition of Aβ at this time did not produce a further inhibitory effect (Figure 7b). However,

after 30 min of continuous washing with normal medium the response of nerve endings to CCh recovered to the respective control level and the addition of Aβ to the perfusion medium produced again an inhibition of the CCh-evoked DA release (Figure 7c).

DISCUSSION

The results reported here show that acute administration of Aβ *in vivo* and *in vitro* impairs the ability of CCh to promote DA release from DA nerve terminals in rat nucleus accumbens. This observation extends previously published data (Itoh *et al,* 1996; Li and Smith, 1996), indicating that Aβ can affect neurotransmitter release in *in vivo* or *in vitro* conditions. In particular, our experiments demonstrate that cholinergic activation by CCh administration elicits a robust DA release from the shell of nucleus accumbens in freely moving adult rats. The CCh effect is mediated by the stimulation of cholinergic muscarinic receptors as indicated by the fact that the increase of DA release is inhibited by Atr and not by Mec. The activation of nicotinic receptors, by means of Epi administration, can also promote the release of DA in the nucleus accumbens, but the extent of the activation is roughly 1/3 of that elicited by muscarinic receptor activation.

The results with isolated nerve terminals confirm and extend the *in vivo* results showing specifically that CCh and Nic can directly induce an increase in DA release by presynaptically activating both muscarinic and nicotinic receptors located on dopaminergic nerve terminals. These findings are also in agreement with previous results showing that both receptors are equally potent in stimulating DA release (Grilli *et al,* 2005; Raiteri *et al,* 1984; Summers *et al,* 1997). However, our results suggest a quantitatively predominant role of muscarinic receptors in the *in vivo* modulation of DA release by CCh. The effect of CCh through muscarinic receptors seems to involve a PKC transduction machinery downstream of the receptors, since it is prevented by the administration of the PKC inhibitor GF109203X. The effect of Aβ 1–40 was persistent when continuously infusing the peptide. On the other hand, when the infusion was stopped and CCh-induced DA release evaluated 180 and 240 min after having stopped the infusion of Aβ, the mean values appeared lower than those observed with CCh alone but the difference did not reach the statistical significance. It should be stressed that at 240 min the peptide had been almost completely cleared from the brain. *In vitro* the effect persisted for 10 but not 30 min after washing the peptide. All together, the results suggest that Aβ 1–40 may give origin to molecular events inhibiting the CCh-induced DA release that may outlast for some time its presence, but are basically reversible. Alternatively, it is possible that the remaining effect is due to small amounts of the peptide tightly bound to the tissue and not cleared after washing. Notably, while the muscarinic receptor-activated DA release was completely counteracted by 1 μM Aβ, the overflow of DA elicited by nicotinic receptors activation through Epi administration was conversely insensitive to Aβ infusion. Similarly, nicotinic stimulation of [³H]DA release from isolated nerve endings was poorly affected. The small inhibitory effect observed with Aβ *in vitro* is consistent with a partial

functional inhibition of α4β2 and α7 nAChRs by Aβ, as observed previously (for a review see Liu and Wu, 2006). It has to be noted (Salminen *et al*, 2004; Quik and McIntosh, 2006) that α7 nAChRs do not seem to be involved in the modulation of DA release in this brain area. Therefore, the modest inhibitory effect we have observed *in vitro* could indeed be due to the multiple nAChRs subtypes, not only α4β2, which are present on DA accumbal nerve ending and are involved in the modulation of DA release.

The reason for the different sensitivity of nicotinic and muscarinic receptor-mediated DA outflow stimulation has not yet been fully elucidated but it is possible that Aβ infusion acutely impairs the PKC transduction machinery as has been suggested by Zhong *et al* (2003) in their study on the disruption by Aβ of the CCh modulation of GABAergic response in prefrontal cortex (PFC). This view is supported by our *ex vivo* data showing that Aβ affects the phospho PKC/PKC ratio. It should be stressed, however, that these data indicate that PKC may mediate the effect of Aβ 1–40 but do not demonstrate it. The mechanism by which PKC inhibition may inhibit DA release may rely on the fact that this kinase phosphorylates presynaptic proteins correlated with neurotransmitter release such as GAP 43 as originally shown in rat hippocampus (Dekker *et al,* 1990) or acting through an increased calcium sensitivity of vesicle fusion as recently suggested by Lou *et al* (2005). The effect of Aβ on DA release is specific since it is not observed with the reverse peptide Aβ 40–1. It is probably due to the passage through the dialysis fiber of soluble Aβ monomers or small molecular weight oligomers, since the cutoff size of the dialysis membrane is 40 000 DA. On the other hand, immunocytochemical inspection indicated that under our experimental conditions, Aβ diffuses through the dialysis membrane to the tissue where it is found in proximity of the dialysis probe. No gross signs of neurodegeneration were observed within the area of amyloid diffusion as shown by Hoechst immunocytochemistry, and as expected within the time frame (from 40 min to few hours) using a low micromolar freshly prepared Aβ 1–40 solution. Notably, in our *in vivo* study, Aβ 1–42 (10 μM) was devoid of the effects observed with Aβ 1–40 prompting us to use Aβ 1–40. However, it is possible that, due to its greater propensity to form aggregates, the adopted conditions were unfavorable for the administration of Aβ 1–42. Indeed when we perfused Aβ 1–42 the amounts found in the nucleus accumbens were much lower than those observed following Aβ 1–40 perfusion. This observation is in agreement with the study by Sabella *et al* (2004), showing a greater propensity of Aβ 1–42 to aggregate in large molecular weight oligomers. When performing *in vitro* experiments, exposing directly the synaptosomes to the peptides, this difference was not observed and Aβ 1–40 and Aβ 1–42 had identical activities (Figure 6a). The choice of Aβ 1–40 was further supported by the observation that the 40-amino-acid long peptide is the most abundant form (Kamenetz *et al*, 2003; Terai *et al*, 2001; Walsh and Selkoe, 2007): the concentration of secreted Aβ 1–42 is about 10% that of Aβ 1–40 (Bitan *et al*, 2003). On the other hand, in other experimental conditions, Aβ 1–40 and Aβ 1–42 may even have different biological actions (Zou *et al*, 2003) and the ratio of their production may be altered by the disease, at least in familial AD (Mayeux *et al*, 1999).

These data are in agreement with the emerging view that amyloid, with mechanisms still to be fully elucidated, may regulate synaptic responses (Kamenetz *et al*, 2003; Kokubo *et al*, 2005; Zhong *et al*, 2003). Moreover, the observation that Aβ *in vivo* can regulate the activity of cholinergic synapses in turn controlling DA release is intriguing for several reasons. First, if Aβ 1–40, under conditions not leading to neurotoxicity, participates in the modulation of the cholinergic control of DA release, a disease associated with a disruption of Aβ production (either an increased production or an altered Aβ 1–40/1–42 ratio) may have functional consequences on the cholinergic regulation of other neurotransmitters before neurotoxicity is observed. In the case of the cholinergic control of DA in nucleus accumbens, these functional effects may involve alterations of adaptive capacities, selective attention or working memory and executive function.

We still do not know whether the effect of Aβ 1–40 on DA release evoked by CCh is specific for nucleus accumbens or can be observed also in other brain areas and in the case of other neurotransmitters. The prediction would be that where the release of a transmitter is modulated by muscarinic cholinergic receptors Aβ can disrupt this regulation. In our hands preliminary studies performed on PFC using the described approach were unsuccessful due to sensitivity problems. On the other hand, Trabace *et al* (2007) using a transverse dialysis probe with a greater exposed surface was able to document an inhibitory action of Aβ intraventricular infusion on DA release also in PFC. This last observation is intriguing since this area plays a critical role in elaborating thoughts, behavior and working memory. Furthermore, dysfunctions of the dopaminergic system in PFC are considered to be major contributing factors to the patho-physiology of mental disease (Trabace *et al*, 2007).

From a clinical point of view, the data obtained suggest the possibility of dysfunctions involving neurotransmitters other than acetylcholine and alterations of cognitive functions not exclusively limited to memory, possibly preceding a neurodegeneration-related loss of function. In turn, this suggests the possibility of investigating by means of neuropsychological tests, functions other than memory in the prodromic phases of the disease and to explore new therapeutic strategies.

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DISCLOSURE

None of the authors has conflict of interest.

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