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Cholinergní neurony
a
Alzheimerova choroba

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Dizertační práce

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PODĚKOVÁNÍ

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SEZNAM ZKRATEK

AC - adenylátcyklasa (též adenylylcyklasa)

AcCoA – acetyl-koenzym A

AD – Alzheimerova choroba

ACh – acetylcholin

AChE - acetylcholinesteráza

APP – prekurzor β-amyloidu (amyloid precursor protein)

APPLP1 - protein podobný prekurzoru β-amyloidu 1 (APP like protein 1)

APPLP2 - protein podobný prekurzoru β-amyloidu 2 (APP like protein 2)

APPswe - gen pro APP se švédskou mutací

APPswe/PS1dE9 - transgenní myší linie nesoucí konstrukt kódující lidské geny pro APPswe a PS1dE9

Aβ - β-amyloid

Aβ, $Aβ_{1-40}$, $Aβ_{1-42}$, $Aβ_{1-43}$ - β-amyloid o délce 40, 42 nebo 43 aminokyselin

BSA - hovězí sérový albumin

BuChE - butyrylcholinesteráza

cAMP - cyklický AMP

CTL1 - protein podobný cholinovému transportéru 1 (choline transporter-like 1)

DAG - diacylglycerol

DHA - dokosahexaenová kyselina

EC – extracelulární

Gs,i,o,q,11 - skupiny G proteinů

EC x.x.x.x – označení enzymu podle International Union of Biochemistry and Molecular Biology

GPCR - receptor spřažený s G-proteiny (G-protein coupled receptor)

GTP - guanosin-5´-trifosfát

GTP-γS - nehydrolyzovatelný analog GTP

Ch1-Ch8 - skupiny cholinergních neuronů v mozku

ChAT - cholinacetyltransferáza

ChE - cholinesteráza

CHO - buněčná linie získaná z fibroblastů vaječníků křečka (chinese hamster ovary)

ChT1 - cholinový transportér 1

IC - intracelulární

IP_3 – inositol-1,4,5-trisfosfát

K_i - inhibiční konstanta

K_t - transportní konstanta

M – muskarinový receptor

M_1 až **M_5** - podtyp 1 až 5 muskarinových receptorů

mAChR – muskarinový acetylcholinový receptor

N – nikotonový receptor

NE – noradrenalin

NG108-15 - cholinergní buněčná linie

NMS - N-metylskopolamin, neselektivní muskarinový antagonista

Pen-2 - součást komplexu γ-sekretázy

PI - fosfatidylinositol

PIP- fosfatidylinozitol-4-fosfát

PIP2 - fosfatidylinositol-4, 5-bisfosfát

PLC – fosfolipáza C

PRBCM - propylbenzilylcholine mustard, neselektivní irreverzibilní antagonista muskarinových receptorů

PS1 - presenilin 1

PS1dE9 - gen pro PS1 s deleční mutací devátého exonu

PS2 - presenilin 2

UK14304 - specifický agonista α_2-adrenergnich receptorů

VAChT - váčkový přenašeč acetylcholinu

β-adrR – beta adrenergní receptor

OBSAH

1 SOUHRN

Alzheimerova choroba je degenerativní onemocnění mozku, jehož výskyt se zvyšuje s rostoucím věkem a které každoročně postihuje vyšší počet osob. Jejím typickým funkčním příznakem jsou poruchy různých mentálních funkcí včetně poruch krátkodobé paměti a rozvoje změn v chování. Je vyvolána zvýšenou produkcí β-amyloidu, která vede k typickému hromadění extracelulárních β-amyloidových plaků. Přirozené stárnutí, a ve zvýrazněné formě i Alzheimerovu nemoc, doprovází úbytek cholinergních neuronů a ochabování cholinergního nervového přenosu v mozku. Ve své práci jsem se zabývala změnami, ke kterým dochází v mozkovém cholinergním systému v průběhu stárnutí u kontrolních myší a u transgeního myšího modelu Alzheimerovy choroby (APPswe/PS1dE9). V této souvislosti jsem se také zabývala vlivem dokosahexaenové kyseliny (ω-3 esenciální mastná kyselina) na udržování cholinergního fenotypu u neuronální cholinergní buněčné linie NG108-15 a v pokusech *ex vivo* na mozkové kůře laboratorního potkana studiem účinku muskarinového agonisty xanomelinu, který byl vyvýjen jako lék pro selektivní posílení přenosu M_1 muskarinovými receptory u Alzheimerovy choroby, na muskarinové M_2 a M_4 receptory.

Z pokusů vyplývá, že transgenní myši APPswe/PS1dE9, produkující zvýšené množství β-amyloidu, vykazují úbytek některých markerů cholinergních synapsí a funkční poškození muskarinové transmise již u mladých zvířat, u kterých se teprve začíná objevovat charakteristická patologie. Poruchy se týkají presynaptické i postsynaptické části synapse. Na modelu cholinergní dediferenciace u neuronální cholinergní buněčné linie NG108-15, vyvolané odstraněním séra z kultivačního média, jsme potvrdili obecný neuroprotektivní účinek dokosahexaenové kyseliny. Navíc jsme však zjistili, že v koncentraci přibližně desetkrát vyšší (ale stále fyziologické), než je koncentrace zajišťující obecnou neuroprotekci, podporuje dokosahexaenová kyselina i expresi cholinergního fenotypu zjišťovanou podle aktivity cholinacetyltransferázy. Při studiu účinku předpokládaného M_1 selektivního muskarinového agonisty xanomelinu, který se ale podle výsledků získaných na heterologně exprimovaných muskarinových receptorech váže na všechny podtypy muskarinových receptorů reversibilně do ortosterického vazebného místa a neodmyvatelně do jiného vazebného místa, jsme zjistili, že v přirozeném preparátu aktivuje i M_2 a M_4 receptory. Aktivace těchto podtypů vyžaduje vytvoření

neodmyvatelné vazby, kterou nelze zablokovat klasickým antagonistou, projevuje se opožděně i v nepřítonosti volného ligandu a má ortosterickou i alosterickou složku účinku.

Výsledky této práce tedy podporují cholinergní hypotézu onemocnění, která předpokládá významnou úlohu oslabování cholinergní transmise již v časné fázi onemocnění. Dále ukazují, že dostatečný příjem dokosahexaenové kyseliny v potravě může mít kromě obecného neuroprotektivního účinku příznivý vliv i na udržování fenotypu cholinergních neuronů. Výsledky funkčních pokusů s xanomelinem poskytují možné vysvětlení jeho vedlejších účinků, které vedly k přerušení klinického testování této látky. Navíc přispívají k pochopení mechanizmu účinku xanomelinu, který by mohl sloužit jako prototyp nového druhu muskarinových agonistů s dlouhodobým působením.

2 SUMMARY IN ENGLISH

Alzheimer's disease is a degenerative brain disorder. The incidence of the disease increases with age and every year the total number of affected persons is higher. The malfunction of various mental functions is a typical feature of Alzheimer's disease, including loss of short-term memory and development of personality changes. It is now generally accepted that the main cause of the disease is increased production of β-amyloid fragments that mediate toxic effects and lead to β-amyloid plaques formation. Alzheimer's disease and also normal aging are accompanied by a loss of cholinergic neurons and weakened cholinergic neurotransmission. In my thesis I dealt with changes in the brain cholinergic system during aging in control mice and in a mouse model of Alzheimer's disease (APPswe/PS1dE9). In this context I also investigated in vitro influence of docosahexaenoic acid (ω-3 essential fatty acid) changes in membrane cholesterol content on the expression of cholinergic phenotype in the NG108-15 cholinergic cell line. I also performed *ex vivo* experiments on rat brain cortex to investigate the characteristics of action of muscarinic agonist xanomeline that was developed as a selective muscarinic agonist to strengthen signalization through the muscarinic M_1 receptor in Alzheimer´s disease.

The experiments on APPswe/PS1dE9 transgenic mice producing increased amount of β-amyloid demonstrated a decrease of some cholinergic markers and functional damage of muscarinic neurotransmission. These changes were already apparent in young animals at the time when increased production of β-amyloid and plaque pathology just started to appear. The disorders of cholinergic markers were present in both presynaptic and postsynaptic compartments of cortical cholinergic synapses. The general neuroprotective effects of docosahexaenoic acid was confirmed in a model of cholinergic dedifferenciation in the NG108-15 cell line cultured in medium without serum. Moreover, we found out that docosahexaenoic acid (in a concentration about 10 times higher than the concentration needed for general neuroprotection) supports expression of cholinergic phenotype manifested as an increase in cholineacetyltransferase activity. Xanomeline was expected to be M_1 selective muscarinic agonist but later was discovered to bind reversibly to the orthosteric binding site on all muscarinic receptors subtypes and also in a wash-resistant manner to another (allsoteric) binding site. We demonstrated that

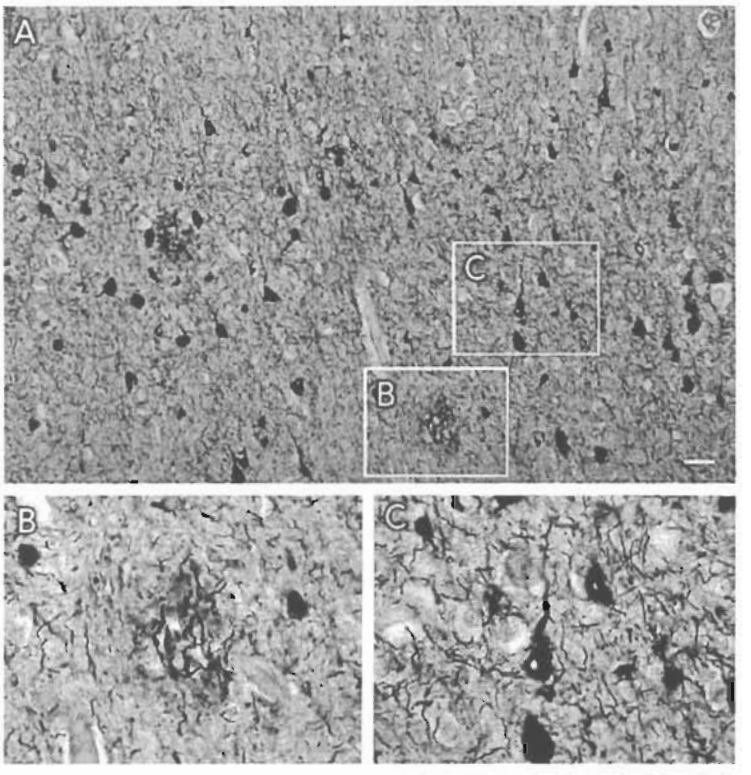
xanomeline also activates M_2 and M_4 muscarinic receptors in rat brain cortex *ex vivo* (i.e. maintained in physiological environment) and that the wash-resistant binding is necessary for this effect. The wash-resistant binding of xanomeline is resistent to blockade by the classical muscarinic antagonist atropine. The stimulatory effects of xanomeline appeared with a time delay, persisted in the absence of free ligand, and exhibited both orthosteric and allosteric components of action.

The cholinergic hypothesis of dementia is based on the major role of cholinergic neurotransmission in the pathogenesis of Alzheimer's disease. Our results provide strong support to this hypothesis by demonstrating an early *in vivo* impairment of cholinergic synapses. Furthermore, our results indicate that sufficient intake of docosahexaenoic acid may improve maintenance of the phenotype of cholinergic neurons, in addition to the known general neuroprotective effects. The results of functional experiments on xanomeline delayed effects offer a plausible explanation for side effect observed during clinical studies. They help to understand the mechanisms of the delayed stimulatory effect of xanomeline that may serve as the prototype of a novel class of muscarinic agonists with long-term action.

3 LITERÁRNÍ PŘEHLED

3.1 ALZHEIMEROVA CHOROBA

Alzheimerova choroba (AD) je degenerativní mozkové onemocnění doprovázené progresivním poškozením paměti, myšlení, chápání a řečových schopností, které v pokročilejším stadiu znemožňuje nemocnému jedinci se o sebe samostatně postarat. Mezi 65. a 85. rokem života se výskyt tohoto onemocnění zdvojnásobuje každých 5 let. Odhaduje se, že v současné době je Alzheimerovou chorobou na světě postiženo 18 milionů osob a do roku 2025 se jejich počet zdvojnásobí (Světová zdravotnická organizace, 2007). Charakteristickým znakem onemocnění je v mozku *post mortem* zjistitelná přítomnost extracelulárních neuritických plaků (obrázek 1 A a B) tvořených β-amyloidem, poškozenými neurity a dalšími proteiny. Neuritické plaky často obsahují mikroglie a jsou obklopeny reaktivními astrocyty. Kromě hromadění β-amyloidových plaků je dalším patologickým nálezem u Alzheimerovy choroby hromadění intracelulárních neurofibrilárních klubíček (obrázek 1 A a C), která jsou tvořena hyperfosforylovaným proteinem tau. Tento protein se vyskytuje především v neuronech, podílí se na stabilizaci mikrotubulů a za patologických podmínek může dojít k jeho hyperfosforylaci, agregaci a vytváření neurofibrilárních klubíček (Selkoe, 2001a; Lopez a DeKosky, 2003). Vytváření neurofibrilárních klubíček ale není specifické pouze pro Alzheimerovu chorobu. Dochází k němu i u řady jiných neurologických onemocnění (Avila, 2004). Mezi patologické nálezy, které se objevují i u jiných onemocnění, patří i další nespecifické příznaky, jako je ztráta neuronů a synapsí, Lewyho tělíska nebo granulovaskulární degenerace (Selkoe, 2001a; Lopez a DeKosky, 2003). Rozlišujeme dvě formy onemocnění Alzheimerovou chorobou: formu sporadickou a formu familiární. Sporadická forma zpravidla začíná po 65. roce věku, představuje většinu případů Azheimerovy choroby a její příčiny jsou neznámé. Familiární forma se zpravidla projevuje již před 65. rokem věku a je podmíněna geneticky. Na celkové populaci postižených Alzheimerovou chorobou se podílí jen několika procenty.



Obrázek 1: Neuritické plaky a neurofibrilární klubíčka. Kůra z temporálního laloku mozku člověka postiženého Alzheimerovou chorobou. Na výřezu B jsou znázorněny neuritické plaky, na výřezu C neurofibrilární klubíčka. Měřítko odpovídá 12,5 μm. (Gotz a spol., 2006)

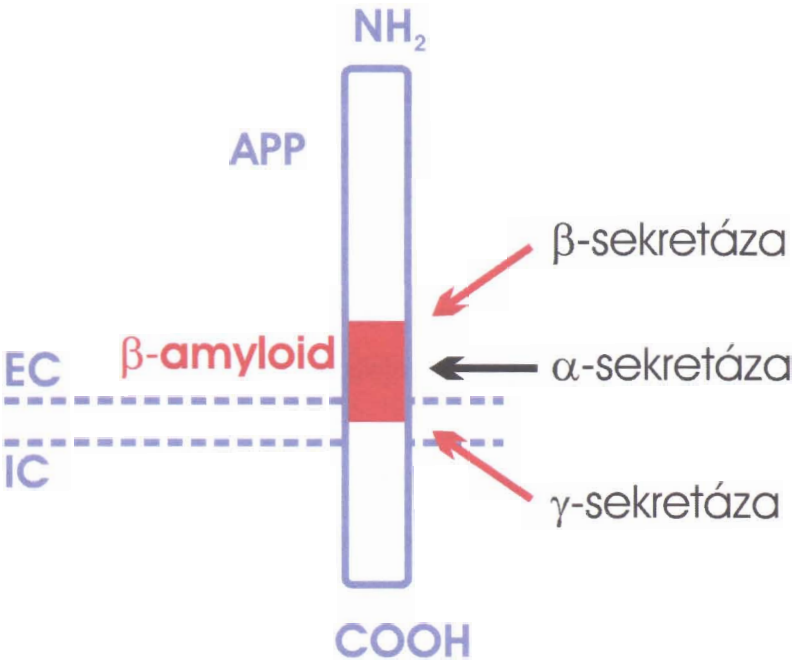
Hlavní složkou neuritických plaků je β-amyloid (Aβ), což je peptid skládající se z 39-43 aminokyselin, který vzniká štěpením většího membránového proteinu nazývaného prekurzor β-amyloidu (APP; amyloid precursor protein). Gen pro tento protein je velký přibližně 400 kb a obsahuje minimálně 18 exonů. Alternativním sestřihem může vznikat protein o velikosti 365 až 770 aminokyselin. Spolu s proteiny podobnými APP 1 a 2 (APPLP1 a APPLP2; APP like protein 1 a 2) patří do stejné proteinové rodiny (conserved type I membrane proteins). V neuronech je exprimován především ve variantě P695 v oblasti postsynaptického ztluštění, na axonech a na dendritech. Jde o membránový glykoprotein s velkou extracelulární N-terminální a malou intracelulární C-terminální doménou. Jeho fyziologická funkce dosud není vyjasněna (Zhang a Koo, 2006). Knockout APP není letální a APP není nepostradatelný pro správný vývoj myši (Zheng a spol., 1996). V důsledku odstranění APP byla u starších myší zaznamenány pouze nižší hmotnost, poruchy kognitivních funkcí a dlouhodobé potenciace, reaktivní glióza a snížení množství synaptických

markerů v kůře a hipokampu (Dawson a spol., 1999). Při odstranění APP jeho funkci zřejmě dokáže nahradit APPLP2 (Heber a spol., 2000). APP/APPLP2 knockout je v 80 % u narozených myší letální v prvním týdnu života (von Koch a spol., 1997). Myši s odstraněnými geny pro APP/APPLP2 mají ve srovnání s těmi s pouze odstraněným APPLP2 cholinový transportér 1 umístěný z velké části na axonech místo na presynaptickém zakončení nervosvalové ploténky a hemicholinium-3 senzitivní transport cholinu do mozkových cholinergních synaptozomů je u těchto zvířat snížen (Wang a spol., 2007).

APP je štěpen proteázami, které jsou označovány na základě místa štěpení jako α-, β- nebo γ-sekretázy (obrázek 2) (Selkoe, 2001a; Doležal a Kašparová, 2003; Racchi a Govoni, 2003; Zhang a Koo, 2006). α-sekretáza štěpí APP uvnitř sekvence β-amyloidu. Je známo několik různých proteáz (ADAM9/MDC-9, ADAM10 a ADAM17/TACE), které mají α-sekretázovou aktivitu (Allinson a spol., 2003). Pokud je tedy APP rozštěpen α-sekretázou, Aβ nemůže vzniknout. Aβ vzniká postupným štěpením β- a γ-sekretázou. β-sekretáza štěpí APP na N-konci a vytváří v membráně vázaný C-terminální fragment APP. Tento fragment štěpí přibližně uprostřed membránového úseku γ-sekretáza a uvolňuje Aβ fragmenty dlouhé 39 až 43 aminokyselin, především $Aβ_{1-40}$ a $Aβ_{1-42}$ (Nunan a Small, 2000; Racchi a Govoni, 2003). Nedávno bylo zjištěno, že ve fyziologických koncentracích $Aβ_{1-42}$ snižuje aktivitu sfingomyelinázy a množství sfingomyelinu, $Aβ_{1-40}$ snižuje aktivitu hydroxymethylglutaryl-CoA syntázy a množství syntetizovaného cholesterolu a familiární mutace presenilinů, které zvyšují poměr $Aβ_{1-42}/Aβ_{1-40}$, snižují množství sfingomyelinu a zvyšují množství cholesterolu (Grimm a spol., 2005).

$Aβ_{1-42}$ má mnohem větší sklony k agregaci a předpokládá se, že se podílí na iniciaci tvorby oligomerů, fibril a plaků (Younkin, 1995). γ-sekretáza je enzymový komplex štěpící v hydrofobní membránové části kromě APP i další proteiny, jako je například Notch (Selkoe, 2001B; De Strooper, 2003; Parks a Curtis, 2007). Důležitou částí γ-sekretázového komplexu jsou presenilin 1 (PS1) a presenilin 2 (PS2). Katalytické jádro komplexu γ-sekretázy tvoří nejméně 4 proteiny: PS1 nebo PS2, nicastrin a dalšími složkami jsou u Caenorhabditis elegans Aph-1 a Pen-2 (Wolfe, 2006). Není překvapivé, že u PS1-/- myši je výrazně narušen embryonální vývoj a její fenotyp je výrazně podobný Notch1 -/- myši (Wong a spol., 1997; Shen a spol., 1997). Zdá se, že inhibice γ-sekretázy, která byla považovaná za slibnou cestu k léčbě Alzheimerovy choroby, nebude použitelná z důvodů vážných vedlejších

účinků způsobených zřejmě inhibicí štěpení proteinu Notch (Searfoss a spol., 2003; Wong a spol., 2004).



Obrázek 2: Schéma štěpení β-amyloidu sekretázami. β - amyloidová doména je zvýrazněna červeně. Pro zjednodušení je pro každý enzym vyznačeno jen jedno místo štěpení.

Hlavní argumanty podporující teorii o významné úloze Aβ při vzniku Alzheimerovy choroby vycházejí z genetických studií. Všechny mutace, o kterých je známo, že způsobují vznik Alzheimerovy choroby, postihují vlastní APP nebo PS1 a PS2, které se jako součást enzymového komplexu γ-sekretázy podílejí na katabolismu APP. V současné době je známo 15 mutací v APP, 120 v PS1 a 12 v PS2, které způsobují vznik Alzheimerovy choroby (Alzheimer Disease & Frontotemporal Dementia Mutation Database). Mutace APP poblíž místa štěpení β-sekretázou zvyšují štěpení APP tímto enzymem. Mutace poblíž γ-sekretázového místa APP a mutace presenilinů zpravidla zvyšují poměr vznikajícího $Aβ_{1-42}$ oproti $Aβ_{1-40}$. Mutace ve vlastní sekvenci Aβ mění jeho vlastnosti a usnadňují jeho agregaci (Wolfe, 2007). Škodlivé mutace v genu pro APP jsou zpravidla umístěny poblíž některé oblasti štěpení sekretázami a vedou ke zvýšení produkce Aβ nebo ke zvýšení produkce $Aβ_{1-42}$ oproti $Aβ_{1-40}$ (Selkoe, 2001a a 2001b). Mezi nejznámější patří takzvaná švédská mutace

(APPswe) způsobující záměnu lysinu a methioninu na pozicích 670 a 671 za kyselinu aspartovou a leucin.

Významný argument podporující význam β-amyloidu jako faktoru, který způsobuje Alzheimerovu chorobu, je odvozen z nálezů u Downova syndromu. U tohoto onemocnění (trisomie 21. chromosomu) je přirozený (nemutovaný) gen pro APP přítomen ve třech kopiích, což se projeví nadprodukcí přirozeného APP a jeho metabolitů (gene dosage effect). Všichni postižení Downovým syndromem onemocní Alzheimerovou chorobou (Mann a Esiri, 1989). K prvním známkám ukládání β-amyloidu u nich dochází již ve 12ti letech věku a degenerace v okolí plaků začíná již kolem třicátého roku věku (Lemere a spol.,1996).

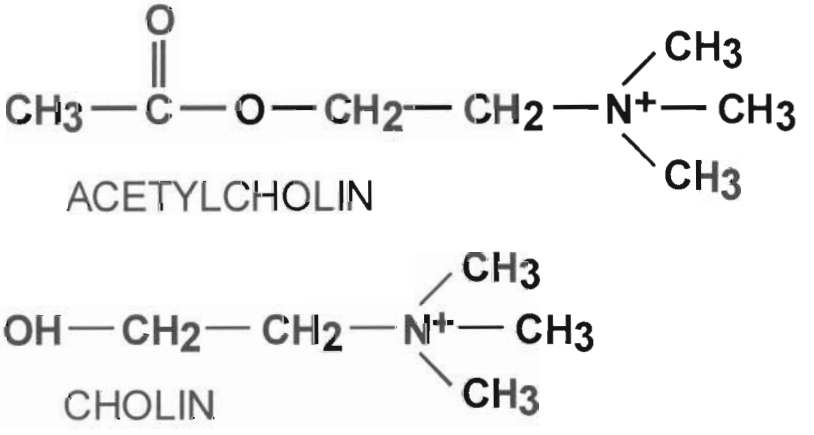
Navzdory tomu, že je zřejmé, že i na vzniku sporadické formy onemocnění se podílí genetické predispozice (Gatz a spol., 1997), byl dosud potvrzen pouze polymorfizmus v genu pro apolipoprotein E jako faktor, který prokazatelně ovlivňuje pravděpodobnost vzniku sporadické formy onemocnění (Strittmatter a spol., 1993 a 1995; Poirier, 2000). Uvažuje se ale o velkém množství dalších genů, které by mohly spolu s environmentálními faktory zvyšovat nebo snižovat pravděpodobnost vzniku Alzheimerovy choroby (Palotas a spol., 2007). Přesto je prvotní příčina vzniku zvýšené produkce β-amyloidu u sporadických případů zatím neznámá. V současné době však existuje shoda, že jedním z prvotních impulzů pro vznik a rozvoj funkčních a patologických změn je zvýšená hladina rozpustných oligomerů β-amyloidu (Aβ), která může být způsobena buď zvýšenou produkcí nebo sníženým odbouráváním Aβ, zvláště $A\beta_{1-42}$, který má větší sklony k oligomerizaci než $A\beta_{1-40}$. Působení těchto oligomerů na synapse má významnou úlohu v rozvoji onemocnění (Selkoe, 2002; Walsh a Selkoe, 2007; Haass a Selkoe, 2007).

U sporadických forem existuje množství hypotéz, které se snaží vysvětlit mechanizmus vzniku onemocnění (Ann N Y Acad Sci, 2000). Jednou z těchto hypotéz je cholinergní hypotéza (Bartus a spol., 1982; Barthus, 2000), kterou se zabývám ve své práci. Tato hypotéza předpokládá vysokou citlivost centrálních cholinergních neuronů ke škodlivému působení Aβ, která se projevuje jejich poškozením u pacientů s Alzheimerovou nemocí, a z účasti muskarinové neurotransmise při neamyloidogenním metabolizmu APP (Nitsch a spol., 1992 a 2000; Rossner a spol., 1998).

3.2 CHOLINERGNÍ NEURONY

3.2.1 Cholinergní synapse

Mediátorem cholinergních synapsí je acetylcholin, který byl prvním objeveným neuropřenašečem. Již v roce 1921 O. Loewi popsal, že stimulace bloudivého nervu (n. vagus) vede v žabím srdci k uvolňování látky, která přenáší fyziologický účinek stimulace, tedy zpomalení frekvence srdečních kontrakcí, a prokázal tím chemický přenos nervového signálu. Tato látka byla později identifikována jako acetylcholin (obrázek 3) (Loewi a Navrátil, 1926; Dale a spol., 1933; Donnerer a Lembeck, 2006). Profesor O. Loewi a sir Henry Dale byli za průkaz chemického přenosu nervového signálu a identifikaci neuromediátoru acetycholinu oceněni v roce 1936 Nobelovou cenou za lékařství.



Obrázek 3: Molekula acetylcholinu a jeho prekurzoru cholinu.

Cholinergní synapse jsou rozmístěny jak v centrálním, tak i periferním nervovém systému. Vlastní cholinergní synapse (obrázek 4) je tvořena z presynaptické a postsynaptické části. Presynaptické zakončení cholinergního neuronu zajišťuje syntézu, skladování a uvolňování acetylcholinu. Na postsynaptické části jsou acetylcholinové receptory, které zprostředkují odpověď na uvolněný acetylcholin. Kromě proteinů potřebných k uvolňování jakéhokoliv mediátoru obsahuje cholinergní zakončení specifické proteiny zajišťující syntézu a skladování acetylcholinu (Tuček, 1978). K těmto specificky cholinergním proteinům patří enzym zajišťující syntézu acetylcholinu cholinacetyltransferáza (ChAT) (Korey a spol., 1951), váčkový transportér acetylcholinu (VAChT; Erickson a spol., 1994; Roghani a

spol., 1994) zajišťující přenos syntetizovaného ACh do synaptických váčků a vysokoafinní cholinový přenašeč transportující z extracelulární tekutiny prekurzor acetylcholinu, tedy cholin (obrázek 3), do cholinergních zakončení (ChT1; Okuda a spol., 2000; Okuda a Haga, 2000; Apparsundaram a spol., 2000). Na základě *in situ* hybridizace a imunohistochemie je známo, že ChT1 se vyskytuje převážně na synapsích cholinergních neuronů a jeho lokalizace je podobná lokalizaci ChAT a váčkového přenašeče pro ACh (Weihe a spol., 1998). Pro správné fungování synapse je také nutná přítomnost enzymů, které ukončí působení mediátoru. Účinek ACh je, na rozdíl od většiny ostatních nízkomolekulárních přenašečů, kde je působení neuromediároru ukončeno jeho vychytáváním, ukončen hydrolýzou, a to na cholin a kyselinu octovou, prostřednictvím enzymů acetylcholinesterázy nebo butyrylcholinesterázy (Massoulié, 2002).

3.2.2 Syntéza a skladování acetylcholinu

Acetylcholin je syntetizován v cytoplazmě cholinergních zakončení enzymem **cholinacetyltransferázou (ChAT**, acetyl CoA:choline O-acetyltransferase EC 2.3.1.6) z cholinu a acetylkoenzymu A (acetyl-CoA):

cholin + acetyl-CoA <--------> acetylcholin + CoASH

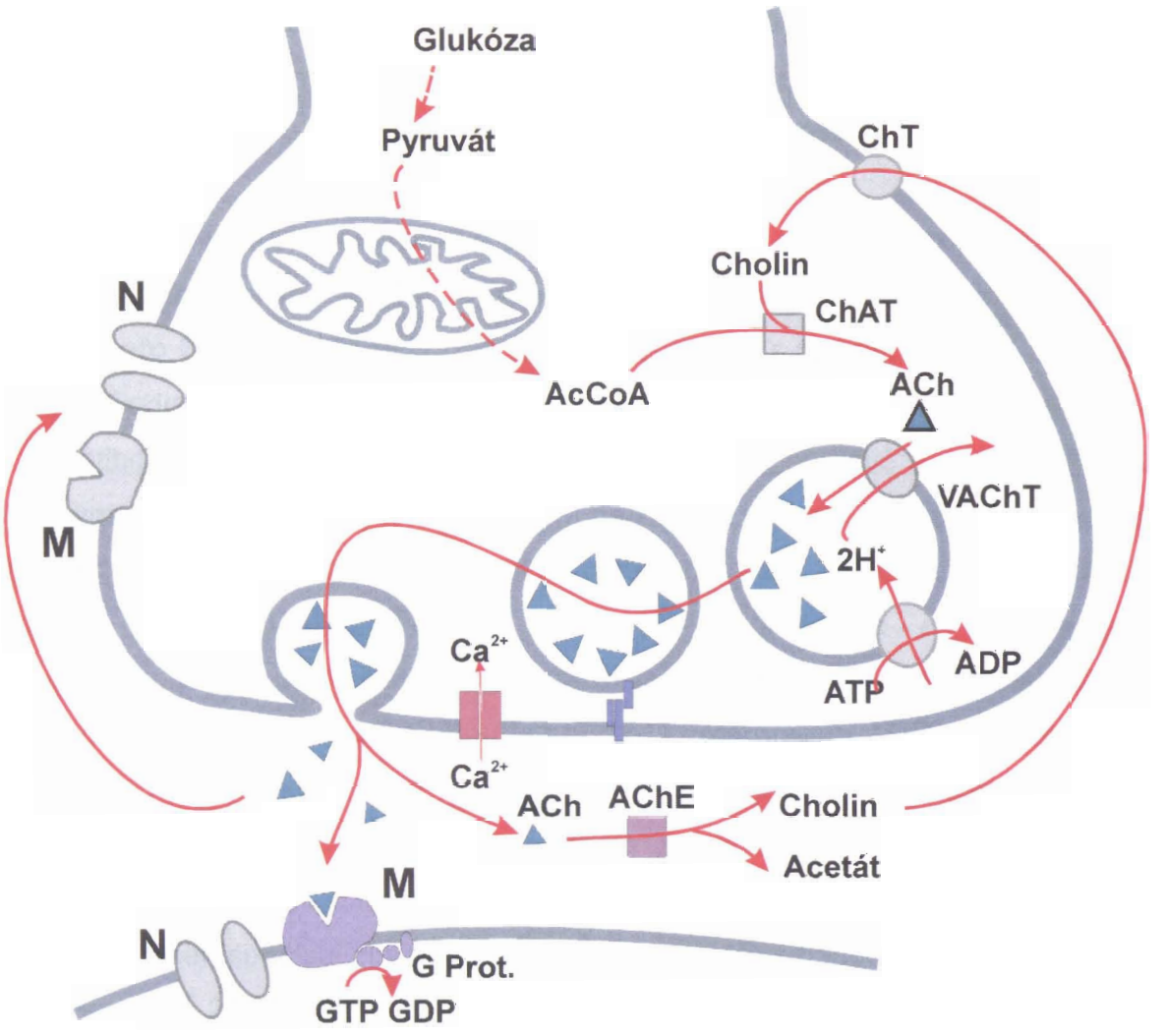
ChAT je nejčastěji používaný marker přítomnosti cholinergních neuronů a cholinergních zakončení jak v centrálním, tak v periferním nervovém systému. Gen pro ChAT se nachází společně s genem pro VAChT na takzvaném cholinergním loku (Bejanin a spol., 1994; Erickson a spol., 1994). Zvláštnost uspořádání tohoto loku spočívá v tom, že gen pro ChAT obsahuje ve svém prvním intronu celý gen pro VAChT a oba geny tedy obsahují i společná regulační místa. Toto uspořádání umožňuje společnou regulaci exprese obou genů. Možná je ale i nezávislá exprese obou proteinů, která se objevuje například během embryonálního a časného postnatálního vývoje (Holler a spol., 1996). Na základě alternativního sestřihu může vznikat protein o velikosti 69 nebo 82 kDa. Enzym se může nacházet v rozpustné formě (80-90%) nebo ve formě vázané na membránu (10-20 %) (Oda, 1999). Má fosforylační místa pro několik různých proteinových kináz. Fosforylace nebo defosforylace těchto míst ovlivňuje jeho aktivitu a přítomnost v membránové nebo cytoplazmatické frakci (Prado a spol., 2002).

Do nitra synaptického váčku, který slouží jako zásobárna uvolnitelného mediátoru, je acetylcholin transportován váčkovým přenašečem pro acetylcholin (VAChT) (Alfonso a spol, 1993; Bejanin a spol., 1994; Erickson a spol., 1994; Roghani a spol., 1994). VAChT je glykoprotein složený z 500 - 600 aminokyselin a je příbuzný váčkovému monoaminovému přenašeči obsahujícímu 12 transmembránových úseků. C a N konec má obrácený směrem do cytoplazmy. K transportu dochází výměnou jedné molekuly acetylcholinu za 2 protony (Nguyen a spol., 1998).

Cholin nezbytný pro syntézu ACh je do cholinergních zakončení dopravován vysokoafinním cholinovým přenašečem 1 (choline transporter 1; ChT1) (Apparsundaram a spol., 2000; Okuda a spol., 2000; Okuda a Haga, 2000), který vykazuje vlastnosti typické pro již dříve farmakologicky definovaný vysokoafinní transport cholinu (high affinity choline transport system; Yamamura a Snyder, 1972 a 1973; Haga a Noda, 1973), tj. K_t v nízké mikromolární oblasti (okolo 2μM), účinnou inhibici hemicholiniem-3 (K_i v rozmezí 2-10 nM) a závislost na Na^+ a Cl^- v extracelulárním prostředí. Kromě ChT1 je dalším předpokládaným cholinovým transportérem vykazujícím některé vlastnosti vysokoafinního transportu cholinu protein CTL1 (choline transporter like protein 1), který ale není typický pro cholinergní nervová zakončení (O'Regan, 2000). Vzhledem k tomu, že koncentrace cholinu je v mozkomíšním moku oproti plazmě nízká (Loffelholz, 1998), mohl by se tento přenašeč uplatňovat zejména v mozku při vychytávání cholinu pro syntézu fosfolipidů nervovými i gliovými buňkami.

3.2.3 Regulace syntézy ACh

Aktivita ChAT měřená v homogenátech je mnohem větší, než je maximálně stimulovaný obrat acetylcholinu zjištěný v mozkových řízcích *in vitro* a mozkové tkáni *in vivo,* a není tedy pravděpodobně omezujícím (rate limiting) krokem při syntéze ACh. Limitujícím faktorem je dostupnost obou substrátů. Syntéza ACh je regulována dodávkou cholinu, který se v cholinergních neuronech nesyntetizuje, prostřednictvím ChT1 (Ribeiro a spol., 2006). Acetyl-CoA je vytvářen v mitochondriích. Převažujícím zdrojem acetyl-CoA, který cholinergní zakončení využívají pro syntézu ACh, je glukóza a její metabolit laktát. Z mitochodrií do cytoplazmy se acetylová skupina dostává převážně jako acetylkarnitin (Doležal a Tuček, 1981; Tuček a spol., 1981). Nedostupnost acetyl-CoA může rovněž omezovat syntézu ACh (Doležal a Tuček, 1982; Trommer a spol., 1982; Maire a Wurtman, 1985).



Obrázek 4: Syntéza, skladování a odbourávání acetylcholinu. Acetylcholin je syntetizován cholinacetyltransferázou (ChAT). Cholin pro tuto syntézu je získáván především transportem z extracelulárního prostředí cholinovým transportérem ChT1. Do synaptických váčků se acetylcholin dostává váčkovým přenašečem pro acetylcholin (VAChT) výměnou za dva protony. Po uvolnění acetylcholinu (ACh) ze synaptických váčků je ACh navázán na nikotinové (N) nebo muskarinové (M) receptory, kde vyvolává příslušnou odpověď nebo je rozštěpen acetylcholinesterázou (AChE).

3.2.4 Acetylcholinové receptory

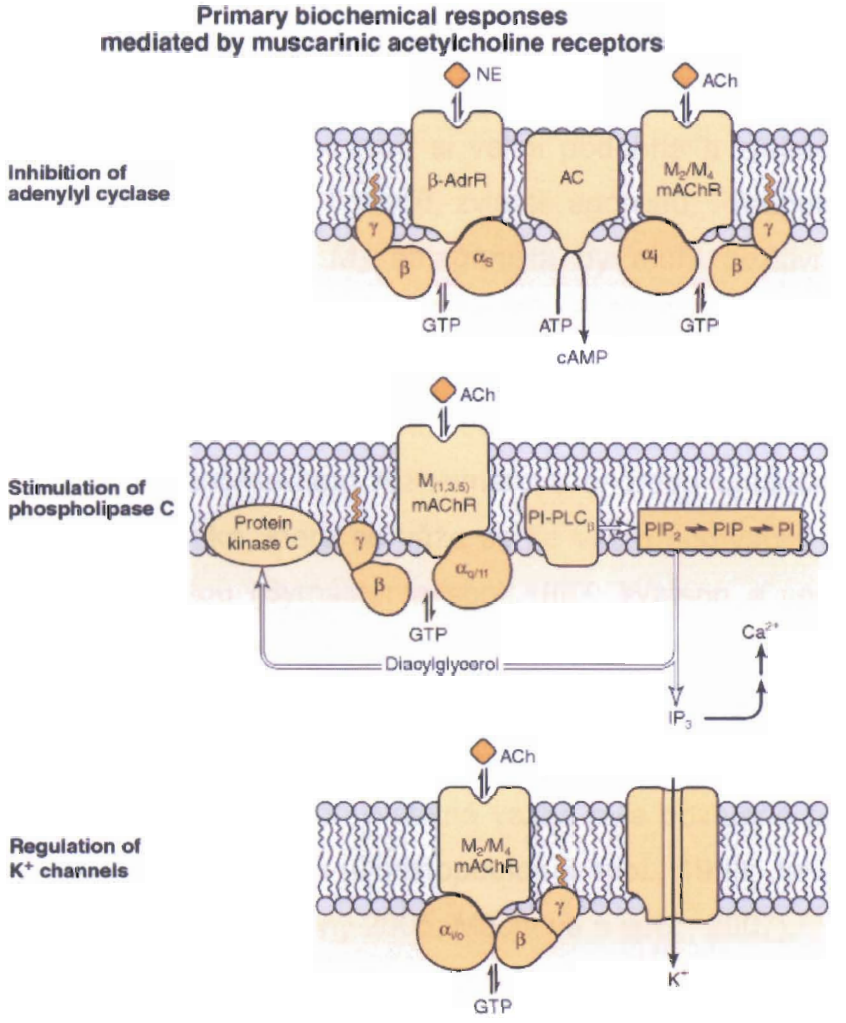
Acetylcholinové receptory se rozlišují na dva základní podtypy, které byly historicky pojmenovány podle farmakologického účinku přírodních agonistů muskarinu a nikotinu. Muskarin (alkaloid z muchomůrky červené *Amanita muscaria*) aktivuje muskarinové receptory a jeho účinek je specificky blokován atropinem (alkaloid obsažený například v rulíku zlomocném *Atropa belladonna*). Naproti tomu

nikotin (alkaloid obsažený v tabáku *Nicotiana tabacum*) aktivuje nikotinové receptory a účinek v podobě stimulace nikotinových receptorů je na nervosvalové ploténce specificky blokován d-tubokurarinem (obsaženo v rostlině *Chondrodendron tomentosum*).

Nikotinové receptory jsou iontové kanály, které jsou tvořeny pěti podjednotkami kruhovitě uspořádanými kolem centrálního póru, který je při otevření kanálu propustný pro kationty. Jde tedy o ionotropní receptory s rychlou aktivací a rychlým účinkem. Podjednotky, které tvoří nikotinový receptor, se označují jako α_{1-x}, β_{1-4}, γ, δ a ε. Nikotinové receptory lze podle lokalizace rozdělit na nervové a svalové. Prvním popsaným nikotinovým receptorem, citlivým na kurare, je svalový nikotinový receptor. Tento receptor se skládá ze dvou podjednotek α_1, dále podjednotek β_1,δ a ε u dospělých savců. V embryonálním období je podjednotka ε nahrazena γ podjednotkou. Neuronální nikotinové receptory jsou pentamery tvořené kombinacemi podjednotek α_{2-10} a β_{2-4}, které mohou být homo- i heteromerní. Většina nikotinových receptorů tvoří neselektivní iontový kanál, ale některé, jako α_7 homopentamer, mají vysokou selektivitu pro vápník. V centrálním nervovém systému se nikotinové receptory hojně vyskytují jako presynaptické nebo preterminální receptory, které mají modulační účinek spočívající ve zvýšeném uvolňování mediátorů (Wonnacott, 1997; Dani a Bertrand, 2007).

Muskarinové receptory jsou receptory spřažené s heterotrimerními G-proteiny (G-protein-coupled receptors, GPCR) (obrázek 5). K aktivaci G-proteinu a k proběhnutí jím vyvolané odpovědi je potřeba více času než k prostému otevření iontového kanálu u nikotinových receptorů. To se projevuje v rychlosti odpovědi zprostředkované těmito receptory, která je oproti nikotinovým receptorům pomalejší o 100-250 ms. Charakteristickým znakem těchto receptorů je sedm transmembránových segmentů, které jsou spojeny třemi extracelulárními a třemi intracelulárními kličkami. N-konec je umístěn extracelulárně a C-konec intracelulárně. Rozlišujeme 5 typů muskarinových receptorů, které označujeme M_1 až M_5 (Bonner a spol., 1987; Peralta a spol., 1987; Bonner a spol., 1988; Bonner, 1989). Podle typu funkční odpovědi dělíme muskarinové receptory do dvou skupin (obrázek 5). Receptory skupiny $M_{1/3/5}$ aktivují prostřednictvím G-proteinů ze skupiny $G_{q/11}$ fosfolipázu C, která z fosfatydylinositolbisfosfátu vytváří druhé posly diacylglycerol (DAG) a inositoltrisfosfát (IP3). IP3 dále zprostředkovává uvolnění Ca^{2+} z intracelulárních zásobáren a DAG aktivuje protein kinázu C. Receptory skupiny $M_{2/4}$

prostřednictvím G-proteinů skupiny $G_{i/o}$ inhibují svou α podjednotkou produkci cAMP adenylcyklázou. Kromě toho komplex podjednotek βγ může bez zapojení druhého posla přímo otvírat draslíkové kanály nebo zabraňovat otevření některých napěťově řízených vápníkových kanálů (Herlitze a spol., 1996). V různých tkáních a na různých částech synapse se nacházejí různé podtypy muskarinových receptorů. Podtyp receptoru a umístění receptoru tedy výrazně ovlivňuje následné účinky acetylcholinu (Wess, 2003).



Obrázek 5: Interakce muskarinových receptorů s G-proteiny. Interakce acetylcholinu s muskarinovým receptorem vyvolá v závislisti na podrypu receptoru různé odpovědi. M_2 a M_4 postyp intereguje s α podjednotkou G-proteinu třídy G_i a vyvolává oddělení této podjednotky od βγ podjednotek. $α_i$ podjednotka poté inhibuje adenylcyklázu. Stejná adenylcykláza může být aktivovatelná jiným receptorem, jako je například β-adrenergní receptor (nahoře). M_1, M_3 a M_5 podtyp muskarinového receptoru po navázáni agonisty interaguje s α podjednotkou třídy G_q nebo G_{11}, která poté aktivuje fosfolipázu C (uprostřed). M_2 a M_4 dále mohou prostřednictvím uvolněných βγ podjednotek G_i nebo G_o G-proteinů regulovat otvírání některých typů draslíkových kanálů (dole). Převzato z Basic Neurochemistry.

3.2.5 Ligandy muskarinových receptorů

Pro nikotinové i muskarinové receptory existuje, kromě přirozeného agonisty ACh, široké spektrum přírodních nebo umělých ligandů s různou mírou specifity pro jednotlivé podtypy receptorů. Jiné ligandy mohou působit přes vlastní vazebné místo pro acetylcholin a poté je nazýváme ortosterické ligandy. Další ligandy mohou vázat do jiného (alosterického) vazebného místa a vytvářet spolu s receptorem a ortosterickým ligandem takzvaný ternární komplex, aniž by kompetovaly o ortosterické vazebné místo. Pokud navázání alosterických ligandů usnadňuje vazbu ortosterického ligandu, jedná se o pozitivní kooperativitu, v opačném případě o negativní kooperativitu. Ortosrerická vazebná místa jednotlivých podtypů muskarinových receptorů jsou si velmi podobná a zřejmě proto existuje poměrně málo jejich specifických ligandů, zvláště agonistů. Předpokládá se, že muskarinový M_1 agonista, případně M_2 antagonista by mohl pozitivně ovlivnit stav pacientů s Alzheimerovou chorobou (Clader a Wang, 2005).

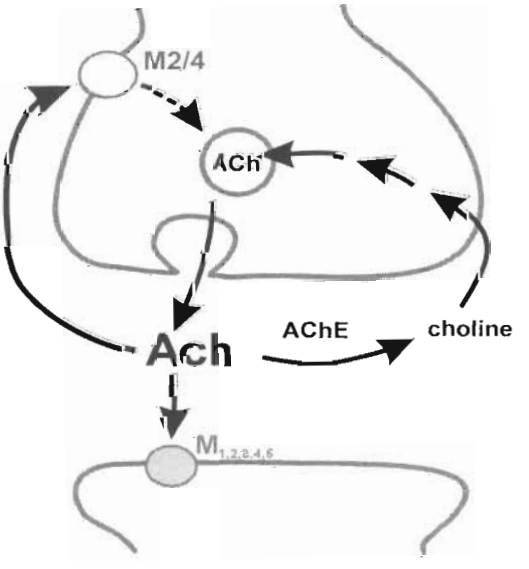
Jedním z předpokládaných selektivních agonistů M_1 muskarinových receptorů byl xanomelin, ale z důvodu výskytu vedlejších účinků v průběhu klinických studií bylo od záměru léčit xanomelinem Alzheimerovu chorobu upuštěno (Mirza a spol., 2003). Xanomelin je látka, která se váže se ke všem podtypům muskarinových receptorů s vysokou afinitou (Bymaster a spol.,1997; Watson a spol., 1989; Wood a spol., 1999; Jakubik a spol., 2006; Machová a spol., 2007), ale vykazuje funkční selektivitu pro M_1 a M_4 receptory (Shannon a spol.,1994; Ward a spol., 1995; Bymaster a spol., 1997 a 1998). Později bylo zjištěno, že vazba xanomelinu má dvě složky: reverzibilní a neodmyvatelnou. Neodmyvatelná vazba byla postupně popsána u všech podtypů muskarinových receptorů (Christopoulos a spol., 1998; Jakubík a spol., 2002, 2004, 2006; Grant a El-Fakahany 2005; Machová a spol., 2007).

3.2.6 Regulace uvolňování ACh muskarinovými autoreceptory

Uvolňování ACh probíhá kvantovým (ze synaptických váčků) nebo nekvantovým způsobem. Klidové uvolňování jednotlivých váčků se na postsynaptické membráně nervosvalového spojení projevuje miniaturními ploténkovými potenciály, zatímco nekvantové uvolňování jako mírná trvalá depolarizace (Katz a Miledi, 1977; Vyskočil a Illes, 1977), která je způsobena vytékáním ACh zprostředkovaným VAChT, který se dostává do membrány nervového zakončení během synaptické aktivity (Doležal a spol., 1983; Edwards a spol., 1985). Stimulované uvolňování, fyziologicky vyvolané

depolarizací nervového zakončení akčním potenciálem, otevřením napěťově řízených vápníkových kanálů a vtokem vápníku, je způsobeno koordinovaným uvolněním ACh z mnoha synaptických váčků. Existuje však i teorie, která ukazuje na existenci póru (mediatophore), který umožňuje na vápníku závislé stimulované uvolňování mediátorů z cytoplazmy (přehled Israel a Dunant, 1998).

Stimulované uvolňování mediátorů je regulováno různými receptory umístěnými na presynaptickém zakončení (Wu a Saggau, 1997; Miller, 1998). Z hlediska možností ovlivňování účinnosti muskarinového přenosu na mozkových cholinergních synapsích jsou důležité presynaptické muskarinové acetylcholinové autoreceptory (obrázek 6), které jsou inhibiční. Farmakologické použití muskarinových agonistů nebo zvyšování hladiny přirozeného ligandu ACh inhibicí cholinesteráz tlumí stimulované uvolňování ACh. Stimulace muskarinových autoreceptorů na centrálních cholinergních zakončeních vede ke snížení uvolňování ACh (Polak a Meeuws, 1966; Polak 1967), které je způsobeno omezením vtoku vápníku napěťově řízenými vápníkovými kanály. Toto snížení vtoku vápníku je vyvoláno interakcí kanálů s dimerem podjednotek βγ (Herlitze a spol., 1996) uvolněným z $G_{i/o}$ heterotrimerických G-proteinů citlivých na působení pertusis toxinu (Doležal a spol., 1989). V mozku hlodavců tuto autoregulaci uvolňování zprostředkovává muskarinový M_4 receptor ve striatu a M_2 receptor v mozkové kůře (Doležal a Tuček, 1998; Zhang a spol., 2002).



Obrázek 6: Schéma presynaptické inhibice uvolňování acetylcholinu. Acetylcholin inhibuje své vlastní uvolňování stimulací muskarinových presynaptických receptorů. Pokud je přidán agonista muskarinových receptorů (acetylcholin, karbachol), je evokované uvolňování acetylcholinu sníženo. Přidání antagonisty (atropin, NMS) zabraňuje inhibici evokovaného uvolňování acetylcholinu.

3.2.7 Ukončení působení acetylcholinu acetylcholinesterázou a butyrylcholinesterázou

Účinek uvolněného acetylcholinu v synaptické štěrbině je ukončen velmi rychlým působením cholinesteráz (ChE), které štěpí acetylcholin na kyselinu octovou a cholin. Cholin může být poté transportován zpět do buňky prostřednictvím ChT1 a být použit pro znovuvytvoření acetylcholinu. Kromě vlastní acetylcholinesterázy (AChE; EC 3.1.1.7) štěpí ACh ještě butyrylcholinesteráza (BuChE; EC 3.1.1.8). Oba enzymy jsou si příbuzné a patří do rodiny α/β-hydroxyláz. Mohou se vyskytovat v rozpustné formě v plazmě nebo v některých hadích jedech, ve formě, kdy je cholinesteráza zakotvena přes glykolipidovou kotvu v membráně například u erytrocytů nebo ve formě zakotvené na kolagenové struktuře nervosvalové ploténky nebo hydrofobně zakotvené k membráně neuronů v mozku. Kromě toho se AChE může vyskytovat v monomerní, dimerní nebo tetramerní formě. Vlastní katalytická doména je ale u všech forem stejná a rozdíl v ostatních částech je způsoben alternativním sestřihem a následnými posttranslačními úpravami. V savčí centrální nervové soustavě se vyskytuje především AChE-T forma, která je asociovaná s dalšími hydrofobními podjednotkami, molekulami kolagenu nebo hydrofobními proteiny (Massoulié, 2000). Na rozdíl od presynaptických proteinů ChAT (Misgeld a spol., 2002; Brandon a spol., 2003) a ChT1 (Ferguson a spol., 2004) však není knockout AChE letální (Li a spol.; 2000), což ukazuje na schopnost BuChE zastat funkci AChE.

3.2.8 Centrální a periferní cholinergní neurony

Centrální cholinergní neurony lze rozdělit podle jejich umístění a funkce na neurony vysílající svá zakončení do periferie, interneurony, lokální projekční neurony a projekční neurony. Mezi neurony vysílající nervová zakončení do periferie patří motoneurony v mozkových jádrech a ventrálních míšních rozích. Dále to jsou preganglivé neurony sympatiku a parasympatiku umístěné v nucleus intermediolateralis. Periferní cholinergní synapse lze tedy nalézt v autonomních gangliích, parasympatických postgangliových synapsích a na nervosvalové ploténce. Interneurony a lokální projekční neurony nacházíme především ve striatu, kde interagují s dopaminergními nervovými zakončeními, které směřují do striata ze substantia nigra. Cholinergní zakončení ve striatu pocházejí především ze striatálních interneuronů ale v menší míře i z bazálního mozku (basal forebrain) (Mesulam, 1990). Projekční neurony se nacházejí především v bazálním mozku.

Protože anatomické uložení cholinergních neuronů v bazálním mozku neodpovídá zcela přesně tradičnímu rozdělení jader, jsou rozdělovány do 8 skupin označovaných Ch1-Ch8. Těla cholinergních neuronů inervujících mozkovou kůru se nacházejí v oblastech Ch1-Ch4 bazálního mozku. Neurony skupiny Ch1 a Ch2 inervují hipokampus, neurony skupiny Ch3 čichový lalok a neurony oblasti Ch4 inervují zbylé části mozkové kůry a amygdalu. Skupiny Ch5-6 inervují thalamická jádra, skupina Ch7 interpedunkulární jádro (nucleus interpeduncularis) a skupina Ch8 horní kolikulus (colliculus superior). V mozku primátů jsou zvláště výrazné oblasti Ch4 (nucleus basalis Meynerti), Ch5 (nucleus pedunculopontinus) a Ch6 (nucleus laterodorsalis). U člověka je zvláště vyvinutá oblast Ch4 (Mesulam, 1990; Mesulam a spol., 1983; Everitt a spol., 1988; Alonso a spol., 1996).

3.3 Cholinergní hypotéza Alzheimerovy choroby

Přirozené stárnutí se projevuje slábnutím mentálních schopností, které je u opic doprovázeno poškozením centrálních cholinergních neuronů v bazálním mozku (Smith a spol., 1999; Conner a spol., 2001). Přirozené oslabování mentálních funkcí v průběhu stárnutí se projevuje i u člověka a ve zvýrazněné podobě, která je již doprovázena nevratnými změnami centrálních cholinergních neuronů, se objevuje u neurodegenerativních onemocnění, jako je například Alzheimerova nemoc (Bartus, 1982).

Existuje množství teorií vysvětlujících příčiny Alzheimerovy choroby (přehledy v Ann N Y Acad Sci, 2000). Jednou z hypotéz vysvětlujících vznik a rozvoj Alzheimerovy choroby je cholinergní hypotéza (Bartus, 1982), kterou se zabývám ve své práci. Tato hypotéza vychází z pravidelně přítomného nálezu poškození cholinergních neuronů u osob postižených Alzheimerovou chorobou. Předpokládá se, že cholinergní neurony jsou vysoce citlivé k toxickému působení Aβ, a že jejich poškození výrazně přispívá k vývoji Alzheimerovy choroby a zhoršení kognitivních schopností ve stáří.

K vytvoření cholinergní hypotézy přispěla zjištění, že u osob zemřelých na Alzheimerovu chorobu byla popsána snížená aktivita acetylcholinesterázy a cholinacetyltransferázy a korelace snížení jejich aktivity s mírou rozvoje Alzheimerovy choroby (Davies a Maloney, 1976; Perry a spol., 1977, 1978a,b). Později ale bylo zjištěno, že snížení aktivity AChE a ChAT nenastávají v rané fázi rozvoje onemocnění (Davis a spol., 1999), či dokonce, že v hipokampu a frontální

kůře dochází u pacientů v počáteční fázi onemocnění ke zvýšení aktivity těchto enzymů (De Kosky a spol., 2002). Nelze ale vyloučit, že zvýšení aktivity jedné části cholinergního systému je kompenzačním mechanizmem poškození jeho jiné části.

V mozkové kůře osob postižených Alzheimerovou chorobou bylo zaznamenáno snížení množství nikotinových receptorů, jmenovitě jejich $\alpha 4$ a $\alpha 7$ podjednotek (Wevers a spol., 1999; Martin-Ruiz a spol., 1999; Burghaus a spol., 2000; Perry a spol., 1995; Banerjee a spol., 2000). Dále byly u lidí s AD popsány změny v množství jednotlivých podtypů muskarinových receptorů v různých oblastech kůry a hipokampu, ale tyto výsledky se mezi jednotlivými studiemi liší (Rodrígues-Puertas a spol., 1997; Flynn a spol., 1995; Nordberg a spol., 1992), a výrazné poruchy spřažení muskarinových receptorů s G-proteiny stanovené jako snížení karbacholem stimulované vazby GTP- γS a aktivity fosfolipázy C (Flynn a spol., 1995; Jope a spol., 1994 a 1997; Tsang a spol., 2006).

Na úlohu cholinergního systému v patogenezi Alzheimerovy choroby lze usuzovat i z toho, že do dnešní doby jsou jedinými, i když jen omezeně účinnými, léky inhibitory acetylcholinesterázy (tacrin, rivastigmin, donepezil, galantamin). Jiné pokusy o léčbu posílením cholinergní transmise, například podávání vysokých dávek cholinu nebo lecithinu (choline loading therapy) analogické k posilování dopaminové transmise u Parkinsonovy nemoci podáváním prekurzoru dopaminu L-dopa, byly ale neúspěšné (Scarpini a spol., 2003).

U myší, primátů i u člověka zhoršují muskarinoví i nikotinoví antagonisté kognitivní výkonnost stejným způsobem jako poškození cholinergních drah vedoucích z bazálního mozku do kůry nebo hipokampu. Naopak byl pozorován příznivý vliv podávání muskarinových agonistů (Terry a Buccafusco, 2003). U osob postižených Alzheimerovou chorobou snižuje selektivní muskarinový M1 agonista AF102B množství Aβ v mozkomíšním moku (Nitsch a spol., 2000).

Je zřejmé, že tato data ukazují na poškození cholinergního systému u osob s Alzheimerovou chorobou. Zatím ale není jasné, jedná-li se o primární nebo o sekundární poškození, tedy jestli se poškození cholinergních neuronů objevuje již v časných stádiích onemocnění, nebo jde o výsledek obecné neurodegenerace v pokročilém stadiu nemoci.

4 CÍLE PRÁCE

Cíle mé dizertační práce vycházejí z nálezů, které ukazují na významnou úlohu ochabování centrální cholinergní transmise v průběhu stárnutí a v patogenezi Alzheimerovy choroby. Ve své práci jsem hledala odpovědi na tyto konkrétní otázky:

1. Lze prokázat časné změny markerů cholinergních neuronů a muskarinové neurotransmise u myšího transgenního modelu Alzheimerovy choroby APPswe/PS1dE9 a urychlení změn v průběhu stárnutí při srovnání s netransgenními kontrolami?

2. Jaký vliv má dokosahexaenová kyselina, která snižuje *in vivo* u myšího transgenního modelu Alzheimerovy choroby APPswe/PS1dE9 produkci β-amyloidu (Oksman a spol., 2006; Hooijmans a spol., 2007), na expresi cholinergního fenotypu u cholinergní buněčné linie NG108-15?

3. Jaký vliv má xanomelin, považovaný původně za M_1 selektivního muskarinového agonistu (Sauerberg a spol., 1992; Shannon a spol., 1994), na autoregulaci uvolňování acetylcholinu M_2 a M_4 muskarinovými receptory z mozkových řezů potkana a jaký je mechanizmus jeho účinku?

5 VÝSLEDKY A DISKUSE

5.1 ZMĚNY MARKERŮ CHOLINERGNÍCH NEURONŮ A MUSKARINOVÉ NEUROTRANSMISE U MYŠÍHO TRANSGENNÍHO MODELU ALZHEIMEROVY CHOROBY APPSWE/PS1DE9 V PRŮBĚHU STÁRNUTÍ PŘI SROVNÁNÍ S NETRANSGENNÍMI KONTROLAMI

Studium mechanizmů vzniku Alzheimerovy choroby je komplikováno nedostupností vzorků postižených tkání člověka. Pokud jsou tyto vzorky k dispozici, jde zpravidla o tkáně z osob již trpících rozvinutou Alzheimerovou chorobou, které jsou získány až několik hodin po smrti. Mechanizmy vzniku Alzheimerovy choroby a kvalita změn v raných fázích postižení tedy zůstávají poměrně nejasné. Pro studium raných fází postižení jsou proto často používány zvířecí, především myší, modely Alzheimerovy choroby. Zpravidla se jedná o zvířata s vloženým genem pro jeden nebo více z proteinů obsahujících mutaci, která u lidí způsobuje familiární formu onemocnění. Problémem těchto modelů je malá možnost srovnání symptomů onemocnění myši a člověka.

Pro naši práci jsme použili myši APPswe/PS1dE9, které produkují zvýšené množství amyloidových fragmentů, zejména $A\beta_{1-42}$, což způsobuje ukládání β-amyloidu a tvorbu neuritických plaků, ale ne hyperfosforylaci proteiny tau a tvorbu neurofibrilárních klubíček (Jankowsky a spol., 2001 a 2004). V kůře a hipokampu myší této transgenní linie dochází ke zvýšení produkce $A\beta_{1-42}$ a k vytváření β-amyloidových plaků již od období mezi 5. a 6. měsícem věku. Změny zřejmé z behaviorálních testů jsou ale patrné až po 12. měsíci (Savonenko a spol., 2005). Myši použité pro tuto studii byly staré 7 měsíců (dále označované jako mladé) a 17 měsíců (dále označované jako staré). Pro pokusy jsme použili samice těchto myší, protože mají rychlejší rozvoj patologie než samci, a parietální kůru, protože vykazuje nejvýraznější akumulaci β-amyloidu (Jankowsky a spol., 2004). Výsledky naší práce jsou podrobně popsány v článku, který je součástí této dizertační práce (Machová a spol., 2008). Sledovali jsme změny presynaptických (aktivita ChAT a množství VAChT) a postsynaptických (aktivita AChE, BuChE, množství muskarinových receptorů a G-proteinů a jako funkční test stimulace vazby GTP-γ^{35}S po aktivaci muskarinových receptorů agonistou acetylcholinových receptorů karbacholem)

markerů cholinergních synapsí. Nalezli jsme změny způsobené věkem myší i změny způsobené přítomností vložených genů.

V souladu s obecně přijímaným ochabováním cholinergní transmise v průběhu stárnutí měly staré kontrolní (netransgenní) myši oproti mladým sníženou aktivitu všech sledovaných markerů s výjimkou počtu muskarinových receptorů. U starých transgenních APPswe/PS1dE9 myší jsme při srovnání s mladými transgenními myšmi pozorovali i snížení denzity muskarinových receptorů, zatímco snížení aktivity AChE, ačkoliv srovnatelného rozsahu jako u kontrolních myší, nebylo signifikantní.

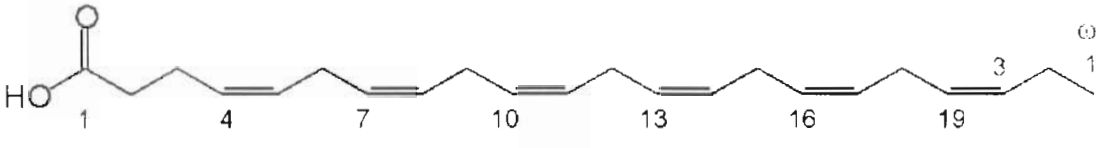
Nejdůležitějším zjištěním získaným v rámci této práce však bylo odhalení poškození, ke kterému dochází v cholinergním systému již u mladých transgenních myší při srovnání s kontrolními a které předchází rozvoji patologie a behaviorálním poruchám a koreluje se zvýšením produkce $A\beta_{1-42}$. Zjistili jsme snížené množství váčkového transportéru pro acetylcholin (stanovené jako vazba radioaktivně značeného specifického ligandu vesamicolu), snížené množství muskarinových receptorů (stanovené jako vazba radioaktivně značeného NMS), sníženou aktivitu BuChE a sníženou aktivaci G-proteinů jakožto odpověď na stimulaci muskarinových receptorů karbacholem (stanovenou jako stimulaci vazby GTP-$\gamma^{35}S$). Popsaná snížení se stávaly méně výrazné u starých myší pravděpodobně v důsledku na věku závislého snižování u kontrolních myší. Nicméně i u starých transgenních myší byla signifikantně snížena hustota muskarinových receptorů a jejich aktivace karbacholem, která se projevila signifikantním zvýšením koncentrace karbacholu potřebného pro aktivaci G-proteinů.

Poškození cholinergního systému je patrné i u mladých APPswe/PS1 myší, kde nacházíme úbytky v podstatě na všech úrovních cholinergní neurotransmise. Na přítomnosti transgenu (zvýšené produkci Aβ, zejména fragmentu $A\beta_{1-42}$) závislé poškození se tedy týká jak presynaptických, tak postsynaptických markerů. Zvláště významné je zjištění, že dochází ke snížení karbacholem stimulované vazby GTP-$\gamma^{35}S$. Tento nález, který je funkčním testem schopnosti přenosu signálu, nelze vysvětlit pouhým snížením množství muskarinových receptorů. Prokazuje sníženou schopnost muskarinových receptorů aktivovat G-proteiny nebo sníženou schopnost G-proteinů být muskarinovými receptory aktivován (přenášt signál do postsynaptické buňky) již v počátečním stadiu rozvoje onemocnění. K podobným výsledkům dospěly i studie provedené v tkáních zemřelých osob s Alzheimerovou chorobou, tedy v terminálním stadiu onemocnění (Flynn a spol.,1995; Jope a spol., 1994 and Jope a

spol., 1997; Tsang a spol., 2005). Obdobné výsledky jsme získali i na APPswe/PS1A246E transgenním modelu Alzheimerovy choroby (nepublikováno). Toto narušení muskarinové neurotransmise přes G-proteiny by mohlo mít výrazné důsledky pro fungování cholinergního systému již ve velmi časném stadiu rozvoje onemocnění.

5.2 Vliv dokosahexaenové kyseliny na expresi cholinergního fenotypu u buněk linie NG108-15.

Dokosahexaenová kyselina (DHA, obrázek 7) je esenciální nenasycená mastná kyselina patřící mezi ω-3 mastné kyseliny. Její molekula obsahuje šest dvojných vazeb. Je součástí buněčných membrán a savčí buňky ji nedokáží syntetizovat *de novo*. Vysoký příjem ω-3 mastných kyselin je asociován se zlepšenými kognitivními funkcemi (Kalmijn a spol., 2004) a snížením rizika vzniku Alzheimerovy choroby (Barberger-Gateau a spol., 2002; Morris a spol., 2003a,; Morris a spol., 2003b). V plazmě pacientů postižených Alzheimerovou chorobou a mírnou kognitivní poruchou je nižší koncentrace DHA (Conquer a spol., 2000). Nedostatek DHA ve stravě způsobuje sníženou výkonnost v behaviorálních testech a toto snížení lze zvrátit podáváním diety s vysokým obsahem DHA (Moriguchi a Salem, 2003), které zlepšuje uvolňování acetylcholinu u starých potkanů (Favreliere a spol., 2003). Obohacení lidských SH-SY5Y neuroblastomových buněk transfekovaných lidským APP se švédskou mutací dokosahexaenovou kyselinou posouvá katabolizmus APP směrem k neamyloidogennímu štěpení (Sahlin a spol., 2007).



Obrázek 7: Konstituční vzorec dokosahexaenové kyseliny.

V našich pokusech jsme využili buněčnou linii NG108-15. Jde o hybridní buněčnou linii (Hamprecht, 1977; Hamprecht a spol.,1985) vykazující cholinergní fenotyp (exprese cholinergního loku a přítomnost zralé ChAT), který lze zvýraznit diferenciací vyvolanou cAMP a dexametazonem (Castel a spol., 2002; Doležal a spol., 2001a, b).

Buňky v buněčné kultuře jsou ve svém růstu odkázány jednak na látky, které jsou přítomny v jejich okolí, to znamená v kultivačním médiu, a dále na látky, které si dokáží nasyntetizovat. Protože běžně dodávané sérum přidávané do kultivačních médií obsahuje nedefinované množství DHA a dalších trofických látek, použili jsme v našich pokusech médium, ve kterém bylo sérum nahrazeno hovězím sérovým albuminem bez mastných kyselin a lipidů, tedy médium, které neobsahovalo DHA, cholesterol ani jiné lipidy nebo mastné kyseliny nebo další trofické látky. Do tohoto média jsme přidávali definované koncentrace DHA.

Naším cílem bylo zjistit, jaký vliv má dodaná DHA na aktivitu ChAT u běžných buněk a buněk diferencovaných směrem ke zvýraznění cholinergního fenotypu a dále srovnat změny exprese cholinergního fenotypu se změnami růstových vlastností buněk vyvolaných přidáním DHA. Výsledky těchto prací jsou shrnuty v práci Machová a spol., 2006b a 2006a.

Před vlastními pokusy s DHA jsme stanovili, jaký vliv má odstranění séra a diferenciace na růstové vlastnosti buněk (aktivita kaspázy-3, obsah proteinu) a aktivitu cholinacetyltransferázy, markeru exprese cholinergního fenotypu. U diferencovaných buněk (v médiu s 200μM dibutyryl-cAMP a 100nM dexametazonem) pěstovaných v médiu se sérem byla oproti nediferencovaným kontrolám více než pětkrát snížena aktivita kaspázy-3, na třetinu snížena rychlost růstu a téměř šestkrát zvýšena aktivita cholinacetyltransferázy.

U kontrolních i diferencovaných buněk způsobilo podle předpokladu nahrazení séra BSA (25 g/l) zpomalení růstu zjišťované podle obsahu proteinů. Toto zpomalení nebylo u nediferencovaných buněk doprovázeno změnami v aktivitě kaspázy-3 a u diferencovaných buněk dokonce došlo k jejímu mírnému snížení. Z toho lze usuzovat, že u buněk nedocházelo k apoptóze. U obou skupin byla snížená aktivita cholinacetyltransferázy, tedy enzymu, který jsme použili jako marker cholinergní diferenciace. Toto snížení bylo mnohem výraznější (více než šestinásobné) u diferencovaných buněk. Konečná aktivita ChAT byla tedy po odstranění séra u diferencovaných buněk jen mírně zvýšená oproti nediferencovaným a buňky rostly obdobně rychle. U buněk pěstovaných v běžném médiu i v médiu bez séra byla diferenciací výrazně snížena aktivita kaspázy-3.

Další pokusy jsme prováděli již jen s buňkami pěstovanými v médiu bez séra, protože sérum obsahuje nedefinované množství DHA a nebylo by tedy možné spolehlivě studovat účinek přidáni DHA za přítomnosti séra. Pokud byla k buňkám

pěstovaným v médiu bez séra přidávána zvyšující se koncentrace DHA, zvyšovala se aktivita ChAT i množství proteinu (růst buněk) a snižovala se aktivita kaspázy-3 u diferencovaných i nediferencovaných buněk. Účinek byl ale výraznější u buněk diferencovaných a to zvláště v případě aktivity ChAT, jejíž zvýšení bylo více než šestkrát větší u diferencovaných buněk. Důležitým výsledkem těchto pokusů bylo zjištění, že závislost účinku na koncentraci DHA měla u diferencovaných buněk stejný průběh u aktivity kaspázy-3 a růstu buněk s EC_{50} přibližně 1μM, zatímco u aktivity ChAT byla hodnota EC_{50} desetkrát vyšší (přibližně 10μM). Tento rozdíl jsme nepozorovali u kontrolních (nediferencovaných) buněk (EC_{50} přibližně 1μM pro všechny tři sledované účinky). Rozdíl mezi účinnými koncentracemi DHA na růst buněk a aktivitu kaspázy-3 na jedné straně a na aktivitu ChAT na druhé straně svědčí pro nezávislost těchto účinků a ukazuje, že DHA podporuje u diferencovaných buněk expresi cholinergního fenotypu (ChAT) mechanizmem nezávislým na růstu buněk.

V dalších pokusech jsme sledovali účinek 100μM DHA (saturující pro růst i pro expresi ChAT) v mediu bez séra u kontrolních a diferenciovaných buněk a srovnávali jsme buňky pěstované bez séra s přidanou DHA a buňku pěstované v přítomnosti séra. Podpora růstu v bezsérovém médiu podle obsahu proteinů byla částečná (78+/-4,3%) u nediferencovaných buněk a nebo úplná (109±5,4%) u diferencovaných. V případě aktivity ChAT byla přidáním DHA výrazněji obnovena její aktivita u kontrolních (67,8±4,9%) než u diferencovaných (17,8±1,4%) buněk, ale absolutní přírůstek aktivity ChAT byl podstatně větší u diferencovaných buněk.

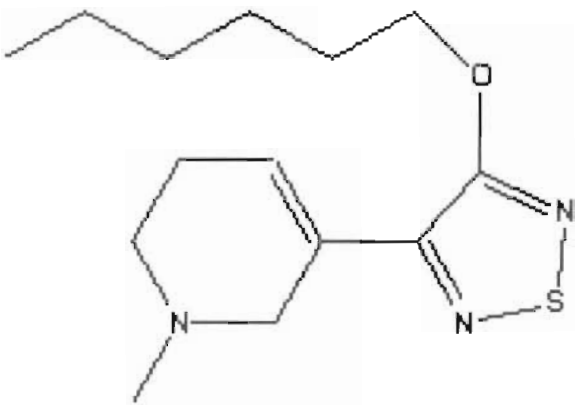
Abychom lépe popsali stav pěstovaných buněk a podpořili nezávislost působení DHA na aktivitu ChAT na obecném metabolickém stavu buněk, provedli jsme další pokusy, ve kterých jsme sledovali oxidativní zatížení buněk (stanovené jako oxidace fluoresceinu), obsah cholesterolu a aktivitu α-sekretázy. Nahrazení séra albuminem u diferencovaných buněk způsobilo zvýšení oxidativní zátěže o 31 procent a tento účinek byl zcela zrušen přidáním 10μM DHA. Přidáni vyšší koncentrace DHA již nemělo další účinek. Nahrazení séra albuminem způsobilo, podle předpokladu, snížení obsahu cholesterolu u diferencovaných (na 59+/-11,6 %) i nediferencovaných (37,4±7,6 %) buněk. Přidáním 10μM DHA se toto snížení částečně zvrátilo (z 37,4±7,6 % na 66,5 ±2,2~~37,4+/-7,6~~ %) pouze u nediferencovaných buněk. Dále jsme sledovali aktivitu α-sekretázy, která nebyla ovlivněna diferenciací. Odstranění séra snižovalo její aktivitu na přibližně polovinu u diferencovaných (na 46.3 ± 5.0 %) i

nediferencovaných (na 50.6 ± 4.5 %) buněk. Snížení aktivity bylo signifikantně, i když pouze částečně, zvráceno přidáním 10μM DHA (na 71.2 ± 3.6 % u kontrolních a 80.1 ± 5.1 % diferencovaných buněk). Zvýšení koncentrace DHA na 100μM již nemělo další účinek.

Naše výsledky demonstrují, že DHA přidaná k buněčné kultuře pěstované v úplně definovaném médiu bez séra (s albuminem) dokáže podpořit růst buněk, upravit obecné metabolické vlastnosti vyvolané odstraněním trofických látek obsažených v séru a zvyšovat aktivitu ChAT. Tyto výsledky jsou v souladu s neuroprotektivním působením DHA (Favreliere a spol., 2003; Hogyes a spol., 2003; Kim a spol., 2001; Minami a spol.,1997). Navíc jsme ukázali, že DHA posiluje expresi cholinergního fenotypu u diferencovaných buněk a že je tento účinek nezávislý na sledovaných obecných protektivních účincích. Naše výsledky podporují důležitost dostatečného dietetického příjmu DHA pro ochranu před neurodegenerativními onemocněními a stárnutím cholinergního systému.

5.3 Vliv xanomelinu na autoregulaci uvolňování acetylcholinu z mozkových řezů potkana zprostředkovanou muskarinovými M_2 a M_4 receptory

V nedávné době se ukázalo, že předpokládaný funkčně selektivní agonista muskarinových M_1 receptorů xanomelin (obrázek 8) se váže nejen reverzibilně do ortosterického vazebného místa pro acetylcholin, ale též neodmyvatelně do jiného vazebného místa všech podtypů muskarinových receptorů. Pokusy charakterizující neodmyvatelnou vazbu byly většinou prováděny na receptorech exprimovaných ve fibroblastech křečka (chinese hamster ovary, CHO buňkách). V naší práci jsme studovali vliv neodmyvatelné vazby xanomelinu na presynaptickou regulaci uvolňování acetylcholinu z korových a striatálních řezů krysy, tedy na modelu, který se podstatně více přibližuje reálným procesům, které probíhají v mozku, než modely využívající expresní systémy v buněčných kulturách. V mozku hlodavců tuto autoregulaci uvolňování zprostředkovává muskarinový M_4 receptor ve striatu (Doležal a Tuček, 1998) a M_2 receptor v mozkové kůře (Zhang a spol., 2002). Výsledky této práce jsou podrobně shrnuty v článku Machová a spol., 2007.



Obrázek 8: Molekula xanomelinu.

Tkáňové řezy byly 30 minut inkubovány s radioaktivně označeným cholinem, z kterého si tkáň sama nasyntetizovala značený acetylcholin. Poté byly inkubovány 15 minut s xanomelinem v 0, 1, 10 nebo 100μM koncentraci a případně s ortosterickým muskarinovým antagonistou. Elektrickou stimulací vyvolané uvolňování ACh jsme měřili v superfuzních pokusech jako uvolňování radioaktivity (radioaktivně označeného ACh) ve čtyřminutových frakcích. Před vlastním měřením byly řízky proplachovány 53 minut médiem bez xanomelinu, aby byl odmyt volný xanomelin.

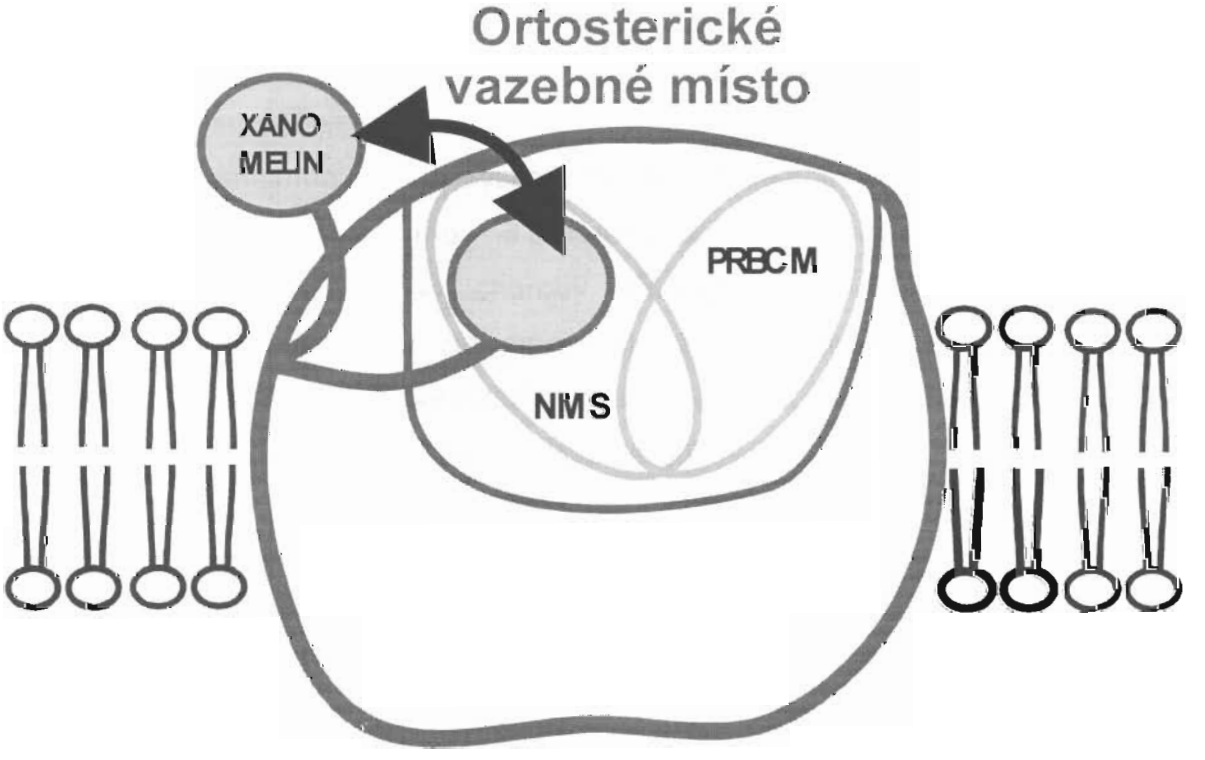
Zjistili jsme, že 10μM xanomelin, pokud je přítomen v průběhu stimulace, nemění uvolňování acetylcholinu z korových ani striatálních řezů a ani nedokáže zabránit snížení uvolňování acetylcholinu vyvolanému cholinergním agonistou karbacholem. Pokud byl ale xanomelin přidán na 15 minut a poté před vlastní stimulací 53 minut odmýván, snižoval v závislosti na koncentraci stimulované uvolňování ACh. Maximální inhibiční účinek xanomelinu byl co do velikosti stejný jako účinek saturující 10μM koncentrace karbacholu. Maximální účinky se nesčítaly. Lze tedy předpokládat, že mechanizmus jejich účinku je stejný. Presynaptické muskarinové receptory na cholinergních zakončeních ovlivňují uvolňování acetylcholinu inhibičně, z čehož je zřejmé, že neodmyvatelně navázaný xanomelin se v našich pokusech chová jako agonista a to jak u M_2, tak i u M_4 receptorů. Účinek neodmyvatelně navázaného xanomelinu je možné zcela (1 a 10μM xanomelin) nebo částečně (100μM xanomelin) zrušit přidáním muskarinového antagonisty NMS. Z toho je zřejmé, že neodmyvatelně navázaný xanomelin účinkuje alepoň z části přes ortosterické vazebné místo.

V dalších pokusech jsme se pokusili zablokovat vznik neodmyvatelné vazby současně s xanomelinem přidaným NMS nebo ireverzibilní inaktivací ortosterického vazebného místa inkubací s antagonistou PRBCM před inkubací s xanomelinem. Ani reverzibilní, ani ireverzibilní antagonita vážící se do klasického vazebného místa však nezabránil účinku xanomelinu, přestože PRBCM ireverzibilně zablokovalo většinu vazebných míst pro NMS. Tyto výsledky prokazují, že pro vytvoření neodmyvatelné vazby xanomelinu není potřeba ortosterické vazebné místo, přesněji jeho část obsazená NMS nebo PRBCM, a že neodmyvatelně navázaný xanomelin může receptor aktivovat, i když je navázán na jiné než klasické vazebné místo (obrázek 9). Tyto výsledky jsou v souladu s pozorováními na M_1 receptorech exprimovaných v CHO buňkách (Jakubík a spol., 2002).

Abychom zjistili, zda je tento účinek specifický pro cholinergní neurony, tedy že nepostihuje některý obecný mechanizmus stimulovaného uvolňování mediátorů společný pro všechny neurony, provedli jsme obdobné pokusy, ve kterých jsme ale stanovovali uvolňování radioaktivně značeného noradrenalinu a jako agonistu a antagonistu jsme použili specifického agonistu presynaptických autoinhibičních α_2-adrenoreceptorů UK14304 a specifického antagonistu yohimbin. V případě uvolňování noradrenalinu neměl 1 a 10μM xanomelin žádný účinek na stimulované uvolňování noradrenalinu, ani nijak neovlivnil rozsah presynaptické inhibice jeho uvolňování. V případě 100μM xanomelinu bylo zvýšeno klidové uvolňování noradrenalinu a lehce ale nesignifikantně sníženo stimulované uvolňování. Regulace uvolňování noradrenalinu presynaptickými α_2-adrenoreceptory však zůstala zachována. Z výsledků těchto pokusů tedy vyplývá, že inhibiční účinek xanomelinu na uvolňování ACh není obecným inhibičním účinkem na stimulované uvolňování mediátorů nebo obecným účinkem na kterékoliv presynaptické inhibiční autoreceptory. Tento závěr se shoduje s vazebnými pokusy na rekonstituovaném systému, které ukazují, že xanomelin se neváže na lipozomy, na purifikovaných muskarinových receptorech vykazuje pouze reverzibilní vazbu a neodmyvatelná vazba se objevuje až po inkorporaci purifikovaných receptorů do lipozomů (Jakubík a spol, 2004).

Naše výsledky na muskarinových receptorech přítomných v jejich přirozeném prostředí potvrzují pozorování odvozená z pokusů na receptorech exprimovaných heterologně v CHO buňkách. Účinek xanomelinu přes M_2 a M_4 receptory vyžaduje jeho neodmyvatelné navázání. Delší doba potřebná k účinku xanomelinu může být

způsobená oproti M_1 pomalejší kinetikou neodmyvatelné vazby xanomelinu, ale hlavně kinetikou aktivace M_2 (Jakubík a spol., 2006) a možná i M_4 receptorů. Opožděná aktivace i jiných podtypů muskarinových receptorů, než je M_1 podtyp, by mohla vysvětlit i vedlejší účinky xanomelinu při klinických testech, které vedly k jejich přerušení. Složitý mechanizmus účinku xanomelinu je ale zajímavý i z jiného důvodu. Xanomelin totiž představuje prototyp látky s dlouhodobým účinkem a mohl by sloužit jako předloha při navrhování struktury specifických ligandů s dlouhodobým působením.



Obrázek 9: Schematické znázornění možného fungování xanomelinu na $M_{2/4}$ receptorech. V oblasti ortosterického vazebného místa jsou schematicky označeny oblasti vazebných míst pro NMS a PRBCM. Xanomelin je neodmyvatelně navázán mimo ortosterické vazebné místo. Jeho účinek je zprostředkován jednak přes ortosterické vazebné místo (blokovatelný NMS), jednak přes jiné vazebné místo.

6 ZÁVĚR

V rámci této práce jsme dospěli k těmto konkrétním výsledkům:

Transgenní myší model Alzheimerovy nemoci APPswe/PS1dE9 vykazuje při srovnání s netransgenními kontrolami ze stejného vrhu signifikantní úbytek markerů cholinergních synapsí a poškození muskarinové transmise již u mladých zvířat, u kterých se teprve začíná objevovat charakteristická patologie v době, kdy teprve dochází k urychlování tvorby β-amyloidu. Tyto poruchy se týkají presynaptické (váčkový přenašeč pro acetylcholin) i postsynaptické (počet muskarinových receptorů) části synapse. Důležitým nálezem je zejména porucha spřažení muskarinových receptorů s G-proteiny, která se u mladých zvířat projevuje snížením maximálního účinku agonisty a u starých zvířat zvýšením jeho koncentrace potřebné pro dosažení maximálního účinku. Význam těchto zjištění spočívá v průkazu časných poruch cholinergního systému s progresivním zhoršováním při stárnutí u tohoto modelu Alzheimerovy choroby. Podporují tak cholinergní hypotézu onemocnění, která předpokládá významnou úlohu oslabování cholinergní transmise již v časné fázi onemocnění, a poskytují podporu pro snahu o ovlivnění Alzheimerovy choroby farmakologickým působením na cholinergní systém.

2. Na modelu cholinergní dediferenciace u neuronální cholinergní buněčné linie NG108-15 vyvolané odstraněním séra z kultivačního média jsme potvrdili obecný neuroprotektivní účinek dokosahexaenové kyseliny. Navíc jsme zjistili, že v koncentraci přibližně desetkrát vyšší (ale stále fyziologické), než je koncentrace zajišťující obecnou neuroprotekci, podporuje dokosahexaenová kyselina i expresi cholinergního fenotypu. Z tohoto pozorování vyplývá, že dostatečný přísun této nenasycené mastné kyseliny v potravě může mít kromě obecného neuroprotektivního účinku příznivý vliv i na udržování fenotypu cholinergních neuronů, který se oslabuje přirozeně během stárnutí a ve zvýrazněné podobě u Alzheimerovy nemoci.

3. Při studiu účinku předpokládaného M_1 selektivního muskarinového agonisty xanomelinu, který se však podle výsledků získaných na heterologně exprimovaných muskarinových receptorech váže na všechny podtypy reverzibilně do ortosterického

vazebného místa a neodmyvatelně do jiného vazebného místa, jsme zjistili, že v přirozeném preparátu xanomelin aktivuje i M_2 a M_4 receptory. Aktivace těchto podtypů vyžaduje vytvoření neodmyvatelné vazby, kterou nelze zablokovat klasickým antagonistou, projevuje se opožděně i v nepřítomosti volného ligandu a má ortosterickou i alosterickou složku účinku. Tato pozorování jsou v souladu se složitým farmakologickými vlastnostmi xanomelinu, který při klinickém testování na nemocných s Alzheimerovou chorobou vedl k výrazným nežádoucím účinkům a přerušení testů. Xanomelin představuje prototyp muskarinového ligandu s dlouhodobým působením a po odhalení molekulárních mechanizmů jeho účinku by mohl sloužit jako předloha pro vývoj skutečně selektivních M_1 agonistů, které se vážou do ektopického místa a mohly by mohly být využity při léčbě Alzheimerovy nemoci k posílení signalizazace prostřednictvím muskarinových receptorů.

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Impairment of muscarinic transmission in transgenic APPswe/PS1dE9 mice

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Abstract

We assessed the integrity of cholinergic neurotransmission in parietal cortex of young adult (7 months) and aged (17 months) transgenic APPswe/PS1dE9 female mice compared to littermate controls. Choline acetyltransferase and acetylcholinesterase activity declined age-dependently in both genotypes, whereas both age- and genotype-dependent decline was found in butyrylcholinesterase activity, vesicular acetylcholine transporter density, muscarinic receptors and carbachol stimulated binding of GTPγS in membranes as a functional indicator of muscarinic receptor coupling to G-proteins. Notably, vesicular acetylcholine transporter levels and muscarinic receptor-G-protein coupling were impaired in transgenic mice already at the age of 7 months compared to wild type littermates. Thus, brain amyloid accumulation in this mouse model is accompanied by a serious deterioration of muscarinic transmission already before the mice manifest significant cognitive deficits.

Keywords: Cholinergic neurotransmission; Choline acetyltransferase; Vesicular acetylcholine transporter; Acetylcholinesterase; Butyrylcholinesterase; Muscarinic receptors; G-protein coupling; Transgenic mouse

1. Introduction

Original neurochemical findings in Alzheimer's brains pointed out disturbances of cholinergic neurotransmission (Bowen et al., 1976; Davies and Maloney, 1976; Francis et al., 1985; Perry et al., 1977a,b; Sims et al., 1981) that were basis for the "cholinergic hypothesis" of Alzheimer's disease (AD) (Bartus et al., 1982; Dolezal and Kasparova, 2003; Francis et al., 1999; Mesulam, 2004). Since then a large body of evidence has accumulated both supporting and questioning the hypothesis (Bartus, 2000). Cholinergic neurotransmission plays an important role in learning and memory. These functions progressively deteriorate as the disease advances and also decline in the course of natural aging. One of the important questions is whether disturbances of cholinergic mechanisms are present early in the pathogenesis of AD or are simply a reflection of a general neurodegeneration which afflicts many neurotransmitter systems in the terminal state of the disease.

It has now been generally accepted that the primary event in the pathogenesis of AD is increased production and aggregation of noxious β-amyloid fragments composed of 39–43 amino acids. Their overproduction in hereditary cases of the disease is due to known genetic defects (Selkoe, 2001) while the reason of increased production in sporadic cases is largely unknown. The active form of β-amyloid consists of soluble oligomeric fragments (Haass and Steiner, 2001; Klein et al., 2001) that appear in the brain earlier than amyloid plaques and neurofibrillary tangles typical for pathological picture in

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the terminal state of the disease. Are these soluble oligomeric β-amyloid fragments also the direct cause of cholinergic transmission deficits in AD? This issue is very pertinent from the viewpoint of potential therapeutic approaches. It was demonstrated that stimulation of M_1 and M_3 subtypes of muscrinic receptors leads to non-amyloidogenic cleavage of the amyloid precursor protein (Buxbaum et al., 1992; Nitsch et al., 1992). Attenuation of cholinergic transmission early in AD pathogenesis may thus result in accelerated progression of amyloid accumulation, which in turn exacerbates degeneration of cholinergic neurons. Alternatively, impaired cholinergic transmission may appear later on during amyloid accumulation as a consequence of indirect mechanisms like impairment of glucose metabolism (Hu et al., 2003; Meier-Ruge et al., 1994; Messier and Gagnon, 1996) or production of reactive oxidative species (Mattson and Pedersen, 1998).

Several studies have indeed demonstrated substantial loss of cholinergic markers in the basal forebrain and hippocampus after intracerebroventricular injection of β-amyloid (Pepeu, 2001). However, acute i.c.v. injection of β-amyloid at relatively high concentrations does not really mimic the gradual accumulation of this peptide in the brain due to a metabolic disturbance as likely happens in the course of human AD. It is not feasible to conduct biochemical investigations in human brains to detect early changes in AD. However, development of transgenic mice models expressing mutated human genes found in hereditary AD enable investigations of various aspects of brain metabolism and functioning in the course of amyloid accumulation. Various transgenic mice modelling the disease display amyloid accumulation and plaque formation but not significant cholinergic deficit, with the exception of anti-NGF mouse (Capsoni et al., 2000, 2002; Ruberti et al., 2000). Loss of basal forebrain neurons was not observed in APP mutant mice and early reports indicated no change in ACh markers such as acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) (Pepeu, 2001). However, moderate morphological changes of cholinergic terminals were reported in the vicinity of plaques in double transgenic APP/PS1 mice (Hu et al., 2003; Wong et al., 1999). Later studies have nonetheless reported moderate decline in cholinergic markers in APP transgenic mice but only after around 16 months of age (Aucoin et al., 2005; Hartmann et al., 2004; Savonenko et al., 2005).

We took advantage of double transgenic APPswe/PS1dE9 mice that overproduce amyloid-β_{1-42} resulting in development of Alzheimer-like pathology (Jankowsky et al., 2001, 2004). This mouse line first develops amyloid plaques around 5–6 months of age in the cortex and hippocampus, but cognitive deficits are present only after 12 months of age (Savonenko et al., 2005). In these experiments we focused on the parietal cortex as this brain area shows the most rapid accumulation of β-amyloid in this mouse model (Jankowsky et al., 2004). Female mice were used as they express faster development of pathology than males (Oksman et al., 2006; Wang et al., 2003). We investigated changes in the density of the vesicular acetylcholine transporter (VAChT) as a presynaptic marker of cholinergic synapse and ability of the full muscarinic agonist carbachol to activate heterotrimeric G-proteins as a functional indicator of cholinergic signal transmission efficiency. We provide evidence that marked deficit in presynaptic cholinergic markers and transmission through muscarinic receptors at a functional level occur much earlier than reported in the literature.

2. Methods

2.1. Animals

The APPswe/PS1dE9 founder mice were obtained from the Johns Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Department of Pathology) and a colony was established at the University of Kuopio. In brief, mice were created by co-injection of chimeric mouse/human APPswe (mouse APP695 harboring a human Aβ domain and mutations K595N and M596L linked to Swedish familial AD pedigrees) and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The two transgenes co-integrate and co-segregate as a single locus (Jankowsky et al., 2004). This line was originally maintained in a hybrid C3HeJ × C57BL6/J F1 background, but the mice of the present study are derived from backcrossing to C57BL6/J for five to six generations. The housing conditions (National Animal Center, Kuopio, Finland) were controlled (temperature 22 °C, light from 07:00 to 19:00; humidity 50–60%), and fresh food and water were freely available. The experiments were conducted according to the Council of Europe (Directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Eastern Finland.

2.2. Sample preparation

Samples were dissected in Kuopio, Finland. The mice were anesthetized with chloralhydrate–pentobarbiturate mixture (each 60 mg/kg, i.p.), transcardially perfused with 50 ml heparinized ice-cold 0.9% saline (10 ml/min), and the brains were rapidly removed. One hemibrain of each brain was immersed in 4% paraformaldehyde in 0.1 M Na-phosphate buffer (pH 7.6) for 4 h and then transferred to a 30% sucrose solution and kept overnight on a shaker table. The hemibrains were then stored in a cryoprotectant in −20 °C for later immunohistology. The other hemibrain was dissected on ice into selected brain areas (hippocampus, frontal and parietal cortex, and cerebellum) that were stored in −70 °C for biochemical assays. Samples of brain parietal cortical tissue from 7- to 17-month-old control and transgenic female mice were transported on dry ice to Prague (Czech Republic). Samples were homogenized in a glass homogenizer (seven strokes at setting 7) in 1 ml of 10 mM sodium phosphate buffer (Na-P-

buffer) containing 1 mM EDTA (ethylenediaminotetraacetic acid), final pH 7.4. All operations were done on ice and samples were then stored at −75 °C before use. Protein content in homogenates ranged between 1.80 and 8.28 mg/ml.

2.3. [^{3}H]-vesamicol binding measurement

Levels of vesicular acetylcholine transporter (VAChT) were estimated as vesamicol (phenylpiperidinocyclohexanol) binding to homogenates. To 100 μl aliquots of homogenates (200–500 μg of protein) was added 100 μl of 50 mM Na-P-buffer (pH 7.4) containing 4 mM CHAPS, 200 nM naloxone to block sigma binding sites (Custers et al., 1997), and tritiated vesamicol (NEN, 34 Ci/mmol) at a final concentration of 7 nM for equilibrium single-point binding assay or at a concentration range of 0–35 nM for saturation analysis. Nonspecific binding was determined in parallel tubes containing in addition 100 μM unlabeled vesamicol. Samples mostly in duplicates or triplicates were incubated for 30 min at 25 °C in a shaking water bath. At the end of incubation free labeled ligand was separated by rapid filtration with a Brandel filtration apparatus through Whatmann GF/F glass filters pretreated for 1 h with 0.25% polyethylenimine. Radioactivity of bound labeled ligand retained on filters was quantified by liquid scintillation counting.

2.4. [^{35}S]GTP-γS binding measurement

Coupling of muscarinic receptors with G-proteins was determined as an increase of GTP-γS binding to membranes induced by the muscarinic receptor agonist carbachol as described earlier (Jakubik et al., 2006). Briefly, crude membranes were prepared by centrifugation of brain cortex homogenates at 30,000 × *g* for 30 min at 4 °C. Supernatants were discarded. To remove traces of EDTA and cytoplasm from original homogenates that would interfere with the assay, pellets were resuspended in a reaction buffer containing NaCl 100 mM, $MgCl_2$ 10 mM, Hepes 20 mM, pH 7.4, and centrifuged once more in the same conditions. Supernatants were again discarded and the resulting pellets were resuspended in the reaction buffer at a final protein concentration of about 1 mg/ml. Suspensions of membranes were used for assay immediately or stored at −75 °C before assayed. For measurement of resting and carbachol-stimulated GTP-γS binding, 50 μl aliquots of membranes containing 2–4 μg protein were incubated for 15 min at 30 °C in 150 μl of reaction buffer containing in addition 1 mM DTT, 50 μM GDP, and the muscarinic agonist carbachol at a concentration range 300 nM to 100 μM. After this preincubation, 50 μl aliquots of [^{35}S]GTP-γS were added to give a final concentration of 500 pM and incubation continued for another 45 min. Total content of G-proteins in membranes was determined as [^{35}S]GTP-γS binding in the absence of GDP. Aliquots of membrane suspension containing 1 μg protein were incubated for 20 min under the same conditions. Nonspecific binding was assessed in the presence of 10 μM unlabeled GTP. Incubations were terminated by rapid vacuum filtration through Whatmann GF/F filters using Tomtec harvester Mach III. Radioactivity retained on filters was measured with Microbeta counter (Wallac).

2.5. [^{3}H]-N-methylscopolamine binding

Density of muscarinic receptors was estimated as specific binding of ^{3}H-*N*-methylscopolamine (^{3}H-NMS) as described previously (Jakubik et al., 1995). Briefly, 50 μl aliquots of homogenates were incubated in triplicates with [^{3}H]-NMS (1 nM, SRA 148 dpm/fmol, NEN) in a final volume 1 ml of incubation buffer (final concentrations: 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4) for 1 h at 25 °C. Free ligand was separated by rapid filtration (Brandel superfusion apparatus) through Whatmann GF/F filter presoaked with 0.25% polyethylenimine. Nonspecific binding was measured in parallel samples in the presence of 10 μM atropine. External standard (membranes of chinese hamster ovary cells expressing M_2 subtype of muscarinic receptors) were included in each filtration to correct results for a possible deviations among individual filtrations.

2.6. Determination of enzyme activities

For determination of enzyme activities, 200 μl aliquots of original homogenates were mixed with 200 μl of choline acetyltransferase assay buffer (final concentrations in mM: 200 mM NaCl, 10 mM Na-P-buffer, 0.2% Triton X-100, pH 7.4) and stored at −75 °C until assayed for activities of choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and butyrylcholinesterase (BuChE). ChAT activity was determined using a modification of Fonnum's method (Berrard et al., 1995; Fonnum, 1969) in 10 μl aliquots of homogenates in three series. Samples were incubated in (final volume) 50 μl ChAT assay buffer containing in addition (final concentrations) 0.2 mM eserine, 2 mM choline, and a mixture of 15 μM unlabeled acetylCoA and ^{3}H-acetylCoA (Amersham, SRA 3 Ci/mmol; final SRA around 140 dpm/pmol) for 15 min at 37 °C. Incubations were stopped by adding 400 μl of ice cold 10 mM N–P-buffer and 400 μl of tetraphenylboron dissolved in butyronitril (10 mg/ml). Synthetized labeled acetylcholine was extracted into organic layer and 250 μl aliquots were taken for scintillation counting. In each series, the same standard sample was measured to verify reproducibility of assay (measured activities in individual series were 1.68, 1.57, and 1.54 pmol/μg protein/15 min). AChE and BuChE activities were estimated using Ellman's method (Ellman et al., 1961). For AChE activity, 5 μl aliquots of samples were incubated for 30 min at 25 °C in (final volume) 200 μl of incubation mixture (55 mM Na-P-buffer, 0.25 mM dithiobisnitrobenzoic acid, 5 mM acetylthiocholine, 0.05% Triton X-100, pH 8.0) and the absorbance was measured at 405 nm. BuChE activity was measured under the same con-

ditions in 50 μl aliquots of samples for 60 min with 5 mM butyrylthiocholine as chromogenic substrate.

2.7. Protein determination

Proteins were determined using Paterson's modification (Peterson, 1977) of Lowry's method (Lowry et al., 1951).

2.8. Immunohistochemisty

The hemibrains were cut in six series of coronal sections (30 μm) using a sliding, freezing microtome. One of the series was stained for Aβ (Mouse anti-human Aβ4-9; 6E10, (Senetek, St. Louis, USA). The sections were first pretreated for 30 min with hot (80 °C) citrate buffer, and then transferred to a solution containing the primary antibody (mouse anti-human Aβ at 1:1000). Following incubation in this solution for 18 h on a shaker table at room temperature (20 °C) in the dark, the sections were rinsed three times in tris buffered saline and Triton (TBS-T) and transferred to the solution containing the secondary antibody (goat anti-mouse-biotin, Sigma). After 2 h, the sections were rinsed three times with TBS-T and transferred to a solution containing mouse ExtrAvidin (Sigma); following rinsing the sections were incubated for approximately 3 min with Ni-enhanced DAB. A second series was stained for AChE as described by Hedreen et al. (1985) with slight modifications. Sections were rinsed in 0.1 M maleate buffer (pH 6.0). After that sections were moved to the reaction solution (Karnovsky & Roots-solution) for 2 h, followed by rinsing five times in 0.05 M Tris buffer (pH 7.6). Finally the sections were intensified in Ni-enhanced 3,3-diaminobenzidine tetrahydrochloride (DAB). All stained sections were mounted on slides and coverslipped.

2.9. Statistical and mathematical evaluation

Curve fitting and statistical evaluation of data were done using Prism 4 (GraphPad Software Inc., CA). A sigmoidal concentration–response curve $Y = (\text{Bottom} + (\text{Top} - \text{Bottom}))/(1 + 10^{(\log EC_{50} - X)})$ was fitted to GTP-γ^{35}S binding data where X is log of carbachol concentration, Y the GTP-γ^{35}S binding expressed as fold over basal, and EC_{50} is concentration of carbachol required to produce half-maximal stimulation. One site saturation binding curve $Y = B_{max} \times X/(K_d + X)$ was fitted to vesamicol binding data where X is vesamicol concentration, Y is vesamicol specific binding, B_{max} is vesamicol maximal binding, and K_d is concentration of vesamicol required to reach half-maximal binding. Variations among measurements were analysed by two-way ANOVA. Statistical significance between transgenic animals and age-matched control or between control and transgenic animals from both age groups was tested by Bonferroni's post-test. Significant age-dependent differences are indicated by crosses (#) and transgene-dependent differences by asterisks (*).

3. Results

3.1. Age and transgene dependent changes of cholinergic markers

Activities of enzymes related to cholinergic neurotransmission and muscarinic receptor density are summarized in Fig. 1. ANOVA revealed an age-related decline in ChAT [$F(1,36) = 16.3$, $p = 0.0003$], AChE [$F(1,36) = 9.1$, $p = 0.0046$], and BuChE [$F(1,35) = 22.1$, $p < 0.0001$] activity. BuChE activity was also reduced in transgenic mice compared to their nontransgenic littermates [$F(1,35) = 9.6$, $p = 0.037$], but no age × genotype interaction were observed in these enzymes. Muscarinic receptor density also decreased age-dependently [$F(1,36) = 8.1$, $p = 0.0074$] and was lower in transgenic mice [$F(1,36) = 25.1$, $p < 0.0001$] resulting in a significant age × genotype interaction [$F(1,36) = 4.3$, $p = 0.0444$].

3.2. VAChT is reduced in young transgenic animals

Because ChAT activity is not a rate-limiting factor in acetylcholine synthesis (Brandon et al., 2004), possible impairment of the presynaptic component of cholinergic synapses was further probed using vesamicol binding. Density of the VAChT in individual samples was determined in equilibrium binding assay with 7 nM ^{3}H-vesamicol. In order to prevent contribution of binding to sigma receptors (Custers et al., 1997) binding measurements were performed in the presence of 500 nM naloxone. As shown in Fig. 2, the density of VAChT exhibited both age and transgene dependent decline. This was verified by the ANOVA, which revealed significant effects of age [$F(1,20) = 73.3$, $p < 0.0001$] and genotype [$F(1,20) = 11.8$, $p < 0.003$]. Furthermore, the age-related decline in vesamicol binding was larger in transgenic mice resulting in age by genotype interaction [$F(1,20) = 4.5$, $p < 0.05$]. These differences of vesamicol binding reflected levels of VAChTs because saturation analysis of vesamicol binding in all groups displayed the same affinity of vesamicol binding (Table 1; in nM: young control, 6.51 ± 0.84; young transgenic, 4.32 ± 0.55; aged control, 5.68 ± 1.90; aged transgenic 4.98 ± 0.50).

3.3. Muscarinic receptor-G-protein coupling is impaired in young transgenic animals

Signal transduction through muscarinic receptors was assessed as the ability of the non-hydrolyzable acetylcholine analogue carbachol to activate coupling of muscarinic receptors with heterotrimeric G-proteins. Stimulation of GTP-γ^{35}S binding in cortical membranes by 10 μM carbachol (Fig. 3, upper graph) was highest in young control mice, followed by aged control, young transgenic and aged transgenic groups. Only the main effect of genotype manifested as a reduction of GTP-γ^{35}S binding was statistically significant [$F(1,18) = 11.9$, $p = 0.003$]. These data

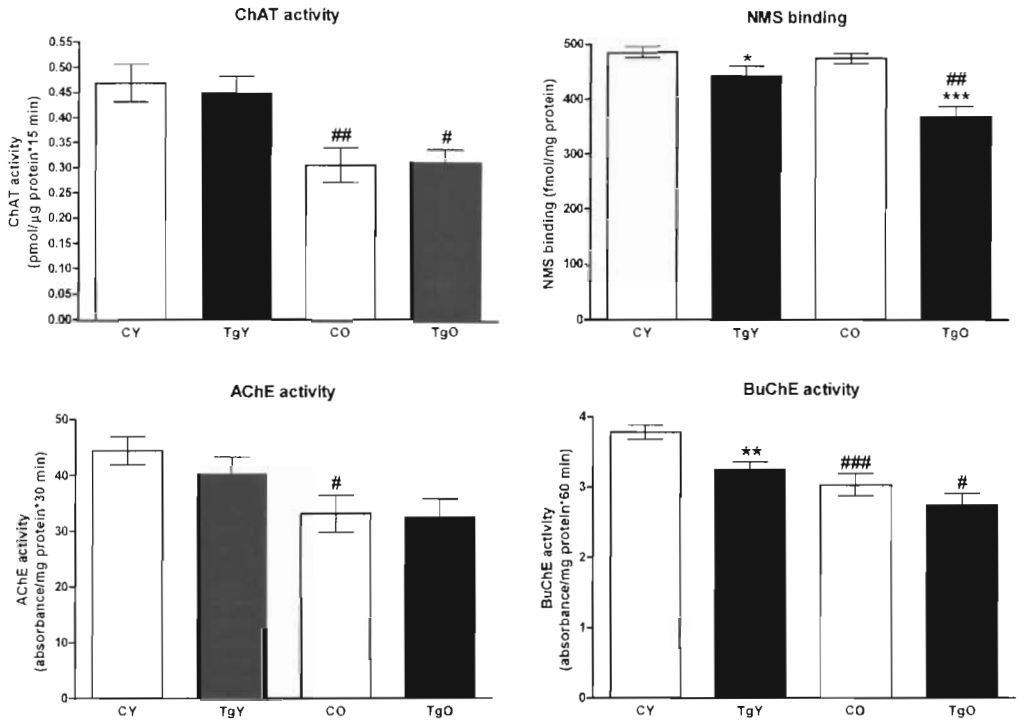

Fig. 1. Age and transgene related changes of cholinergic markers in parietal cortex. *Upper left*: Choline acetyltransferase activity (ChAT) is expressed in pmol ACh/μg protein/15 min. $^{\#}p<0.05$, $^{\#\#}p<0.01$, significantly different from corresponding young animals. *Upper right*: Density of muscarinic receptors was determined as ^{3}H-NMS binding and is expressed in fmol/mg protein. $^{*}p<0.05$, $^{***}p<0.001$, significantly different from age-matched controls; $^{\#\#}p<0.01$, significantly different from young transgenic animals. *Lower left*: AChE activity is expressed as the increase of absorbance/mg protein/30 min. $^{\#}p<0.05$, significantly different from young controls. *Lower right*: BuChE activity is expressed as the increase of absorbance/mg protein/60 min. $^{**}p<0.01$, significantly different from age-matched controls; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$, significantly different from corresponding young animals. CY, 6-month-old control mice; TgY, 6-month-old transgenic mice; CO, 16-month-old control mice; TgY, 16-month-old transgenic mice. In all graphs, columns represent mean ± S.E.M. of samples derived from 14, 10, 8 and 7 (in the case of BuChE activity in TgY only 6) animals, respectively. Statistical significance between age-matched or control transgenic animals was determined by two-way-ANOVA followed by Bonferroni's posttest.

Table 1
Parameters of vesamicol binding

	B_{max} (fmol/mg protein)	K_d (nM)
Control 7 months	93.6 ± 4.4 (82.3–105.0)	6.51 ± 0.84 (4.34–8.68)
Transgenic 7 months	69.1 ± 4.3 (61.9–76.4)	4.32 ± 0.55 (2.91–5.73)
Control 17 months	72.0 ± 5.7 (50.5–93.5)	5.68 ± 1.90 (0.79–10.56)
Transgenic 17 months	65.2 ± 5.0 (58.8–72.4)	4.98 ± 0.50 (3.58–6.38)

Data represent mean ± S.E.M. of binding parameters derived from experiment shown in Fig. 2. In parentheses are shown 95% limits of confidence.

implied serious impairment of muscarinic signal transduction efficacy in young transgenic animals. To get more detailed insight into malfunction of muscarinic receptor-G-proteins coupling we performed a concentration–response analysis of carbachol stimulation of GTP-γ^{35}S binding in pooled samples of individual groups (Fig. 3, lower part). Results demonstrate age-dependent decline in both efficacy (E_{max}) [$F(1,8)=65.3$, $p<0.0001$] and potency (EC_{50}) [$F(1,8)=46.0$, $p=0.0001$] of carbachol in activating coupling of muscarinic receptors with G-proteins (Table 2). The main effect of transgene on these parameters was also significant (E_{max}, $p=0.001$; EC_{50}, $p<0.0001$). In addition, the decline in both parameters was more robust in transgenic compared to control mice (age × genotype interaction for both E_{max}

Table 2
Efficacy (E_{max}) and potency (EC_{50}) of carbachol in stimulating GTP-γ^{35}S binding

	E_{max} (fold increase)	EC_{50} (μM)
Control 7 months	1.85 ± 0.07	5.22 ± 0.79
Transgenic 7 months	1.48 ± 0.01$^{\#\#\#}$	8.46 ± 1.08
Control 17 months	1.35 ± 0.01***	7.58 ± 0.43
Transgenic 17 months	1.34 ± 0.02	24.43 ± 2.43$^{***,\#\#\#}$

Data represent mean ± S.E.M. of three independent measurements done in quadruplicates as that shown in Fig. 3. $^{***}p<0.001$, significantly different from young controls; $^{\#\#\#}p<0.001$, significantly different from age-matched controls by two-way-ANOVA and Bonferroni's post-test.

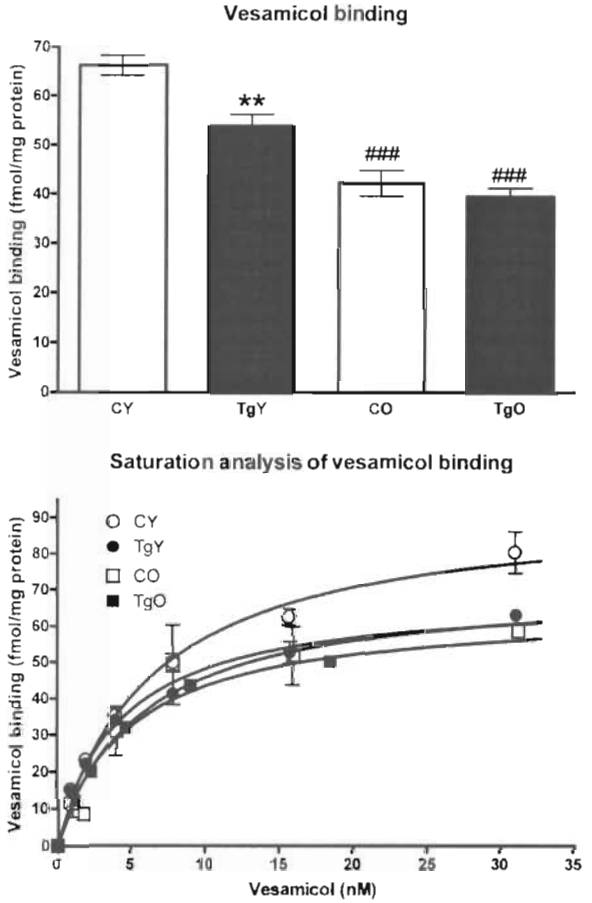

Fig. 2. Density of VAChT. *Upper graph*: Vesamicol binding in fmol/mg protein (ordinate) in individual samples was determined in triplicates with 7.0 nM ^{3}H-vesamicol. Columns represent mean ± S.E.M. of six samples in each group. $^{**}p < 0.01$, significantly different from age-matched controls; $^{***}p < 0.001$, significantly different from corresponding young controls by ANOVA followed by Bonferroni's posttest. *Lower graph*: Pooled samples were used for saturation analysis. Data are derived from a single measurement in each group done in duplicates and expressed in fmol/mg protein (ordinate). Error bars show individual values. Abscissa: concentration of vesamicol. Parameters of saturation curves are given in Table 1. CY, 7-month-old control mice; TgY, 7-month-old transgenic mice; CO, 17-month-old control mice; TgY, 17-month-old transgenic mice.

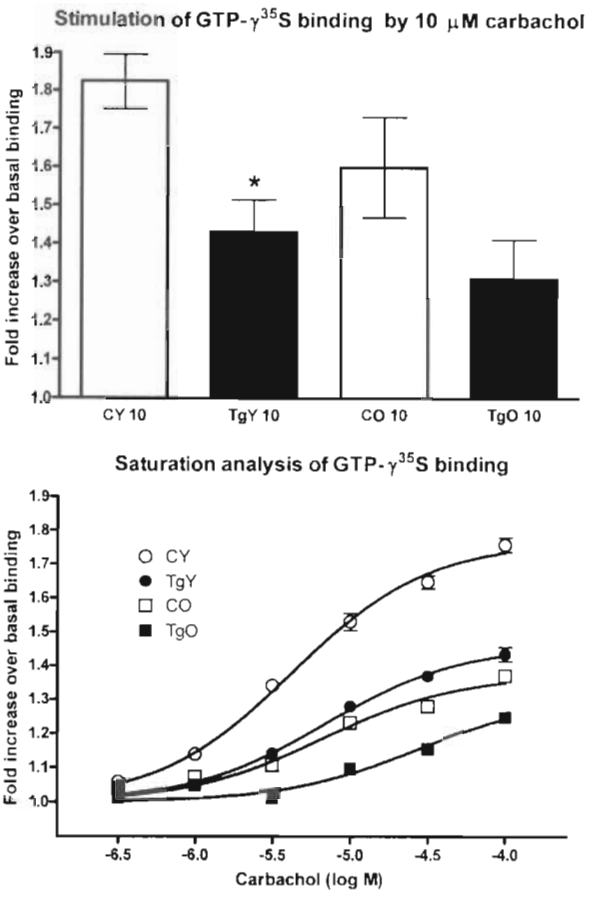

Fig. 3. Carbachol stimulation of GTP-γ^{35}S binding. *Upper graph*: Stimulation of GTP-γ^{35}S binding in individual samples by 10 μM carbachol is expressed as fold increase over basal binding in the absence of agonist. Columns represent mean ± S.E.M. of six samples in each group. $^{*}p < 0.05$, significantly different from age-matched control by two-way-ANOVA followed by Bonferroni's posttest. Basal binding was not significantly different among groups (183 ± 35, 273 ± 48, 317 ± 59, and 288 ± 67 fmol/mg protein in CY, TgY, CO, and TgO groups, respectively). *Lower graph*: Pooled samples were used for concentration–response analysis. Data shown are representative of three experiments run in quadruplicates. Values expressed as fold increase of GTP-γ^{35}S binding (ordinate) induced by carbachol (abscissa, log *M* concentration) represent mean ± S.E.M. Parameters of concentration–response curves are given in Table 2. CY, 7-month-old control mice; TgY, 7-month-old transgenic mice; CO, 17-month-old control mice; TgY, 17-month-old transgenic mice.

and EC_{50}, $p = 0.001$). Total content of G-proteins in membranes determined as GTP-γ^{35}S binding in the absence of GDP was not changed either in transgenic or aged animals (Table 3).

Table 3
Content of G-proteins in brain cortex membranes

	G-protein content (pmol/mg protein)
Control 7 months	37.7 ± 3.5
Transgenic 7 months	33.7 ± 0.5
Control 17 months	35.7 ± 2.8
Transgenic 17 months	34.1 ± 2.4

Data represent mean ± S.E.M. of three samples done in quadruplicates.

3.4. Development of amyloid plaques and AChE staining

Progressive plaque formation between 7 and 17 months of age in APPswe/PS1dE9 mice is illustrated in Fig. 4. As reported earlier (Savonenko et al., 2005), the parietal cortex displayed amyloid plaques already at 7 months of age. Nevertheless, both plaque number and size substantially increased at 17 months of age. The advancing plaque pathology was not accompanied by a systematic weakening of AChE immunopositivity. However, as the lower part of the figure with high magnification indicates, amyloid plaques caused local derangement of AChE positive fibers. Some

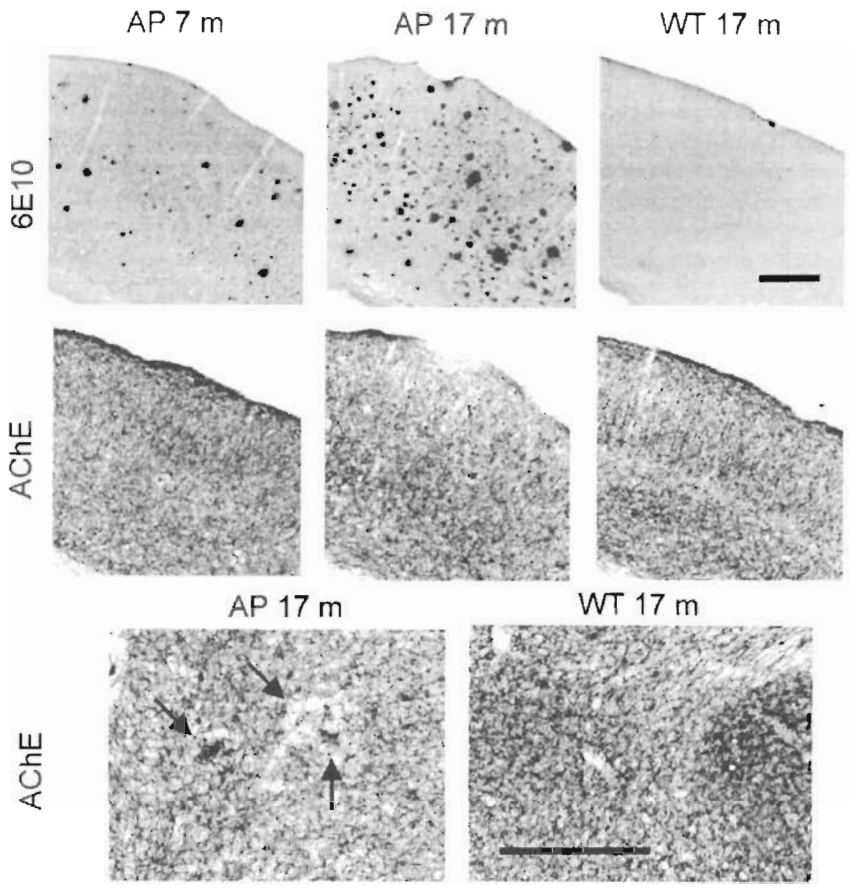

Fig. 4. Immunohistochemical staining for human specific β-amyloid (6E10) and cholinergic synapses (AChE) in 7- and 17-month-old APPswe/PS1dE9 mice and aged wild type mouse. The bottom row shows high magnification of cortical AChE-positive fibers. Note how amyloid plaques (arrows) show up as either higher or lower density spots than the surrounding tissue. The scale bar = 200 μm.

plaques appeared pale and devoid of AChE positive terminals, while others showed increased immunopositivity. This most likely simply reflects pushing the cholinergic terminals aside at the cutting plane.

4. Discussion

The most important finding of our experiments is presynaptic as well as postsynaptic impairment of cholinergic synapses in parietal cortex of young adult transgenic mice. The observed modest age-dependent decline of esterases activities and transgene-dependent reduction of BuChE activity in young animals are not likely to have major impact on strength of cholinergic transmission, considering abundance of these enzymes, and may therefore represent an adaptive response. Evidence for the functional significance of BuChE comes from AChE knockout in mice which are viable till adulthood when fed liquid diet (Duysen et al., 2002; Xie et al., 2000). These findings indicate that AChE is not essential for embryonic development and survival, and can be substituted by BuChE. Moreover, selective inhibition of BuChE increased brain ACh concentration and improved cognitive performance in aged rats, and decreased $A\beta_{1-40}$ and $A\beta_{1-42}$ production in transgenic APPswe + PS1 mice (Greig et al., 2005). Our finding of the transgene-dependent reduction in BuChE activity is in concert with its decrease in Alzheimer's disease brain cortex determined in vivo by positron emission tomography (Kuhl et al., 2006).

On the other hand, the transgene-dependent decrease of VAChT in young animals and muscarinic receptors in aged animals point to relaxation of synaptic strength. These observations are consistent with the notion that damage of synaptic transmission plays a crucial role at the very beginning of pathogenesis of Alzheimer's disease (Bartus et al., 1982; Dolezal and Kasparova, 2003; Francis et al., 1985; Mesulam, 2004; Selkoe, 2001). VAChT that we used together with ChAT as a presynaptic marker serves to pack ACh into synaptic vesicles. Its decrease in young transgenic animals thus represents damage that may limit the size of the releasable pool of ACh during ongoing activity. Because we did not find a change in vesamicol binding affinity, the decrease of its binding in single point measurements reflects lower levels of VAChT protein. This conclusion is further supported by the

significant decrease of calculated maximal vesamicol binding in saturation experiments (Fig. 2; Table 1). The lower VAChT number could indicate a decrease in the density of cholinergic terminals. However, this explanation does not seem likely because we did not observe a concomitant decrease of ChAT activity in the same samples from young animals (Fig. 1). Although ChAT and VAChT are transcribed from a common gene locus (Erickson et al., 1994) and their transcription is co-regulated (Berrard et al., 1995; Berse and Blusztajn, 1995; Cervini et al., 1995), an independent regulation of the expression of the two proteins at a posttranscriptional step, i.e. at the level of protein translation or breakdown, has been demonstrated during late embryogenesis and early postnatal life (Holler et al., 1996). This dissociation of the levels of the two proteins in transgenic animals resembles the situation in late embryogenesis and may be the result of cholinergic neuron dedifferentiation (Blusztajn and Berse, 2000) induced by increased production of β-amyloid fragments, whereas concomitant reduction of both proteins observed in aged mice may reflect a loss of cholinergic synapses similar to that found in parietal cortex of 18-month-old $hAPP_{Sw,Ind}$ transgenic mice (Aucoin et al., 2005). Results shown in Fig. 4, however, are not in favor of the latter possibility. Our findings of both age- and transgene-related decrease of VAChT density in parietal cortex compares well with the reported decrease of cortical VAChT in AD patients (Efange et al., 1997). The decrease of VAChT density may offer a plausible explanation for reduced ACh content observed in APPsw mice (Ikarashi et al., 2004).

In addition to ACh release, an integral step in cholinergic transmission is transduction of extracellular chemical signal represented by released ACh to intracellular signalling pathways mediated by ACh receptors. Our finding of both age- and transgene-related decline of the capability of carbachol to activate G-proteins demonstrates serious impairment of muscarinic signal propagation across the cell membrane. This observation may explain the apparent discrepancy between cognitive impairment and preserved or even increased expression of the cholinergic marker ChAT observed in post-mortem samples of patients diagnosed with mild cognitive impairment or mild AD (DeKosky et al., 2002; Frolich, 2002). We found no change in the density of muscarinic receptors in the parietal cortex between young and old control animals and detected a reduction by only 9% in young and 22% in aged transgenic animals compared to age-matched controls. The large (about 50%) decrease in G-protein activation efficacy we observed in young transgenic and aged control animals compared to young controls thus could not be due to a decrease in muscarinic receptors density. This is further supported by the finding that the small reduction of muscarinic receptors number in aged transgenic animals compared to aged controls was not connected with further appreciable decrease of carbachol efficacy in activating G-proteins. In concert with reported observations in human brain (Li et al., 1996; McLaughlin et al., 1991) we did not find any appreciable reduction of G-proteins level measured as a total number of GTP binding sites in membrane preparation in transgenic animals compared to corresponding controls or in aged compared to young controls that would explain observed decrease of carbachol efficacy. However, a possibility of selective depletion of a class of G-proteins will need further investigation. Another important feature of the impairment of signal transduction was the detection of more than three-fold decrease of carbachol potency in aged transgenic animals in comparison to all other groups. These data are in line with attenuated muscarinic receptor-stimulated phosphatidylinositol hydrolysis in rat primary cortical neuron cultures exposed to β-amyloid fragments (Kelly et al., 1996) and with a decrease of low K_m GTPase activity stimulated by muscarinic agonists in hippocampus and striatum of aged rats (Joseph et al., 1993). The deficit of muscarinic receptor/G-protein coupling estimated as a decrease of muscarinic receptor-stimulated phosphatidylinositol hydrolysis (Jope et al., 1994; Jope et al., 1997) and a loss of high affinity M_1 muscarinic receptor binding sites has also been demonstrated in membranes prepared from post-mortem human cortex in the terminal state of AD (Tsang et al., 2005).

All these observations point to a rather complex malfunction of muscarinic receptor/G-protein coupling than to a selective depletion of specific muscarinic receptor subtype and/or specific G-protein. The large reduction of carbachol efficacy without change of potency in young transgenic animals could derive from a selective reduction of G_s or $G_{q/11}$ G-proteins that preferentially couple with prevailing M_1 receptor in the cortex and/or of the M_1 receptor itself. This mechanism, however, cannot explain the large reduction of carbachol potency in aged transgenic animals but the same efficacy in young and aged transgenic animals (Table 2). It has been shown that coupling specificity of muscarinic receptor subtypes is not absolute and activation of non-conventional G-proteins and their signalling pathways with respect to muscarinic receptor subtype could take place under certain conditions (Jakubik et al., 2006; Michal et al., 2001). Considering complexity of agonist-stimulated receptor/G protein coupling and functional outcome of receptor stimulation (Kenakin, 2004; Tucek et al., 2002; Urban et al., 2006), it is apparent that clarification of mechanism(s) responsible for the observed attenuation of muscarinic signal transduction will require further studies.

The observed early changes in cholinergic neurotransmission in APPswe/PS1dE9 mice are probably not unique to this particular mouse line. Early studies in the common APPswe (Tg2576) line were not able to detect any cholinergic neuropathology up to 23 months of age (Gau et al., 2002), whereas a more recent study reported decreased ACh levels in several brain areas at 10 months but not yet at 6 months of age (Ikarashi et al., 2004). Additionally, changes in cholinergic presynaptic markers have been reported in mouse lines carrying the APP717(Lon/Ind) mutation alone or combined with PS1 mutation from 14 months of age on (Aucoin et al., 2005; German et al., 2003; Hartmann et al., 2004). None of the earlier reports have addressed components of cholin-

ergic neurotransmission to the same detail as our current study, so these functional changes may have gone undetected. It is also possible that APPswe/PS1dE9 mouse line has a particularly aggressive pathology among APP overexpressing mouse lines, which renders the cholinergic system more vulnerable at an early age. Compared to the Tg 2576 line, APPswe/PS1dE9 mice show a more rapid formation of amyloid plaques with age, so that 7-month-old APPswe/PS1dE9 mice have similar amyloid burden as APPswe (Tg2576) show around 12 months of age (Hsiao et al., 1996). The lines also differ slightly in terms of APP processing. Both overexpress human APP, but the expression is about six-fold compared to the endogenous levels in Tg2576 mice (Hsiao et al., 1996) while it is only about three-fold in our mice (our unpublished observation). Second, whereas APPswe mutation alone leads mainly to production of β-amyloid 1–40 ($A\beta_{1-40}$ to $A\beta_{1-42}$ ratio in Tg2576 mice is 2–4), the presence of PS1 mutation modifies γ-secretase activity so that about two times more $A\beta_{1-42}$ peptide than $A\beta_{1-40}$ peptide is produced in the APPswe/PS1dE9 double mutant line (Jankowsky et al., 2004; Oksman et al., 2006; Savonenko et al., 2005). These comparisons suggest that accumulating $A\beta_{1-42}$ plays an important role in the age- and transgene-related deterioration of cholinergic neurotransmission. However, the role of other APP degradation products as a cause for cholinergic degeneration cannot be ruled out.

Changes in APPswe/PS1dE9 mice compared to wild type littermates resemble precipitated age-related changes observed in wild type mice. This fits with the idea of accelerated aging in amyloid overproducing mice. Accumulation of β-amyloid may induce metabolic changes in the brain such as increase of oxidative stress (Aucoin et al., 2005; Mattson and Pedersen, 1998) or disturbances of glucose metabolism (Hoyer, 2000; Meier-Ruge et al., 1994; Messier and Gagnon, 1996) that accompany aging. These metabolic changes may render the cholinergic system more vulnerable to toxic effects of β-amyloid. However, it is known from clinical studies, e.g. the famous religious order's study on centenarian nuns, that many individuals may carry remarkable amyloid load in the brain without any impairment in neuropsychological tests (Snowdon, 2003). Deficit in muscarinic transmission that we observed provides a new promising tool to study the interaction between genetic and life-style factors in the pathophysiology of AD. For instance, Jankowsky et al. (2005) demonstrated that environmental enrichment enhances amyloid accumulation, but results in improved cognition in APPswe/PS1dE9 mice. It would be interesting to see effects of such manipulations on strength of muscarinic transmission.

In conclusion, the most widely used cholinergic markers AChE and ChAT exhibit only age-related decreases in the present study. However, our data convincingly demonstrate extensive functional decline of cholinergic signal transmission at the muscarinic receptor level in the transgenic APPswe/PS1dE9 mouse model of Alzheimer's disease reminiscent of accelerated aging. This decline is already apparent in young adult (7-month-old) transgenic animals that display rapid accumulation of β-amyloid deposits in the brain but no cognitive decline yet (Savonenko et al., 2005), and deteriorate with aging as evidenced by the decrease of carbachol potency to activate G-proteins (Table 2). We also showed reduction of VAChT level that is not a consequence of cholinergic denervation. Our findings thus provide evidence for both presynaptic and postsynaptic defects of cortical cholinergic synapses in young transgenic APPswe/PS1dE9 mice and unequivocally prove early malfunction of muscarinic transmission in this transgenic model of Alzheimer's disease.

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Wash-Resistantly Bound Xanomeline Inhibits Acetylcholine Release by Persistent Activation of Presynaptic M_2 and M_4 Muscarinic Receptors in Rat Brain

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ABSTRACT

We studied the effects of 3-[3-hexyloxy-1,2,5-thiadiazo-4-yl]-1,2,5,6-tetrahydro-1-methylpyridine (xanomeline) wash-resistant binding on presynaptic muscarinic regulation of electrically evoked [^{3}H]acetylcholine (ACh) release from rat brain slices. In both cortical and striatal tissues that possess M_2 and M_4 autoreceptors, respectively, immediate application of 10 μM xanomeline had no effect on evoked [^{3}H]ACh release or its inhibition by 10 μM carbachol. In contrast, preincubation with 1, 10, or 100 μM xanomeline for 15 min decreased evoked release of ACh measured after 53 min of washing in xanomeline-free medium in a concentration-dependent manner. The maximal inhibitory effect equaled the immediate effect of the muscarinic full agonist carbachol, and it was completely (at 1 and 10 μM xanomeline) or partially (at 100 μM xanomeline) blocked by 1 μM *N*-methylscopolamine. Neither presence of *N*-methylscopolamine during 100 μM xanomeline treatment nor previous irreversible inactivation of the classical receptor binding site using propylbenzylcholine mustard in cortical slices prevented the inhibitory effect of wash-resistantly bound xanomeline. Treatment of cortical slices with xanomeline slightly decreased the number of muscarinic binding sites, and it markedly decreased affinity for *N*-methylscopolamine. When applied as in acetylcholine release experiments, xanomeline did not impair presynaptic α_2-adrenoceptor-mediated regulation of noradrenaline release. The functional studies in brain tissue reported in this work demonstrate that xanomeline can function as a wash-resistant agonist of native presynaptic muscarinic M_2 and M_4 receptors with both competitive and allosteric components of action.

3-[3-Hexyloxy-1,2,5-thiadiazo-4-yl]-1,2,5,6-tetrahydro-1-methylpyridine (xanomeline) is an atypical agonist that binds to all muscarinic receptor subtypes with high affinity (Bymaster et al., 1997; Watson et al., 1998; Wood et al., 1999; Jakubík et al., 2006), but it displays functional selectivity for the M_1 and M_4 receptors (Shannon et al., 1994; Ward et al., 1995; Bymaster et al., 1997, 1998). Xanomeline was supposed to be developed as an M_1 receptor-selective drug for treatment of Alzheimer's disease. However, original clinical trials revealed frequent peripheral side effects that were not consistent with M_1 selectivity and led to interruption of testing (Mirza et al., 2003). More recent behavioral studies in rodents, primates, and clinical studies have pointed to potential profitable antipsychotic effects that are ascribed to the M_1/M_4 agonistic profile of the drug (Bymaster et al., 2002; Andersen et al., 2003).

We observed previously that xanomeline interacts with two binding sites on the muscarinic receptor, i.e., the orthosteric binding site and another distinct site where it binds in a wash-resistant manner (Christopoulos et al., 1998; Jakubík et al., 2002, 2004). Wash-resistantly bound xanomeline activates guanosine 5′-*O*-(3-thio)triphosphate binding at the M_1 receptor, and, although with lower efficacy but similar affinity, also at the M_2 receptor (Jakubík et al., 2006). Likewise, wash-resistantly bound xanomeline induces durable antagonism of M_5 receptor activation (Grant and El-Fakahany, 2005). Together, these findings strongly indicate that the complicated pharmacological profile of xanomeline action may be due not only to the selective stimulation of M_1/M_4 receptors but also to not yet well characterized effects of wash-resistant xanomeline at all subtypes of muscarinic receptors thus far tested.

All these observations on the effects of wash-resistant xa-

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ABBREVIATIONS: ACh, acetylcholine; PRBCM, propylbenzylcholine mustard; NMS, *N*-methylscopolamine; NA, noradrenaline; CHO, Chinese hamster ovary; UK-14,304, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine.

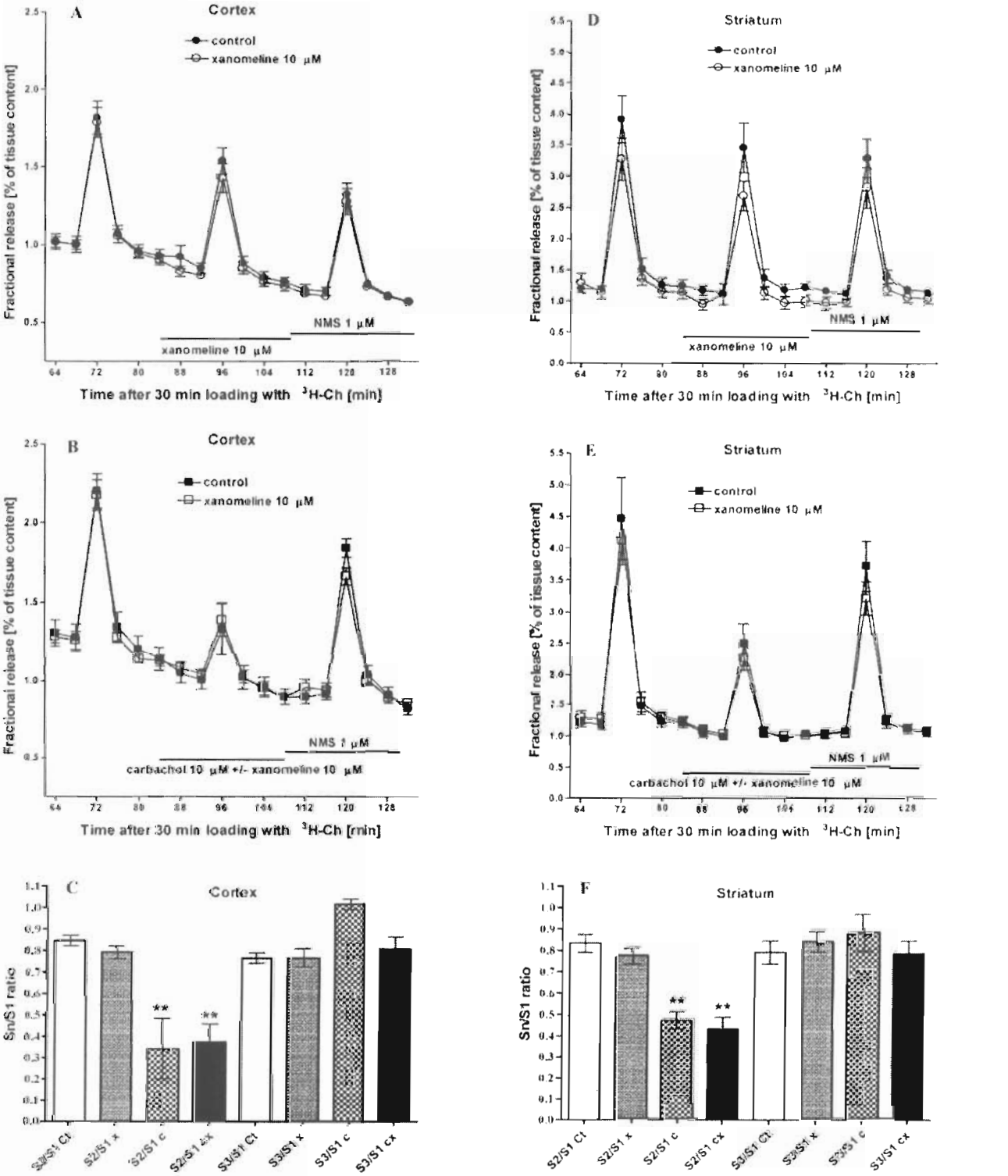

Fig. 1. Immediate application of xanomeline does not influence electrically evoked [^{3}H]ACh release and its presynaptic M_2 (cortex) and M_4 (striatum) receptor-mediated inhibition. Brain cortical (left column) and striatal (right column) slices were stimulated (2-ms monopolar pulses at 1 Hz for 1 min at 25 mA) at the beginning of the 3rd, 9th, and 15th collection fraction (abscissa). A and D, no drug (closed circles) or 10 μM xanomeline (open circles) was present 4 min before and during the second stimulation and 10 μM NMS 4 min before and during the third stimulation as indicated. [^{3}H]ACh release is expressed as fractional release in percentage of tissue content of radioactivity (ordinate). B and E, slices were stimulated as in described in previous experiments but in the presence of 10 μM carbachol (closed squares) or 10 μM carbachol and 10 μM xanomeline together during the second stimulation. Points are mean ± S.E.M. of five to nine samples from two (cortex) or three (striatum) animals in independent experiments. Ordinate, fractional release of radioactivity. Abscissa, time from the end of loading. C and F, influence of tested drugs is expressed (ordinate) as S_x/S_1 ratios relative to the first, always control, stimulation; letters Ct, x, c, and cx denote superfusion with control medium, 10 μM xanomeline, 10 μM carbachol, or 10 μM xanomeline and 10 μM carbachol together before and during the second stimulation, respectively). **, $p < 0.01$, significantly different from corresponding preceding control stimulation and following stimulation in the presence of 1 μM NMS by ANOVA followed by Tukey's test.

meline on receptor function were derived in experiments human muscarinic receptors heterologously expressed in broblasts (Chinese hamster ovary cells). It is therefore very portant to elucidate whether such unusual effects of xameline are also evident in case of receptors expressed in eir natural environment. To approach this question, we rformed ex vivo experiments with rat brain cortical and riatal tissue. We investigated delayed wash-resistant efcts of xanomeline on the regulation of stimulation-evoked lease of acetylcholine (ACh) that is mediated by M_2 receprs in brain cortex and M_4 receptors in striatum (Doležal d Tuček, 1998; Zhang et al., 2002; Bymaster et al., 2003). e show that wash-resistant xanomeline binding decreases oked ACh release by stimulating both M_2 and M_4 receptors d that both the allosteric and orthosteric binding sites rticipate in this effect.

Materials and Methods

Release Experiments. Brain cortical and striatal prisms were epared from 2- to 3-month-old male Wistar rats using a McIlwain ssue chopper set at a width of 0.35 mm. Superfusion experiments ere done essentially as described previously (Lazareno et al., 2004). brief, brain cortical slices were loaded with [^{3}H]choline (specific dioactivity, 82 Ci/mmol; Amersham, Little Chalfont, Buckinghamire, UK) in Krebs' buffer (138 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, mM $MgCl_2$ 1, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 10 mM ucose; for noradrenaline release experiments in addition 0.03 mM DTA and 0.06 mM ascorbic acid) for 30 min. Superfusion medium ntained 10 μM hemicholinium-3 in ACh release experiments to event reuptake of labeled choline, and 1 μM desipramine was dded in noradrenaline release experiments to prevent reuptake of beled noradrenaline. Domperidone (500 nM) was included to preent dopamine D_2 receptor-mediated inhibition of acetylcholine rease ((Doležal et al., 1992). The release was evoked electrically by pplying 60 2-ms rectangular monopolar pulses (1 Hz; 25 mA) at the eginning of the 3rd, 9th, and 15th 4-min fraction denoted S_1, S_2, and S_3, respectively. Immediate effects of tested drugs were determined y adding them to the superfusion medium 8 min before respective timulation. Tested drugs remained in the medium as indicated in e figures. Effects of wash-resistantly bound xanomeline were studd in experiments in which brain prisms were preincubated for 15 in in the presence of 1 to 100 μM xanomeline, thoroughly washed ith fresh medium, and superfused using xanomeline-free medium r 53 min before the first electrical stimulation. In some experients, 100 nM propylbenzylcholine mustard (PRBCM) was added uring the last 15 min of loading with [^{3}H]choline to irreversibly activate muscarinic receptor orthosteric binding sites (Fig. 5B). electivity of xanomeline pretreatment on regulation of acetylchoe release was verified in analogous experiments after loading ssue with [^{3}H]noradrenaline (specific radioactivity, 36 Ci/mmol; mersham) and evaluating functionality of the α_2-adrenoceptor-meiated presynaptic inhibition of noradrenaline release.

The evoked release of tritiated ACh and noradrenaline is expressed as fraction of tissue content of radioactivity present at the eginning of individual collected fractions during a 4-min collection eriod over background. The latter was calculated by subtracting alues immediately preceding and following the evoked liberation. mmediate influence of xanomeline on evoked [^{3}H]ACh release (Fig.) was estimated from changes of S_n/S_1 ratios ([^{3}H]ACh release oked by the second and third stimulation in the presence of drugs elative to the first stimulation) and as changes of the evoked fracional release of [^{3}H]ACh in xanomeline-pretreated compared with ontrol slices in other experiments.

Binding Experiments. [^{3}H]*N*-methylscopolamine binding and ash-resistant xanomeline binding were determined as described by

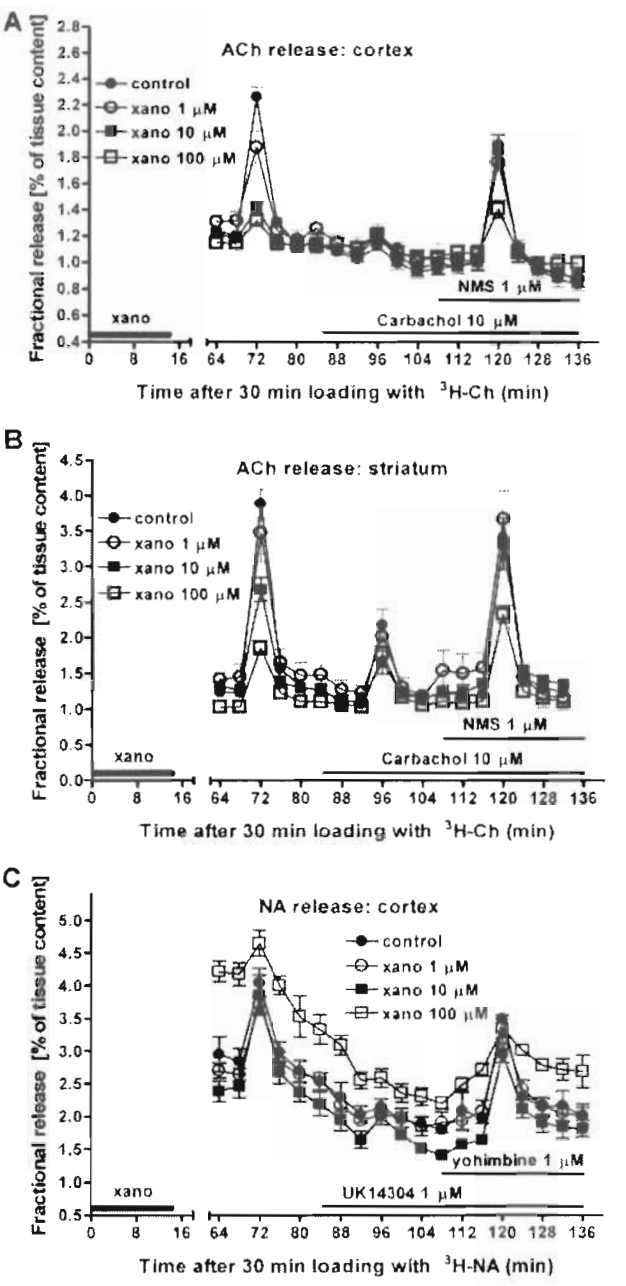

Fig. 2. Concentration-response relationship of wash-resistant xanomeline effects on electrically evoked [^{3}H]ACh and [^{3}H]NA release. Cortical (A) and striatal (B) slices were loaded with tritiated choline and then preincubated for 15 min without or with 1 to 100 μM xanomeline as indicated and then superfused with xanomeline-free medium. Three consecutive electrical stimulation were applied as in experiments shown in Fig. 1. Electrically evoked [^{3}H]ACh release was measured during superfusion in control medium, in the presence of 10 μM carbachol, and in the presence of 10 μM carbachol and 1 μM NMS together, respectively. C, cortical slices were loaded with [^{3}H]NA, treated for 15 min without or with 1 to 100 μM xanomeline as indicated, and then superfused in medium containing 1 μM desipramine. Electrically evoked [^{3}H]NA release was measured with time as in A and B. The α_2-adrenoceptor agonist UK-14,304 (1 μM) was present during the second stimulation and UK-14,304 together with 1 μM α_2-adrenoceptor antagonist yohimbine during the third stimulation. Ordinate, fractional release of transmitter. Abscissa, time from the end of loading. Each point is mean ± S.E.M. of samples derived from at least two animals. Values of evoked [^{3}H]ACh and [^{3}H]NA release, number of observations, and statistical evaluation are given in Table 1.

Jakubík et al. (2006). Curve fitting and statistical evaluation of data were done using Prism 4 (GraphPad Software Inc., San Diego, CA).

Chemicals. Xanomeline was kindly supplied by Dr. C. Felder (Eli Lilly & Co., Indianapolis, IN). Hemicholinium-3, carbachol, *N*-methylscopolamine (NMS), desipramine, UK-14,304, domperidone, and yohimbine were from Sigma (Prague, Czech Republic), and PRBCM was from NEN (Boston, MA). All other chemicals were of highest available purity.

All experiments were performed on male albino Wistar rats bred in the animal house of the Institute of Physiology (Czech Academy of Sciences, Prague, Czech Republic). The experiments were approved by the Animal Care and Use Committee of the Institute of Physiology to be in agreement with the Animal Protection Law of the Czech Republic that is fully compatible with European Community Council Directives 86/609/EEC.

Results

In control slices, sequential stimulations applied at the 9th and 33rd min of superfusion (third and ninth collected fractions) evoked the release of [^{3}H]ACh that slightly declined from 0.92 ± 0.08 to 0.78 ± 0.06% of tissue content of radioactivity (mean ± S.E.M.; n = 6) in cortex and from 2.99 ± 0.44 to 2.53 ± 0.41 (n = 9) in striatum (Fig. 1, A and D). Addition of 10 μM xanomeline to the superfusion medium had no effect on either basal outflow of radioactivity (0.85 ± 0.04 versus 0.80 ± 0.02% of tissue content of radioactivity in cortex and 1.13 ± 0.07 versus 1.11 ± 0.18 in striatum for control and xanomeline treatments, respectively) or electrically evoked release of [^{3}H]ACh evaluated as S_2/S_1 (Fig. 1C for cortex and F for striatum). As expected, 10 μM carbachol significantly inhibited the evoked release of [^{3}H]ACh and 1 μM NMS abolished its inhibitory influence in both tissues. Xanomeline at a concentration of 10 μM present together with carbachol did not influence its inhibitory effect (Fig. 1, B and C, for cortex and E and F, for striatum).

Pretreatment of choline-loaded cortical and striatal slices with 1, 10, or 100 μM xanomeline for 15 min followed by extensive washing had no effect on basal outflow of radioactivity, but it resulted in a concentration-dependent inhibition of evoked [^{3}H]ACh release from cortical slices by 49, 77, and 86% and striatal slices by 23, 48, and 67%, respectively (Fig. 2, A and B; Table 1). Carbachol at 10 μM further caused a significant decrease in cortical slices pretreated with 1 μM xanomeline and in striatal slices pretreated with 1 and 10 μM xanomeline. In both tissues, 1 μM NMS fully prevented the inhibition of ACh release induced by pretreatment with 1 and 10 μM xanomeline, but it only partially reversed the inhibition induced by pretreatment with 100 μM xanomeline. Unlike [^{3}H]ACh release, pretreatment with up to 10 μM xanomeline had no influence on either basal outflow of radioactivity or evoked release of [^{3}H]NA and its regulation by presynaptic α_2-adrenoceptors (Fig. 2C; Table 1). Compared with all other groups, pretreatment with 100 μM xanomeline significantly increased basal outflow of ^{3}H radioactivity to 4.18 ± 0.17% of tissue content of radioactivity from 2.84 ± 0.20, 2.64 ± 0.09, and 2.46 ± 0.18 in controls and after pretreatment with 1 and 10 μM xanomeline, respectively ($p < 0.001$ compared with all other groups by ANOVA and Tukey's test). As in ACh release experiments, pretreatment with 100 μM xanomeline significantly reduced by approximately 35% evoked release of [^{3}H]NA. However, regulation of evoked [^{3}H]NA release by presynaptic α_2-adrenoceptors was preserved as demonstrated by inhibition of evoked [^{3}H]NA release by the selective agonist of α_2-adrenoceptors, UK-14,304, and full reversal of this effect by the α_2-adrenoceptors antagonist yohimbine.

Binding experiments with membranes of CHO cells expressing human M_1–M_5 subtypes of muscarinic receptors demonstrated that wash-resistant xanomeline binding occurs with similar potency at all subtypes (Fig. 3). Likewise, pretreatment of cortical slices with 100 μM xanomeline followed by washing as in superfusion experiments slightly decreased maximal binding of [^{3}H]NMS determined in corti-

TABLE 1

Concentration-response relationship of wash-resistant xanomeline inhibitory effect on the evoked [^{3}H]ACh release from cortical slices, striatal slices, and lack of effect on [^{3}H]noradreneline release in cortical slices

Data are derived from experiments shown in Fig. 2, A to C. The number of observations is given within parentheses.

Stimulation	Evoked Transmitter Release in % of Tissue Content of Radioactivity		
	S1	S2	S3
Cortex: ACh release			
Drug during stimulation	Control	10 μM Carbachol	1 μM NMS
Drug during preincubation			
None	1.23 ± 0.07 (6)	0.16 ± 0.02* (6)	1.02 ± 0.07‡ (6)
Xanomeline, 1 μM	0.63 ± 0.13* (6)	0.18 ± 0.04* (6)	0.89 ± 0.08‡ (6)
Xanomeline, 10 μM	0.28 ± 0.03* (6)	0.23 ± 0.04 (6)	1.00 ± 0.12‡ (6)
Xanomeline, 100 μM	0.17 ± 0.03* (6)	0.14 ± 0.01 (6)	0.42 ± 0.04*,‡ (6)
Striatum: ACh release			
Drug during stimulation	Control	10 μM Carbachol	1 μM NMS
Drug during preincubation			
None	2.87 ± 0.22 (16)	0.87 ± 0.09* (16)	2.43 ± 0.21‡ (16)
Xanomeline, 1 μM	2.21 ± 0.43 (8)	0.92 ± 0.20* (8)	2,35 ± 0.43‡ (8)
Xanomeline, 10 μM	1.49 ± 0.21* (8)	0.57 ± 0.18* (8)	2.11 ± 0.45‡ (8)
Xanomeline, 100 μM	0.95 ± 0.11*(19)	0.87 ± 0.09 (19)	1.28 ± 0.11*,‡ (19)
Cortex: NA release			
Drug during stimulation	Control	1 μM UK-14,304	1 μM Yohimbine
Drug during preincubation			
None	1.48 ± 0.07 (6)	0.18 ± 0.04* (6)	1.72 ± 0.15‡ (5)
Xanomeline, 1 μM	1.24 ± 0.12 (6)	0.24 ± 0.07* (6)	1.52 ± 0.07‡ (6)
Xanomeline, 10 μM	1.69 ± 0.31 (6)	0.56 ± 0.16* (6)	1.54 ± 0.18‡ (6)
Xanomeline, 100 μM	1.07 ± 0.15 (5)	0.09 ± 0.09* (5)	0.88 ± 0.12*,‡ (4)

By columns, *$p < 0.01$, significantly different from control-evoked transmitter release in the absence of xanomeline during preincubation in respective stimulations by ANOVA and Tukey's test. By rows, ‡$p < 0.01$, significantly different from both preceding S1 and following S3 stimulation, and ‡$p < 0.01$, significantly different from preceding S2 stimulation by repeated measures ANOVA and Tukey's test.

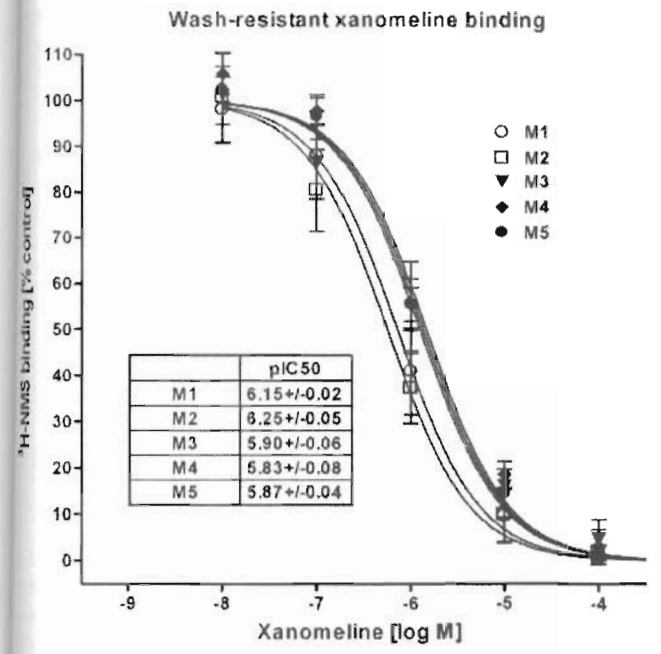

Fig. 3. Wash-resistant xanomeline binding to the M_1-M_5 subtypes of muscarinic receptors. Membranes prepared from M_3 (triangles), M_4 (diamonds), and M_5 (closed circles) receptor expressing CHO cells were preincubated 60 min with increasing concentrations of xanomeline (abscissa), extensively washed, and then incubated with [^{3}H]NMS. Wash-resistant binding of xanomeline to muscarinic receptors was determined by its ability to decrease binding of 1 nM [^{3}H]NMS (ordinate, specific binding in percentage of control) as described in Jakubík et al. (2006). Nonspecific binding was determined in the presence of 10 μM NMS. Incubation with [^{3}H]NMS was terminated after 60 min by filtration through glass fiber filters. Data are means ± S.E.M. of three to four independent experiments performed in triplicates. Hill slopes are not significantly different from unity. Inset, pIC_{50} values. Data for M_1 and M_2 receptors (open circles and open squares, respectively) were taken from Jakubík et al. (2006) for comparison.

cal membranes by 28% (from 675 to 486 fmol/mg protein), but it induced a large (approximately 10 times) decrease of [^{3}H]NMS affinity from 495 to 4847 pM (Fig. 5C; Table 2).

In the next experiments, we further investigated features of the irreversible agonistic effects of pretreatment with 100 μM xanomeline in cortical slices, i.e., at M_2 receptors. As shown in Fig. 4A and Table 3, the release of acetylcholine after xanomeline pretreatment was stable for at least three stimulations. Neither presence of 1 μM NMS during xanomeline treatment (Fig. 4B; Table 3) nor continuous washing with 1 μM NMS for 57 min before stimulation (Fig. 4C; Table 3) prevented inhibition of ACh release. In experiments summarized in Fig. 5, A and B and Table 4, we irreversibly inactivated the orthosteric binding site using propylbenzylcholine mustard to further support the finding that activation of the M_2 receptor by prolonged pretreatment with 100 μM xanomeline does not involve the orthosteric binding site. Treatment of slices with 100 nM PRBCM for 15 min reduced specific binding of the orthosteric ligand [^{3}H]NMS at 2 nM to

TABLE 2

Parameters of [^{3}H]*N*-methylscopolamine binding to cortical membranes after treatment with xanomeline

Data shown in Fig. 5C were evaluated as described by Jakubik et al. (2006). Results are mean ± S.E.M. of four independent samples run in triplicates.

Pretreatment	B_{max}	K_d
	fmol/mg protein	*pM*
None, control	675 ± 54	495 ± 36
Xanomeline, 1 μM	663 ± 20	605 ± 14
Xanomeline, 10 μM	619 ± 47	1170 ± 164
Xanomeline, 100 μM	486 ± 46*	4847 ± 572*

* $p < 0.01$, significantly different from controls by ANOVA and Tukey's test.

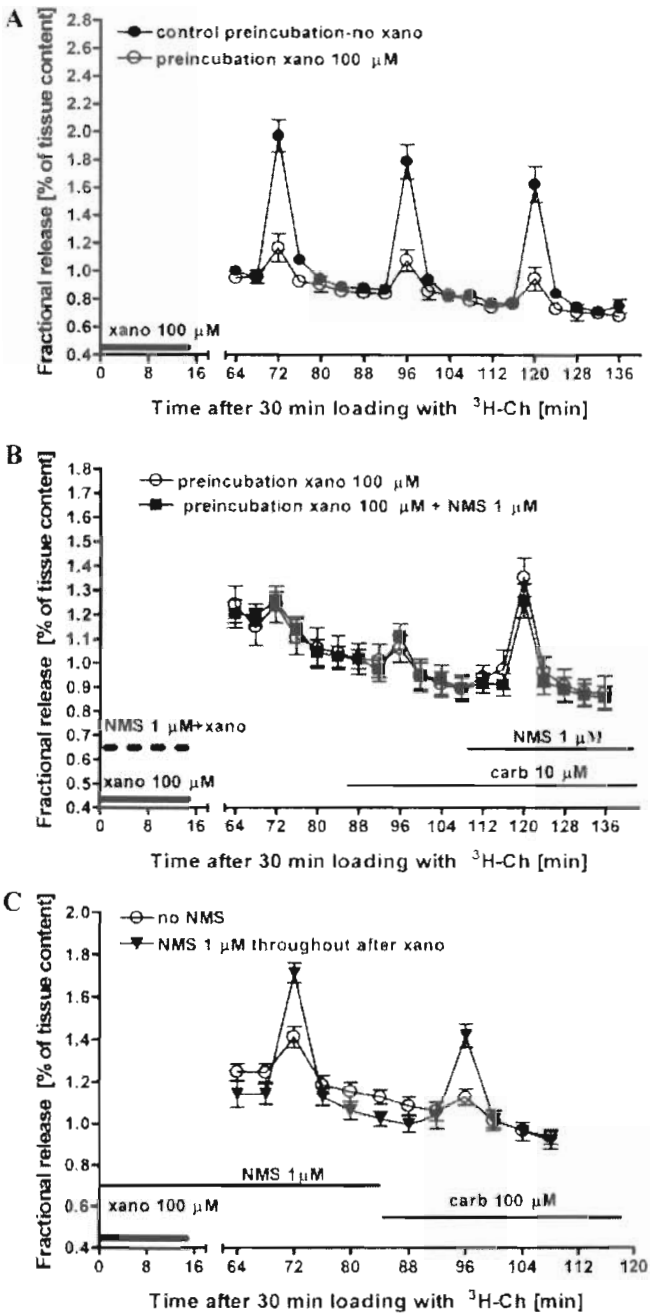

Fig. 4. *N*-Methylscopolamine does not prevent wash-resistant xanomeline effect. A, three consecutive control stimulations (in the absence of muscarinic ligands) evoke comparable [^{3}H]ACh release in control slices as well as in 100 μM xanomeline-treated slices. B, 1 μM NMS present during xanomeline treatment (closed squares) does not influence the inhibitory effect of wash-resistant xanomeline on [^{3}H]ACh release. C, extensive washing of slices in medium containing 1 μM NMS (closed triangles) prevents only partially the inhibitory effect of wash-resistant xanomeline. Ordinate, fractional release of transmitter. Abscissa, time from the end of loading. Points are mean ± S.E.M. of samples derived from two independent experiments. Values of evoked [^{3}H]ACh release, number of observations, and statistical evaluation are given in Table 3.

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ition of electrically evoked [^{3}H]ACh release by wash-resistantly bound xanomeline in cortical slices
re derived from experiments shown in Fig. 4, A to C. The number of observations is given in parentheses.

Stimulation	Evoked ACh Release in % of Tissue Content of Radioactivity		
	S1	S2	S3
Drug during stimulation	Control	Control	Control
Drug during preincubation			
None	1.26 ± 0.06 (6)	1.08 ± 0.10 (6)	1.01 ± 0.10 (6)
Xanomeline, 100 μM	0.23 ± 0.07* (6)	0.27 ± 0.07* (6)	0.22 ± 0.04* (6)
Drug during stimulation	Control	10 μM Carbachol	1 μM NMS
Drug during preincubation			
Xanomeline, 100 μM	0.14 ± 0.03 (6)	0.06 ± 0.02 (6)	0.44 ± 0.03†,‡ (6)
Xanomeline, 100 μM, + NMS, 1 μM	0.16 ± 0.03 (6)	0.15 ± 0.03 (6)	0.38 ± 0.05†,‡ (6)
Drug during stimulation	1 μM NMS	100 μM Carbachol	
Drug during preincubation			
Xanomeline, 100 μM	0.20 ± 0.04 (6)	0.11 ± 0.03 (6)	
Xanomeline, 100 μM, then washing with NMS	0.64 ± 0.05* (6)	0.44 ± 0.06* (6)	

< 0.01, significantly different from control evoked ACh release (in the absence of drugs during preincubation, by columns) in individual stimulations by *t* test.
< 0.01, significantly different from preceding S2 stimulation in the presence of carbachol.
< 0.01 significantly different from preceding S1 stimulation in the absence of drug (by rows) by ANOVA and Tukey's test.

: 0.8% of control. As expected, inactivation of the orthoc binding site by PRBCM treatment markedly attenupresynaptic modulation of ACh release by carbachol 5A; Table 4). The small remaining response to carbachol not receptor-mediated, because it was not blocked by 5. However, in line with our observation that NMS ent together with xanomeline does not prevent its de- inhibitory effects on ACh release, preincubation of CM-treated slices with 100 μM xanomeline for 15 min strongly inhibited ACh release, and this inhibition was reversed in the presence of NMS during stimulation, rary to what is expected in case of drugs acting through orthosteric binding site.

Discussion

nomeline is a muscarinic agonist that binds equally well l subtypes of muscarinic receptors, but its immediate ication in functional assays in vitro points to M_1/M_4 tivity (Bymaster et al., 1997). Another remarkable fea- of xanomeline interaction with muscarinic receptors is ash-resistant binding demonstrated at M_1 (Christopou- t al., 1998, 1999; Jakubík et al., 2002, 2004), M_2 (Jakubík ., 2006), and M_5 (Grant and El-Fakahany, 2005) recep- We also demonstrate wash-resistant binding of xanome- to the M_3 and M_4 receptor subtypes that has similar ity with other subtypes (Fig. 3). In addition, it has been onstrated that in the absence of free ligand, wash-resis- y bound xanomeline exhibits different potency, time se, and efficacy in activating M_1 and M_2 receptors (Jaku- t al., 2006) and antagonizes the M_5 subtype (Grant and akahany, 2005). However, all these observations were ned using muscarinic receptors heterogenously ex- sed in cell lines. The main finding of the present exper- ts is the confirmation of the presence of persistent wash- tant effects of xanomeline at M_2 and M_4 receptors essed in their natural environment.

line with previous findings on M_2 receptors expressed in branes of CHO cells, xanomeline at concentrations that rate the orthosteric binding site had no immediate effect ither basal efflux of radioactivity or evoked release of ed ACh release from cortical slices. Conversely, preintion of cortical slices for 15 min with xanomeline induced a delayed concentration-dependent decrease of evoked ACh release estimated 53 min after xanomeline washout. The half-maximal inhibition of evoked ACh release by wash-resistant xanomeline was reached at around 1 μM. The maximal effect of xanomeline amounted to that of the full agonist carbachol and maximal effects of xanomeline and carbachol were not additive, indicating common mechanism of action, i.e., activation of M_2 receptors. Although the potency of wash-resistant xanomeline in inhibiting ACh release and in inducing coupling of M_2 receptor expressed in CHO cell membranes to $G_{i/o}$ G proteins (Jakubík et al., 2006) is reasonably comparable, its efficacy in inhibiting evoked acetylcholine release is higher. This discrepancy may be due to receptor reserve of autoreceptors on cholinergic endings.

Selectivity of xanomeline pretreatment with regard to muscarinic receptor-mediated effects was tested using presynaptic α_2-adrenoceptors that mediate presynaptic inhibition of evoked noradrenaline release (Starke, 2001). Noradrenaline release from rat brain cortex in vivo is not influenced by administration of xanomeline (Perry et al., 2001). In concert, as shown in Fig. 2C and Table 1, xanomeline pretreatment at concentrations up to 10 μM had no appreciable effect on evoked noradrenaline release or on its inhibition by the α_2-adrenoceptor agonist UK-14,304. Pretreatment with 100 μM xanomeline, however, significantly increased basal outflow of radioactivity and somehow reduced evoked noradrenaline release. However, the inhibition of evoked release by an α_2-adrenergic agonist remained preserved.

An interesting feature of wash-resistant xanomeline inhibitory action was observed in experiments testing the involvement of the orthosteric site in xanomeline effects on ACh release. Presence of the orthosteric antagonist *N*-methylscopolamine in the medium during stimulation abolishes inhibition of ACh release observed after treatment with low concentrations of xanomeline (1 and 10 μM), whereas only partial prevention was found after treatment with 100 μM xanomeline for both cortex and striatum. The inhibition of ACh release from cortical slices induced by pretreatment with 100 μM xanomeline was not abolished by either presence of *N*-methylscopolamine during pretreatment or extensive washing in the presence of *N*-methylscopolamine. Like-

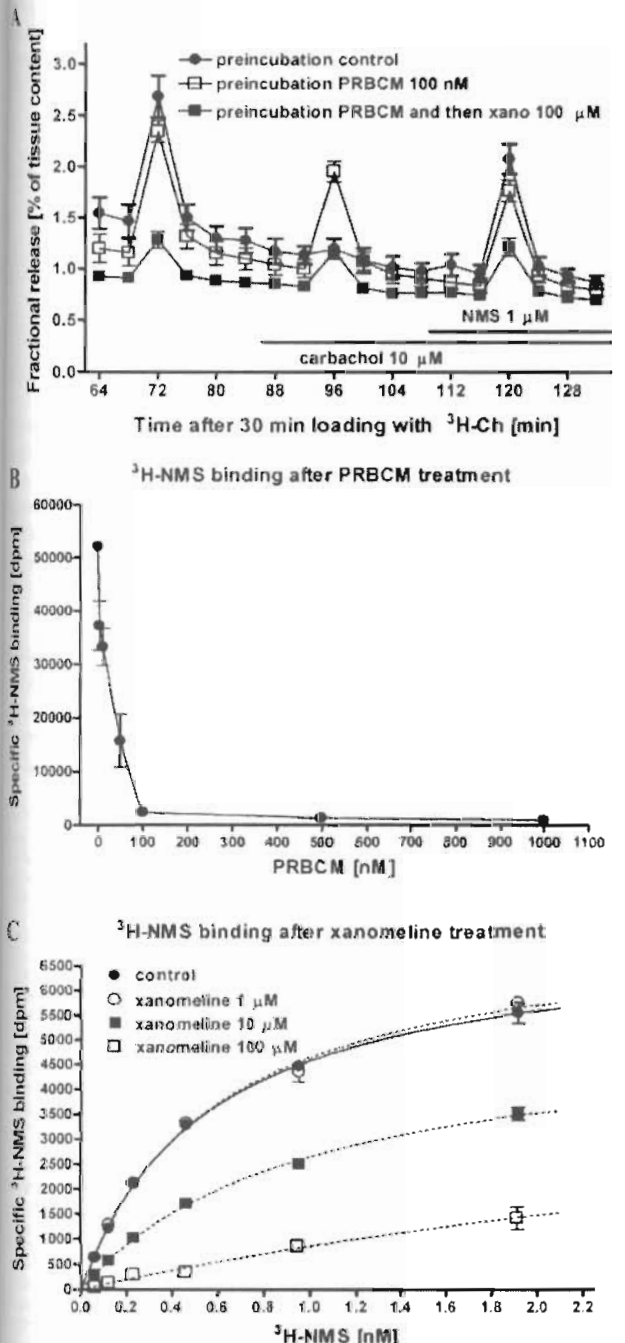

Fig. 5. A, inhibition of electrically evoked [^{3}H]ACh release by wash-resistantly bound xanomeline after irreversible blockade of the muscarinic receptor orthosteric binding site. Cortical slices were treated during the last 15 min of loading with [^{3}H]Ch with 100 nM PRBCM to irreversibly inactivate the muscarinic orthosteric binding site and afterward without (closed squares) or with (opened squares) 100 μM xanomeline for another 15 min. Slices were then superfused in the absence of free xanomeline and stimulated in control medium, then in the presence of 10 μM carbachol, and finally in the presence of 10 μM carbachol and 1 μM NMS together as described in Fig. 2A. Each point is mean ± S.E.M. of samples derived from at least two independent experiments. Ordinate, fractional release of radioactivity. Abscissa, time from the end of loading. Values of evoked [^{3}H]ACh release and statistical evaluation are given in Table 4. B, effect of treatment with increasing concentrations of PRBCM for 15 min on [^{3}H]NMS binding to brain cortex membranes prepared after 53 min of washing. Abscissa: concentration of PRBCM during treatment. Ordinate: [^{3}H]NMS-specific binding is expressed in dpm per aliquot of 500 μg of protein. Each point is mean ± range of two measurements in triplicates. Control binding was 52,139 ± 23 dpm (mean ± range). C, effects of increasing concentraions of xanomeline applied for 15 min on [^{3}H]NMS binding to brain cortex membranes prepared after 53 min of washing. [^{3}H]NMS-specific binding (ordinate) to brain cortex membranes is expressed in dpm per aliquot of 50 to 60 μg of protein. Points are mean ± S.E.M. of representative measurement in triplicates. Abscissa, [^{3}H]NMS in nanomolar. Parameters of [^{3}H]NMS binding are summarized in Table 2.

wise, irreversible inactivation of the orthosteric binding site using propylbenzylcholine mustard before xanomeline treatment reduced [^{3}H]*N*-methylscopolamine binding by more than 95% and abolished carbachol-induced inhibition of evoked ACh release, but it did not prevent the inhibitory action of wash-resistantly bound xanomeline. These results apparently indicate that binding of xanomeline to the orthosteric site is not imperative for formation of its wash-resistant interaction with the receptor. Furthermore, they demonstrate that wash-resistant xanomeline activates the receptor even when the orthosteric site is obstructed as evidenced by abolition of *N*-methylscopolamine binding and inhibition of evoked ACh release by an orthosteric agonist after propylbenzylcholine treatment.

The inhibitory action of xanomeline on ACh release was similar in cortex and striatum in that it did not display immediate effects; the concentration-responses were roughly the same, in line with comparable affinity of wash-resistant binding (Fig. 3); and the delayed inhibitory effects were not prevented by pretreatment with xanomeline in the presence of antagonist (data not shown for striatum). This observation was a bit surprising in striatum because of reported M_1/M_4 agonistic profile (Bymaster et al., 2002, 2003). Because striatum contains cholinergic interneurons, we verified that the inhibitory action of xanomeline on ACh release was effected through presynaptic M_4 receptors in experiments with 50 mM potassium stimulation that precludes a possible involvement of action potential propagation from cell bodies. As with electrical stimulation, xanomeline had no immediate effect when applied 8 min before and during potassium stimulation. However, preincubation with 100 μM xanomeline for 15 min followed by 53-min washing significantly reduced evoked ACh release (data not shown). We speculate that wash-resistant xanomeline binding is necessary for agonistic effects of xanomeline. It may be a matter of kinetics of wash-resistant binding formation that is very fast in M_1 receptor; therefore, receptor activation seems immediately. This is in contrast to much slower onset in case of the M_2 subtype (Jakubík et al., 2006) and perhaps also in the M_4 subtype. Alternatively, free xanomeline acting only through the orthosteric binding site might have antagonistic effects. This possibility is unlikely because continuous presence of xanomeline does not interfere with carbachol inhibitory influence on ACh release (Fig. 1, B and E). Moreover, we did not observe a reduction of wash-resistant xanomeline-induced receptor activation by free xanomeline in M_1 and M_2 receptors (Jakubík et al., 2006).

In conclusion, results of our experiments demonstrate wash-resistant delayed agonistic effects of xanomeline at muscarinic M_2 and M_4 receptors in functional tests using natural brain tissue. They are in line with observations obtained in binding and functional experiments in CHO cells expressing individual subtypes of muscarinic receptors, and they provide evidence for a complex mode of xanomeline action that encompasses both orthosteric and allosteric components. Interaction of xanomeline at an allosteric site on the

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valent modification of muscarinic receptors orthosteric binding site with PRBCM does not prevent wash-resistant inhibitory effect of nomeline on evoked [^{3}H]ACh release

s are from experiments shown in Fig. 5A. Number of observations is given in parentheses.

Stimulation	Evoked ACh Release in % of Tissue Content of Radioactivity		
	S1	S2	S3
Drug during stimulation	Control	10 μM Carbachol	1 μM NMS
Drug during preincubation			
None	1.43 ± 0.08 (6)	0.13 ± 0.05* (6)	1.22 ± 0.14 (6)
PRBCM, 100 nM	1.36 ± 0.06 (12)	1.09 ± 0.06* (12)	1.03 ± 0.08 (12)
PRBCM, 100 nM, then Xanomeline, 100 μM	0.42 ± 0.07* (6)	0.39 ± 0.04* (6)	0.54 ± 0.08* (6)

* $p < 0.01$, by columns, significantly different from [^{3}H]ACh release in the absence of drugs during preincubation in respective stimulations by ANOVA and Tukey's test
$p < 0.01$, by rows, significantly different from both preceding S1 control stimulation and following S3 stimulation in the presence of antagonist by repeated measures OVA and Tukey's test.

uscarinic receptor is probably involved in its wash-resistnt binding and agonistic effects (Jakubík et al., 2002). hus, our findings support the potential of allosteric agonists azareno and Birdsall, 1995; Jakubík et al., 1996, 1998, 06; Sur et al., 2003; Langmead et al., 2006), and they ovide an example of an agonist drug with prolonged activy that lingers in the absence of free ligand.

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ORIGINAL PAPER

The Increase of Choline Acetyltransferase Activity by Docosahexaenoic Acid in NG108-15 Cells Grown in Serum-free Medium is Independent of its Effect on Cell Growth

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Abstract We investigated the influence of the polyunsaturated docosahexaenoic acid (22:6*n*-3; DHA) on the constitutive expression of choline acetyltransferase (ChAT) in native and induced expression in differentiated cholinergic cells NG108-15 grown in serum-free medium. Elimination of serum-derived trophic support resulted in growth arrest and a strong decrease of ChAT activity. In either conditions, DHA largely rescued general indicators of cell growth and function, and partially prevented the decrease of ChAT activity. However, the maximal effect on general cell state in native and differentiated cells, and ChAT activity in native cells, was reached at or below 10 μmol/l of DHA. In contrast, maximal induction of ChAT activity in differentiated cells required about six times higher concentrations of DHA. These data thus demonstrate stimulatory effect of DHA on ChAT activity that is independent of its general cell protective properties.

Keywords Cholinergic neuron · Docosahexaenoic acid · Trophic support · Cell growth · Choline acetyltransferase · Alzheimer's disease · Calcium influx · Oxidative load · α-Secretase · Caspase-3 · Cholesterol

Introduction

The polyunsaturated docosahexaenoic acid (22:6*n*-3; DHA) is a natural component of cell membranes that is particularly abundant in brain, testes, and liver. In mammals, DHA is an essential fatty acid that cannot be synthesized de novo. DHA itself or its immediate precursors thus must be supplied as indispensable constituents of food. A large body of evidence indicates that adequate intake of polyunsaturated fatty acids is essential for brain development in early ontogenesis and positively impacts various pathological states connected with aging, including, among others, neurodegenerative diseases [1–4]. It has been reported that inadequate intake of DHA results in depletion of its brain content and disturbances of acetylcholine metabolism in rat hippocampus [5]. Similarly, DHA-rich diet rectifies deficits of behavioral performance accompanying DHA depletion [6] and restores lipid composition and acetylcholine release in hippocampus of old animals [7]. Increased intake of DHA has also been shown to prevent excitotoxic injury of cholinergic neurons in basal forebrain [8]. Altogether, these findings demonstrate general neuroprotective effects of DHA intake that might involve protection of cholinergic neurons and cholinergic function. However, they do not elucidate existence of such presumed direct effects on cholinergic neurons.

Hybridoma cell line NG108-15 [9, 10] demonstrates a phenotype in that these cells transcribe cholinergic gene locus [11] and express mature choline acetyltransferase (ChAT) protein. We have reported previously that differentiation of NG108-15 cells induced by cAMP and dexamethasone leads to morphological differentiation accompanied by a marked enhancement of the cholinergic gene locus transcription, the expression of ChAT activity, and depolarization-induced calcium influx through neuronal voltage-operated calcium channels [12–14]. In the present

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experiments we utilized these cells to investigate a presumed effect of DHA on the expression of their cholinergic phenotype represented by ChAT activity.

Growth of most mammalian cell lines strictly depends on the presence of poorly defined components of serum encompassing low molecular weight substances like DHA or cholesterol, and various trophic factors. Because commercially available defined culture media and lipid medium supplements do not contain defined concentrations of DHA we employed for experiments fully defined medium in which fatty acid-free serum albumin replaced serum as DHA carrier. The cells were therefore devoid of any possible trophic factors and all cholesterol needed for membrane formation and cell growth had to be synthesized de novo. Our main aim was to determine the influence of exogenous DHA on the constitutive expression of ChAT activity in native cells and on its induced expression in cells differentiated to enhance their neuronal and cholinergic phenotype. Because of deprivation of trophic support from serum we also followed effects of DHA supplementation on additional parameters important for cell functioning that may be involved in neurodegeneration involving depolarization-induced calcium transients, cell cholesterol content, α-secretase activity caspase-3 activity, and oxidative state of cells, respectively.

Experimental procedure

Cell cultures

NG108-15 cells (kindly donated by Prof. B. Hamprecht) were propagated in 75 cm^2 culture flasks in Dulbecco's modified Eagle's medium containing in addition 5% non-inactivated fetal calf serum, 1% HAT supplement (Sigma, Prague, Czech Republic), 3 μM glycine, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (basal medium), under an atmosphere of 5% CO_2/95% humidified air at 37°C. For experiments, cells were seeded in basal medium (serum containing). Drugs were added the next day after seeding and the cells were grown in their presence for 3–5 days either in the same medium or in medium containing 25 g/l fatty acid-free bovine serum albumin (Sigma, Prague, Czech Republic) in place of serum (serum-free medium) as a carrier of DHA (*cis*-4,7,10,13,16,19-DHA, Sigma, Prague, Czech Republic) without change of the medium before being used for experiments. DHA was dissolved at thousand times of desired final concentration in ethanol. Controls received only ethanol. Cells for intracellular calcium measurements were seeded on round glass coverslips placed in 3 cm diameter plastic Petri dishes at a density 20,000 cells per dish and cultured in 3 ml of basal or serum-free Dulbecco's modified Eagle's medium. For all other experiments, 20,000 cells were seeded into each well of 24-well-plate and grown in 2 ml of basal or serum-free Dulbecco's modified Eagle's medium. Differentiation of cells was induced by 0.2 mM dibutyryl-cAMP and 100 nM dexamethasone [12–14].

Biochemical measurements

Oxidative load of intact cells and caspase-3 activity were determined as described earlier [15].

Choline acetyltransferase activity was determined using a modification of Fonnum's method [16, 17]. Briefly, cells grown in 24-well-plates were homogenized by trituration in 200 μl of ChAT assay buffer (Na_2HPO_4/NaH_2PO_4 buffer 10 mM, NaCl 200 mM, Triton X-100 0.2%, pH = 7.4). Aliquots of homogenates (10–30 μl) were incubated in 50 μl of ChAT assay buffer (final volume) containing in addition (final concentrations) 0.2 mM eserine, 2 mM choline, and a mixture of 15 μM cold acetylCoA and ^{3}H-acetylCoA (Amersham, SRA 3 Ci/mmol; final SRA around 140 dpm/pmol) for 15 min at 37°C. Incubations were stopped by adding 400 μl of ice-cold 10 mM Na_2HPO_4/NaH_2PO_4 buffer with 200 μM acetylcholine as a carrier (pH = 7.4) and 400 μl of tetraphenylboron dissolved in butyronitril (10 mg/ml). Synthesized labeled acetylcholine was extracted into organic layer and 250 μl aliquots were taken for scintillation counting.

Activity of α-secretase was determined using α-Secretase Activity Kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Enzyme activities are corrected for protein content.

Cholesterol content of cells grown in 24-well-plates was determined with Amplex® Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR, USA) according to manufacturer's instructions. Results are corrected for protein content.

Proteins were determined using Peterson's modification [18] of Lowry's method [19] with human serum albumin as standard.

Microfluorimetry

Intracellular calcium measurements were done essentially as described previously [20]. Briefly, cells on coverslips were washed in Krebs–Hepes buffer and incubated at 37°C for 30–60 min in 1 ml of the same buffer supplemented with 5–10 μM Fura-2-AM (Molecular Probes, Eugene, OR, USA). The coverslips

were then washed again and installed into a superfusion chamber on an inverted fluorescence microscope (Olympus). The cells were superfused at room temperature (22–27°C) at a rate close to 0.5 ml/min using an application system that enabled extremely fast switches of the superfusion media. They were stimulated by 2.3 s lasting exposures to a medium containing 73 mM KCl which had been isoosmotically substituted for NaCl. For fluorescence measurements, cells were alternately illuminated at 340 and 380 nm wavelengths and light emission at 510 nm was recorded using a CCD camera. Data were collected every 1.2 s and processed with Metafluor software (Universal Imaging Corporation, West Chester, PA, USA). Influence of the treatment during cultivation on potassium depolarization-evoked increases of $[Ca^{2+}]_i$ was determined by comparing the increases of fluorescence ratio F340/F380.

Evaluation of data

Data were analyzed and statistical tests were performed using GraphPad 4.0 software. Sigmoidal concentration–response curve $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1+10^{(\log EC_{50}-X)})$ is used to fit concentration–response data. Statistical significance was evaluated using one-way ANOVA followed by Tukey's test or Student's *t*-test.

Results

Effects of DHA on cell growth and ChAT activity in serum-free medium

Differentiation of NG108-15 cells induced by 100 nM dexamethasone and 0.2 mM dibutyryl-cAMP in presence of serum increased ChAT activity more than five times and attenuated cell growth estimated as a decrease of protein content by 34% (Table 1) in line with previous findings [12, 13]. Deceleration of cell growth was connected with strong reduction of executioner caspase-3 activity. Replacement of serum in native cells by fatty acid-free albumin (2.5 g/100 ml) resulted in growth arrest and significant reduction of ChAT activity while caspase-3 activity remained unchanged. The presence of differentiating drugs in serum-free medium had no effect on cell growth while slightly but significantly increased ChAT activity and reduced caspase-3 activity. The extent of these changes in serum-free medium induced by differentiation was, however, much smaller than that observed in serum-containing medium (5.9-fold vs 1.6-fold increase of ChAT activity and 5.6-fold vs 1.3-fold decrease of caspase-3 activity in native and differentiated cells, respectively).

Supplementation of serum-free medium with DHA resulted in a concentration-dependent increase of cell growth demonstrated by an increase of protein content, ChAT activity and a decrease of caspase-3 activity in both native and differentiated cells compared to control values in the absence of DHA (Fig. 1; Table 2). The potency of DHA in supporting cell growth and reducing caspase-3 activity (Table 2) was similar for control ($EC_{50} = 1.9$ and 1.2 μM for cell growth and caspase activity, respectively) and differentiating cells ($EC_{50} = 0.9$ and 0.7 μM for cell growth and caspase activity, respectively). With regard to these effects on cell growth and caspase activity, DHA was slightly more potent in differentiated cells. In contrast, EC_{50} of the increase of ChAT activity in differentiated cells was significantly more than six times higher than in control cells (8.8 μM vs 1.3 μM).

The extent of the recovery of cell growth and ChAT activity was verified in separate experiments with 100 μM DHA that is saturating with respect to both cell growth and expression of ChAT activity. In three independent experiments done in quadruplicates the recovery of cell growth was only partial in native cells (protein content 78.0 ± 4.3% of controls grown in serum-containing medium) while differentiated cells

Table 1 Effects of differentiation on ChAT activity, protein content, and caspase-3 activity in serum-containing and serum-free medium

	Control cells		Differentiated cells	
	Serum	Albumin	Serum	Albumin
ChAT activity (pmol/mg protein × 30 min)	120.0 ± 6.3 (12)	71.7 ± 4.9* (21)	706.0 ± 33.4** (12)	114.0 ± 10.8*,** (22)
Protein content (μg/well)	296.0 ± 10.2 (12)	93.7 ± 6.8* (21)	195.0 ± 6.4** (12)	101.8 ± 6.9* (22)
Caspase-3 activity (AU/μg protein × 1 h)	118.0 ± 12.0 (12)	122 ± 2.9 (12)	21.2 ± 1.6** (12)	16.4 ± 1.2*,** (12)

Data are mean ± SEM of number of independent observations on cells from at least three independent seedings

AU arbitrary units

* $p < 0.01$ significantly different from control values in serum-containing medium

** $p < 0.01$ significantly different from corresponding values in native cells by unpaired *t*-test with Welch's correction

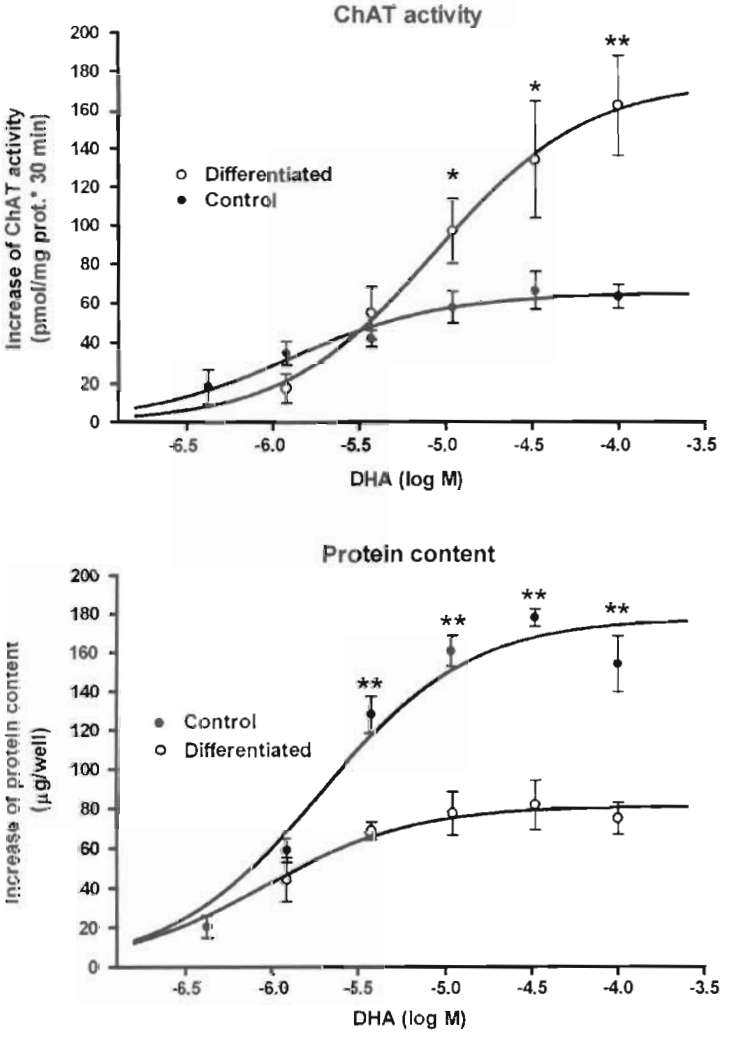

Fig. 1 Concentration–response of DHA effect on protein content and ChAT activity in NG108-15 cells. Native (*full symbols*) or differentiated cells (*closed symbols*) were grown in medium with serum-free albumin and indicated concentrations of DHA (*abscissa*). *Ordinate*—increase of ChAT activity (*upper graph*) and protein content (*lower graph*) over controls grown in the absence of DHA is shown. *Points* are means ± SEM of 4–12 values obtained in experiments on cells from at least two independent seedings. Sigmoidal concentration–response curve $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{(\log EC_{50} - X)})$ is fitted to data. Parameters are given in Table 2. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from corresponding native cells by Student's *t*-test

Table 2 EC_{50} and E_{max} of DHA effect on ChAT activity, protein content, and caspase-3 activity

	Control cells		Differentiated cells	
	EC_{50} (log *M*)	E_{max}	EC_{50} (log *M*)	E_{max}
Increase of ChAT activity	−5.89 ± 0.09 (0.7–2.3 µM)	64.9 ± 2.70 (57.3–72.5)	−5.05 ± 0.04 (6.6–11.8 µM)	174.1 ± 4.5 (159.9–188.2)
Increase of protein content	−5.72 ± 0.13 (0.9–4.4 µM)	177.5 ± 11.3 (146.2–208.8)	−6.07 ± 0.10 (0.4–1.9 µM)	81.3 ± 3.0 (71.8–90.7)
Decrease of caspase-3 activity	−5.92 ± 0.02 (1.1–1.4 µM)	266.8 ± 2.4 (263.9–273.7)	−6.14 ± 0.05 (0.6–0.9 µM)	23.3 ± 0.4 (22.4–24.6)

Values of EC_{50} and E_{max} indicating mean ± SEM and 95% confidence interval given in parentheses are derived from experiments shown in Fig. 1

demonstrated full recovery (109.0 ± 5.4% of controls grown in serum-containing medium). Although the recovery of ChAT activity by 100 μM DHA was more complete in native than differentiated cells (to 68.8 ± 4.9% vs 17.8 ± 1.4% of native and differentiated controls in grown in serum-containing medium, respectively; $p < 0.001$ by unpaired t-test with Welch's correction), the net increase of ChAT activity was more than two times bigger in differentiated than in control cells (59.1 ± 9.8 and 24.9 ± 3.3 pmol/mg protein × 30 min, respectively; $p < 0.01$ by unpaired t-test with Welch's correction). These results correlate well with more than two times higher ChAT activity in differentiated cells calculated from E_{max} values (Table 2).

DHA prevents increase of oxidative load and decrease of calcium influx consequent to serum withdrawal

Observations in differentiated cells indicated that the effects of DHA on ChAT activity and cell growth are independent. In order to get further insight into effects of DHA on general cell performance, we determined in differentiated cells the influence of 10 μM DHA that fully supports cell growth but has only about half maximal effect with respect to the expression of ChAT, on oxidative activity and depolarization-evoked calcium influx. As is shown in Fig. 2, replacement of serum by albumin increased oxidative load deduced from fluorescein oxidation by 31%. This increase was fully reversed by 10 μM DHA present in culture medium during cultivation. Three times higher concentration of DHA had no further effect. Similarly, withdrawal of serum reduced potassium depolarization-evoked influx of calcium determined as the increase of Fura-2 fluorescence ratio and 10 μM DHA abolished this effect. In three independent experiments, the increase of fluorescence ratio evoked by depolarization was 0.788 ± 0.038 ($n = 114$), 0.596 ± 0.035 ($n = 132$; $p < 0.05$, significantly different from both other groups by ANOVA followed by Tukey's test), and 0.743 ± 0.041 ($n = 109$) in cells grown in serum-containing medium, serum-free medium, and serum-free medium supplemented with 10 μM DHA, respectively.

Influence of DHA on cholesterol content and α-secretase activity

Availability of cholesterol is essential for membrane production and renewal. In addition, cell cholesterol content plays an important role in signal transduction and links lipid homeostasis with β-amyloid formation. We therefore estimated the influence of 10 μM DHA, a concentration which already provides a maximal effect in supporting cell growth, on cell cholesterol content and α-secretase activity (Table 3). Withdrawal of serum induced a decrease of cholesterol content in both native and differentiated cells compared to corresponding controls grown in the presence of serum. However, cell cholesterol content in differentiated cells was higher than in native cells, i.e., differentiation in serum-free medium could increase cholesterol content. Supplementation of serum-free medium by DHA significantly augmented cholesterol content in native cells but had no additional effect in differentiated cells. Unlike cholesterol content, differentiation did not change α-secretase activity. Removal of serum significantly diminished α-secretase activity in control as well as differentiated cells to a similar extent. Addition of 10 μM DHA significantly augmented α-secretase activity in both conditions to a similar level which was, however, still significantly smaller than that in cells grown in serum-containing medium. Increasing the concentration of DHA to 100 μM did not cause further effect in either condition.

Discussion

Serum contains in addition to a variable amount of fatty acids other undefined trophic factors. The most important finding of our experiments is that DHA alone was able to support growth and functionality of neuronal NG108-15 cells in a fully defined serum-free medium, i.e., in the absence of any additional trophic support. Serum withdrawal had as expected adverse effects on cell growth and functional characteristics like an increase of oxidative load and a decrease of depolarization-induced calcium influx. The attenuation of growth induced by differentiation or growth arrest consequent to serum deprivation apparently was not connected with induction or enhancement of apoptosis because differentiation of cells markedly diminished activity of executioner caspase-3 activity while omission of serum both in control and differentiated cells had no appreciable effect in either condition. At physiologically relevant concentrations DHA largely or fully prevented these deficits and diminished caspase-3 activity. These results are consistent with reported neuroprotective effects of in vivo administration of polyunsaturated fatty acids, namely DHA [7, 8, 21–24].

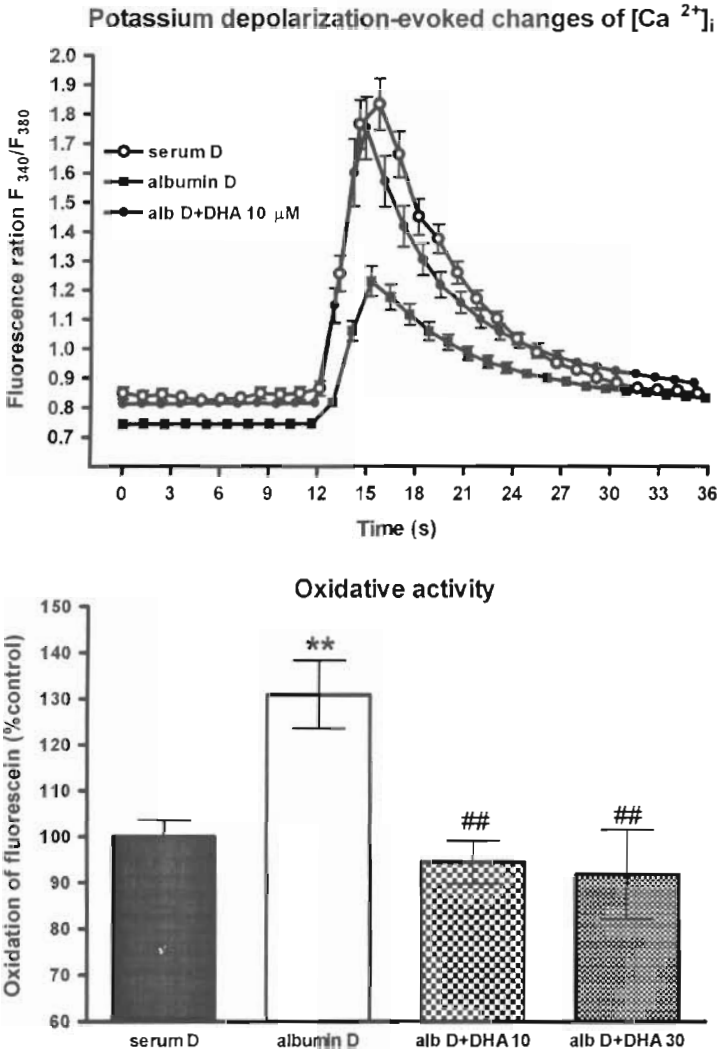

Fig. 2 Influence of DHA on potassium depolarization-evoked calcium influx and oxidative activity in differentiated NG108-15 cells. *Upper graph*—cells were grown in differentiating conditions in medium containing serum (*open circles*), serum-free medium with albumin (*closed squares*), or serum-free medium with albumin and 10 μM DHA (*closed circles*). They were stimulated by 2.3 s lasting exposures to a medium containing 73 mM KCl which had been isoosmotically substituted for NaCl. *Abscissa*—time in second. *Ordinate*—the changes of intracellular-free calcium concentration are expressed as changes of fluorescence ratio F_{340}/F_{380}. Points are mean ± SEM of 29, 40, and 22 cells grown in serum-containing medium, serum-free medium, and serum-free medium supplemented with 10 μM DHA, respectively. Shown is representative of three experiments on cells from independent seedings. *Lower graph*—cells were grown in differentiating conditions in medium containing serum (*black column*), serum-free medium with albumin (*open column*), or serum-free medium with albumin and 10 or 30 μM DHA as indicated. *Ordinate*—results are expressed in percent of control cells grown in serum-containing medium as mean ± SEM of 12–31 values obtained in experiment on cells from two (30 μM DHA) or seven independent seedings. $^{**}p < 0.01$, significantly different from cells grown in the presence of serum; $^{\#\#}p < 0.01$, significantly different from cells grown in the presence of albumin by ANOVA followed by Tukey's multiple comparison test

Another important finding is the ability of DHA to induce an increase of ChAT activity in the absence of any other trophic factor. However, the concentration dependency of this effect is different in native cells than in cells induced to differentiate. While the maximal effect of DHA in native cells is reached with EC_{50} close to that supporting cell growth in either condition, the EC_{50} in differentiated cells is about five times higher. It indicates that the increase of ChAT activity by DHA in differentiated cells is independent of its effects on cell growth and protection. Deficits in mental performance during aging or Alzheimer's diseases are regularly accompanied by a decline of ChAT activity and cholinergic transmission in general [for

Table 3 Influence of DHA on α-secretase activity and cholesterol content in NG108–15 cells grown in serum-free medium

	α-Secretase activity (% control)	Cholesterol content
Native	100.0 ± 1.9 (17)	100.0 ± 5.8 (8)
Albumin	46.3 ± 5.0 (15)*	37.4 ± 7.6 (8)*
DHA 10 μM	80.1 ± 5.1 (13)*,**	66.5 ± 2.2 (8)*,**
DHA 100 μM	70.4 ± 4.5 (12)*,**	n.m.
Differentiated	100.0 ± 1.9 (14)	100.0 ± 4.5 (8)
Albumin	50.6 ± 4.5 (12)*	59.0 ± 11.6 (6)*
DHA 10 μM	71.2 ± 3.6 (10)*,**	50.0 ± 3.9 (–8)*
DHA 100 μM	67.1 ± 5.3 (10)*,**	n.m.

Results are given as percentage of values obtained in control native or differentiated cells from the same seeding grown in medium with serum and represent mean ± SEM of number of observations given in parentheses obtained on cells from at least two independent seedings. Control values of α-secretase activity were 134.7 ± 22 and 145.1 ± 23.4 of arbitrary units/μg protein × 2 h and that for cholesterol content were 18.2 ± 1.4 and 23.6 ± 1.1 nmol/mg protein ($p < 0.01$ by Student's t-test) for native and differentiated cells, respectively

n.m. not measured

* $p < 0.01$, significantly different from respective controls grown in serum-containing medium

** $p < 0.01$, significantly different from cells grown in serum-free medium by ANOVA followed by Tukey's multiple comparison test within native or differentiated group

reviews see references 25–27]. It has been demonstrated that the reduction of ChAT expressing neurons in aging brain and Alzheimer's disease is due to a loss of trophic support that leads at the beginning to their dedifferentiation [5, 28–34]. The increased expression of ChAT activity by DHA offers a plausible mechanism of action by which food DHA supplementation ameliorates behavioral performance and cholinergic activity [6, 7, 21, 35, 36].

An important component of all cell membranes is cholesterol that determines membrane fluidity. Cholesterol in membrane microdomains plays a crucial role in signal transduction [37]. In addition, its deficiency was shown to inhibit dendrite outgrowth and decrease microtubules stability [38]. Under the conditions of our experiments there was no supply of exogenous cholesterol and cell needs thus had to be covered by de novo synthesis. Results indicate that withdrawal of serum lead to the diminution of cell cholesterol content. This decrease was significantly smaller in differentiated cells. Addition of DHA in control cells reduced this deficit to the level of differentiated cells demonstrating the ability of DHA to ameliorate cholesterol homeostasis. Cell cholesterol content and its distribution influence amyloid precursor protein breakdown [39] and conversely the products of β and γ cleavage of amyloid precursor protein are involved in the regulation of cholesterol and sphingomyelin metabolism [40]. Increased production of amyloidogenic fragments of amyloid precursor protein is the primary pathogenic event in the development of Alzheimer's disease. Therefore it was of interest to know the influence of DHA treatment on the activity of α-secretase that mediates non-amyloidogenic breakdown of amyloid precursor protein. Our results evidence that the decrease of α-secretase activity consequent to serum deprivation is significantly prevented by DHA treatment in control as well as differentiated cells.

In summary, we demonstrated that DHA alone applied in defined medium in vitro supports growth of cholinergic NG108-15 cells. This effect is accompanied by maintenance of general functional properties. Independently of effects on cell growth, it also sustains with lower potency the expression of ChAT activity in differentiated cells. Nevertheless, both of these effects plateau at physiologically relevant levels demonstrating a potential benefit of DHA supplementation in the maintenance of cholinergic phenotype and confirm its general protective potency.

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ORIGINAL ARTICLE

Docosahexaenoic Acid Supports Cell Growth and Expression of Choline Acetyltransferase and Muscarinic Receptors in NG108-15 Cell Line

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A large body of evidence indicates that adequate intake of polyunsaturated fatty acids is essential for brain development in early ontogenesis and positively impacts various pathological states connected with aging, as well as other neurodegenerative diseases (Jump, 2002; Bazan, 2003; Ruxton et al., 2004). In the present experiments, we investigated the possible effects of polyunsaturated docosahexanoic acid (DHA [22:6, $n = 3$]) on the expression of cholinergic phenotype-represented by choline acetyltransferase (ChAT) activity and a number of surface muscarinic receptors-as well as on cell growth in the cholinergic cell line NG108-15 (Hamprecht, 1977; Hamprecht et al., 1985). However, chemical composition of different batches of sera is neither stable nor defined, and this fact complicates investigations on in vitro effects of substances that are natural constituents of serum. To avoid this restraint we employed defined medium in which fatty acid–free bovine albumin as a carrier of DHA replaced serum.

Growth of most cell lines, as well as cells in primary cultures, depends strictly on the presence of serum in growth medium. As expected, withdrawal of serum resulted in growth arrest exemplified by a decrease in protein content compared with control cells grown in the presence of serum and also caused a decrease in ChAT activity (Fig. 1, lower left). DHA, at a concentration of 10 μmol/L, largely prevented both growth arrest in defined medium with fatty acid–free bovine albumin as a carrier of DHA and the attenuation of ChAT activity. DHA at concentrations 10 times higher had no further effect. At a concentration of 100 μmol/L, DHA also significantly increased the number of surface muscarinic receptors compared with cells grown in serum-containing as well as serum-free medium (Fig. 1, upper right). These data demonstrate the ability of DHA at low micromolar concentrations to support cell growth and expression of ChAT activity. Although it is not possible to stipulate a mechanism of action on the expression of ChAT and muscarinic receptors, a plausible explanation could be prevention of apoptosis, evidenced by a sharp decrease in executive caspase-3 activity (Fig. 1, lower right). Apoptosis is a process with a high requirement for energy. An improved metabolic state of cells consequent to suppression of apoptosis might thus better fulfill requirements for protein synthesis and targeting.

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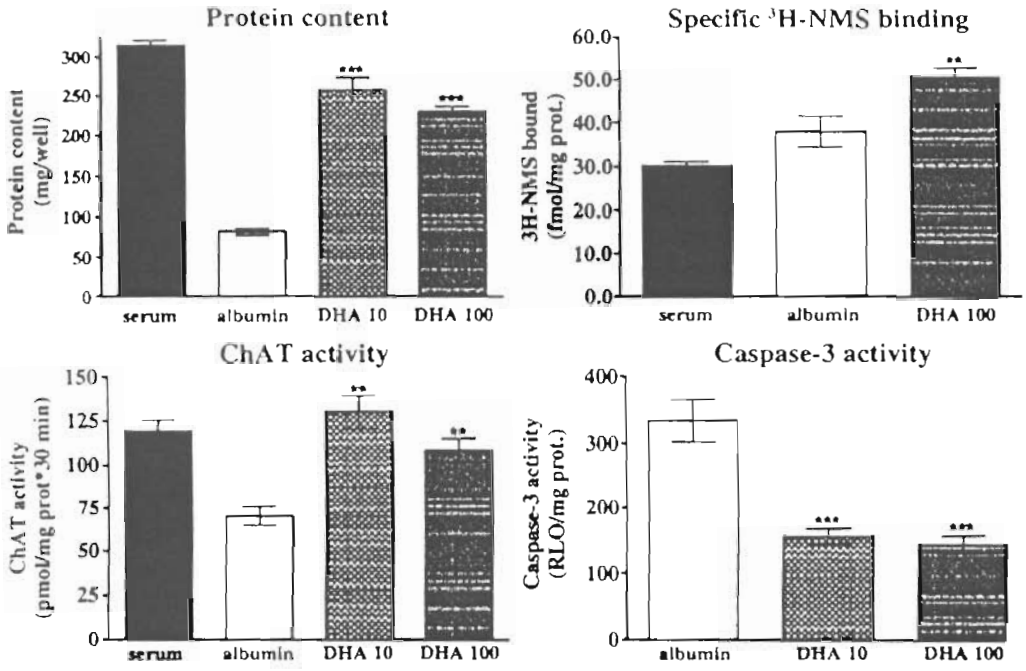

Fig. 1. NG108-15 cells were seeded in serum containing DMEM in 24-well-plates at a density of 20,000 cells/well. Serum-containing medium was replaced the next day by defined growth medium with 2.5 g/100 mL fatty acid-free serum albumin instead of serum (open columns). DHA (shaded columns) at indicated concentrations was added at this time. Control cells were left in serum-containing medium (black columns). Cells were grown for an additional 3–4 d and used for assays. Individual graphs show the influence of DHA (numbers indicate concentrations in μmol/L) on protein content in serum-free medium (upper left), ChAT activity (lower left), number of muscarinic receptors measured as specific ^{3}H-*N*-methylscopolamine (NMS) binding (upper right), and caspase-3 activity in cell lysates (lower right). Protein content was measured using Lowry's method; ChAT activity was determined using Fonnum's method; number of plasma-membrane muscarinic receptors was estimated as specific NMS binding in intact cells; and caspase activity was determined in cell lysates fluorometrically using DEVD-AMC as a substrate. Results are expressed as mean ± S.E.M. of 7–40 observations obtained in cells from at least two independent seedings. (**) $p < 0.01$; (***) $p < 0.001$, significantly different from controls grown in medium containing only albumin by one-way ANOVA, followed by Tukey's test.

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Research Report

Chronic treatment with amyloid β_{1-42} inhibits non-cholinergic high-affinity choline transport in NG108-15 cells through protein kinase C signaling

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Abstract

We investigated the influence of the amyloid-β-peptide$_{1-42}$ on hemicholinum-3-sensitive high-affinity choline uptake in NG108-15 cells. RT-PCR analysis revealed the presence of mRNA for a choline transporter-like protein but not for cholinergic high-affinity choline transporter. Differentiation of cells increased both hemicholinum-3-sensitive choline uptake and high-affinity hemicholinium-3 binding. This transport was not influenced by tenfold excess of carnitine. Continuous presence of submicromolar concentrations of amyloid-β-peptide$_{1-42}$ during differentiation resulted in a decrease of both choline uptake and hemicholinium-3 binding. These effects were not present when amyloid-β-peptide$_{1-42}$ was added 5 min prior to measurements. Neither differentiation nor amyloid-β-peptide$_{1-42}$ treatment changed levels of choline transporter-like protein mRNA. Protein kinase C inhibition by staurosporine or its inactivation by continuous presence of tetradecanoyl phorbol acetate prevented the inhibitory effect of amyloid-β-peptide$_{1-42}$ treatment on choline uptake. Activation of protein kinase C by tetradecanoyl phorbol acetate during measurement had inhibitory effect on choline uptake in control but not amyloid-β-peptide$_{1-42}$-treated cells. The concentration of amyloid-β-peptide$_{1-42}$ maximally effective on hemicholinium-3-sensitive choline uptake had no effect on cell growth, oxidative activity, membrane integrity, number of surface muscarinic receptors, caspase-3 and -8 activities, or uptake of deoxyglucose. Results demonstrate that long-term treatment with non-toxic concentrations of amyloid-β-peptide$_{1-42}$ downregulates choline uptake presumably mediated by a choline transporter-like protein through activation of protein kinase C signaling. The decrease of choline uptake may have relevance to the pathogenesis of Alzheimer's disease.

Theme: Disorders of the nervous system
Topic: Degenerative disease: Alzheimer's beta amyloid

Keywords: β-amyloid; High-affinity choline transport; Hemicholinium-3 binding; Choline transporter 1; Choline transporter-like protein; Protein kinase C

1. Introduction

It is generally accepted that malfunction in the metabolism of amyloid precursor protein (APP) plays a fundamental role in the pathogenesis of Alzheimer's disease [28,45]. A large body of evidence indicates that an increased production or decreased clearance of Aβ fragments or a combination of both processes leads to the development of Alzheimer's disease [6,45]. Most recent findings are consistent with a notion that soluble oligomeric Aβ fragments and not insoluble amyloid plaques are involved in the initiation and progression of the disease [19,25,46]. Probably, the most toxic species of Aβ fragments is that comprising 42 amino acid residues (Aβ_{1-42}). In spite of a substantial experimental effort, however, there is no general consensus as to what constitutes the early pathogenic influence of Aβ_{1-42} on neuronal functions [20].

Abbreviations: Amyloid β-peptide1–42, Aβ_{1-42}; Amyloid Precursor Protein, APP; Hemicholinum-3, HC-3; Choline Transporter-like Protein 1, CTL1; High-affinity choline transporter 1, ChT1; N-methylscopolamine, NMS; Protein Kinase C, PKC; Tetradecanoyl Phorbol Acetate, TPA

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Impairment of cholinergic neurons in basal forebrain innervating cerebral cortex and hippocampus is invariably found in Alzheimer's brains at autopsy. It is not known whether this damage is due to a general neuronal degeneration occurring at a late stage of the disease or whether cholinergic neurons are more sensitive to Aβ toxicity and involved in the pathogenesis of the disease [3,11,18]. Several aspects of cholinergic neurotransmission have been shown to be targets of physiologically relevant concentrations of Aβ [2]. We have observed in previous experiments that submicromolar concentrations of $A\beta_{1-42}$ present in the culture medium during differentiation of the cholinergic cell line NG108–15 [23,24] inhibited N-type calcium channels [31]. The differentiation also increased transcription of genes embedded in the cholinergic gene locus and enhanced general neuronal phenotype [7,10,12,13,15].

These findings led us to investigate the influence of $A\beta_{1-42}$ on a high-affinity choline uptake in NG108-15 cells. Using RT-PCR analysis we found that these cells do not express the specific cholinergic high-affinity choline transporter ChT1 [1,40,41]. However, we have identified expression of a hemicholinium-3-sensitive (HC-3) choline transporter-like protein (CTL1) that is similar to ChT1 [39]. CTL1 is widely expressed in the brain and its overexpression in neuroblastoma cells has been shown to increase choline uptake [48]. We demonstrate that chronic but not acute treatment with non-toxic concentrations of $A\beta_{1-42}$ diminishes both HC-3-sensitive choline uptake and high-affinity ^{3}H-HC-3 binding occurs downstream of gene transcription and involves protein kinase C signaling.

2. Materials and methods

2.1. Cell culture

NG108-15 cells were cultured as described [11]. Briefly, they were grown in Dulbecco's modified Eagle's medium containing 5% non-inactivated foetal calf serum, 1% HAT supplement (Sigma; containing hypoxanthine, aminopterin and thymidine), 3 μmol/l glycine, 2 mmol/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, under an atmosphere of 5% CO_2/95% humidified air at 37 °C. For hemicholinium-3 (HC-3) binding and choline uptake experiments they were seeded on 10 cm Petri dishes at a density of 200,000 cells per dish in 10 ml of supplemented DMEM. For all other experiments, they were seeded in 24-well plates at a density 20,000 cells per well in 2 ml of supplemented DMEM. Drugs were added the next day as indicated and the cells were grown in their presence for 4–5 days without change of the medium. Differentiation was induced by 0.2 mmol/l dibutyryl cAMP and 100 nmol/l dexamethasone. $A\beta_{1-42}$ was dissolved in redestilled water at a concentration of 100 μmol/l at room temperature and stored frozen in aliquots before use. It has been demonstrated that formations of the toxic soluble oligomers is reached within a few minutes and ageing does not increase its toxicity [14].

2.2. ^{3}H-Hemicholinium-3 binding

Medium was removed and cells were released into 6 ml of Krebs–HEPES buffer (final concentrations in mmol/l: NaCl 138, KCl 4, $CaCl_2$ 1.3, $MgCl_2$ 1.2, NaH_2PO_4 1.2, glucose 11, HEPES 10, pH 7.4) and collected by centrifugation (5 min at 200 × *g*). Cell pellets were resuspended in fresh Krebs–HEPES buffer (300–500 μl per one Petri dish) and aliquots (50 μl) of the cell suspension were added in triplicate to eppendorf test tubes containing 50 μl of the buffer with labeled HC-3 and incubated 30 min at 37 °C. At the end of incubation, they were chilled on ice and pelleted for 5 min at 2000 g in a refrigerated centrifuge. Supernatants were carefully removed, the cell pellets were surface washed with 200 μl of ice-cold Krebs–HEPES buffer and centrifuged again for 2 min at 2000 × *g*. Supernatants were discarded and cell pellets were dissolved in 100 μl aliquots of 1 mol/l sodium hydroxide. 60 μl aliquots were used for scintillation counting and 20 μl aliquots for protein determination. Non-specific binding was measured in the presence of 20 μmol/l unlabeled HC-3 in single point determinations or 10 mmol/l choline in kinetic experiments. The displaceable binding of HC-3 ranged between about 40% of total binding in low concentrations and 10% in high concentrations of tracer. For this reason, the single point measurements were performed using 5–10 nmol/l of ^{3}H-HC-3 and the results are expressed as percent of controls in individual experiments.

2.3. ^{3}H-Choline uptake

Cells were treated and harvested as described for HC-3 binding. The cell pellet was resuspended in 10 ml of Krebs–HEPES buffer and incubated for 30 min at 37 °C in a Petri dish to deplete endogenous choline. Cells were collected again, resuspended in Krebs–HEPES buffer (300–500 μl per one Petri dish) and 50 μl aliquots of cell suspension were added to Eppendorf test tubes in ice. 50 μl aliquots of buffer with ^{3}H-choline were added and samples were incubated in most cases (see text to figures) for 4 min at 37 °C. Incubation was stopped by transferring samples to an ice bath and adding 500 μl of ice cold Krebs–HEPES buffer containing 10 μmol/l HC-3. The samples were than processed as described for HC-3 binding. Non-specific uptake of choline was determined in parallel samples that contained 10 μmol/l HC-3 during the uptake period.

2.4. ^{3}H-NMS binding

Medium was removed and 1 ml of Krebs–HEPES buffer containing 2 nmol/l ^{3}H-NMS or ^{3}H-NMS plus 5 μmol/l atropine was added into the wells in triplicate. The plates

were incubated for 1 h at 37 °C. The medium was then removed and each well was washed with 1 ml of Krebs–HEPES solution at room temperature. The cells in each well were dissolved in 300 µl of 1 mol/l sodium hydroxide. 200 µl aliquots were used for scintillation counting and 30 µl aliquots for protein determination.

2.5. ^{3}H-deoxyglucose uptake

Culture medium was removed and 0.3 ml of Krebs–HEPES buffer containing ^{3}H-deoxyglucose was added to each well. The plates were incubated for 60 min at 37 °C. At the end of incubation the medium containing ^{3}H-deoxyglucose was carefully removed and each well was washed. Cells were dissolved in 200 µl of 1 M sodium hydroxide. One hundred fifty-µl aliquots were used for scintillation counting and 5-µl aliquots for protein determination.

2.6. Cell viability assay

Cell viability was estimated using uptake and retention of the fluorescent probe calcein AM. Cells grown for 4 days in the absence or presence of $A\beta_{1-42}$ were incubated for 2 h at 37 °C in Krebs–HEPES buffer containing the probe at a concentration of 10 µmol/l. After incubation, the emission of light at 535 nm excited by 485 nm light was followed using a Victor™ plate reader. A signal from extracellular calcein was quenched using 0.1 mmol/l manganese chloride.

2.7. Oxidative activity of cells

Oxidative load of intact cells was deduced from the oxidation of fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate. Cells grown for 4 days in the absence or presence of $A\beta_{1-42}$ were incubated for 1 h at 37 °C in Krebs–HEPES buffer containing the probe at a concentration of 10 µmol/l and then the emission of light at 535 nm excited by 485 nm light was followed using Victor™ plate reader.

2.8. Determination of enzyme activities

Activities of caspase-3 and caspase-8 were determined in cell lysates fluorometrically using acDVLD-AMC and acIETD-AMC substrates (Sigma, Prague). Cells were grown in 24-well-plates. Cultivation medium was removed and each well was washed with 1 ml of PBS (155 mmol/l NaCl, 10 mmol/l sodium phosphate buffer, pH = 7.4). Cells were homogenized on ice in 200 µl of lysis buffer (50 mmol/l HEPES, 0.1% CHAPS, 0.1 mmol/l EDTA, 1 mmol/l DTT, pH = 7.4) by trituration and then left on ice for 30 min. Afterwards, the homogenates were centrifuged at 14,000 × *g* and 0 °C for 10 min. Resulting supernatants were used for determination of caspase activities and protein content. 50 µl aliquots of cell lysates in a final volume of 100 µl made up using lysis buffer with 10 µmol/l (final concentration) fluorescent probe in 96-well-plates were used for measurement. Fluorescence response due to substrate cleavage was measured in Victor™ plate reader using umbelliferone filters. Enzyme activities are expressed as light output corrected for protein content in cell lysates. Protein content of cell lysates was determined after precipitation of proteins by trichloroacetic acid to remove dithiothreitol. Proteins in samples were determined using Peterson's modification of Lowry's method with human serum albumin as standard.

2.9. RT-PCR and real time PCR analysis

Total RNA from control and differentiated NG108-15 cells, and from different tissues as indicated in Figs. 1 and 5, was isolated using RNAwiz (Ambion, UK) and further processed using DNA-free™ kit (Ambion, UK). The purity was checked spectrophotometrically at 260 nm and 280 nm. The Following primers were used to investigate a presence of ChT and CTL1 by RT-PCR: rat ChT-forward 5′-GGA CAC CCG GAC CCC TAA ATC-3′, reverse 5′-CAA TGT CTC GGC CCC CAA CTA T-3′, product 244 bp, GeneBank accession no. NM053521; mouse ChT-forward 5′-CTG TGT ATG GGC TGT GGT ACC-3′, reverse 5′-TCA TTG TAA GTT ATC TTC AGT CCC-3′, product 542 bp, GeneBank accession no. AJ401467; rat CTL1-forward 5′-GGG CCT TTG CAG TAC ATG TGG-3′, reverse 5′- CCT ACT GTT

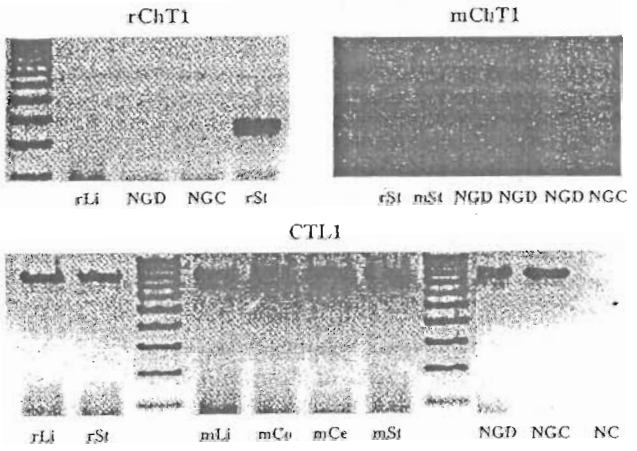

Fig. 1. NG108-15 cells do not express the cholinergic high-affinity choline transporter (ChT1). RT-PCR analysis of total RNA was performed in NG108-15 cells grown for 3–4 days in control (NGC) or differentiating medium (NGD). Rat and mouse tissues were used as positive and negative controls. rChT1: product of anticipated size (244 bp, 31 cycles) was found using a rat high-affinity choline transporter primers in rat striatum (rSt) but not in either control or differentiated NG108-15 cells, or rat liver (rLi). mChT1: the primers designed to recognize both mouse and rat sequence of high-affinity choline transporter gave expected products (542 bp, 28 cycles) in rat (rSt) and mouse (mSt) striatum but not in either control or differentiated NG108-15 cells. CTL1: the primers for CTL1 gene [39] produced a product of the expected size (756 bp, 25 cycles) in all tissues examined (mLi, mouse liver; mCo, mouse cerebral cortex; mCe, mouse cerebellum; mSt, mouse striatum; NC, negative PCR control).

TTC CAC AAA CTC CAT-3′, product 756 bp, GeneBank accession no. AJ245619. Primers used for real time PCR were: rat CTL1-forward 5′-GAA TGC ATA CAC AGC CAC AG-3′; reverse 5′- GTT GAG CAG CAT AAT CCC AG-3′, product 187 bp, GeneBank accession no. AJ245619; rat GAPDH-forward 5′-GAA CAT CAT CCC TGC ATC C-3′, reverse 5′-GCT TCA CCA CCT TCT TGA TG-3′, product 179 bp, GeneBank accession no. M17701. Reverse transcription of isolated RNA was performed using a commercial kit from Roche (C. therm. Polymerase for Reverse Transcription in Two-Step RT-PCR) with 1 μg of isolated RNA in a final volume of 10 μl at 60 °C for 30 min and 94 °C for 3 min. The PCR reaction was done using the commercial kit HotStarTaq™ PCR (Qiagen), usually with 1 μl of reverse transcription mixture, for the indicated number of cycles (activation 15 min at 94 °C, 45 s at 58 °C and 40 s at 75 °C; PCR cycle 30 s at 94 °C; 40 s at 58 °C; 40 s at 72 °C; termination 7 min at 72 °C). The PCR product was separated on a 1.9% agarose gel. Isolation of cDNA from gels was done using a commercial kit (Gel Extraction QIAquick, Qiagen). Cloning of the PCR product was accomplished using supercompetent bacteria XL1 (Invitrogen, TOPO TA Cloning) after ligation of PCR product by means of the commercial kit pGEM-T Easy (Promega). Plasmids were isolated using Plasmid Mini/Midi Kit (Qiagen) according to manufacturer's protocol. Sequencing was done commercially. Real-time PCR was accomplished using QuantiTect™ SYBR Green PCR kit (Qiagen) in LightCycler apparatus (Roche) and results were processed using software LightCycler3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

2.10. Chemicals

[Methyl-^{3}H]choline chloride (SRA 81 Ci/mmol), 1-[N-methyl-^{3}H]scopolamine methyl chloride (SRA 79 Ci/mmol), and 2-deoxy-D-[2,6-^{3}H]glucose (SRA 43 Ci/mmol) were from Amersham, UK. [Methyl-^{3}H]hemicholinium-3 diacetate salt (SRA 127.8 Ci/mmol) came from NEN. Amyloid β peptide 1–42 was purchased from US Peptides (Rancho Cucamonga, CA) or from Sigma (Prague, Czech Republic). Calcein AM and 2′,7′-dichlorodihydrofluorescein diacetate were supplied by Molecular Probes (Eugene, OR). All other reagents and media were from Sigma (Prague, Czech Republic).

3. Results

3.1. NG108-15 cells express the high-affinity choline transporter CTL1

Differentiation of NG108-15 cells in the presence of cAMP and dexamethasone increased high-affinity HC-3-sensitive choline uptake [11] and high-affinity ^{3}H-HC-3 binding (Fig. 3B). RT-PCR analysis of total mRNA isolated from control and differentiated NG108-15 cells did not detect a presence of the cholinergic high-affinity choline transporter ChT1 (Figs. 1A and B). However, in concert with previously reported findings, both undifferentiated and differentiated cells contained mRNA for CTL1, another putative high-affinity choline transporter (Fig. 1C). The specificity of the high-affinity choline transporter in control NG108-15 cells was further verified by competition with carnitine to exclude possible involvement of carnitine transporters. Carnitine at a concentration 0.1 mmol/l had no influence on the transport of 10 μmol/l choline (96.2 ± 1.6% of control choline transport in two experiments with cells from independent seedings). Similarly, transport of 10 μmol/l carnitine was not inhibited by 0.1 mmol/l choline (110.0 ± 1.4% of control carnitine transport in two experiments with cells from independent seedings).

3.2. Chronic treatment with $A\beta_{1-42}$ attenuates high-affinity choline uptake

HC-3-sensitive choline uptake in differentiated cells was concentration dependent (Fig. 2B) with half saturation in the range of 5–10 μmol/l choline and proceeded linearly for at least 12 min (Fig. 2A). Cultivation of differentiated cells in medium containing 100 nmol/l $A\beta_{1-42}$ for 4 days induced a slight reduction of HC-3-sensitive choline uptake when measured at the concentration of 2 μmol/l and a significant decrease when measured at the concentration of 10 μmol/l (Fig. 2C). This effect required persistent presence of $A\beta_{1-42}$ during growth because it was not apparent when $A\beta_{1-42}$ was added 5 min before choline uptake measurement (Fig. 2D). Comparable results were observed in experiments on specific high-affinity ^{3}H-HC-3 binding (Fig. 3). In differentiated cells $A\beta_{1-42}$ present for 4 days induced a concentration-dependent decrease of ^{3}H-HC-3 binding with an EC_{50} of about 1 nmol/l (Fig. 3A). This inhibitory effect was not found in cells that were not differentiated (Fig. 3B) or in differentiated cells when $A\beta_{1-42}$ was added 5 min prior to the binding assay (Fig. 3D). In three independent experiments (Fig. 3C) ^{3}H-HC-3 bound to intact differentiated cells with K_d 10.5 ± 2.1 nmol/l. Four days of treatment with 100 nmol/l $A\beta_{1-42}$ reduced B_{max} to 51.3 ± 5.4% of control (mean ± SEM, $P < 0.05$ by t test) while K_d remained unchanged (8.7 ± 1.9 nmol/l).

3.3. Influence of protein kinase C activity modulators on high-affinity choline transport

A role of protein kinase C in the inhibitory effect of 100 nmol/l $A\beta_{1-42}$ on choline transport was investigated in differentiated cells. The protein kinase C inhibitor staurosporine at a concentration of 100 pmol/l present 5 min before and during choline uptake measurement (determined at the concentration of 10 μmol/l) significantly

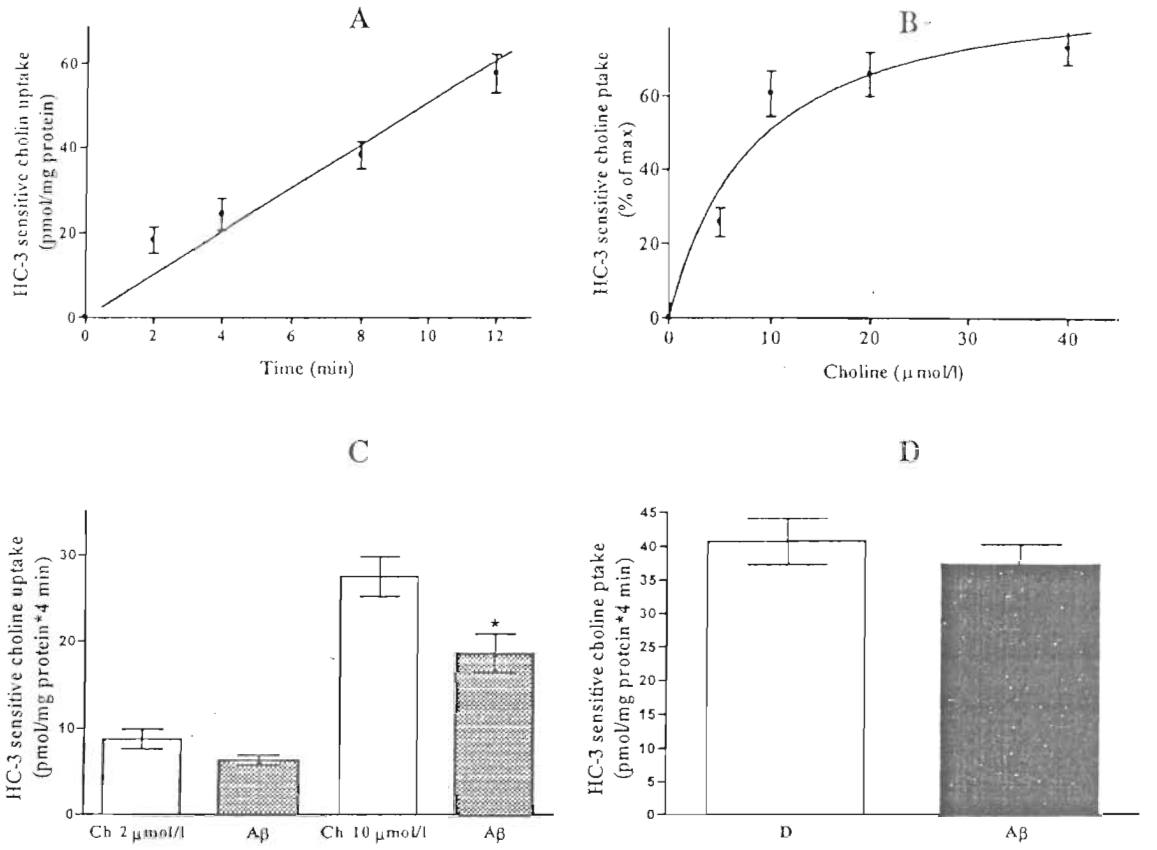

Fig. 2. Influence of chronic treatment with $A\beta_{1-42}$ on hemicholinium-3-sensitive choline uptake in differentiated NG108-15 cells. (A) The uptake of choline measured at a concentration of 10 μmol/l linearly increased for at least 12 min. Ordinate: HC-3-sensitive uptake of choline in pmol/mg of protein. Each point represents the mean ± SEM of nine observations made on cells from three independent seedings with the exception of the last point which is derived from a single experiment. (B) Concentration dependence of choline uptake during 4-min incubations. Abscissa: concentration of ^{3}H-choline in μmol/l. Ordinate: HC-3-sensitive uptake of choline calculated as percent of maximal uptake (mean ± SEM was 67.8 ± 13.3 pmol/mg protein) in four individual experiments. Each point represents the mean ± SEM of 11–12 observations on cells from four independent seedings. Choline transport proceeds with K_m around 5–10 μmol/l. (C) Treatment of differentiated cells with 100 nmol/l Aβ for 4 days attenuates the uptake of choline during 4-min incubations measured at 2 μmol/l choline (left pair of columns) and significantly decreases the uptake at 10 μmol/l choline (right pair of columns). Ordinate: choline uptake in pmol/mg protein at 4 min. Each column represents 13–15 observations derived from experiments on cells from five independent seedings. $^*P < 0.05$ significantly different from controls (Student's t test). D: $A\beta_{1-42}$ at a concentration of 100 nmol/l has no immediate effect on hemicholinium-3-sensitive choline uptake. Differentiated cells (D) were preincubated for 5 min with 100 nmol/l $A\beta_{1-42}$ which was also present during assay. Columns represent 24 values derived from experiments on cells from eight independent seedings expressed in pmol/mg protein at 4 min.

increased choline uptake (Fig. 4A) while 1 μmol/l of the protein kinase C activator TPA significantly reduced choline uptake (Fig. 4B). Staurosporine prevented the inhibitory effect of the persistent presence of $A\beta_{1-42}$. In contrast, TPA lost its inhibitory effect in $A\beta_{1-42}$-treated cells. Staurosporine present during the cultivation and measurement also increased choline uptake both in the absence and presence of 100 nmol/l $A\beta_{1-42}$ (Fig. 4C). Similar to the effects of staurosporine, TPA treatment during cultivation downregulated PKC activity and resulted in an increase of choline uptake in control as well as $A\beta_{1-42}$-treated cells (Fig. 4D).

3.4. *Differentiation and chronic treatment with $A\beta_{1-42}$ do not change level of CTL1 mRNA*

Differentiation of cells in the presence of dbcAMP and dexamethasone had no effect on the level of CTL1 mRNA over a time interval of 1–4 days (Fig. 5A). Similarly, 100 and 1000 nmol/l $A\beta_{1-42}$ did not change CTL1 mRNA level after treatments lasting 1 or 3 days (Fig. 5B).

3.5. *Chronic treatment with $A\beta_{1-42}$ at submicromolar concentrations has no general adverse cellular effects*

In the next experiments, we examined whether $A\beta_{1-42}$ under experimental conditions has general toxic effects. Oxidative load of differentiated cells was estimated according to the extent of dihydrofluorescein oxidation. In control non-differentiated cells permanent presence of 1 μmol/l but not 100 nmol/l $A\beta_{1-42}$ significantly increased oxidative activity (data not shown), whereas in differentiated cells no influence at both tested concentrations of $A\beta_{1-42}$ was detected (Table 1). Capacity of the fluorescent probe to sense increased signal was verified at the end of measurements by adding

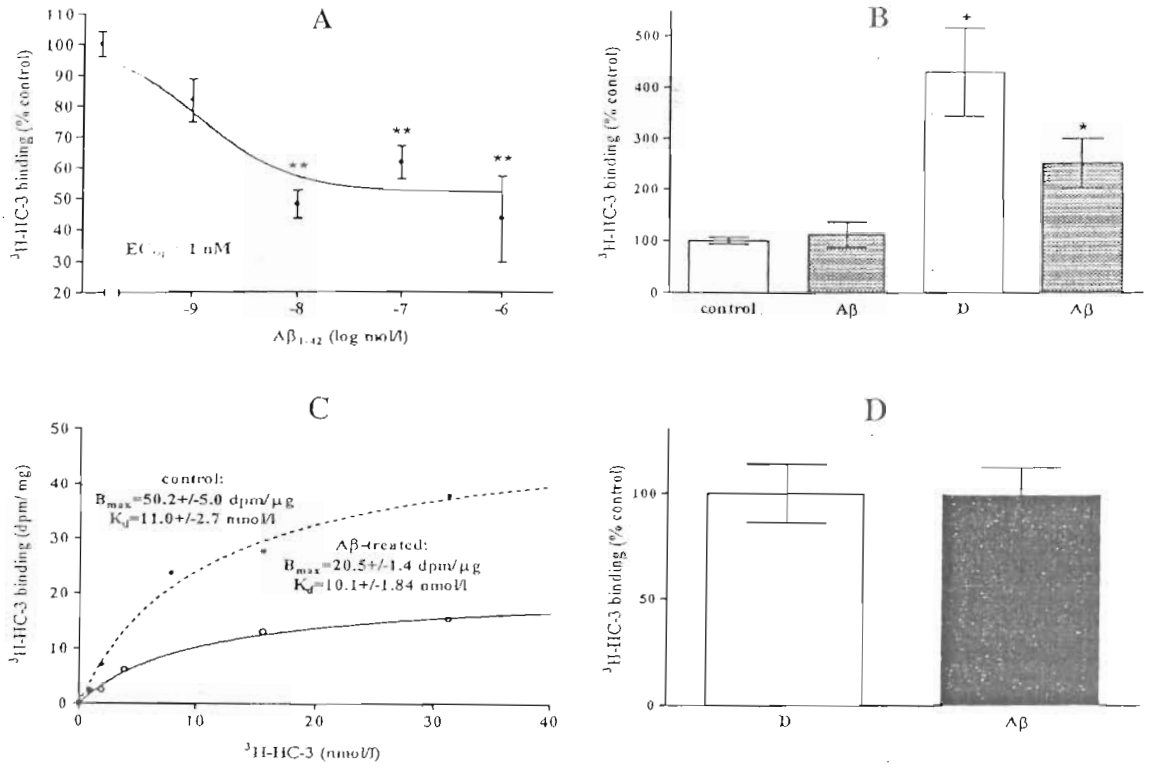

Fig. 3. Influence of chronic treatment with $A\beta_{1-42}$ on high-affinity saturable binding of hemicholinium-3 in differentiated NG108-15 cells. (A) $A\beta_{1-42}$ present from the beginning of cultivation decreased specific binding of HC-3 in a concentration dependent manner with EC_{50} of approximately 1 nmol/l. Results are expressed in percent of control (ordinate). Each point is the mean ± SEM of 6–12 values derived from 2 to 4 independent experiments. $^{**}P < 0.01$, significantly different from control cells grown in the absence of $A\beta_{1-42}$ by ANOVA followed by Dunnett's multiple comparison test. (B) Differentiation of NG108-15 cells increased the number of high-affinity HC-3 binding sites. Presence of 100 nmol/l $A\beta_{1-42}$ for 4 days decreased the binding of HC-3 in differentiated (right pair of columns) but not in control (left pair of columns) cells. Specific HC-3 binding is expressed as percent of control values (control, non-differentiated cells). Each column represents 9–15 values derived from 3–5 independent experiments. Control values were 8.1 ± 1.6 dpm/μg protein (mean ± SEM, $n = 14$). $^{*}P < 0.01$ significantly different from control; $^{*}P < 0.05$ significantly different from control differentiated cells (D) by ANOVA followed by Tukey's multiple comparison test. (C) 4-day treatment of differentiated cells with 100 nmol/l $A\beta_{1-42}$ had no effect on the affinity of specific HC-3 binding. Data shown are representative of three experiments on cells from independent seedings that yielded affinity 10.6 ± 2.9 nmol/l and 9.4 ± 2.5 nmol/l in control and $A\beta_{1-42}$-treated cells, respectively. B_{max} values were more variable, ranging from 17 to 72 dpm/μg protein in control differentiated cells. However, $A\beta_{1-42}$-treated cells showed a consistent reduction in B_{max} in individual experiments (58, 41, and 55% of control values). (D) $A\beta_{1-42}$ at a concentration of 100 nmol/l has no immediate effect on the specific binding of HC-3. Differentiated cells were preincubated for 5 min with 100 nmol/l $A\beta_{1-42}$ that was also present during assay. Columns represent values derived from experiments on cells from three independent seedings and are expressed as percent of control (4.7 ± 0.8 dpm/μg protein, mean ± SEM, $n = 9$).

hydrogen peroxide (data not shown). Integrity of plasma membranes was checked by the ability of cells to take up calcein AM and retain deesterified (fluorescent) calcein. $A\beta_{1-42}$ up to 1 μmol/l did not change calcein AM uptake and hydrolysis as well as calcein retention inside the cells that was measured after quenching extracellular signal by manganese. Capacity of the method to detect a possible change was checked by treatment with 1 μmol/l monomycin for 60 min (data not shown). Additional experiments summarized in Table 1 show that 100 nmol/l $A\beta_{1-42}$ applied for 4 days did not affect cell growth determined as protein content, the number of surface muscarinic receptor measured as specific binding of ^{3}H-N-methylscopolamine on intact cells, and uptake of ^{3}H-deoxyglucose. At concentration of 1 μmol/l, $A\beta_{1-42}$ slightly but significantly augmented protein content and significantly reduced the uptake of ^{3}H-deoxyglucose. None of the tested concentrations of $A\beta_{1-42}$ changed activity of extracellular signal regulated caspase-8 or executioner caspase-3 activities.

4. Discussion

The most important finding of our experiments is the attenuation of the HC-3-sensitive high-affinity choline transport and the density of saturable HC-3 binding sites caused by chronic but not acute treatment with submicromolar concentrations of $A\beta_{1-42}$. The kinetic parameters of HC-3 binding (K_d around 10 nmol/l) and choline uptake (K_m around 5–10 μmol/l) are similar to that of the high-affinity choline transporter ChT1. However, our data demonstrate that NG108-15 cells do not express the ChT1 gene. On the other hand, we have confirmed the expression of another recently cloned high-affinity choline transporter, CTL1. This choline transporter can also be inhibited by low

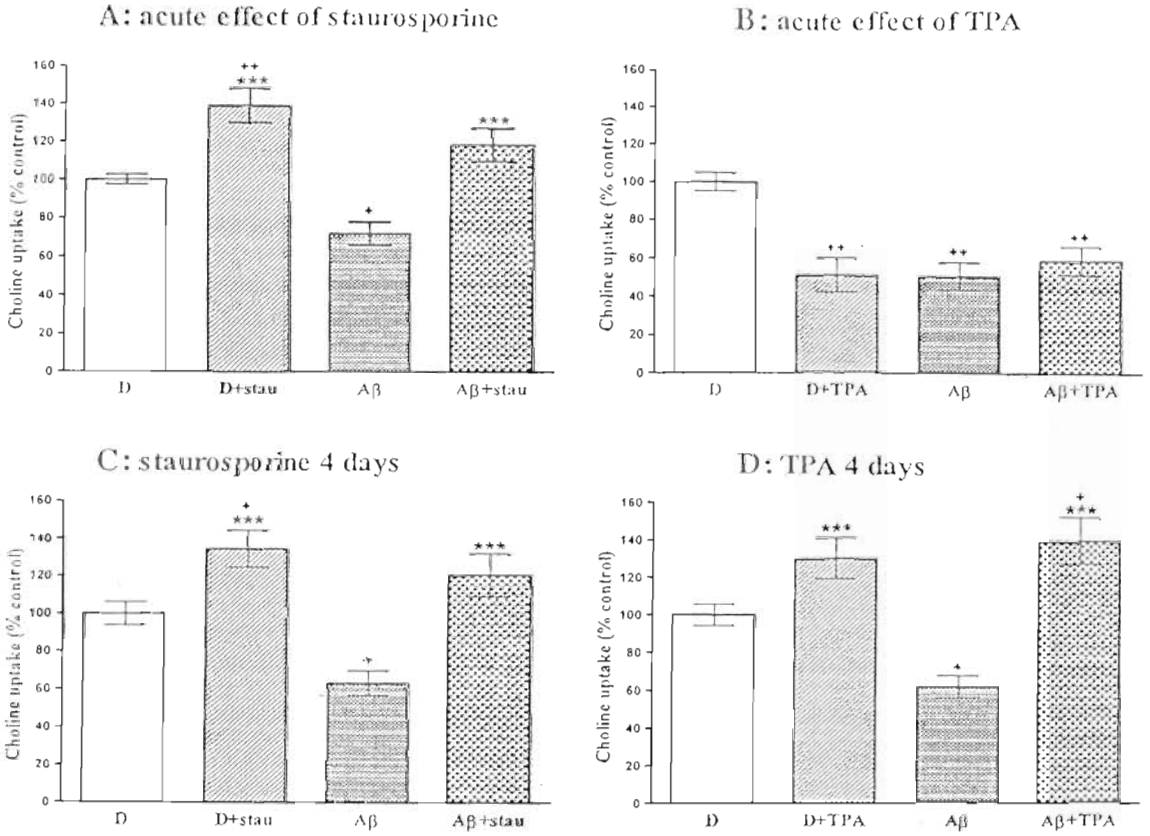

Fig. 4. Effect of $A\beta_{1-42}$ on HC-3-sensitive choline uptake in differentiated NG108-15 cells in the absence or in the presence of protein kinase C modulators. (A) The inhibitor of protein kinase C staurosporine (stau) at a concentration of 100 pmol/l added 5 min before measurement increases hemicholinium-3-sensitive choline uptake and prevents the inhibitory effect of 100 nmol/l $A\beta_{1-42}$ present during cultivation. (B) The activator of protein kinase C tetradecanoyl phorbolacetate (TPA) at a concentration of 1 μmol/l added 5 min before measurement decreases hemicholinium-3-sensitive choline uptake while this reduction is not apparent in cells grown in the presence of 100 nmol/l $A\beta_{1-42}$. (C) Continuous presence of staurosporine during cultivation increases choline uptake and prevents inhibitory effect of $A\beta_{1-42}$. (D) Inhibitory effect of $A\beta_{1-42}$ on HC-3-sensitive choline uptake is abolished by continuous presence of TPA during cultivation. Data are expressed as percent of control uptake. Control values were 37.3 ± 3.8, $n = 9$, 41.2 ± 2.1, $n = 6$, 41.2 ± 4.8, $n = 9$, 38.4 ± 2.7 (pmol/mg protein at 4 min) in A, B, C, and D, respectively. Columns represent the mean ± SEM of 6–9 observations derived from experiments on cells from two (B) or three (A, C, D) independent seedings. $^{***}P < 0.001$, significantly different from $A\beta_{1-42}$ alone (Aβ), $^{*}P < 0.05$, $^{++}P < 0.01$, significantly different from control (D) by ANOVA followed by Tukey's multiple comparison test. D, control cells grown in differentiating conditions.

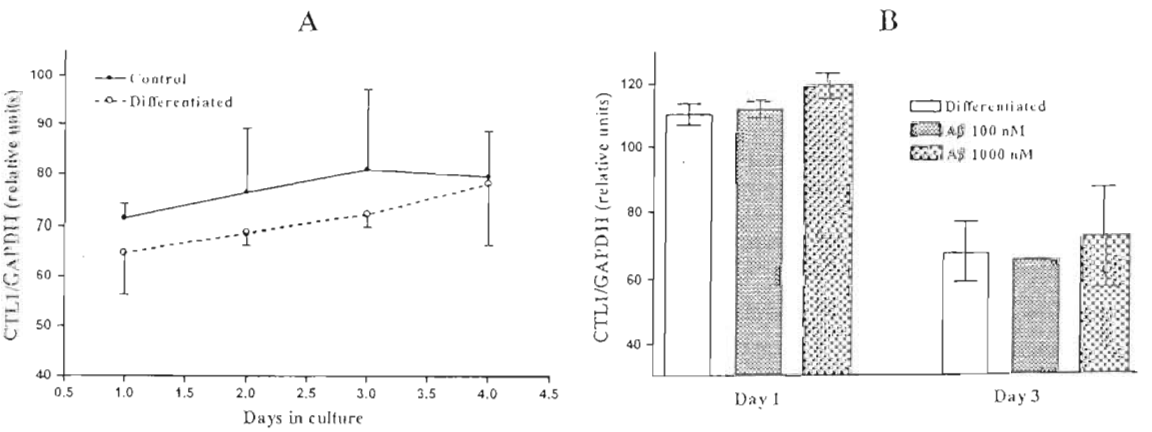

Fig. 5. Lack of effect of differentiation and chronic treatment with $A\beta_{1-42}$ in differentiated cells on CTL1 gene expression. (A) Cells were grown in for 1–4 days in control conditions (full circles) or differentiated in the presence of dbcAMP and dexamethasone (open circles). The amount of CTL1 mRNA is expressed relative to GAPDH. Each point represents the mean ± SEM of samples from three independent seedings. (B) Cells were grown for one or 3 days in the presence of 100 or 1000 nmol/l $A\beta_{1-42}$ (shaded columns) in differentiating conditions. Each bar represents the mean ± range of values obtained on cells from two independent seedings.

Table 1
Influence of 4-day treatment with $A\beta_{1-42}$ on cell growth, number of muscarinic receptors, caspase-3 and 8 activities, ^{3}H-deoxyglucose uptake, membrane integrity, and oxidative activity in differentiated NG108-15 cells

	Control	Aβ 0.1 μmol/l (% control)	Aβ 1 μmol/l (% control)
Protein content (μg/well)	255 ± 6 (52)	101 ± 2 (43)	111 ± 5* (31)
muscarinic receptors (fmol/mg protein)	29.4 ± 2.0 (12)	107 ± 5 (9)	105 ± 9 (6)
Caspase-3 activity (AU/μg prot*120 min)	65.9 ± 14.0 (7)	119 ± 12 (8)	99 ± 9 (6)
Caspase-8 activity (AU/μg prot*120 min)	615 ± 119 (8)	112 ± 6 (8)	119 ± 7 (8)
^{3}H-DOG uptake (fmol/mg protein*60 min)	0.72 ± 0.05 (16)	95 ± 3 (16)	79 ± 3** (16)
Calcein retention (AU/μg prot*120 min)	558 ± 48 (16)	110 ± 7 (12)	109 ± 6 (12)
Dihydrofluorescein oxidation (AU/μg prot*60 min)	68.2 ± 3.3 (26)	102 ± 2 (16)	101 ± 2 (16)

Results are given in percentage of control values shown in the first column as the mean ± SEM of the number of observations given in parentheses. Each experiment was done at least twice on cells from independent seedings. *$P < 0.05$, **$P < 0.01$, significantly different from control by t test. AU, arbitrary units.

concentrations of HC-3, has distinct ionic requirements, and was found in all cell lines tested so far [38]. The specificity of the investigated transporter for choline is further supported by its insensitivity to inhibition by carnitine.

The absence of ChT1 has offered a unique possibility to study in isolation the effect of $A\beta_{1-42}$ on specific high-affinity choline transport mediated presumably by the CTL1 protein that is largely expressed in the nervous system [37,48]. Lack of a direct effect on choline transport implies that, unlike ChT1 [29,30], $A\beta_{1-42}$ does not interact directly with the transporter but rather influences its gene expression or mechanisms downstream of gene expression. Our results are in favor of the second possibility. We did not find any change of CTL1 gene expression induced by either differentiation or 1–3 days of treatment with $A\beta_{1-42}$ in concentrations that caused about fifty percent reduction of choline transport (Fig. 5).

It has been repeatedly demonstrated that β-amyloid exhibits toxic effects in in vitro experiments including induction of oxidative stress, damage of cell membranes and initiation of apoptotic cell death. However, these effects are usually demonstrated only when relatively high concentrations of β-amyloid in the micromolar range are used. In control experiments we did not find any indications of overt cell toxicity brought about by 100 nmol/l $A\beta_{1-42}$, a concentration that maximally inhibits choline transport (Table 1). An increase of oxidative load in native NG108-15 cells (data not shown) and a decrease of deoxyglucose transport in differentiated cells after treatment with 1 μmol/l $A\beta_{1-42}$ evidenced that treatment using higher concentrations of β-amyloid can provoke toxic effects. In our previous work, we demonstrated that chronic treatment of differentiated NG108-15 cells with 100 nmol/l $A\beta_{1-42}$ leads to a disappearance of calcium influx through N-type channels while the activity of a specific cholinergic neuronal marker, choline acetyltransferase, remained unchanged [31]. Taken together, these findings indicate that submicromolar concentrations of $A\beta_{1-42}$ do not demonstrate general cell toxicity but rather show certain specificity in compromising distinct physiological functions like choline transport and calcium N-type channel functioning.

An interesting feature of the high-affinity choline transport in NG108-15 cells is its sensitivity to modulators of protein kinase C activity. Disturbance of protein kinase C signaling has been amply demonstrated in post mortem Alzheimer's brains. This might be a reflection of the terminal state of the disease and cannot be thus considered indicative of early pathophysiological events. However, changes in levels of various isoforms of PKC [44] and disturbances of its signaling [50] were observed at different ages in brains of transgenic mice overexpressing β-amyloid as well as in skin fibroblasts obtained from Alzheimer's patients [16,49]. Similarly, low concentrations of β-amyloid have been shown to induce activation of PKC in primary cell cultures and cell lines [27,32,33,47]. Altogether, these findings point to the involvement of PKC signaling as a plausible pathogenic event operating early in the genesis of the disease. Our observations are in line with this view. We found that the decrease of choline uptake is counteracted by the protein kinase C inhibitor staurosporine or by chronic treatment with the protein kinase C activator TPA that eventually downregulates PKC activity. In concert, acute addition of TPA decreases HC-3-sensitive choline uptake in control but not $A\beta_{1-42}$-treated cells. In addition, the inhibitory effect of chronic treatment with $A\beta_{1-42}$ is not just a general deleterious effect on transport mechanisms and has certain specificity for choline uptake because glucose transport was not influenced by the concentration of $A\beta_{1-42}$ that displayed full inhibitory effect on choline uptake and HC-3 binding. The inhibition of glucose transport only appeared after the treatment of cells with one order of magnitude higher concentrations of $A\beta_{1-42}$ (Table 1) confirming the sensitivity of glucose transport to high concentrations of β-amyloid [4].

Suppression of non-cholinergic high-affinity choline uptake may have a severe impact particularly in the brain. Choline is an essential component of all cell membranes. Although humans can synthesize a small proportion of choline de novo, the predominant portion of required choline must be supplied as a constituent of food [5]. Choline concentration in brain extracellular fluid is kept lower than in the rest of the body. This suggests relevance of a high-affinity transport mechanism in brain cells that may be of critical importance to fuel phospholipid metabolism necessary for membrane synthesis and renewal during development of the brain neuronal network and in the

plasticity of neuronal cells or their repair after damage or injury. Indeed, it has been demonstrated that prenatal supplementation with choline results in acceleration of brain development, increased resistance against toxicity, and improvement of mental performance that lasts until senescence [5,8,21,22,34–36,43]. These effects are unlikely to be due to improved delivery of choline for acetylcholine synthesis and cholinergic activity because proteins imperative for presynaptic cholinergic activity, such as choline acetyltransferase and vesicular acetylcholine transporter in the brain are not fully expressed until several days after birth [26]. In addition, experiments on knockout mice have indicated that the functional ChT1 becomes indispensable after but not before delivery [17]. Further evidence of the involvement of CTL1 in membrane repair comes from studies showing upregulation of CTL1 expression after cranial motor nerve transection that returns to normal level after axon regeneration [9], while in a similar situation ChT1 expression in neuronal cell bodies decays and renews only when synaptic contact is being re-established [42].

In summary, our data demonstrate that non-toxic submicromolar concentrations of $A\beta_{1-42}$ acting for an extended period of time impair hemicholinium-3-sensitive high-affinity choline uptake presumably mediated by CTL1. This transport is distinct from uptake mediated by the specific cholinergic transporter ChT1, does not occur through carnitine carriers, and its regulation involves protein kinase C signaling. Inhibition of protein kinase C prevents suppression of high-affinity choline transport by $A\beta_{1-42}$. However, the molecular mechanism that link protein kinase C and the inhibitory effect of $A\beta_{1-42}$ on choline transport require further investigation. Regardless of the molecular mechanisms involved, these findings may have important implications in early stages of Alzheimer's disease with regard to the importance of choline supply for membrane resynthesis and renewal.

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