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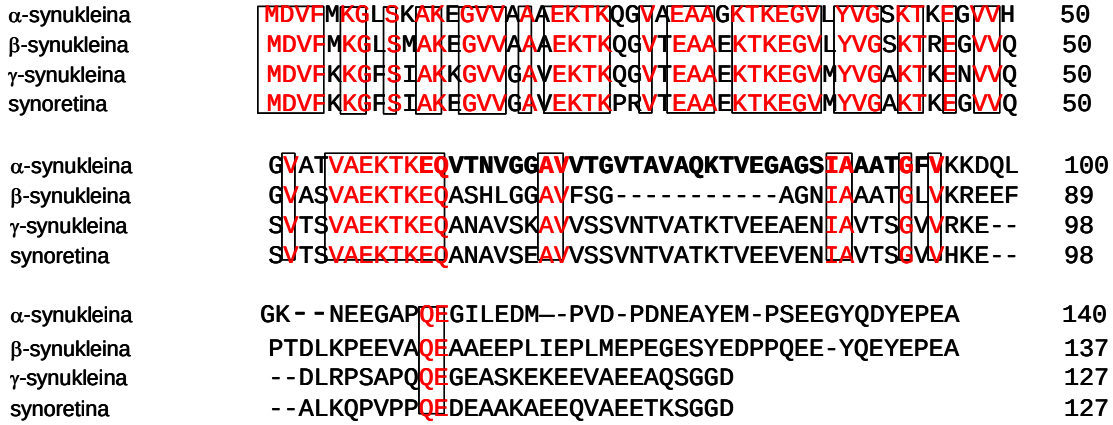
ZNACZENIE ALFA-SYNUKLEINY W FUNKCJI UKŁADU DOPAMINERGICZNEGO ORAZ W MOLEKULARNYCH MECHANIZMACH CYTOTOKSYCZNOŚCI

WARSZAWA 2009

SPIS TREŚCI

I. WSTĘP

Synukleiny, to rodzina małych (15-20 kDa), rozpuszczalnych, wysoce konserwatywnych białek, które obficie występują w neuronach. Do rodziny tej należą cztery izoformy: α-, β-, γ-synukleina oraz synoretina (Rycina 1) (von Bohlen und Halbach, 2004).

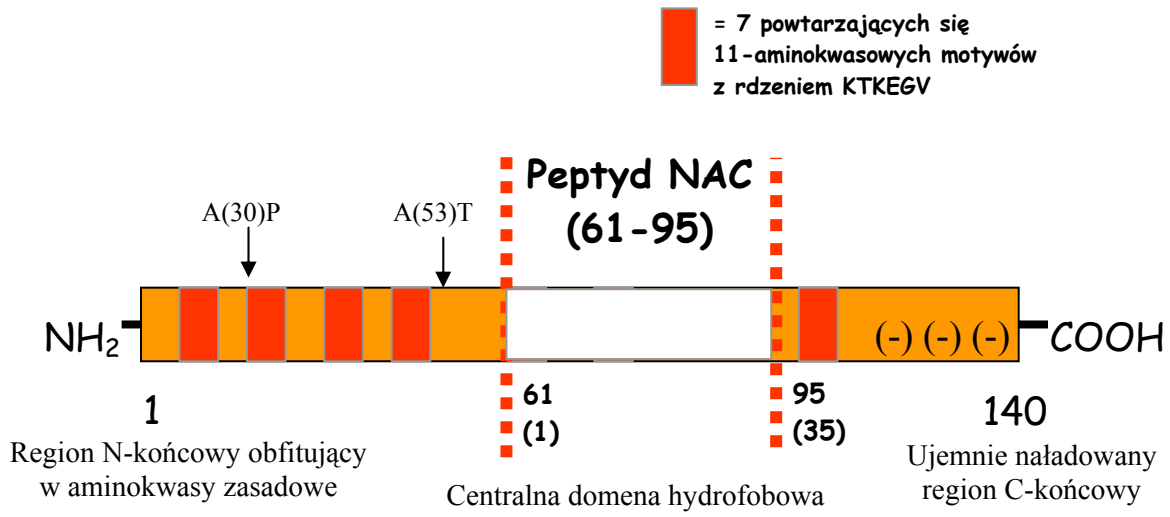


Rycina 1. Sekwencje aminokwasowe ludzkich białek z rodziny synuklein.

W ramkach na czerwono zaznaczono fragmenty konserwatywne we wszystkich izoformach.

α-Synukleinę (**ASN**) wyizolowano po raz pierwszy w 1988 roku z pęcherzyków synaptycznych narządu elektrycznego morskiej ryby z gatunku *Torpedo californica* i nazwano "syn-nukleina" ze względu na jej obecność w synapsach (ang. ***syn****apse*) jak i w jądrze komórkowym (ang. ***nucle****us*) (Maroteaux i wsp., 1988). Obecnie wiadomo, że ASN występuje w zakończeniach presynaptycznych, cytozolu, mitochondriach, jądrze komórkowym oraz w aksonach neuronów (Liu i wsp., 2009a, Liu i wsp., 2009b, Zhang i wsp., 2008). Ponadto, badania ostatnich kilku lat wskazują na obecność ASN w przestrzeni zewnątrzkomórkowej zarówno w hodowlach komórkowych jak i w płynie mózgowo-rdzeniowym oraz w osoczu krwi (Borghi i wsp., 2000, El-Agnaf i wsp., 2003, Lee i wsp., 2008 (a), Lee i wsp., 2008 (b), Lee 2008, Liu i wsp., 2009).

W roku 1993 z płytek starczych mózgów alzheimerowskich wyizolowano 35-cio aminokwasowy peptyd NAC (ang. ***n****on-****a****myloid β* ***c****omponent of Alzheimer's disease plaques*), czyli "nie będący białkiem β-amyloidowym składnik płytek starczych" (Ueda i wsp., 1993) (Rycina 2).



Rycina 2 Struktura α-synukleiny i peptydu NAC

W strukturze pierwszorzędowej α–synukleiny można wyróżnić trzy domeny. Region N-końcowy obfitujący w aminokwasy zasadowe (aminokwasy 1-60) zawiera cztery jedenasto-aminokwasowe, nieregularnie powtarzające się motywy, których rdzeń stanowi konserwatywna sześcio-aminokwasowa sekwencja (KTKEGV). Te powtórzenia są oddzielone odcinkami 5-8 reszt aminokwasowych. W rejonie tym znajdują się miejsca obydwu mutacji związanych z rodzinnym wariantem choroby Parkinsona. Centralny, silnie hydrofobowy odcinek (aminokwasy 61-95) stanowi amyloidogenną sekwencję ***NAC****. Zawiera on dwa kolejne powtórzenia motywu (KTKEGV). Region C-końcowy (aminokwasy 96 do 140) obfituje w prolinę oraz aminokwasy kwaśne, które nadają mu ujemny ładunek, co warunkuje przyjmowanie przez białko struktury nieuporządkowanej na tym odcinku.*

Następnie zsekwencjonowano ludzkie cDNA kodujące białko prekursorowe peptydu NAC, które nazwano NACP, (ang. ***n****on-****a****myloid* β *component* ***p****recursor*). Wykazano, że sekwencja aminokwasowa NACP i ludzkiej ASN jest identyczna (Iwai i wsp., 1995). Zainteresowanie naukowców tym białkiem wzrosło, gdy wykazano związek pomiędzy występowaniem rzadkiej, dziedzicznej postaci choroby Parkinsona, a mutacjami w genie kodującym ASN. W genie tym wykryto niezależnie dwie mutacje typu podstawienia, które powodują odpowiednio: zamianę alaniny w pozycji 30 na prolinę (A30P) oraz zamianę alaniny w pozycji 53 na treoninę (A53T) (Li i wsp., 2001). Efektem obydwu mutacji jest ujawniająca się w młodym wieku choroba Parkinsona, dziedziczona w sposób autosomalny dominujący (Iwai, 2000). W ostatnim czasie opisano kolejną mutację w białku ASN, E46K, która wpływa na oddziaływania ASN z lipidami i na jej agregację (Choi i wsp., 2004, Greenbaum i wsp., 2005). Obecnie już wiadomo, że ASN stanowi główny składnik nierozpuszczalnych złogów białkowych

tzw. ciał Lewy'ego, które występują w chorobie Parkinsona (Crowther i wsp., 2000), ale również w wielu innych chorobach neurodegeneracyjnych: w otępieniu z ciałami Lewy'ego (Spillantini i wsp., 1998), chorobie Alzheimera (Giasson i wsp., 2000), zespole Downa (Raghavan i wsp. 1993), dziecięcej dystrofii neuroaksonalnej (Hayashi i wsp., 1992), zwyrodnieniu ośrodkowego układu nerwowego typu I z odkładaniem się żelaza w mózgu (Neumann i wsp., 2000) czy podostrym stwardniającym zapaleniu mózgu (Gibb i wsp., 1990). Uważa się, że ASN pełni istotną rolę w patomechanizmie chorób neurodegeneracyjnych, które określa się mianem synukleinopatii (Pountney i wsp., 2004). Jednak mimo intensywnych badań prowadzonych w czołowych ośrodkach badawczych na świecie mechanizmy toksyczności tego białka pozostają nadal nie w pełni wyjaśnione. Podobnie, fizjologiczna funkcja ASN nie jest do końca poznana. Uważa się, że znaczenie tego białka w fizjologii oraz w neurodegeneracji zależne jest od jego stężenia oraz oligomeryzacji i stopnia agregacji (Norris i wsp., 2004). ASN obecna w mózgu w stężeniu nanomolarnym reguluje transport pęcherzykowy, uwalnianie neuroprzekaźników, wpływa na przekaźnictwo informacji zależne od fosfolipazy Cβ, uczestniczy w kształtowaniu plastyczności synaptycznej (Sidhu i wsp., 2004 (a), Sidhu i wsp., 2004 (b), Steidl i Gomez, 2003, Cabin i wsp., 2002, Liu i wsp., 2004, Narayanan i wsp., 2005) oraz pełni funkcje białka opiekuńczego i antyapoptotycznego (Alves Da Costa i wsp., 2002, Jensen i wsp., 2003). W wysokim stężeniu oraz w postaci oligomerów/agregatów ASN jest cytotoksyczna (Recchia i wsp., 2004, Bodles i Irvine 2004, Martin i wsp., 2004, Cookson 2009). Białko to wykazuje tendencję do samoistnego formowania agregatów *in vitro* (Madine i wsp., 2004). Pierwszym etapem tego procesu jest zmiana formy kłębka statystycznego (ang. *random coil*) na strukturę β-harmonijki, która łatwo tworzy włókna. W początkowym etapie agregacji powstają rozpuszczalne oligomery złożone z kilku, kilkunastu cząsteczek ASN (tzw. protofibryle) (Ding i wsp., 2002). Oligomery pełnią prawdopodobnie kluczowe znaczenie w cytotoksyczności ASN (Sharon i wsp., 2003, Kim i wsp., 2009). Postępująca agregacja ASN może być mechanizmem chroniącym komórkę przed szkodliwym działaniem oligomerów, a jednocześnie prowadzi do powstania nierozpuszczalnych złogów, które uszkadzają komórki w sposób mechaniczny (Jellinger, 2003). Liczne badania wykazały udział reaktywnych form tlenu (ROS, *ang.* ***r****eactive* ***o****xygen* ***s****pecies*) oraz azotu (RNS, *ang.* ***r****eactive* ***n****itrogen* ***s****pecies*) w stymulacji agregacji ASN. Nadtlenek wodoru i jony metali: Fe^{2+} i Cu^{2+} oraz Ca^{2+} stymulują agregację tego białka (Norris i wsp., 2003, Yamin i wsp., 2003, Lowe i wsp., 2004). Za czynniki stymulujące agregację ASN uważa się również jej zwiększoną ekspresję oraz

mutacje (Rockenstein i wsp., 2001, Lansbury i Brice 2002, Shtilerman i Ding, 2002). W ostatnich latach poszukiwano intensywnie związków zapobiegających oligomeryzacji/agregacji ASN, które mogłyby mieć znaczenie w leczeniu synukleinopatii (El-Agnaf i wsp., 2004, Klucken i wsp., 2004, Hashimoto i wsp., 2004, McLean i wsp., 2004). Fizjologiczna funkcja ASN, mechanizmy jej agregacji oraz udział w procesie neurodegeneracji zostały wyczerpująco przedstawione w pracy poglądowej (Solecka i wsp., 2005) oraz w rozdziale książki pt. „Mózg a stres" (Adamczyk i wsp., 2008). Najnowsze i nieliczne jeszcze badania ostatnich kilku lat wskazują na istotne znaczenie zewnątrzkomórkowej ASN w procesie cytotoksyczności (Lee i wsp., 2008 (a), Lee i wsp., 2008 (b), Lee 2008, Liu i wsp., 2009). ASN zarówno w postaci monomerycznej jak i zagregowanej może być wydzielana do przestrzeni zewnątrzkomórkowej i uszkadzać funkcje komórek sąsiadujących (Lee i wsp., 2005). Badania El-Agnaf i wsp., 2006 wykazały podwyższony poziom oligomerów ASN w osoczu krwi u pacjentów z chorobą Parkinsona, sugerując, że mogą to być potencjalne „biomarkery" choroby Parkinsona. Monomeryczna ASN oraz pre-fibrylarne agregaty mogą być transportowane z przestrzeni zewnątrzkomórkowej do wnętrza komórek przez nie wyjaśniony dotąd mechanizm (Bucciantini i wsp., 2004, Ahn i wsp., 2006). Ostatnie badania sugerują, że wychwyt zwrotny zewnątrzkomórkowej ASN do wnętrza komórek odbywa się na drodze endocytozy lub bezpośrednio przez błonę komórkową w zależności od stopnia agregacji tego białka (Lee i wsp., 2008 (a)). Oligomery i agregaty są pobierane na drodze endocytozy, formy monomeryczne mogą zaś swobodnie dyfundować do komórki przez błonę komórkową. Najnowsze badania wskazują, że zewnątrzkomórkowa ASN po przedostaniu się do wnętrza komórki może być na nowo wydzielana z neuronów do przestrzeni zewnątrzkomórkowej przez proces zależny od białka rab11a (Liu i wsp., 2009). Kolejne badania wskazują na przemieszczanie się oligomerów ASN między komórkami, oraz sugerują, że zewnątrzkomórkowe oligomery ASN mogą indukować agregację wewnątrzkomórkowej puli ASN (Danzer i wsp., 2009). Obecnie sugeruje się, że zewnątrzkomórkowa ASN może mieć istotne znaczenie w patomechanizmie choroby Parkinsona, ale mechanizm jej toksyczności pozostaje dotąd nie w pełni wyjaśniony. Wyłącznie badania *in vitro* wykazały, że zewnątrzkomórkowe oligomery ASN mogą tworzyć pory w błonie komórkowej (Volles i wsp., 2001), co sugeruje udział tych form ASN w zaburzeniu przepuszczalności komponenty białkowo-lipidowej. W mechanizm cytotoksyczności zewnątrzkomórkowej ASN prawdopodobnie zaangażowane są białka z rodziny Bcl 2. Wykazano, że ASN obniża ekspresję białka Bcl-xL oraz zwiększa poziom białka BAX

prowadząc do uwalniania cytochromu c i aktywacji kaspaz (Seo i wsp., 2002). Obumieranie komórek dopaminergicznych w hodowli pierwotnej neuronalno-glejowej śródmózgowia zaobserwowano w wyniku działania zewnątrzkomórkowej ASN. Sugeruje się, że uszkodzenie neuronów dopaminergicznych prowadzi do uwolnienia zagregowanej ASN, która z kolei aktywuje mikroglej i produkcję pro-zapalnych mediatorów, a w konsekwencji prowadzi do neurodegeneracji. W mechanizm ten ma być zaangażowana NADPH oksydaza i produkcja anionorodnika ponadtlenkowego (Zhang i wsp., 2005). Ponadto, wykazano udział ASN oraz peptydu NAC w aktywacji procesów wolnorodnikowych oraz czynnika transkrypcyjnego kappa-B (NF-κB) (Turnbull i wsp., 2001, Tanaka i wsp., 2002, Tabner i wsp., 2002). Z drugiej strony, wiadomo jest, że wolne rodniki, a przede wszystkim O_2^- i NO pełnią istotną rolę w kaskadzie zdarzeń prowadzących do obumierania neuronów w chorobach neurodegeneracyjnych (Singh i Dikshit 2007, Watanabe i wsp., 2008, Padovan-Neto i wsp. 2009, Westermann 2009). Podsumowując, istnieje powszechnie zaakceptowana hipoteza, że ASN w postaci oligomerów lub agregatów pełni istotną rolę w procesie neurodegeneracji i może być odpowiedzialna za obumieranie neuronów dopaminergicznych (Cookson i Brug 2008). Obecnie uważa się, że forma oligomeryczna ASN jest najbardziej toksyczna (Kim i wsp., 2009). ASN w zmienionej konformacji może tworzyć pory w błonach pęcherzyków synaptycznych, szczególnie układu dopaminergicznego i zaburzać homeostazę dopaminy (Volles i Lansbury, 2003). Kolejna hipoteza pozostaje w ścisłym związku z omówioną powyżej i zwraca uwagę na udział ASN w uszkodzeniu funkcji siateczki śródplazmatycznej i pęcherzyków Golgiego, z ang., „ER-Golgi stress"(Gosavi i wsp., 2002, Cooper i wsp., 2006, Smith i wsp., 2005). Istotne znaczenie w toksyczności przypisuje się również dysfunkcji mitochondriów wywołanej przez ASN, jednak dane dotyczące mechanizmów działania ASN na funkcję tych organelli są nieliczne. Sugeruje się, że ASN może być transportowana do mitochondriów i wchodzić w interakcję z oksydazą cytochromową (Elkon i wsp., 2002). W ostatnich latach zwrócono uwagę na znaczenie zaburzeń układu ubikwityna-proteasom (ang. *ubiquitin-proteasome system* – UPS) w patogenezie choroby Parkinsona. Uważa się, że ASN może być odpowiedzialna za uszkodzenie funkcji tego układu (Snyder i wsp., 2003) bądź też za zaburzenie degradacji niepożądanych białek na drodze lizosomalnej. Powszechnie przyjęta jest również hipoteza toksyczności ASN zależna od aktywacji stresu oksydacyjnego. Jednak, żadna z tych teorii nie jest w pełni wyjaśniona i mechanizmy toksyczności ASN są nadal w centrum zainteresowania wiodących ośrodków badawczych na świecie. Należy zwrócić

również uwagę, że chociaż powszechnie wiadomo, że ASN pełni istotną rolę w patomechanizmie chorób neurodegeneracyjnych związanych ze starzeniem się organizmu, to dotychczas nie istniały dane dotyczące ekspresji ASN oraz poziomu białka w różnych strukturach mózgu starczego oraz nie wyjaśniona była funkcja tego białka w mózgu starczym. Badania prowadzące do zrozumienia zmian w poziomie, strukturze i rozmieszczeniu ASN w różnych częściach mózgu starczego, a także wyjaśnienie mechanizmów działania ASN zwiększają szanse na wprowadzenie efektywnej terapii w leczeniu choroby Parkinsona i innych chorób neurodegeneracyjnych z grupy synukleinopatii.

II. CEL BADAŃ OBJĘTYCH ROZPRAWĄ HABILITACYJNĄ

Przeprowadzone badania miały **na celu** określenie udziału ASN w funkcji układu dopaminergicznego oraz w molekularnych mechanizmach cytotoksyczności, a także poszukiwanie nowych związków cytoprotekcyjnych.

CELE SZCZEGÓŁOWE

1. Zbadanie ekspresji genu i poziomu białka ASN w różnych częściach mózgu dojrzałego i starczego.

2. Wyjaśnienie molekularnych mechanizmów działania ASN na funkcję transportera dopaminy (DAT).

3. Zbadanie wpływu ASN na aktywację kaskady wolnorodnikowej, funkcję kanałów wapniowych i utrzymanie homeostazy wapniowej w mózgu oraz na regulację wapniowo-zależnych białek zaangażowanych w procesy przeżycia/obumierania komórek.

4. Ocena udziału ASN w uwalnianiu i toksyczności peptydów amyloidu beta (Aβ) oraz wyjaśnienie molekularnych mechanizmów interakcji tych peptydów/białek. Poszukiwanie wspólnych szlaków działania ASN i Aβ oraz związków zapobiegających toksyczności wywołanej współistnieniem ASN i Aβ.

III. EKSPRESJA ASN W MÓZGU DOJRZAŁYM I STARCZYM ORAZ JEJ UDZIAŁ W AKTYWACJI NAPŁYWU WAPNIA I MODULACJI SYNTAZY TLENKU AZOTU

Adamczyk A., Solecka J., Strosznajder JB. Expression of α-synuclein in different brain parts of adult and aged rats. J. Physiol. Pharmacol., 2005, 56, 1, 29-37.

Adamczyk A., Strosznajder JB. Alpha-synuclein potentiates Ca^{2+} influx through voltage-dependent Ca^{2+} channels. NeuroReport 2006, 17 (18), 1883-1886.

Adamczyk A., Czapski GA., Kaźmierczak A., Strosznajder JB. Effect of *N*-methyl *D*-aspartate (NMDA) receptor antagonists on α-synuclein-evoked neuronal nitric oxide synthase activation in the rat brain. Pharmacol Rep. 2009, 61 (6), 385-393.

Znaczenie ASN w procesach fizjologicznych mózgu i w chorobach neurodegeneracyjnych jest niezwykle ważne i jest ostatnio przedmiotem badań wielu ośrodków naukowych. We wstępie niniejszej rozprawy przedstawiono, że ASN pełni istotną rolę w funkcji zakończeń synaptycznych oraz w przekaźnictwie informacji (Clayton i George 1998, Abeliovich i wsp., 2000, Purisai i wsp., 2005). Zaprezentowane przeze mnie po raz pierwszy zmiany w ekspresji oraz poziomie białka monomerycznej formy ASN w mózgu starczym w porównaniu do dojrzałego mogą wpływać na strukturę i funkcję zakończeń synaptycznych i ewentualnie na propagację procesów neurodegeneracyjnych. Analiza ekspresji i poziomu białka ASN w różnych częściach mózgu dojrzałego wykazała, że poziom mRNA oraz immunoreaktywność tego białka jest zbliżona w korze mózgu, w hipokampie oraz w prążkowiu, natomiast znacząco niższa w móżdżku w porównaniu do pozostałych struktur mózgu. W mózgu starczym obniżony jest poziom mRNA ASN w prążkowiu oraz w móżdżku w porównaniu do odpowiednich struktur mózgu dojrzałego. Ponadto, immunoreaktywność tego białka obniża się we wszystkich badanych strukturach mózgu starczego (Adamczyk i wsp., 2005, Adamczyk i Strosznajder 2005, Adamczyk i wsp., 2006). Proces starzenia pozostawał bez wpływu na ekspresję β-synukleiny (BSN), która uważana jest za nietoksyczną izoformę w rodzinie synuklein. Wskazuje to na szczególną wrażliwość ASN na zmiany wywołane starzeniem. Spadek poziomu monomerycznej formy ASN w starczej korze mózgu i w hipokampie bez zmian w ekspresji genu może świadczyć o aktywacji procesu degradacji i/lub inhibicji syntezy ASN na poziomie translacji, w grę

mogą wchodzić również modyfikacje konformacyjne białka ASN. Być może w tych strukturach mózgu w procesie starzenia dochodzi do oligomeryzacji/agregacji ASN, a w konsekwencji do obniżenia poziomu jej monomerycznej formy. Zmiana konformacji ASN w mózgu starczym może również wynikać ze zwiększonej fosforylacji tego białka na serynie 129 (Saito i wsp., 2003). W mózgach starczych zaburzeniu ulega również transport aksonalny ASN oraz zwiększa się jej agregacja (Li i wsp., 2004). Stres oksydacyjny/nitrozacyjny w strukturach starczego mózgu może stymulować oligomeryzację/agregację ASN i w konsekwencji może prowadzić do obniżenia monomerycznej formy tego białka (Choi i wsp., 2008, Esteves i wsp., 2009). Najnowsze dane literaturowe opublikowane w bieżącym roku wskazują na obniżenie ekspresji ASN w mózgu starczym myszy bez zmian w ekspresji BSN (Mak i wsp., 2009). Badania Mak'a i współpracowników potwierdzają wysuniętą przeze mnie hipotezę, że obniżenie poziomu monomerycznej formy ASN w mózgu starczym może uszkadzać funkcje ASN jako białka antyapoptotycznego oraz opiekuńczego.

Wyniki badań własnych oraz dane wskazujące na obecność ASN w przestrzeni zewnątrzkomórkowej i jej znaczenie w procesie neurodegeneracji wpłynęły na podjęcie zagadnień mających na celu wyjaśnienie udziału zewnątrzkomórkowej ASN w formie monomeryczno-oligomerycznej w funkcji zakończeń synaptycznych. Obumieranie neuronów może rozpoczynać się od degeneracji synaps w procesie zwanym „synaptozą". Jednym z czynników zaangażowanych w proces neurodegeneracji jest zaburzenie homeostazy wapniowej (Wojda i wsp., 2008, Kawahara i wsp., 2009, Small i wsp., 2009). Nieprawidłowa gospodarka wapniowa związana jest z dysfunkcją mitochondriów i siateczki śródplazmatycznej, uszkodzeniem układów buforujących, ekscytotoksycznością glutaminianu oraz nieprawidłowym działaniem kanałów wapniowych. Dane literaturowe wskazują, że Aβ, który podobnie jak ASN należy do rodziny białek amyloidogennych stymuluje napływ wapnia do wnętrza komórek przez kanały wapniowe zależne od potencjału (ang., *voltage-operated calcium channels*, VOCC) (Samochocki i wsp., 1998, Ho i wsp., 2001, MacManus i wsp., 2000, Ueda i wsp., 1997). Dotychczas nie istniały jednak dane wskazujące na udział ASN w modulacji funkcji tych kanałów. Wyniki badań prezentowane w tym opracowaniu wskazują, że ASN oraz jej centralny fragment, peptyd NAC stymuluje napływ wapnia $[^{45}]Ca^{2+}$ do synaptoneurosomów. Natomiast, BSN nie posiadająca w swojej strukturze fragmentu NAC nie powoduje zmian w napływie $[^{45}]Ca^{2+}$, co wskazuje, że efekt ten jest specyficzny jedynie dla ASN. Użycie specyficznych blokerów kanałów wapniowych zależnych od potencjału typu N wskazuje, że w głównej mierze dochodzi do aktywacji

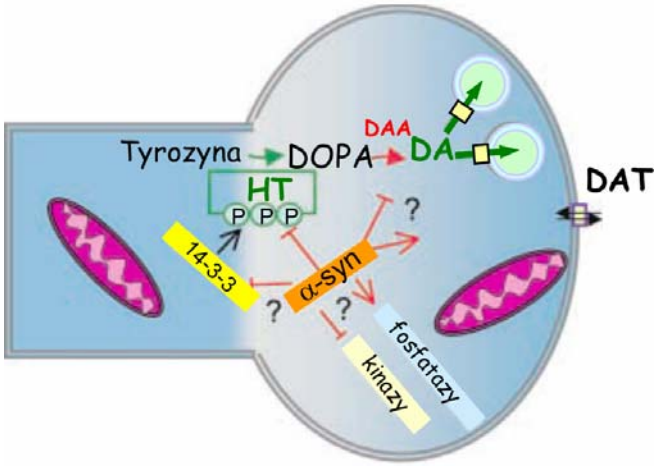
napływu [^{45}]Ca^{2+} przez kanał N. Podobny efekt stymulujący wykazywały również peptydy Aβ, jednak istnieją rozbieżne dane dotyczące typu kanału modulowanego przez ten peptyd. Istnieją dane wskazujące na aktywację kanałów VOCC zarówno typu L, jak i N przez Aβ (Ueda i wsp., 1997, Ho i wsp., 2001, MacManus i wsp., 2000). Dane literaturowe wskazują na udział reaktywnych form tlenu oraz azotu w modulacji VOCC (Ueda i wsp., 1997). Moje badania wykluczyły jednak udział NO oraz innych wolnych rodników w aktywacji VOCC wywołanej działaniem ASN. Sugeruje się natomiast bezpośrednią interakcję ASN z białkiem kanału i jego modyfikację. Kolejne przeprowadzone badania wykazały aktywację napływu wapnia przez peptyd NAC, co w połączeniu z danymi literaturowymi wskazuje, że interakcja białko : białko pomiędzy domeną NAC synukleiny, a białkiem kanału może mieć istotne znaczenie w modulacji VOCC typu N. Zwiększony napływ Ca^{2+} i aktywacja licznych enzymów może być czynnikiem inicjującym proces neurodegeneracji. Konsekwencją napływu Ca^{2+} jest między innymi aktywacja syntazy tlenku azotu (NOS). Wzrost syntezy NO prowadzi do oksydacji makrocząsteczek, nitrozylacji licznych białek i uszkodzenia mitochondriów oraz DNA. Procesy te pośredniczą w neurotoksyczności peptydów Aβ (Keil i wsp., 2004), w patomechanizmie choroby Parkinsona (Chalimoniuk i wsp., 2006, Watanabe i wsp., 2008) oraz w innych chorobach neurodegeneracyjnych (Malinski 2007, Ali i wsp., 2009, Westermann 2009). Badania przedstawione w tej rozprawie wykazały, że zewnątrzkomórkowa ASN oraz peptyd NAC stymulują aktywność NOS odpowiednio o około 70 i 40% w skrawkach z mózgu szczura. BSN pozostawała bez wpływu na aktywność NOS, co potwierdza, że i ten efekt jest specyficzny dla izoformy alfa. Zarówno ASN jak i NAC stymulowały wyłącznie konstytutywną neuronalną izoformę NOS (nNOS), a pozostawały bez wpływu na izoformę śródbłonkową (eNOS) oraz indukowalną (iNOS). Kolejne badania wykazały udział receptora NMDA (*N*-metylo-*D*-asparaginian) w stymulacji nNOS wywołanej przez ASN oraz peptyd NAC. Badania te wskazują, że ASN stymuluje syntezę NO w sposób zależny od aktywacji napływu Ca^{2+} przez receptor NMDA. Chociaż wiadomo jest, że nadmierna aktywacja receptora NMDA skutkuje cytotoksycznością (Bojarski i wsp., 2008) i stwierdzona jest w procesie neurodegeneracji, to nie istniały dotychczas dane dotyczące udziału ASN w aktywacji receptora NMDA. Wieloletnie i liczne badania wskazują jedynie na istotny udział peptydów Aβ w modulacji tego receptora. Istnieją dane wskazujące, że peptydy Aβ mogą wchodzić w interakcję z receptorami NMDA i zwiększać związaną z tymi receptorami ekscytotoksyczność (Windblad i wsp., 2002). Wykazano też, że Aβ aktywuje nNOS w korze mózgu i w hipokampie (Pákáski i Kálmán, 2008, Stepanichev i

wsp., 2008). Wydaje się, że ASN, która podobnie jak Aβ jest białkiem amyloidogennym i należy do rodziny tzw. „białek konformacyjnych" może wchodzić w interakcję z receptorem NMDA i aktywować napływ Ca^{2+}, rozpoczynając kaskadę zdarzeń prowadzącą w konsekwencji do cytotoksyczności. NO jest słabo reaktywnym wolnym rodnikiem, jednak w wyniku reakcji z anionorodnikiem ponadtlenkowym (O_2^{-}▪) tworzy wysoce reaktywny nadtlenoazotyn ($ONOO^-$). $ONOO^-$ oraz inne reaktywne formy azotu mogą zaburzać funkcję łańcucha oddechowego (Ebadi i Dharma, 2003) oraz modyfikować białka poprzez ich nitrację i S-nitrozylację, co może prowadzić do uszkodzenia funkcji neuronów, a w konsekwencji do ich obumierania (Cho i wsp., 2009, Lipton 2006, Uehara i wsp., 2006). Dotychczasowe dane literaturowe wskazywały, że ASN aktywuje NOS w wyizolowanych mitochondriach, mechanizm jednak nie został wyjaśniony (Parihar i wsp., 2008). Wyniki badań przedstawione w niniejszej rozprawie wskazują, że inaktywacja receptora NMDA zapobiega całkowicie stymulacji NOS wywołanej przez ASN i NAC. Wiadomo jest, że ASN może wchodzić w interakcję z komponentą białkowo-lipidową poprzez domenę NAC i w ten sposób może modulować funkcje białek błon synaptoplazmatycznych (Wersinger i wsp., 2003). Sugeruje się, że ASN w wyniku interakcji z błoną synaptoplazmatyczną poprzez domenę NAC prowadzi do zmiany struktury receptora NMDA, a w konsekwencji do jego aktywacji. Stymulacja receptorów glutaminianergicznych była obserwowana w przypadku działania peptydów Aβ (Pellistri i wsp., 2008). Ostatnie dane wykazały, że Aβ aktywuje również metabotropowe receptory glutaminianergiczne (Casley i wsp., 2009). Ponadto, wykazano, że Aβ zwiększa uwalnianie glutaminianu i hamuje jego wychwyt (Kabogo i wsp., 2008, Li i wsp., 2009, Matos i wsp., 2008). Badania zaprezentowane w niniejszej rozprawie wskazują, że ASN oraz jej fragment NAC stymulują aktywność nNOS poprzez mechanizm zależny od pobudzenia receptora NMDA. Syntetyzowany w nadmiarze NO w wyniku reakcji z O_2^{-}▪ i utworzenia wysoce reaktywnego $ONOO^-$ doprowadza do oksydacji białek, lipidów oraz DNA.

IV. UDZIAŁ ASN W MODULACJI TRANSPORTERA DOPAMINY (DAT)

<u>Adamczyk A.,</u> Kaźmierczak A., Strosznajder JB. alpha-Synuclein and its neurotoxic fragment inhibit dopamine uptake into rat striatal synaptosomes. Relationship to nitric oxide. Neurochem Int. 2006, 49, 407-412.

Lokalizacja ASN w presynaptycznych zakończeniach nerwowych oraz w pęcherzykach synaptycznych wskazuje na udział tego białka w regulacji neurotransmisji. Przede wszystkim przypisuje się istotne znaczenie ASN w regulacji przekaźnictwa dopaminergicznego. Wykazano, że w warunkach fizjologicznych ASN, w niskim stężeniu oraz w formie rozpuszczalnej, utrzymuje homeostazę dopaminy (DA) w ośrodkowym układzie nerwowym i reguluje przebieg neurotransmisji dopaminergicznej na wielu etapach (Rycina 3) (Abeliovich i wsp., 2000, Al-Wandi i wsp., 2008, Perez i wsp., 2002). ASN moduluje biosyntezę DA, wpływa na magazynowanie i uwalnianie tego neuroprzekaźnika z pęcherzyków synaptycznych oraz na wychwyt zwrotny DA z przestrzeni międzysynaptycznej (Rycina 3) (Perez i wsp., 2002, Perez i Hastings 2004, Yu i wsp., 2005, Kaźmierczak i wsp., 2007). Nieliczne i rozbieżne dane dotyczą udziału ASN w funkcji transportera DA (DAT) (Lee i wsp., 2001, Wersinger i wsp., 2003).

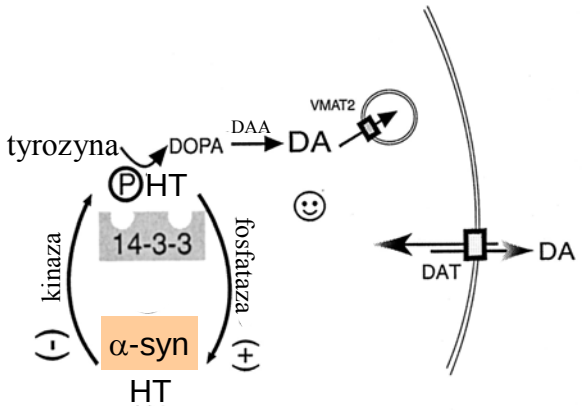


Rycina 3 Udział α-synukleiny w utrzymaniu homeostazy DA w warunkach fizjologicznych. Wg. Perez RG, Hastings TG. J Neurochem. 2004, 89(6), 1318-24.

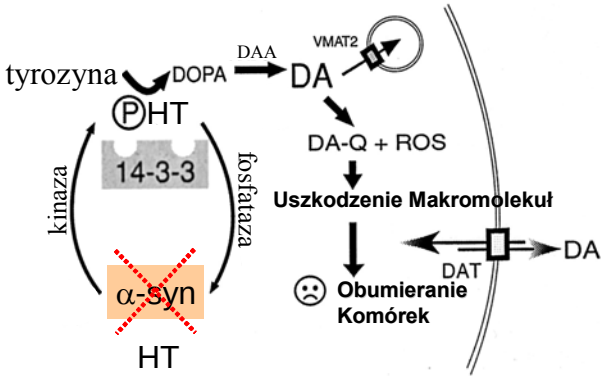
*W warunkach fizjologicznych α-synukleina (**α-syn**), w formie rozpuszczalnej, utrzymuje homeostazę dopaminy (**DA**) w ośrodkowym układzie nerwowym. Obniża biosyntezę DA poprzez hamujący wpływ na aktywność hydroksylazy tyrozynowej (**HT**), reguluje proces transportu DA do pęcherzyków synaptycznych oraz wpływa na wychwyt zwrotny DA za pośrednictwem transportera (**DAT**).*

W warunkach fizjologicznych ASN reguluje syntezę DA poprzez hamowanie aktywności hydroksylazy tyrozynowej (HT), kluczowego enzymu w syntezie DA (Rycina 3 i 4A). Zmiana ekspresji lub konformacji ASN prowadzi do zaburzenia jej funkcji fizjologicznych. ASN w postaci oligomerów lub agregatów traci swoje funkcje fizjologiczne i kontrolę nad wewnątrzkomórkowym stężeniem DA (Rycina 4B).

(A) Wpływ ASN na biosyntezę DA poprzez regulację aktywności hydroksylazy tyrozynowej (HT) w warunkach fizjologicznych



(B) Zaburzenie regulacji i wzrost aktywności HT wywołany spadkiem poziomu monomerycznej formy ASN



Rycina 4. Udział α-synukleiny w regulacji aktywności hydroksylazy tyrozynowej (HT) w fizjologii (A) i w procesie neurodegeneracji (B). Wg. Perez RG, Waymire JC, Lin E, Liu JJ, Guo F, Zigmond MJ. J Neurosci. 2002, 22(8), 3090-9.

*(**A**) Dopamina powstaje z aminokwasu tyrozyny na drodze wielostopniowego procesu. Początkowo tyrozyna przekształcana jest przez ufosforylowaną hydroksylazę tyrozynową (**HT**) do 3,4-dihydroksyfenyloalaniny (**DOPA**), z której potem w wyniku działania dekarboksylazy aromatycznych aminokwasów (**DAA**) powstaje dopamina (**DA**). Białko opiekuńcze 14-3-3 wiąże się z ufosforylowaną formą HT, natomiast α-synukleina (**ASN**) z formą nie ufosforylowaną. Cząsteczki te wykazują antagonistyczne działanie w regulacji aktywności HT, co prowadzi do utrzymania optymalnego stężenia dopaminy w komórce. Z cytozolu DA jest natychmiast przenoszona do pęcherzyków synaptycznych za pomocą jednej z izoform pęcherzykowego transportera amin*

*katecholowych - **VMAT 2** (ang. **V**esicular **M**ono**a**mine **T**ransporter). ASN hamuje aktywność HT bezpośrednio, poprzez wiązanie się z cząsteczką enzymu, albo pośrednio, poprzez aktywację (+) fosfataz i inhibicję (-) kinaz.*
*(**B**) Spadek stężenia rozpuszczalnej formy ASN w wyniku obniżenia ekspresji ASN lub jej oligomeryzacji/agregacji, prowadzi do zaburzenia regulacji oraz do nadmiernej aktywności HT. Dochodzi do wzrostu stężenia DA w cytozolu, a w konsekwencji do nadmiernej produkcji wolnych rodników (**ROS**) oraz semichinonów i chinonów (**DA-Q**), które prowadzą do uszkodzenia licznych makromolekuł i śmierci komórki.*

Zarówno u myszy transgenicznych, jak i w komórkach transfekowanych genem kodującym ASN, stwierdzono znaczne obniżenie aktywności HT oraz wewnątrzkomórkowego stężenia DA (Perez i wsp., 2002). ASN może wpływać na HT bezpośrednio, poprzez interakcję białko-białko lub pośrednio, poprzez aktywację specyficznych fosfataz lub hamowanie kinaz regulujących aktywność HT (Rycina 4A). Istotną rolę w regulacji aktywności HT pełni oddziaływanie pomiędzy ASN, a białkiem 14-3-3 (Xu i wsp., 2002). Opiekuńcze białko 14-3-3 wiąże się z ufosforylowaną formą HT i zapobiega jej defosforylacji, natomiast ASN wiąże się z nieufosforylowaną formą HT i utrzymuje enzym w formie nieaktywnej, co powoduje znaczne obniżenie syntezy DA (Maguire-Zeiss i wsp., 2005) (Rycina 4A). Z kolei udział ASN w procesie magazynowania i uwalniania dopaminy z pęcherzyków synaptycznych zachodzi poprzez regulację aktywności fosfolipazy D_2 i uwalniania kwasu fosfatydowego, który inicjuje tworzenie pęcherzyków synaptycznych z błon plazmatycznych (Liscovitch i wsp., 2000). Poza tym sugeruje się udział ASN w aksonalnym transporcie pęcherzyków synaptycznych (Lucking i Brice 2000) poprzez interakcję z białkami cytoszkieletu (Alim i wsp., 2002, Jansen i wsp., 2000, D'andrea i wsp., 2001, Chung i wsp., 2001, Sharma i wsp., 2001). ASN wpływa również na wewnątrzkomórkowe stężenie DA poprzez regulację ilości pęcherzykowego transportera dopaminy VMAT2 (ang. ***V**esicular **M**ono**a**mine **T**ransporter 2*) na powierzchni pęcherzyków synaptycznych (Lotharius i Brundin 2002). Wykazano również, że oligomery ASN wchodzą w interakcję z błoną pęcherzyków synaptycznych zwiększając jej przepuszczalność i tworząc pory, przez które uwalniana jest DA do cytozolu (Volles i Lansbury, 2002). Po aktywacji specyficznych receptorów na błonie post-synaptycznej i przekazaniu sygnału, DA jest wychwytywana z przestrzeni synaptycznej do cytozolu części pre-synaptycznej zakończeń nerwowych przez transporter DA (DAT) (ang. ***D**opa**m**ine **T**ransporter*), kluczowe białko regulujące przekaźnictwo dopaminergiczne. Wcześniejsze rozbieżne i nieliczne dane literaturowe wskazywały na udział ASN w modulacji funkcji DAT. Zaobserwowano zarówno efekt hamujący jak i pobudzający ASN na aktywność tego białka (Lee i wsp., 2001, Wersinger i wsp., 2003). Badania własne wykazały, że

aktywność DAT jest obniżona w starczym prążkowiu o około 50% w porównaniu do tej struktury z mózgu dojrzałego. Jednocześnie, w prążkowiu starczym obniżony jest poziom białka ASN w błonach synaptoplazmatycznych. Na podstawie tych wyników badań oraz w świetle danych literaturowych wskazujących na istotną rolę ASN w modulacji funkcji DAT i jej działania jako białka opiekuńczego sugeruje się, że obniżenie poziomu monomerycznej formy ASN w starczym prążkowiu może być odpowiedzialne za zaburzenie funkcji DAT obserwowane w tej strukturze mózgu. Ponadto, zewnątrzkomórkowa ASN w postaci monomeryczno-oligomerycznej w wysokim (10 μM) stężeniu oraz jej fragment NAC (10 μM) obniżają aktywność DAT o około 30%. Zarówno ASN jak i peptyd NAC stymulują generację wolnych rodników w synaptosomach prążkowia, odpowiednio o 20 i 80%. Ponadto, ASN zwiększa aktywność NOS w tej strukturze mózgu o około 20%. Donor tlenku azotu, nitroprusydek sodu (SNP, 10 μM) oraz stres oksydacyjny wywołany działaniem $FeCl_2$ (25 μM) w obecności kwasu askorbinowego (250 μM) powodował znaczącą inhibicję aktywności DAT. Inhibitor konstytutywnych izoform NOS, N-nitro-L-arginina (NNLA, 100 μM), zapobiega dysregulacji DAT wywołanej działaniem ASN. Natomiast przeciwutleniacz Trolox, pochodna tokoferolu (kwas 6-hydroksy-2,5,7,8-tetrametylchroman-2-karboksylowy) oraz Tempol, związek o właściwościach dysmutazy ponadtlenkowej, pozostają bez wpływu na funkcję DAT zaburzoną przez ASN oraz NAC. Uzyskane wyniki badań po raz pierwszy wykazały, że zewnątrzkomórkowa ASN w postaci monomeryczno-oligomerycznej obniża aktywność DAT i wychwyt zwrotny DA, prowadząc tym samym do obniżenia poziomu tego neuroprzekaźnika w cytozolu zakończeń presynaptycznych. W wyniku tego dochodzi do zaburzenia homeostazy DA oraz uszkodzenia funkcji układu dopaminergicznego w prążkowiu i może mieć kluczowe znaczenie w chorobie Parkinsona, w której dochodzi do spadku poziomu DA w neuronach szlaku czarno-prążkowiowego. Badania Wersinger i wsp., 2003 prowadzone na liniach komórkowych COS-7 i HEK293 transfekowanych ASN również wskazywały, że ASN obniża aktywność DAT. Dane literaturowe wskazują, że ROS oraz RNS modulują funkcję DAT (Park i wsp., 2002, Kiss i wsp., 2004, Barrier i wsp., 2003). Badania własne wykazały, że ASN aktywuje uwalnianie NO oraz ROS. Ponadto, inhibitor NOS, NNLA skutecznie zapobiega inhibicji DAT, co wskazuje na udział NO w modulacji tego transportera. W zależności od stanu redoks NO może występować w formie wolnego rodnika (NO▪) lub powstających z niego w wyniku przemian metabolicznych kationu nitrozoniowego (NO+) oraz anionu nitroksylowego (NO–). Ponadto, NO▪ w wyniku reakcji z

anionorodnikiem ponadtlenkowym ($O_2^{-\bullet}$) tworzy wysoce reaktywny nadtlenoazotyn ($ONOO^-$). Stwierdzono ponadto, że silne przeciwutleniacze, takie jak Trolox i Tempol nie zapobiegają inhibicji DAT wywołanej działaniem ASN, co sugeruje, że $O_2^{-\bullet}$ oraz $^{\bullet}OH$ nie są odpowiedzialne za zaburzenie funkcji DAT. Podsumowując, uzyskane wyniki badań wskazują, że w starczym prążkowiu dochodzi do zaburzenia funkcji DAT w wyniku obniżenia poziomu monomerycznej formy ASN oraz pod wpływem działania NO uwalnianego przez zewnątrzkomórkową pulę ASN. W mechanizmie działania ASN nie należy jednak wykluczać bezpośredniej interakcji ASN poprzez domenę NAC z białkiem DAT.

V. MECHANIZMY TOKSYCZNOŚCI ASN W KOMÓRKACH DOPAMINERGICZNYCH I JEJ ZNACZENIE W UWALNIANIU I DZIAŁANIU PEPTYDÓW Aβ

Kaźmierczak A., Strosznajder JB., Adamczyk A. alpha-Synuclein enhances secretion and toxicity of amyloid beta peptides in PC12 cells. Neurochem Int. 2008, 53 (6-8), 263-269.

Badania przeprowadzone na frakcji zakończeń synaptycznych (synaptosomach i synaptoneurosomach) oraz na skrawkach z mózgów szczurzych wykazały, że ASN już w krótkim czasie działania (30 min) wywołuje zaburzenie funkcji układu dopaminergicznego. W celu lepszego zrozumienia oraz wyjaśnienia mechanizmów działania ASN na komórki dopaminergiczne kolejne badania przeprowadzono na linii komórek dopaminergicznych PC12. Wyniki badań na tej linii komórkowej hodowanej w obecności ASN w czasie 12-48 godzin wykazały, że ASN w sposób zależny od stężenia oraz stopnia agregacji wywołuje uszkodzenie mitochondriów i obumieranie komórek dopaminergicznych PC12. Po 48 godzinach inkubacji w obecności ASN w formie rozpuszczalnych monomerów-oligomerów przeżywa około 50% komórek. Analogiczny efekt na przeżywalność komórek PC12 wywierał peptyd NAC (Kaźmierczak i wsp., 2009). Ponieważ dotychczasowe badania wykazały, że ASN znacząco zwiększa syntezę NO w mózgach szczurzych (Adamczyk i wsp., 2009 (a)) oraz stymuluje aktywność i ekspresję NOS w komórkach neuronalnych mysiego hipokampa HT22 (Adamczyk i wsp., 2009 (b)) zbadano udział NO w procesie obumierania komórek PC12 poddanych działaniu ASN. Istotne działanie protekcyjne zaobserwowano w obecności niespecyficznego inhibitora izoform NOS zależnych od Ca^{2+} (NNLA, 100 μM), specyficznego inhibitora neuronalnej izoformy NOS (NAAN, 50 μM) oraz w obecności inhibitora indukowalnej izoformy NOS (1400W, 100 μM). Podobnie, cyklosporyna A, (CsA, 2μM), która blokuje otwarcie megakanału mitochondrialnego wykazywała działanie protekcyjne, co wskazuje na zmiany w przepuszczalności mitochondriów w komórkach PC12 traktowanych ASN. Konsekwencją zaburzenia funkcji mitochondriów jest aktywacja kaspazy-3, która wydaje się być najważniejszą spośród kaspaz wykonawczych w mechanizmie programowanej śmierci komórki. Aktywacja tego enzymu prowadzi do cięcia białka PARP, a w konsekwencji do uszkodzenia jego funkcji naprawczych (dane nie

prezentowane w tej pracy). Analogicznie jak CsA, inhibitor kaspazy 3 (Z-DEVD-FMK 100μM) częściowo zapobiegał śmierci komórek PC12 wywołanej przez ASN.

Uzyskane wyniki badań wykazały, że zewnątrzkomórkowa ASN wywołuje zaburzenie funkcji mitochondriów oraz aktywuje zależną od kaspaz programowaną śmierć komórek dopaminergicznych PC12. Podobnie, jak w przypadku badań prowadzonych na materiale z mózgów szczurzych uszkodzenie komórek PC12 zaobserwowano wyłącznie pod wpływem działania ASN monomeryczno-oligomerycznej. ASN w formie dojrzałych agregatów nie powodowała cytotoksyczności. Prawdopodobnie jest to wynikiem natychmiastowej degradacji zagregowanej formy ASN w lizosomach. Chociaż mechanizm transportu zewnątrzkomórkowej ASN do wnętrza komórek nie jest wyjaśniony, to sugeruje się, że zewnątrzkomórkowe agregaty ASN transportowane są do wnętrza komórki na drodze endocytozy i natychmiast degradowane w lizosomach, natomiast ASN w formie rozpuszczalnej swobodnie „dyfunduje" przez komponentę białkowo-lipidową i tylko w niewielkim stopniu ulega degradacji, nagromadzona wewnątrz komórki może inicjować i propagować proces degeneracji (Lee 2008, Lee i wsp., 2008 (a)). Przedstawione w niniejszej pracy wyniki badań wskazują, że rodzaj śmierci komórek PC12 poddanych działaniu ASN nie jest w pełni określony, około 25% komórek z całej puli obumierających (50%) podlega śmierci apoptotycznej, pozostałe 25% wchodzi na inną drogę programowanej śmierci. Podsumowując, uzyskane wyniki badań wykazały, że pula ASN uwalniana do przestrzeni zewnątrzkomórkowej prowadzi za pośrednictwem NO do zaburzenia funkcji mitochondriów oraz programowanej śmierci komórek zależnej od kaspaz. Uzyskane wyniki badań wykluczają udział ASN w śmierci komórek dopaminergicznych na szlaku zależnym od aktywacji PARP i uwalniania czynnika indukującego apoptozę (AIF).

Ponieważ ASN należy do rodziny białek „konformacyjnych" oraz występuje u około 60% pacjentów z rodzinną i sporadyczną postacią choroby Alzheimera (AD) zbadano udział tego białka w uwalnianiu peptydów Aβ oraz w molekularnych mechanizmach toksyczności Aβ. Badania przeprowadzono na komórkach PC12 transfekowanych ludzkim genem białka prekursorowego Aβ (APP) noszącym podwójną szwedzką mutację – K670M/N671L (APPsw), które uwalniają około pięciokrotnie więcej Aβ w porównaniu z komórkami kontrolnymi PC12. Uzyskane wyniki z użyciem metody ELISA wykazały, że zewnątrzkomórkowo podana ASN około dwukrotnie zwiększa uwalnianie peptydów Aβ z komórek PC12 oraz APPsw do medium inkubacyjnego. Ponadto test redukcji błękitu tiazolowego (MTT), który jest

wyznacznikiem funkcji mitochondriów oraz wskaźnikiem liczby żywych komórek wykazał, że nadmierne uwalnianie peptydów Aβ w komórkach APPsw prowadzi do obumierania tych komórek, a efekt ten jest dodatkowo zwiększony w wyniku działania ASN. Analiza morfologiczna komórek w mikroskopie fluorescencyjnym przy użyciu barwnika fluorescencyjnego Hoechst 33342 wykazała, że komórki kontrolne PC12 jak też APPsw traktowane ASN wykazywały typowe cechy komórek apoptotycznych: kondensacja chromatyny, fragmentacja jąder, oraz tworzenie ciał apoptotycznych. Ilość komórek apoptotycznych wzrastała w komórkach APPsw traktowanych ASN w porównaniu do komórek PC12. W przeciwieństwie do komórek PC12 kontrolnych badane inhibitory izoform NOS nie zapobiegały cytotoksyczności ASN w komórkach APPsw. Podobnie, CsA pozostawała bez efektu na obumieranie komórek APPsw traktowanych ASN, co wskazuje, na nieodwracalne uszkodzenie mitochondriów w komórkach APPsw z nadmierną produkcją Aβ oraz dodatkowo traktowanych ASN. Wykazano, że ASN aktywuje kaspazę-3 w komórkach APPsw. Inhibitor kaspazy 3 (Z-DEVD-FMK 100μM) częściowo zapobiegał toksyczności Aβ w komórkach APPsw, natomiast nie zwiększał przeżywalności komórek APPsw w wyniku równoczesnego działania peptydów Aβ i ASN.

W niniejszej pracy po raz pierwszy wykazano, że ASN zwiększa uwalnianie i toksyczność peptydów Aβ. Badania własne oraz dane literaturowe wskazują, że zarówno ASN jak i Aβ zwiększają uwalnianie wolnych rodników oraz syntezę NO. Jednak zahamowanie aktywności NOS w komórkach APPsw ze zwiększoną produkcją Aβ, w przeciwieństwie do komórek PC12 traktowanych ASN nie zapobiegało śmierci wywołanej działaniem ASN. Wskazuje to na znaczenie interakcji ASN/Aβ w obumieraniu komórek ze zwiększoną ekspresją APP. Ponieważ CsA oraz inhibitor kaspazy 3 zapobiegały częściowo śmierci komórek PC12 traktowanych ASN oraz wywierały efekt protekcyjny na komórki APPsw ze zwiększonym uwalnianiem Aβ, a pozostawały bez wpływu na komórki APPsw traktowane ASN wskazuje to, że zaburzenie funkcji mitochondriów w przypadku współdziałania ASN i Aβ jest procesem nieodwracalnym.

VI. PODSUMOWANIE I WNIOSKI

Istotne znaczenie ASN w patogenezie choroby Parkinsona oraz innych chorób neurodegeneracyjnych jest coraz lepiej udokumentowywane i znajduje potwierdzenie w pojawiających się ostatnio danych literaturowych (Cookson 2005, Ono i wsp., 2008, Maguire-Zeiss 2008, Kim i wsp., 2009). Jednak mimo intensywnych badań nad mechanizmami toksyczności ASN prowadzonych w czołowych ośrodkach badawczych na świecie do chwili obecnej nie są w pełni wyjaśnione molekularne mechanizmy działania tego białka. Nieliczne jeszcze badania ostatnich kilku lat wskazują na kluczowe znaczenie zewnątrzkomórkowej ASN oraz peptydu NAC w procesie cytotoksyczności (Lee i wsp., 2008 (a), Lee i wsp., 2008 (b), Lee 2008). Uważa się, że zewnątrzkomórkowa ASN zarówno w postaci monomerów jak i oligomerycznej jest biomarkerem choroby Parkinsona (Borghi i wsp., 2000, El-Agnaf i wsp., 2003, Lee 2008). Badania własne wykazały, że w mózgu starczym obniża się poziom rozpuszczalnej monomerycznej formy ASN, z drugiej zaś strony ASN uwalniana jest z zakończeń synaptycznych do przestrzeni zewnątrzkomórkowej pod wpływem stresu oksydacyjnego i nitrozacyjnego (Adamczyk i wsp., 2007). Wskazuje to, że starzenie, czynnik ryzyka chorób neurodegeneracyjnych oraz stres oksydacyjny/nitrozacyjny związany z procesem starzenia i neurodegeneracji powoduje zaburzenie konformacji i funkcji ASN. Dysfunkcja tego białka polegająca na oligomeryzacji może rozpoczynać proces cytotoksyczności, a w konsekwencji prowadzić do rozwoju synukleinopatii. Najnowsze dane literaturowe wskazują, że to właśnie zewnątrzkomórkowa pula ASN pełni kluczowe znaczenie w procesie neurodegeneracji, a mechanizm działania zależny jest od stężenia tego białka w przestrzeni zewnątrzkomórkowej. Zgodnie z wynikami (Zhou i wsp., 2009) ASN w stężeniu 0,1-5 μM wykazuje właściwości neuroprotekcyjne, natomiast ASN w stężeniu 10-40 μM wywiera efekt neurotoksyczny. W 2001 roku po raz pierwszy wykazano neurotoksyczne działanie zewnątrzkomórkowej ASN na komórkach dopaminergicznych oraz komórkach neuronalnych H19-7 (Sung i wsp., 2001). Ponadto, zaobserwowano, że zewnątrzkomórkowa ASN aktywuje mikroglej oraz astroglej (Zhang i wsp., 2005, Lee 2008, Lee i wsp., 2009) prowadząc do uwolnienia czynników prozapalnych oraz ROS. ASN w formie monomerycznej jak też zagregowanej jest prawdopodobnie uwalniana do przestrzeni zewnątrzkomórkowej na drodze egzocytozy z pęcherzyków synaptycznych (Lee i wsp., 2005). Może być też uwalniana z uszkodzonych komórek w rozpoczynającym się procesie neurodegeneracji. W przestrzeni zewnątrzkomórkowej ASN może ulegać degradacji proteolitycznej przez

metaloproteinazy, głównie przez metaloproteinazę 3 (MMP-3) (Sung i wsp., 2005) lub być transportowana do sąsiednich komórek. Może też prowadzić do aktywacji mikrogleju i zależnej od tego neurodegeneracji (Lee 2008). Toksyczność zewnątrzkomórkowej ASN może być związana z jej transportem do wnętrza sąsiadujących komórek. Rodzaj transportu zależny jest od konformacji ASN i stopnia jej agregacji. ASN w postaci fibrylli transportowana jest na drodze endocytozy i ulega degradacji w lizosomach. Rozpuszczalna ASN transportowana jest bezpośrednio przez błonę komórkową do wnętrza komórek i natychmiast usuwana na zewnątrz komórki zanim zostanie zdegradowana przez wewnątrzkomórkowy system proteolizy (Lee i wsp., 2008). Dotychczas nie istniały jednak dane w literaturze dotyczące mechanizmów działania zewnątrzkomórkowej ASN na zakończenia synaptyczne, gdzie prawdopodobnie rozpoczyna się proces neurodegeneracji oraz nie wyjaśnione były mechanizmy toksyczności zewnątrzkomórkowej ASN w komórkach dopaminergicznych. Badania, które złożyły się na niniejszą rozprawę wykazały, że białko to zaangażowane jest w istotny sposób w procesy obumierania komórek dopaminergicznych, a za jego toksyczność może być odpowiedzialny centralny fragment: peptyd NAC. Badania własne przeprowadzone na skrawkach oraz frakcji zakończeń synaptycznych z mózgów szczurzych wykazały, że ASN oraz peptyd NAC zaburzają homeostazę wapniową poprzez zwiększenie napływu Ca^{2+} do zakończeń synaptycznych przez kanały wapniowe zależne od potencjału typu N. Ponadto, ASN i NAC aktywują receptory glutaminianergiczne NMDA. W wyniku zwiększonego napływu wapnia przez receptor NMDA dochodzi do aktywacji nNOS i nadmiernego uwalniania NO, który jest istotnym modulatorem procesów neurodegeneracyjnych. Uwalniany w wyniku działania ASN, NO prowadzi do dysregulacji transportera DA. Konsekwencją zaburzenia homeostazy wapniowej, nadmiernego uwalniania NO oraz aktywacji procesów wolnorodnikowych jest zaburzenie funkcji mitochondriów i obumieranie komórek dopaminergicznych. Obumieranie wywołane działaniem ASN zachodzi w wyniku aktywacji programowanej śmierci komórek (PCD) zależnej od kaspaz. Przeprowadzone przeze mnie badania wyjaśniły szlak toksyczności zewnątrzkomórkowej ASN w komórkach dopaminergicznych. Na podstawie uzyskanych wyników badań można zasugerować, że obumieranie neuronów dopaminergicznych m in. w chorobie Parkinsona może wynikać z obecności ASN wewnątrz komórek jak również w przestrzeni międzykomórkowej. Mechanizmy cytotoksyczności tego białka są bardzo złożone i wynikają ze współdziałania wielu czynników, m.in. aktywacji kanałów wapniowych, stymulacji uwalniania wolnych

rodników oraz NO, aktywacji kaspaz oraz zaburzenia funkcji mitochondriów. Zastosowanie inhibitorów NOS, inhibitora kaspazy-3 oraz zablokowanie kanałów mitochondrialnych chroni neurony dopaminergiczne przed obumieraniem wywołanym działaniem ASN. To zaś wskazuje, że rozwojowi cytotoksyczności można będzie zapobiegać na różnych etapach działania ASN, a zaobserwowane właściwości cytoprotekcyjne stosowanych związków mogą być pomocne w opracowaniu skutecznych środków terapeutycznych. Ponadto, po raz pierwszy wykazano, że ASN zwiększa uwalnianie oraz toksyczność peptydów Aβ, wskazując na istotną rolę ASN w patomechanizmie choroby Alzheimera. Tym samym daje to nowe spojrzenie na mechanizm(y) współdziałania ASN i peptydów Aβ oraz sugeruje wspólne bądź analogiczne mechanizmy cytotoksyczności obu peptydów, ASN i Aβ. Równoczesne działanie ASN i Aβ może prowadzić do nieodwracalnych zaburzeń funkcji mitochondriów, a zapobieganie interakcji ASN/Aβ wydaje się być ważną strategią cytoprotekcyjną.

WNIOSKI

1. Proces starzenia mózgu znacząco obniża ekspresję ASN oraz poziom monomerycznej rozpuszczalnej formy tego białka w badanych częściach mózgu. Zmiany w ekspresji ASN oraz jej oligomeryzacja/agregacja mogą prowadzić do zaburzeń funkcji zakończeń synaptycznych i predysponować mózg starczy w kierunku rozwoju synukleinopatii i innych neurodegeneracji.

2. Podczas starzenia mózgu dochodzi do obniżenia aktywności transportera dopaminy DAT. Dysfunkcja tego białka w mózgu starczym może być związana ze spadkiem poziomu rozpuszczalnej monomerycznej formy ASN na skutek jej oligomeryzacji/agregacji. Działająca zewnątrzkomórkowo ASN w wysokim stężeniu w formie oligomerycznej obniża aktywność DAT prawdopodobnie poprzez mechanizm zależny od uwalniania NO.

3. ASN indukuje napływ wapnia do zakończeń synaptycznych przez kanały wapniowe zależne od potencjału (VOCC) typu N oraz przez receptor glutaminianergiczny NMDA. Przyczyną zaburzeń aktywności kanałów wapniowych może być bezpośrednia interakcja ASN z białkiem kanału lub z komponentą białkowo-lipidową.

4. ASN w wyniku zaburzenia homeostazy wapniowej powoduje nadmierne uwalnianie NO oraz aktywację procesów wolnorodnikowych, a w konsekwencji uszkodzenie funkcji mitochondriów i aktywację programowanej śmierci komórek (PCD) zależnej od kaspaz. Powyższe badania pozwalają na lepsze zrozumienie molekularnych mechanizmów cytotoksyczności ASN i wskazują na punkty uchwytu dla nowych środków cytoprotekcyjnych.

5. ASN zwiększa uwalnianie oraz toksyczność peptydów Aβ prowadząc w wyniku wzajemnych oddziaływań do nieodwracalnych zmian w funkcji mitochondriów i obumierania komórek. Przeciwdziałanie interakcji ASN/Aβ może mieć znaczenie w zapobieganiu cytotoksyczności tych peptydów.

VII. WYKAZ PUBLIKACJI STANOWIĄCYCH ROZPRAWĘ HABILITACYJNĄ

1) **Adamczyk A.,** Solecka J., Strosznajder J.B. Expression of α-synuclein in different brain parts of adult and aged rats. J Physiol. Pharmacol. 2005, 56, 29-37 (IF = 2,212).

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3) **Adamczyk A.,** Czapski GA., Kaźmierczak A., Strosznajder JB. Effect of N-methyl D-aspartate (NMDA) receptor antagonists on α-synuclein-evoked neuronal nitric oxide synthase activation in the rat brain. Pharmacol Rep. 2009, 61 (6), 385-393 (IF = 2,167).

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5) Kaźmierczak A, Strosznajder JB, **Adamczyk A**. alpha-Synuclein enhances secretion and toxicity of amyloid beta peptides in PC12 cells. Neurochem Int. 2008, 53(6-8), 263-269 (IF = 3,228).

VIII. KOPIE PUBLIKACJI WYMIENIONYCH W WYKAZIE VII

IX. PIŚMIENNICTWO

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EXPRESSION OF α-SYNUCLEIN IN DIFFERENT BRAIN PARTS OF ADULT AND AGED RATS.

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The synucleins are a family of presynaptic proteins that are abundant in neurons and include α-, β-, and γ-synuclein. α-Synuclein (ASN) is involved in several neurodegenerative age-related disorders but its relevance in physiological aging is unknown. In the present study we investigated the expression of ASN mRNA and protein in the different brain parts of the adult (4-month-old) and aged (24-month-old) rats by using RT-PCR technique and Western blot, respectively. Our results indicated that mRNA expression and immunoreactivity of ASN is similar in brain cortex, hippocampus and striatum but markedly lower in cerebellum comparing to the other brain parts. Aging lowers ASN mRNA expression in striatum and cerebellum by about 40%. The immunoreactivity of ASN in synaptic plasma membranes (SPM) from aged brain cortex, hippocampus and cerebellum is significantly lower comparing to adult by 39%, 24% and 65%, respectively. β-synuclein (BSN) was not changed in aged brain comparing to adult. Age-related alteration of ASN may affect the nerve terminals structure and function.

Keywords: *α-synuclein, mRNA expression, immunoreactivity, brain, aging*

INTRODUCTION

α-Synuclein (ASN) belongs to a larger family of molecules, including β-synuclein (BSN) (or phosphoneuroprotein 14) (1), γ-synuclein (or breast carcinoma-specific factor) and synoretin (2, 3). ASN, a 140 amino acid synaptic

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molecule, was originally identified in human brain as the precursor protein of the non-amyloid β-protein (Aβ) component of Alzheimer's disease (AD) amyloid (NAC). NAC is a highly hydrophobic 35 amino acid central domain (residues 61-95) within the ASN molecule liberated by till unknown mechanism and it is the component of senile plaques in AD (4-6). Highly conserved amino-terminal domain (residues 1-60) of ASN is responsible for association with phospholipid bilayers and C-terminal region (residues 96-140) is rich in Pro, and the acidic residues Glu and Asp (7-9). The functions of the ASN and other members of this family of proteins are poorly understood, however several lines of evidence suggest potential roles in synaptic and neuronal plasticity. ASN is involved in regulation of several enzymes, transporters, presynaptic vesicle dynamic and neurotransmitter release. Moreover, ASN interacts with a variety of proteins including 14-3-3 protein, PKC isozymes, BAD, ERK, MAPKs, Aβ and tau and with several divalent cations (10, 11). It is suggested that soluble nonaggregated ASN decreases caspase-3 activity, modulates bcl2 expression and inactivats the c-Jun N-terminal kinase stress-signaling pathway and through this action it may play antiapoptotic function (12). However, the ASN molecule is capable of self-aggregating to form oligomers and polymers. Polymerization includes formation of protofibrils, nucleation (13), and fibril formation (14). Oxidative stress mediated by iron, cytochrome c and copper promoted ASN aggregation (15-20). ASN polymerization could be trigger by mutations associated with familial parkinsonism, by binding to lipid membrane vesicles and by interactions with Aβ peptide (21-30). Aggregated form of ASN leading to the Lewy bodies formation has been implicated in the pathophysiology of neurodegenerative disorders including Parkinson's disease, dementia with Lewy bodies, Lewy body variant of AD and other α-synucleinopathies (9, 31). In Lewy bodies the ratio of BSN to ASN is altered (32) and it is suggested that a balance between these two synucleins might be critical in neurodegenerative disorders. Hashimoto et al. (17) indicated that BSN might block ASN accumulation and amyloidogenesis. Till now, little is known about the expression of ASN and BSN in physiological aging of the brain. Our preliminary data indicated that aging significantly decrease ASN but not BSN protein amount in the brain (33, 34). In this study we investigated ASN and BSN mRNA and protein expression in different brain parts of adult and aged rats.

MATERIALS AND METHODS

Animals

Males adult (4-month-old) and aged (24-month-old) Wistar rats were supplied from the Animal Breeding House of the Medical Research Center, Warsaw, Poland. The Institutional Ethics Committee accepted the research project.

Preparation of homogenate and synaptic plasma membranes

Animals were decapitated and the brain cortex, hippocampus, striatum and cerebellum immediately isolated. A 10% homogenate from each brain parts was obtained by homogenization in a Dounce glass-glass homogenizer in 0.32 M sucrose with 10 mM Tris-HCl buffer, pH 7.4. Then homogenate was used for preparation of synaptic plasma membranes (SPM) as described previously (35).

RNA extraction and RT-PCR

Total RNA was extracted from brain cortex, hippocampus, striatum and cerebellum from adult and aged animals using a TRI REAGENT isolation kit (Sigma, St. Louis, MO, U.S.A.) according to manufacturer's procedure. The yield and quality of the RNA were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer. First-strand cDNA was synthesized from 8µg of total RNA by reverse transcription (RT), using Reverse Transcription System (Promega Corporation, Madison, WI, U.S.A.). Reaction was performed in final volume of 20µl using 1.500 units of AMV reverse transcriptase, 0.5µg oligo$(dT)_5$', 2500 units of Rnasin(inh), 1mM each deoxyribonucleotide, 5mM $MgCl_2$ and RT buffer in one cycle: 42°C for 1h and 99°C for 5 min with subsequent cooling to 4°C.

Polymerase chain reaction (PCR) was carried out using *Taq* PCR Master Mix Kit (Qiagen, GmbH, Germany) according to the manufacturer's procedure in total volume of 50µl with 20pmol of each primer. The primer sequences for ASN were 5'-TGCTGTGGATATTGTTGTGG-3' (forward) and 5'-AGGTGCGTAGTCTCATGCTC-3' (reverse). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was coamplified using primer sequences 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' (forward) and 5'-CATGTGGGCCATGAGGTCCACCAC-3' (reverse). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and extension at 72°C for 2 min, followed by a final 10-min extension at 72°C. PCR amplification was carried out for 20 cycles for both ASN and GAPDH using a Perkin-Elmer GeneAmp 2400 thermal cycler. The conditions for each PCR amplification resulted in an exponential amplification range for quantification of each mRNA. After amplification, samples were separated on 2% agarose gel containing 200µg/l ethidium bromide in 0.5x Tris-borate-EDTA buffer. The intensity of ASN and GAPDH bands was estimated by densitometric analysis of the gel in UV light using NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

Western blot analysis

Aliquots containing equal amounts of protein from homogenate and SPM were diluted 1:1 in 2 x electrophoresis sample buffer (2xSB) and incubated at 95°C for 5 min. Then, proteins were loaded onto 15% polyacrylamide denaturing gels (40µg per lane) and subjected to electrophoresis (applied constant voltage, 100 V). After protein separation, each gel was placed on a nitrocellulose membrane (Amersham, Hybond-Extra C) in transfer buffer, and the proteins were transfered to the membrane by the application of 100 V for 2 h. After the transfer was completed, equal protein loading and protein transfer was confirmed by Ponceau S staining. In order to block non-specific binding, the membrane was incubated at room temperature (RT) for 1 h in 5% dried skimmed milk and 0.3% Tween 20 in phosphate-buffered saline (PBS), pH 7.4. Incubation with primary antibody (anti-synuclein α developped in rabbit, 1:1000 dilution, anti-β-synuclein (PNP-14), 2µg/ml dilution, Sigma Immunochemicals, St. Louis, MO, U.S.A.) in PBS with 0.1% Tween 20 and 1% bovine serum albumin (BSA) was performed overnight at 4°C. Then blots were washed three times for 15 min at RT in PBS with 0.1% Tween 20. The next membranes were incubated with

horseradish peroxidase (HRP) conjugated anti-rabbit IgG secondary antibody diluted 1:8000 (Sigma, St. Louis, MO, U.S.A.) for 1 h at RT. After that blots were washed as described above and bound antibodies visualized by enhanced chemiluminescence (Amersham, U.K.). Incubation with anti-β actin antibody confirmed additionally equal protein loading. Quantification of immunoblots was performed with the NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

RESULTS

To determine the expression of ASN mRNA and protein in rat brain parts RT-PCR and Western blot in brain cortex, hippocampus, striatum and cerebellum was performed. The results indicated that ASN mRNA expression in adult brain was similar in cerebral cortex and hippocampus, slightly lower in striatum and significantly lower in cerebellum comparing to brain cortex and hippocampus (*Fig. 1*). ASN protein expression in the homogenate from brain cortex, hippocampus and striatum was similar but was significantly lower in cerebellum by about 70% comparing to the other investigated brain parts (*Fig. 1*). Brain aging significantly decreased ASN mRNA expression in striatum and cerebellum by 39% and 47%, respectively (*Fig. 2*). ASN protein level in SPM from aged brain cortex, hippocampus and cerebellum decreased by 39%, 24% and 65%, respectively comparing to adult (*Fig 3*). BSN immunoreactivity was not altered by brain aging (data not shown).

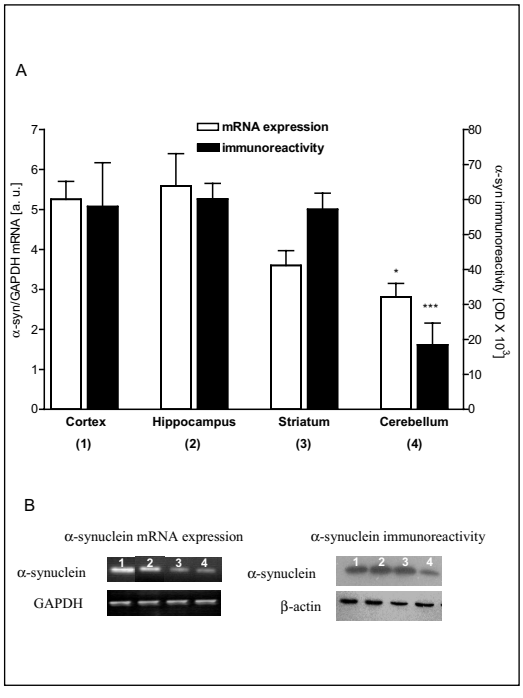

Fig. 1 ASN mRNA and protein expression in the adult rat brain parts.
(A) ASN mRNA expression and its immunoreactivity in brain cortex (1), hippocampus (2), striatum (3) and cerebellum (4) isolated from adult animals. The mRNA ratios were calculated by dividing ASN mRNA levels with the corresponding GAPDH mRNA levels measured in the same experiment. For each experiment, four rats per group were used and all values are expressed as the means ± SEM. Differences were analyzed using one-way ANOVA with the different brain parts as the independent factor with Tukey post-hoc test, ***$p<0.001$, *$p<0,05$ compared with other brain parts.
(B) A representative gel showing the expression of ASN mRNA and GAPDH as an internal control (left panel), and a immunoblots showing ASN immunoreactivity and β-actin as a control (right panel) in brain parts; 1 - brain cortex, 2 - hippocampus, 3 - striatum, 4 - cerebellum.

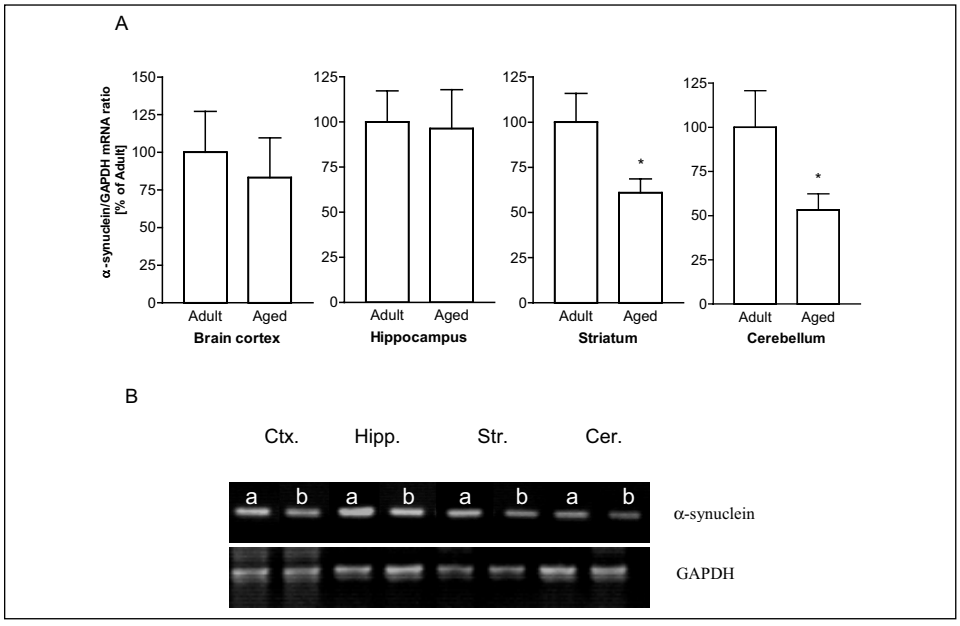

Fig. 2 The effect of aging on ASN mRNA expression in the rat brain parts.
(A) ASN mRNA expression in brain cortex, hippocampus, striatum and cerebellum from adult and aged animals. Adult animals were used as a control. For the experiment, six to eight rats per group were used and all values are expressed as the means ± SEM. Differences between adult and aged animals were analyzed using paired *t-test*. *$p<0.05$, compared with adult.
(B) A representative gel showing the expression of ASN mRNA and GAPDH as an internal control in brain cortex (Ctx.), hippocampus (Hipp.), striatum (Str.), and cerebellum (Cer.) from adult (a) and aged (b) animals.

DISCUSSION

Our study indicated similar ASN expression on mRNA and protein level in brain cortex, hippocampus and striatum with a markedly lower level in cerebellum comparing to the other brain parts. The difference in protein level in this brain structure versus the others is much more pronounced than in mRNA level. These data suggested that not only lower mRNA expression but also other factors might be responsible for the low ASN protein concentration in cerebellum. It could be caused by the lack of the dopaminergic and cholinergic synapses in this structure, cerebellum is enriched only in noradrenergic neurons containing ASN. Axons of dopaminergic, cholinergic and noradrenergic neurons form synaptic connections in brain cortex and hippocampus where ASN protein level is very high. In striatum ASN is probably localized in dopaminergic endings of neurons from substantia nigra and in short internal cholinergic neurons. Moreover, post-translational modification of ASN protein in cerebellum including proteolysis or conformational changes should be taken into

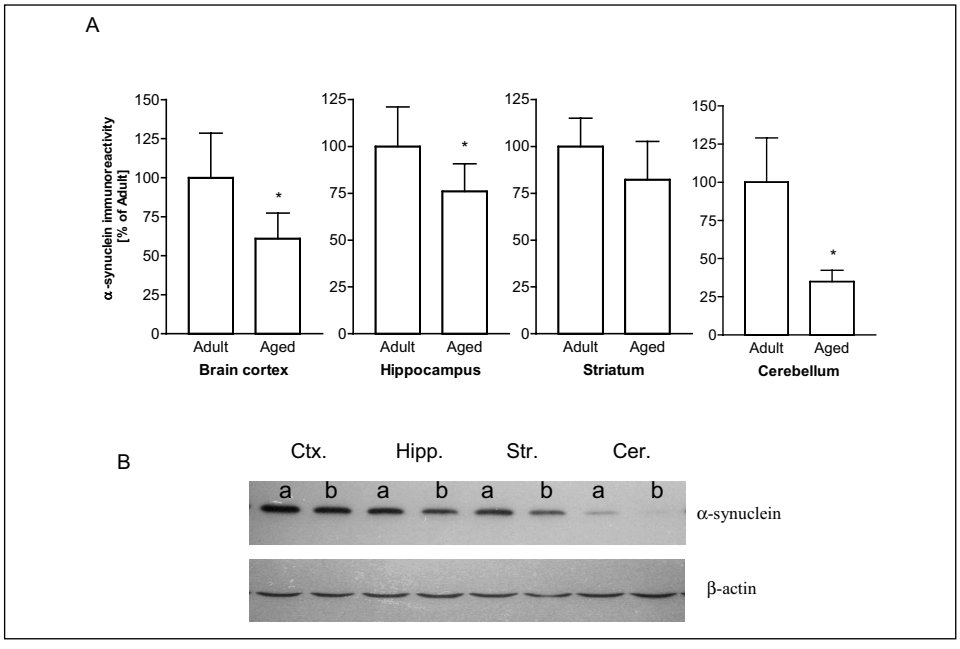

Fig. 3 Aging significantly decreased ASN immunoreactivity in rat brain.
(A) ASN immunoreactivity in brain cortex, hippocampus, striatum, and cerebellum from adult and aged animals. Adult animals were used as a control. For each experiment, six rats per group were used and all values are expressed as the means ± SEM. Differences between adult and aged animals were analyzed using paired *t-test*, *$p<0.05$, compared with adult.
(B) A representative immunoblot showing ASN immunoreactivity and β-actin as a control in brain cortex (Ctx.), hippocampus (Hipp.), striatum (Str.), and cerebellum (Cer.) from adult (a) and aged (b) animals.

consideration. A discrepancy between mRNA and protein levels were described for the other presynaptic proteins, including synaptotagmin I, synapsin I and synaptophysin (36). Our results indicated that aging affected ASN expression in the brain. The most significant alteration of ASN mRNA expression and its immunoreactivity was observed in aged cerebellum comparing to adult. These data indicated that cerebellum is mainly affected by age-related events. Expression of ASN in this part of the brain is altered on the gene and protein level. Future studies will reveal the mechanism(s) of this modification. In brain cortex and hippocampus aging significantly decreased ASN immunoreactivity with no effect on its gene expression. This alteration could be caused by several factors like higher degradation, lower biosynthesis or conformational changes. The low efficiency of translation process and in consequence decrease of protein amount could be also responsible for these changes. The last data on transgenic mice indicated that axonal transport of ASN slows with aging and could result in longer half-life of this protein creating greater opportunities for aggregation-

promoting modifications (37). It is possible that oxidative stress generated in aged brain cortex and hippocampus lead to ASN aggregation and to the lowering of soluble form of ASN. It was observed that oxidative stress is generated during brain aging and that protein oxidation significantly increased in aged brain. Moreover, it was founded that oxidized proteins lose their physiological function (38, 39). Several studies have shown a correlation between generation of reactive oxygen species (ROS) and aggregation of ASN (16, 20, 23, 24, 25). Oxidative stress could be responsible for ASN alteration in aged brain. In aged striatum a lower ASN mRNA expression not correlated with the protein changes. This discrepancy between mRNA and protein level is not fully understand and need future investigation. Aging had no effect on BSN indicated the higher ASN vulnerability for the age-related processes. Our results indicated for the first time that aging significantly decreased ASN expression in the investigated brain parts. This alteration could significantly affect synaptic function and could promote the aged brain for α-synucleinopathy and neurodegeneration.

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Alpha-synuclein potentiates Ca^{2+} influx through voltage-dependent Ca^{2+} channels

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Alpha-synuclein localized in synaptic terminals plays an important role in the pathogenesis of neurodegenerative diseases. The central domain of the protein, the nonamyloid component, is probably responsible for α-synuclein toxicity. Here, we report that α-synuclein and its nonamyloid component induced Ca^{2+} influx in rat synaptoneurosomes. The effect of α-synuclein was eliminated by the N-type specific Ca^{2+} channel blocker, ω-conotoxin GVIA. The antioxidant, resveratrol, and the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine, did not prevent α-synuclein-induced Ca^{2+} influx. Our findings indicate that α-synuclein stimulated Ca^{2+} influx through N-type voltage-dependent Ca^{2+} channels by a mechanism other than free radicals. A direct interaction between α-synuclein and N-type Ca^{2+} channels could be responsible for their effects on Ca^{2+} influx through voltage-dependent Ca^{2+} channels. *NeuroReport* 17:1883–1886 © 2006 Lippincott Williams & Wilkins.

Keywords: alpha-synuclein, Ca^{2+} influx, synaptoneurosomes, voltage-dependent Ca^{2+} channels

Introduction

Alpha-synuclein is a small, acidic synaptic protein composed of 140 amino acid residues, including seven incomplete repeats of 11 amino acids and a core sequence of KTKEGV at the amino terminus [1]. Under physiological conditions, α-synuclein participates in synaptic function [2]. Like other amyloidogenic proteins and peptides, α-synuclein, however, undergoes aberrant folding in neurodegenerative disorders. Aggregated α-synuclein is believed to be involved in the pathogenesis of Parkinson's disease, dementia with Lewy bodies, multiple system atrophy, and other synucleinopathies [3–5]. The mechanisms of α-synuclein toxicity are not known. Aggregation and toxicity of α-synuclein may be related to its 35-amino acid central domain (residues 61–95) known as the nonamyloid component [6]. Recent data suggest that α-synuclein is liberated into extracellular space under pathogenic conditions [7], and our previous results indicate that extracellular α-synuclein affects the function of presynaptic terminals via nitric oxide (NO)-dependent inhibition of dopamine transporter activity [8]. Growing evidence points to a disruption of intracellular Ca^{2+} homeostasis in neurodegenerative diseases [9], and elevated intracellular Ca^{2+} levels are known to trigger apoptosis and/or excessive phosphorylation of key proteins that leads to cell death [10]. Amyloid beta (Aβ) peptide coexists with α-synuclein in neurodegenerative 'conformational' disorders [11] and is involved in dysregulation of Ca^{2+} homeostasis [12]. Thus, α-synuclein may exert its neurotoxic effects through modulation of Ca^{2+} influx. In the present study, we examined the effects of extracellular α-synuclein on the function of voltage-dependent Ca^{2+} channels in rat synaptoneurosomes that are enriched in released presynaptic structures (synaptosomes) with attached sealed postsynaptic entities.

Methods

Preparation of synaptoneurosomes

All animal experiments were approved by the Polish National Ethics Committee and were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Four-month-old male Wistar rats (250–300 g) were killed, and their brains were rapidly removed. The cerebral cortex, including the hippocampus, was manually dissected with a chilled razor blade on an ice-chilled Petri dish. Synaptoneurosomes were prepared according to Hollingsworth *et al.* [13] with the modifications of Strosznajder and Samochocki [14] (Fig. 1). Briefly, brain slices were homogenized by hand (five strokes) in 7 ml Krebs–Henseleit buffer, pH 7.4 [120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose equilibrated with O_2/CO_2 (95/5)], using a Dounce-type glass homogenizer. The homogenate was diluted with 28 ml Krebs–Henseleit buffer and centrifuged at 1100*g* for 15 min. The supernatant was decanted, and the pellet was resuspended in 5 ml of the

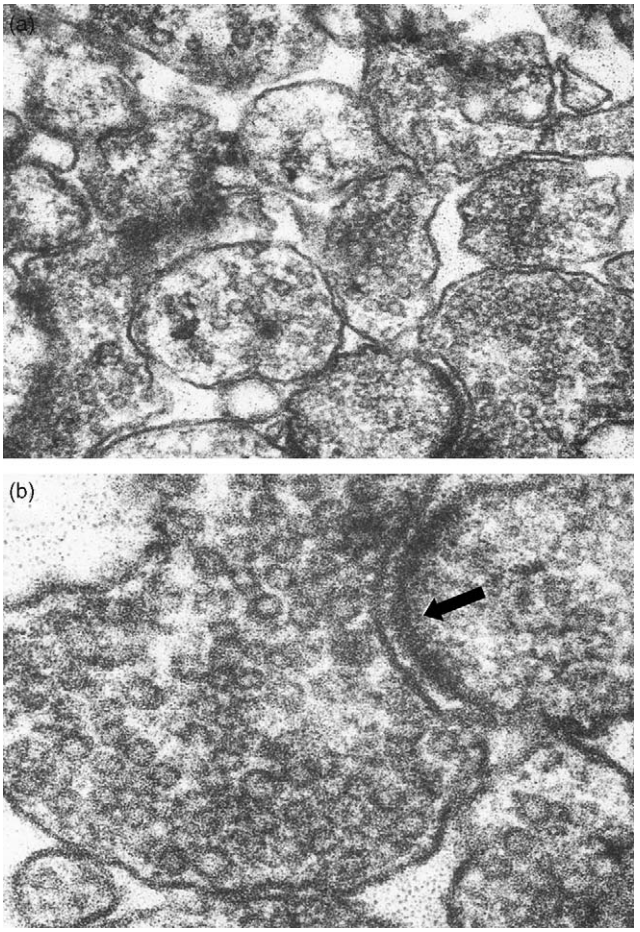

Fig. 1 Electron micrograph showing unfiltered particulate preparation from rat brain cortex. (a) Enrichment of preparation with synaptoneurosomes. × 20 000. (b) A typical profile of a synaptoneurosome. The presynaptic profile with characteristic vesicles is attached at the postsynaptic density to a postsynaptic structure (see arrow). × 40 000.

assay buffer, with 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid equilibrated to pH 7.0 with Tris base. The preparation was preincubated at 37°C for 30 min in an O_2/CO_2 atmosphere.

Assay of $[^{45}]Ca^{2+}$ influx

Approximately 0.7 mg of the synaptoneurosome preparation in 0.1 ml assay buffer with 1 mM $CaCl_2$ was preincubated at 37°C for 30 min either alone or with α-synuclein, its nonamyloid component, or β-synuclein at a final concentration of 10 μM. In selected experiments, synaptoneurosomes were first preincubated with 0.01–1 mM nimodipine, 10 μM ω-conotoxin GVIA, 100 μM N^{ω}-nitro-L-arginine, or 50 μM resveratrol for 5 min before the addition of α-synuclein. The reaction was started by adding 0.1 μCi $[^{45}]CaCl_2$ (522 MBq/mg $CaCl_2$) and incubating at 37°C for 30 s. The samples were diluted with 5 ml ice-cold assay buffer with 5 mM ethylene glycol-bis (*b*-aminoethyl ether), immediately filtered through Whatman GF/C filters under vacuum pressure, and washed three times in 5 ml assay buffer. Filters were placed in 8 ml Bray's scintillation fluid, and intrasynaptoneurosome $[^{45}]Ca^{2+}$ was measured in an LKB Wallac 1409 counter.

Measurement of cytosolic $[Ca^{2+}]_i$

Synaptoneurosomes were incubated with 5 μM Fura-2/AM for 1 h at 30°C in oxygenated Krebs–Henseleit buffer. Fura-2/AM-loaded synaptoneurosomes were centrifuged at 12 300*g* for 5 min, and the pellet was resuspended in Krebs–Henseleit buffer to a final concentration of 1 mg/ml and incubated for 30 min at 30°C with or without 10 μM α-synuclein. The synaptoneurosomes (0.4 ml) were placed in a cuvette in a spectrophotometer. Experiments were performed at 30°C. Changes in $[Ca^{2+}]_i$ were determined by the ratio of fluorescence at 340 and 380 nm and a constant 510 nm emission, as described previously [15]. The maximum fluorescence ratio (R_{max}) was achieved by lysing synaptoneurosomes with 5 μl 10% Triton X-100, and the minimum fluorescence ratio (R_{min}) was achieved by adding 50 μl of 0.5 M ethylene glycol-bis (*b*-aminoethyl ether). Correction for autofluorescence in the synaptoneurosome preparation was made before making final determinations of $[Ca^{2+}]_i$.

Results

We evaluated the effect of 10 μM extracellular α-synuclein, its neurotoxic nonamyloid component, and β-synuclein on $[^{45}]Ca^{2+}$ influx in rat synaptoneurosomes. Data are reported as mean ± SEM. In the presence of 5 mM KCl, the basal $[^{45}]Ca^{2+}$ influx in freshly prepared synaptoneurosomes was maintained at 991 ± 94 dpm/mg protein (control). A strong membrane depolarization, evoked by the addition of 75 mM KCl, produced a significant increase in $[^{45}]Ca^{2+}$ influx (1508 ± 129 dpm/mg protein). Alpha-synuclein and its neurotoxic, nonamyloid component enhanced basal $[^{45}]Ca^{2+}$ influx in synaptoneurosomes during the 30-min preincubation by 42 and 56% (Fig. 2), and the same concentration of β-synuclein had a negligible effect (Fig. 2). The activation of $[^{45}]Ca^{2+}$ influx by α-synuclein was reduced by 10 μM ω-conotoxin GVIA, an N-type specific voltage-dependent Ca^{2+} channel blocker (Fig. 3). Addition of 75 mM KCl enhanced $[^{45}]Ca^{2+}$ influx in synaptoneurosomes, and the effect was decreased by ω-conotoxin GVIA. Inhibition of L-type voltage-dependent Ca^{2+} channels by 10 μM nimodi-

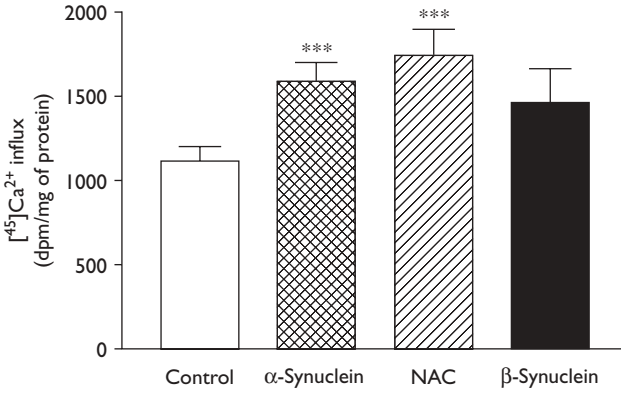

Fig. 2 Alpha-synuclein and nonamyloid component induce $[^{45}]Ca^{2+}$ influx in rat brain synaptoneurosomes. Data represent the mean ± SEM. $[^{45}]Ca^{2+}$ influx from 3 to 14 animals. Each experiment was carried out in triplicate. The data were analysed with one-way analysis of variance followed by Newman–Keuls test. ***$P < 0.001$. NAC, nonamyloid component of α-synuclein.

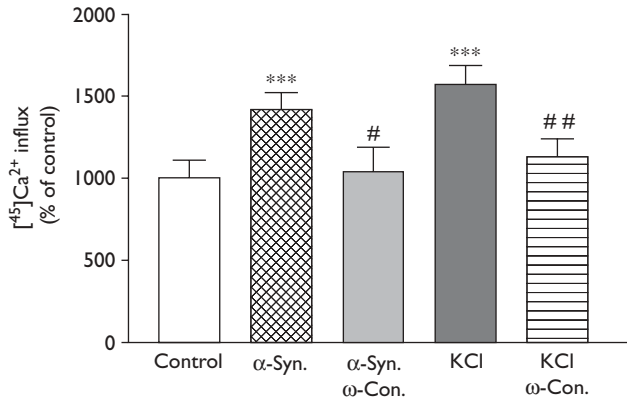

Fig. 3 Omega-conotoxin GVIA prevents α-synuclein-induced $[^{45}]Ca^{2+}$ influx in rat brain synaptoneurosomes. Data represent the mean ± SEM. $[^{45}]Ca^{2+}$ influx from 2 to 12 animals. Each experiment was carried out in triplicate. Data were analysed with one-way analysis of variance followed by a Newman–Keuls test. $^{***}P < 0.001$ versus control, $^{\#}P < 0.05$ versus α-synuclein, $^{\#\#}P < 0.05$ versus KCl. α-syn., α-synuclein; ω-con., ω-conotoxin GVIA.

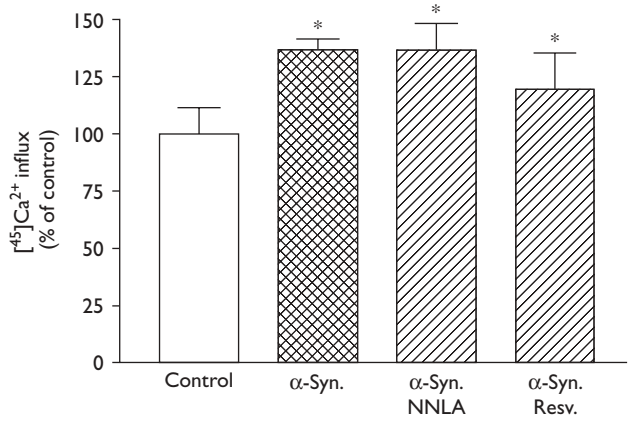

Fig. 4 Effect of N^{ω}-nitro-L-arginine and resveratrol on α-synuclein-induced $[^{45}]Ca^{2+}$ influx in rat brain synaptoneurosomes. Data represent the mean ± SEM. $[^{45}]Ca^{2+}$ influx from three animals. Each experiment was carried out in triplicate. Data were analysed with one-way analysis of variance and a Newman–Keuls test. $^{*}P < 0.05$ versus control. α-syn., α-synuclein; NNLA. N^{ω}-nitro-L-arginine; Resv., resveratrol.

pine did not prevent $[^{45}]Ca^{2+}$ influx evoked by α-synuclein. High concentrations of nimodipine (e.g. 1 mM) act at voltage-dependent Ca^{2+} channels other than L-type channels and prevent $[^{45}]Ca^{2+}$ influx evoked by α-synuclein (data not shown). We previously showed that α-synuclein activated nitric oxide synthase (NOS) and free radical generation [8]. To determine whether reactive oxygen species, including NO, are involved in α-synuclein-induced Ca^{2+} influx, we evaluated the effects of the antioxidant, resveratrol (50 μM) and the NOS inhibitor, N^{ω}-nitro-L-arginine (100 μM). Neither compound affected $[^{45}]Ca^{2+}$ influx evoked by α-synuclein (Fig. 4). Under the same experimental conditions, α-synuclein and its nonamyloid component did not elevate cytosolic Ca^{2+} concentration as measured by Fura-2/AM (data not shown).

Discussion

In the present study, we demonstrate for the first time that α-synuclein induces Ca^{2+} influx in rat synaptoneurosomes. Similar effects on Ca^{2+} entry have been reported using Aβ peptide. It is, however, not clear what specific type of Ca^{2+} channel is modulated by Aβ. Ueda *et al.* [12] reported that Aβ 25–35 increased Ca^{2+} influx in cultured rat cortical and hippocampal neurons via L-type voltage-dependent Ca^{2+} channels but not through N-type and P/Q-type channels. Similarly, our previous data showed that Aβ 25–35 in its aggregated form activated L-type voltage-dependent Ca^{2+} channels [16]. MacManus and coworkers [17] reported that Aβ 1–40 caused a significant increase in Ca^{2+} influx in rat cortical synaptosomes via activation of L-type and N-type voltage-dependent Ca^{2+} channels. In the present study, we attempted to identify the types of voltage-dependent Ca^{2+} channels that are modulated by α-synuclein and to clarify the mechanism of α-synuclein modulation. As the stimulatory effects of α-synuclein on Ca^{2+} influx in synaptoneurosomes in the present study were blocked by ω-conotoxin GVIA, we concluded that N-type voltage-dependent Ca^{2+} channels were involved in α-synuclein-induced increases in Ca^{2+} influx in our preparation. Reactive oxygen species have been suggested to be involved in Aβ-mediated Ca^{2+} influx [12]. The mechanism of α-synuclein-induced enhancement of Ca^{2+} influx, however, remains unclear. Furthermore, the action of NO on Ca^{2+} influx in neurons is not fully understood, and controversial results have accumulated, some demonstrating inhibition [18–20], some stimulation [21], and some even absence of effect of NO on Ca^{2+} influx [22]. Our previous study showed that α-synuclein induces free radical generation [8]. The present findings, however, indicate that the antioxidant, resveratrol, and the NOS inhibitor, N^{ω}-nitro-L-arginine, did not prevent α-synuclein-induced voltage-dependent Ca^{2+} channel stimulation. On the basis of these findings, we suggest that free radicals are not involved in α-synuclein modification of voltage-dependent Ca^{2+} channels. It is possible that α-synuclein, which interacts with plasma membranes through its nonamyloid component, activated voltage-dependent Ca^{2+} channels by protein–protein interactions. As α-synuclein had no effect on cytosolic calcium concentrations measured by Fura-2/AM, we suggest that Ca^{2+} entering through voltage-dependent Ca^{2+} channels is immediately loaded into intrasynaptoneurosomal calcium storage site, such as the endoplasmic reticulum and mitochondria.

Conclusion

Our findings indicate that α-synuclein induces Ca^{2+} influx in rat synaptoneurosomes through N-type voltage-dependent Ca^{2+} channels by a mechanism independent of free radical generation. The direct interaction between α-synuclein and N-type Ca^{2+} channel protein could be responsible for changes in the activity of the channels.

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Effect of N-methyl-D-aspartate (NMDA) receptor antagonists on α-synuclein-evoked neuronal nitric oxide synthase activation in the rat brain

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Abstract:
α-Synuclein (ASN), a small presynaptic protein that is abundant in the brain, is implicated in the pathogenesis of neurodegenerative disorders including Parkinson's and Alzheimer's disease. The central domain of α-synuclein, the non-amyloid β component of the Alzheimer's disease amyloid (NAC) is probably responsible for its toxicity. However, the molecular mechanism of α-synuclein action remains largely elusive. The present study examined the effect of α-synuclein and the NAC peptide on nitric oxide synthase (NOS) activity in rat brain cortical and hippocampal slices using a radiochemical technique. Moreover, nitrite levels in brain slices incubated in the presence of α-synuclein were measured using the Griess reaction. ASN and the NAC stimulated NOS activity by about 70% and 40%, respectively. β-Synuclein, a homologous protein of ASN that lacks the NAC domain, had no effect on NOS activity. Under the same experimental conditions, α-synuclein increased nitrite levels by 27%. α-Synuclein and the NAC affected the activity of constitutive neuronal isoform of NOS, but had no impact on the endothelial or inducible NOS isoforms. The effect of α-synuclein and the NAC peptide on NOS activity was inhibited by MK-801 and APV, antagonists of the NMDA receptor. These results indicate that the NMDA receptor plays an important role in α-synuclein-evoked nitric oxide synthesis. We suggest that nitric oxide liberated by the over-activated neuronal isoform of NOS could react with superoxide to form peroxynitrite, which modulates the function of a variety of biomolecules including proteins, lipids, and DNA.

Key words:
α-synuclein, NAC peptide, nitric oxide synthase, NMDA receptor, MK-801, APV

Abbreviations: APV – ((2R)-amino-5-phosphonovaleric acid; (2R)-amino-5-phosphonopentanoate); ASN – α-synuclein; Aβ – amyloid β-peptide; BSN – β-synuclein; MK-801 – (dizocilpine: (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate); MPTP – (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine); NAC – non-amyloid β component of Alzheimer's disease amyloid; NMDA – N-methyl-D-aspartate; NNLA – N^G-nitro-L-arginine; NO – nitric oxide; NOS – nitric oxide synthases (EC 1.14.13.39); O_2^{-} – superoxide; $^{\cdot}OH$ – hydroxyl radical; $ONOO^{-}$ – peroxynitrite; PD – Parkinson's disease

Introduction

α-Synuclein (ASN), a small (140 amino acids) acidic synaptic protein, is recognized in various cell types, but is primarily present in the central nervous system and is especially abundant in presynaptic terminals [37]. The function of ASN under normal physiological conditions, as well as its role in neurodegenerative

diseases, depends on its concentration and assembly state [31]. At nanomolar concentrations, ASN protects neurons against oxidative stress and inhibits apoptosis; it also plays important roles in synaptic plasticity and the regulation of vesicle transport and acts as a chaperone protein [37]. Conversely, ASN overexpressed at micromolar concentration in the cell results in cytotoxicity [5, 14]. Because ASN is a classical cytosolic protein, it was generally assumed that either its protective or pathogenic effect was limited to the intracellular organelles and proteins.

However, recent data has emphasized the significance of extracellular ASN and its internal hydrophobic fragment, the non-amyloid β component of the Alzheimer's disease amyloid (NAC) [8, 23, 25, 27]. In humans, ASN is present in blood plasma and cerebrospinal fluid in both monomeric and oligomeric forms [10, 18, 26, 42]. Moreover, intravesicular ASN could be secreted from cells *via* exocytosis [24]. Our previous data indicated that ASN is liberated from synaptoneurosomes into extracellular space during oxidative stress evoked by $FeCl_2$/ascorbate, hydrogen peroxide, and the nitric oxide (NO) donor sodium nitroprusside [2]. Seo and co-workers demonstrated that extracellular ASN increased the level of Bax protein and decreased Bcl-xL in PC12 cells leading to mitochondrial failure, cytochrome c release, caspase cascade activation, and cell death [36]. Moreover, our previous results showed that ASN enhanced the release and toxicity of the amyloid β-peptide (Aβ) leading to NO-mediated irreversible mitochondrial dysfunction and caspase-dependent programmed cell death [23]. Extracellular ASN and the NAC were shown to potentiate Ca^{2+} influx in rat synaptoneurosomes [4] and inhibit dopamine uptake into rat striatal synaptosomes by altering NO-mediated dopamine transporter activity [3].

Multiple lines of evidence indicate that increased NO synthesis leads to macromolecular oxidation as well as mitochondrial and DNA damage, which are common pathogenic mechanisms involved in Parkinson's disease (PD) and other neurodegenerative disorders. Pathologic studies in *post-mortem* PD brains and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice suggest that NO indeed plays an important role in PD [12, 43]. It was reported that neuronal NOS (nNOS) overexpression enhances the susceptibility of SH-SY5Y neuroblastoma cells to rotenone treatment [6]. In addition to the studies on PD-related neurotoxins, it has been demonstrated that NO evokes structural changes in the ASN protein. Giasson detected an accumulation of nitrated ASN in Lewy bodies of the PD brain [19]. Thus, nitration of ASN might enhance the formation of Lewy bodies in PD patients. Other studies show that parkin can be S-nitrosylated by NO, and this modification reduces parkin's E3 ligase activity [13, 45]. Taken together, it has been documented that NO, when converted to peroxynitrite ($ONOO^-$), becomes a powerful detrimental oxidant with direct neuropathological consequences.

Our previous results indicated that ASN and the NAC peptide stimulated the generation of NO and free radicals [3]. However, it is still far from clear how these toxic proteins contribute to NO overproduction. In the present work we have focused our attention on the role of particular NOS isoforms in extracellular ASN- and NAC-evoked enhancement of NO synthesis in the rat brain slices. Moreover, the study reports that the uncompetitive and competitive NMDA-receptor antagonists MK-801 and APV, respectively, elicit a protective effect against ASN- and NAC-evoked NO over-production.

Materials and Methods

All experiments on animals were accepted by the Polish National Ethics Committee, and were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Materials

ASN and β-synuclein (BSN) were obtained from rPeptide (Bogart, GA, USA). Protease inhibitor cocktails were from Roche (Mannheim, Germany). [^{14}C(U)]L-arginine (360 mCi/mmol), NAC peptide, and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

Preparation of ASN and evaluation of its oligomerization

ASN protein was dissolved in filtered, deionized, 18.2-MΩ water at a stock solution of 100 μM. For evaluation of ASN oligomerization, 3.6 μl of the stock solution, containing 5 μg of protein, was mixed with 2X SDS sample buffer and boiled for 5 min at

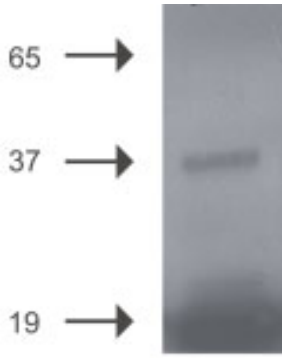

Fig. 1. Western blot analysis of ASN conformation. ASN was prepared and analyzed as described in Materials and Methods section. The western blot analysis with anti-ASN antibody indicates that ASN used in experiments is in monomeric (ca. 19 kDa)/ oligomeric (ca. 37 kDa) form

100°C. The electrophoretic mobility of ASN analyzed in this study was determined by SDS-polyacrylamide gel electrophoresis using 15% acrylamide concentrations. After electrophoresis, the proteins were transferred onto polyvinylidene fluoride membranes that were then blocked for 1 h in a solution of 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.3% Tween-20 (PBS-T). The membrane was then incubated overnight at 4°C with ASN antibody (1:1000) in 1% BSA solution in 0.1% PBS-T. After treatment for 1 h with anti-rabbit horseradish peroxidase-coupled secondary antibody (1:8000), the protein bands were visualized with enhanced chemiluminescence (GE Healthcare, UK) and light emission was detected by using Hyperfilm ECL (Kodak). The western blot analysis indicated that ASN used in these experiments is in monomeric (ca. 19 kDa)/oligomeric (ca. 37 kDa) form (Fig 1).

Preparation of brain slices

Four-month-old male Wistar rats (250–300 g) were decapitated and their brains were rapidly removed. For each rat, the cerebral cortex, including the hippocampus, was dissected and chopped into 0.35-mm sections in both the sagittal and coronal planes using a tissue chopper. Slices were placed in ice-cold calcium-free KRBS buffer (in mM: NaCl, 120; KCl, 5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 10) equilibrated with 5% CO_2 in 95% O_2 to maintain a pH of 7.4. Slices were then washed by decantation and stabilized in KRBS buffer under 5% CO_2 in 95% O_2 atmosphere at 37°C for 90 min.

Determination of the effects of ASN, BSN, and NAC on NOS activity

After stabilization, slices were washed twice with pre-warmed, magnesium-free KRBS containing 2 mM calcium and 1 μM glycine, and pre-incubated in the presence of 10 μM ASN, BSN, or NAC for 30 min at 37°C. In experiments analyzing response to a NMDA receptor antagonist (MK-801 [10 μM] or APV [10 μM]) [7, 40], an additional 5 min pre-incubation was performed.

At this point, slices were centrifuged at 1731 g for 10 min at 4°C and homogenized in 2 ml of ice-cold lysis buffer, pH 7.2 (in mM: Tris-HCl, 50; saccharose, 320; EDTA, 1; DTT, 1; protease inhibitor cocktail). NOS activity in the lysate was assayed by measuring the conversion of radiolabeled arginine to citrulline using ion-exchange separation as described by Czapski et al. [15]. Lysate (ca. 300 μg of protein) was incubated in a solution of 50 mM Tris-HCl pH 7.4, 100 μM [^{14}C]L-arginine (0.1 μCi), 2 mM $CaCl_2$, 1 μM calmodulin, 15 μM FAD, 10 μM tetrahydrobiopterin, 1 mM NADPH, 1 mM EDTA, and 1 mM DTT in a final volume of 300 μl. A separate set of experiments was designed to evaluate the activity of each NOS isoform. After pre-incubation with ASN or NAC, a NOS assay was performed for each sample in conditions inhibiting activity of particular NOS isoforms: A) in the absence of any inhibitor for determination of total NOS activity; B) in the presence of 10 μM 7-nitroindazole (7-NI) for inhibition of nNOS activity; C) in the absence of Ca^{2+} and in the presence of 2 mM EGTA and 100 μM N^Gnitro-L-arginine (NNLA) for inhibition of constitutive NOS activity (Table 1). These inhibitors of NOS were added 5 min before incubation. The mixture was incubated for 20 min at 37°C. Reactions were stopped by adding 1 ml 100 mM Tris-HCl buffer, pH 5.5, containing 10 mM EDTA. After centrifugation at 3000 g for 10 min, 0.5 ml of supernatant was passed through 0.5 ml of Dowex™ 50WX-8 (Serva, Germany) column (Na^+ form) and [^{14}C]L-citrulline was eluted with 2 1 ml H_2O. The radioactivity of eluted and [^{14}C]L-citrulline was determined using Bray's scintillation solution in a Wallac 1409 counter (Wallac Oy, Finland).

Determination of the effect of ASN on nitrite level

Nitrite levels in brain slices were determined using Griess reagent [20]. Slices from the brain cortex in-

Tab. 1. Calculation of activity of separate NOS isoforms in rat brain slices

	Inhibitors	Description	Control	ASN	NAC
			NOS activity [pmol mg protein^{-1} min^{-1}]		
A	–	total NOS	11.51 ± 1.49	18.88 ± 1.36**	15.59 ± 0.63*
B	7NI	nNOS-devoid	2.21 ± 0.32	2.91 ± 0.17	2.59 ± 0.19
C	NNLA + EGTA	iNOS	0.45 ± 0.23	0.44 ± 0.20	1.00 ± 0.45

Activities of separate NOS isoforms were calculated according to formulas: nNOS act. = A – B; iNOS act. = C; eNOS act. = A – nNOS act. – iNOS act., respectively. Data represent the mean value ± SEM for 3 separate experiments, each carried out in triplicate. Significance of data was determined with one-way ANOVA followed by Bonferoni *post-hoc* test, *, **p < 0.05, 0.01 *vs.* control

cluding hippocampus were prepared for this determination in the same manner as for the NOS activity assay. After a 30-min pre-incubation in the presence of 10 μM ASN or BSN, slices were homogenized and centrifuged at 21,000 g for 20 min. Supernatant (100 μl) or serial dilution of $NaNO_2$ standard (linear range 0–100 μM) were applied to a microtiter plate well. The total nitrite in each sample was then determined by adding 50 μl Griess reagent 1 (1% sulfanilamide in 1M HCl), followed by Griess reagent 2 (0.1% N-(1-napthyl)-ethylenediamine). The mixture was incubated for 10 min at room temperature in the dark. Optical density was measured spectrophotometrically at 540 nm. Nitrite level was expressed as nanomoles per mg of tissue protein.

Statistical analysis

Analyses among multigroup data were conducted using one-way analysis of variance (ANOVA), followed by a Bonferoni *post-hoc* test. Differences among groups were considered significant if the probability of error was less than 5%. The data represent the mean ± SEM. Results presented as percent of control were calculated as crude data, statistically analyzed and then re-calculated and expressed as % of control, considering value of control group as 100%.

Results

This study analyzed the effects of extracellular monomeric/oligomeric ASN and NAC peptides on NOS activity in rat brain slices. As shown in Figure 2, 10 μM ASN or the toxic NAC fragment significantly

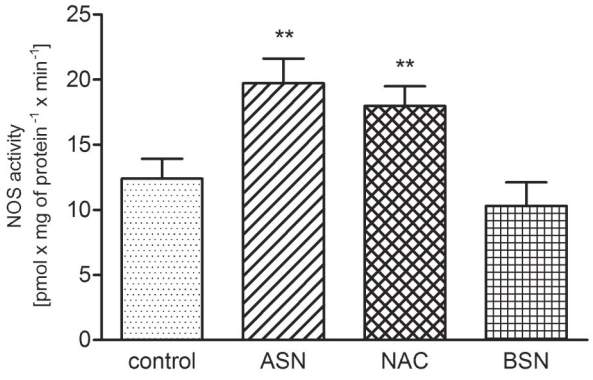

Fig. 2. The effects of ASN, BSN, and NAC on NOS activity in rat brain slices. NOS activity was measured in rat brain slices after 30-min incubation in the absence (control) and presence of 10 ĘM ASN, BSN, or NAC. Data represent the mean value ± SEM for 5 separate experiments, each carried out in triplicate. Significance of the data was determined with one-way ANOVA followed by the Bonferoni *post-hoc* test, ** p < 0.01 *vs.* control

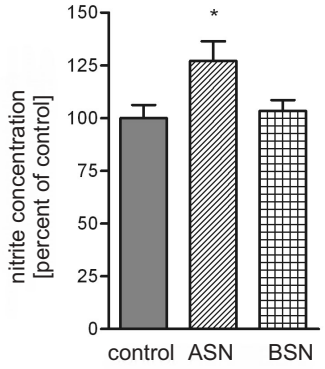

Fig. 3. ASN increases nitrite formation in rat brain slices. Nitrite level was measured in rat brain cortex including hippocampus after 30-min pre-incubation in the absence (control) and presence of 10 μM ASN or BSN. Data represent the mean values ± SEM, expressed as % of control value, for 2–8 determinations. Significance of the data was determined with one-way ANOVA followed by the Bonferoni *post-hoc* test, * p < 0.05 *vs.* control

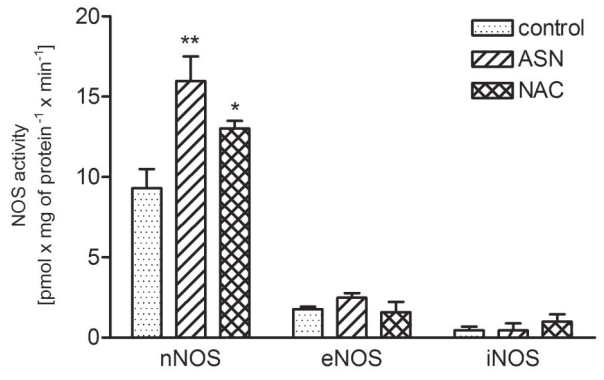

Fig. 4. The effects of ASN and NAC on the activity of separate NOS isoforms in rat brain slices. NOS activity was measured in rat brain slices after 30-min incubation in the absence (control) and presence of 10 μM ASN or NAC. Data represent the mean value ± SEM for 3 separate experiments, each carried out in triplicate. Significance of the data was determined with one-way ANOVA followed by the Bonferoni *post-hoc* test; *, ** $p < 0.05$, 0.01 *vs.* control

stimulated total NOS activity during the 30-min pre-incubation period. Treatment of brain slices with 10 μM BSN that lacks the NAC region did not change NOS activity, indicating the effect to be specific to the isoform of synuclein (Fig. 2). Under the same experimental conditions, ASN increased the level of nitrite in the brain slices by 27%, whereas BSN had no effect

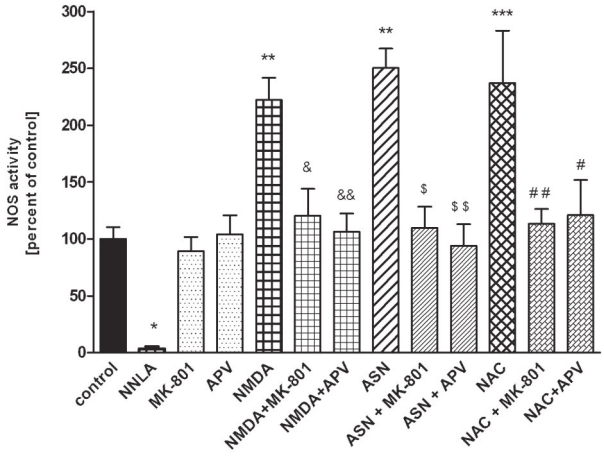

Fig. 5. Involvement of NMDA receptor in ASN and NAC-evoked NOS activation. NOS activity was measured in rat hippocampal slices. Data represent the mean value ± SEM (expressed as % of control value) for 3–8 separate experiments, each carried out in triplicate. NNLA was used as a positive control. Significance of the data was determined with one-way ANOVA followed by the Bonferoni *post-hoc* test; *, **, *** $p < 0.05$, 0.01 and 0.001 *vs.* control, $^{\$}$, $^{\$\$}$ $p < 0.05$ and 0.01 *vs.* ASN $^{\#}$, $^{\#\#}$ $p < 0.05$ and 0.01 *vs.* NAC $^{\&}$, $^{\&\&}$ $p < 0.05$ and 0.01 *vs.* NMDA

(Fig. 3). To analyze which NOS isoform was activated by ASN and NAC peptides, the NOS assays were performed in conditions enabling calculation of the activity of each isoform, as presented in Table 1. As shown in Figure 4, the investigated proteins exclusively affected the constitutively active neuronal isoform of NOS (nNOS) without affecting the endothelial or inducible NOS isoforms. ASN and the NAC stimulated nNOS by about 70% and 40%, respectively (Fig. 4). Because the primary pool of nNOS is physically and functionally connected to the NMDA receptor, we further investigated the involvement of this receptor in nNOS stimulation evoked by ASN and the NAC. Both NMDA receptor antagonists, competitive APV (10 μM) and uncompetitive MK-801 (10 μM) prevented NOS stimulation evoked by ASN and the NAC, suggesting that activation of NMDA receptor may be responsible for this peptide-evoked NOS activation (Fig. 5).

Discussion

ASN is a small presynaptic protein that is released by neurons into extracellular space, both as a part of its normal cellular processing [18] and under oxidative/nitrosative stress and neurodegeneration [2, 41]. In this study, we have shown for the first time that extracellular ASN in monomeric/oligomeric form activates NMDA receptor-mediated NO synthesis in rat brain slices. Moreover, BSN, which is characterized by the lack of the NAC domain, had no effect on NOS activity. Prolonged action of ASN on cells in culture (8–24 h) leads to increase of nNOS mRNA expression without effect on the endothelial or inducible protein isoforms (data not shown). Our findings are consistent with previous data showing that the action of ASN is related to its internal NAC domain [3, 4, 17]. Similar effects of nNOS activation in the cerebral cortex and hippocampus were observed in the presence of amyloid β-peptide (Aβ), which is an amyloidogenic protein and may interact with ASN [32, 39]. Overactivation of NMDA receptors may trigger cytotoxicity and cell death [9]. The increased intracellular Ca^{2+} concentration leads to activation of many Ca^{2+} dependent enzymes, including nNOS. The main product of NOS-catalyzed reactions is NO, but in some conditions (low levels of the substrate arginine or its

co-factor, tetrahydrobiopterin) the superoxide radical (O_2^-) and H_2O_2 can also be produced by NOS. NO alone is a rather low-reactivity radical; it can diffuse freely in tissue, but may react with O_2^- to form $ONOO^-$, a very potent oxidizing agent. It is now well documented that NO and its toxic metabolite, $ONOO^-$, can inhibit components of the mitochondrial respiratory chain leading to a cellular energy deficiency [16]. Our previous results indicated that the NAC peptide activated generation of free radicals and caused DNA fragmentation in rat brain slices; enhanced $ONOO^-$ production might be responsible for this DNA damage [1].

$ONOO^-$ and other reactive nitrogen species can modify protein functions by nitration and nitrosylation, which may result in cell damage and death. Schulz et al. [35] have shown that systemic administration of 7-nitroindazole attenuates lesions produced by striatal malonate injections or systemic treatment with 3-nitropropionic acid or MPTP. Furthermore, 7-nitroindazole attenuates increases in lactate production and hydroxyl radical (OH) and 3-nitrotyrosine generation *in vivo,* which may be a consequence of $ONOO^-$ formation.

ASN stimulates NOS activity in isolated mitochondria and increases the intramitochondrial calcium concentration $[Ca^{2+}]_m$ [33]. However, until now, the detailed mechanism of how ASN induces NOS stimulation and NO liberation has been unknown. In the present study we identified that ASN and NAC exclusively affect nNOS. Our previous findings indicated that ASN induces Ca^{2+} influx in rat synaptoneurosomes [4]. The present data demonstrate that NMDA receptor inactivation by specific antagonists MK-801 and APV prevents NOS stimulation evoked by ASN and the NAC.

It has been suggested that accumulation of the Aβ peptide, which displays many similarities to ASN, is partly responsible for triggering neurodegeneration *via* glutamate-mediated excitotoxicity in AD brain. This is supported by observations that toxicity induced by Aβ in cultured neurons and in the adult rat brain is mediated by activation of NMDA receptors. Harkany and co-workers indicated that NMDA receptor antagonist MK-801 protected against the neurotoxic events evoked by administration of Aβ 1–42 into the magnocellular nucleus basalis of rats [21]. Additionally, recent clinical studies have shown that memantine, an uncompetitive NMDA receptor antagonist, can significantly improve cognitive functions in some AD patients [30]. The last data demonstrated that Aβ (1–42)-induced toxicity in rat primary cortical cultured neurons is accompanied by increased extracellular and decreased intracellular glutamate levels. Memantine treatment significantly protected cultured neurons against Aβ-induced toxicity by attenuating phosphorylation and its associated signaling mechanisms [38]. Moreover, nitromemantine, a second-generation memantine derivative, is highly neuroprotective in both *in vitro* and *in vivo* animal models [29].

The previous investigations showed that ASN, through the NAC region, interacts with membrane proteins and through this interaction can modulate the function of synaptic terminals [44]. The detailed mechanism of how ASN activates NMDA receptor-mediated NO synthesis remains to be elucidated. However, on the basis of the present data we suggest that extracellular ASN in monomeric/oligomeric form interacts with plasma membranes, probably through the NAC domain, and activates NMDA channels, leading to an increase in the intracellular Ca^{2+} concentration and nNOS activation. Since both competitive and uncompetitive antagonists of the NMDA receptor prevented ASN-evoked NOS activation, we suggest that this protein could interact with lipid bilayers and consequently affect the NMDA receptor structure leading to its activation. Similar non-specific activation of glutamatergic receptors was recently observed for prefibrillar aggregates of Aβ [34]. Moreover, it was presented recently that Aβ up-regulates metabotropic glutamate receptors [11]. Another possibility is that ASN may affect extracellular glutamate levels. As was recently demonstrated, Aβ can increase glutamate release and inhibit glutamate uptake [22, 28].

In summary, our findings indicate that ASN and its fragment NAC stimulate nNOS activity *via* the NMDA receptor-mediated pathway. NO liberated by over-activated nNOS could react with O_2^- to form $ONOO^-$, which modulates the function of a variety of biomolecules including proteins, lipids, and DNA. Chronic stimulation of NMDA receptor-dependent signaling by ASN could contribute to the development of neurodegenerative processes.

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α-Synuclein and its neurotoxic fragment inhibit dopamine uptake into rat striatal synaptosomes Relationship to nitric oxide

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Abstract

α-Synuclein (ASN), a 140-amino acid protein, is richly expressed in presynaptic terminals in the central nervous system, where it plays a role in synaptic vesicle function. However, if it is altered and accumulated it is involved in neurodegeneration as Parkinson's disease (PD). ASN contained 35-amino acid domain known as non-amyloid beta component of Alzheimer's disease amyloid (NAC) that is probably responsible for its aggregation and toxicity. Up till now the role of ASN in dopaminergic system function and in pathogenesis of PD is unknown. The aim of this study was to determine the effect of brain aging and the role of ASN and NAC peptide on striatal dopamine transporter (DAT) function. The study was carried out using radiochemical and spectrofluorimetrical determination. It was found that DAT activity assessed by measuring [^{3}H]-dopamine (DA) uptake into striatal synaptosomes significantly decreased in 24-month-old rats comparing to 4-month-old. ASN and NAC peptide at 10 μM concentration inhibited DAT activity by 30%. Both molecules evoked intrasynaptosomal generation of reactive oxygen species measured by fluorogenic probe, 2′7′-dichlorofluorescin diacetate. In addition, ASN activated striatal cytosolic nitric oxide synthase (NOS) by 20%. Nitric oxide (NO) donor, sodium nitroprusside (SNP) (10 μM) and oxidative stress evoked by $FeCl_2$ (25 μM) reduced [^{3}H]DA uptake by 28 and 41%, respectively. Potent antioxidants: Trolox and 4-hydroxy-Tempo had no effect on DAT function but NOS inhibitor Nω-nitro-L-arginine (100 μM), prevented ASN-evoked DAT down-regulation. These data indicated an important role of ASN in alteration of DA synaptic homeostasis, probably by NO mediated DAT alteration.

Keywords: Alpha-synuclein; Dopamine transporter; Striatum; Nitric oxide

1. Introduction

Alpha-synuclein (ASN) is an ubiquitous protein that is especially abundant in the brain and has been postulated to play a central role in the pathogenesis of Parkinson's disease (PD), dementia with Lewy bodies (DLB), neurodegeneration with brain iron accumulation type 1 (NBIA-1), and other age-related neurodegenerative disorders which collectively are termed synucleinopathies. The link between neurodegenerative disease and ASN, however, was first reported for Alzheimer's disease (AD) when the small peptide termed 'NAC' was purified from AD brain, leading to the cloning of the non-amyloid beta component precursor protein (NACP, Ueda et al., 1993), now known to be ASN. More recent studies revealed that NAC peptide, which is a 35-amino acid fragment of ASN (residues 61–95) could be responsible for its aggregation and toxicity (Bodles et al., 2000; Giasson et al., 2001). Accumulating data showed close relationship between ASN and dopaminergic neurotransmission involved in the control of locomotor activity and regulation of goal-oriented behavior and reward (Schultz, 2002). It is suggested that ASN in native soluble form modulates dopamine (DA) biosynthesis, storage into vesicles and release, as well as the reuptake of this neurotransmitter (Perez and Hastings, 2004; Yu et al., 2005). Aggregation of ASN into protofibrils causes loss in its normal functions and dysregulation of dopaminergic neurotransmission. Increased level of DA could activate free radicals formation. Enzymatic deamination of DA by monoamine oxidases (MAO) leads to the production of the non-toxic 3,4-dihydroxyphenylacetic acid

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(DOPAC) and hydrogen peroxide (H_2O_2) (Maker et al., 1981). In turn, H_2O_2 can be converted to highly toxic hydroxyl radicals. DA can also undergo spontaneous auto-oxidation into toxic and reactive DA-quinones, superoxide free radicals and H_2O_2 (Graham et al., 1978). On the other hand, the reactive oxygen species (ROS) produced by DA metabolism could promote aggregation and toxicity of ASN (Conway et al., 2001; Xu et al., 2002). It was reported by Lee et al. (2001) that DA transporter (DAT) activity is stimulated by ASN. However, Wersinger et al. (2003) observed inhibitory effect. The last data showed that ASN is not only accumulated intracellular but also can acts extracellularly (Seo et al., 2002; Zhang et al., 2005; Sung et al., 2005). Moreover, full-length ASN was found in cerebrospinal fluid (CSF) from PD patients and also in age-related control (Borghi et al., 2000). These all results indicated that ASN could be present extracellularly and exert toxicity. The aim of our study was to investigate the role of ASN and its neurotoxic fragment NAC on DA uptake into striatal synaptosomes. Moreover, the relationship between DA uptake and ASN alteration in aged brain was evaluated.

2. Materials and methods

2.1. Materials

The following products were used: [2,5,6-^{3}H]DA, 8.50 Ci/mmol (Amersham Biosciences, UK), α-synuclein (rPeptide, USA). All other chemicals were purchased from Sigma.

2.2. Animals

Male Wistar rats adult (4-month-old) and old (24-month-old) obtained from the Animal Breeding House of the Medical Research Centre, Warsaw, Poland were used in all experiments. Animals were kept behind the barrier system and were specific pathogen free (SPF). The Institutional Ethics Committee accepted the research project. After animals' decapitation, brains were rapidly removed and the striatum dissected on ice-cooled dish according to Glowinski and Iversen (1966).

2.3. Preparation of striatal synaptosomal fraction

Synaptosomes were prepared as previously described by Morel et al. (1998), with slight modifications. Striatum were homogenized in 10 vol. of 0.32 M sucrose with 10 mM Tris–HCl and 1 mM EDTA, pH 7.4 using 12 up- and down-strokes of glass–glass Dounce homogenizer. The homogenates were then centrifuged at 1000 × *g* for 10 min at 4 °C to obtain a crude nuclear pellet. Supernatants were stored at 4 °C, and the pellet was re-suspended in 10 vol. of homogenizing buffer and centrifuged for 10 min at 1000 × *g*. The two supernatants were pooled and centrifuged at 17,500 × *g* for 30 min at 4 °C. The resulting supernatant was discarded and the synaptosomal fraction was re-suspended in ice-cold Krebs–Ringer buffer, pH 7.6 (NaCl, 120 mM; KCl, 5 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; glucose, 10 mM). Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.4. Preparation of striatal plasma membranes and cytosol

Striatum was homogenized using 14 up- and down-strokes of glass–glass Dounce homogenizer in 0.32 M sucrose containing 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT) and protease inhibitors (Roche Diagnostics GmbH, Germany). The homogenate was then centrifuged at 900 × *g* for 3 min at 4 °C. Resulting pellet (P_1) was discarded and the supernatant (S_1) was centrifuged at 100,000 × *g* for 60 min at 4 °C to obtain cytosolic fraction (S_2). Plasma membranes (PM) were obtained as described previously (Zambrzycka et al., 2000).

2.5. Measurement of DA uptake

DA uptake was assayed as previously described by Morel et al. (1998), using tritiated ([^{3}H]DA) and non-labeled DA to obtain a final concentration 400 nM. Synaptosomes were pre-incubated for 30 min at 37 °C in 2 ml Krebs–Ringer buffer. Then $CaCl_2$ and pargyline was added to obtain 2 mM and 100 μM concentration, respectively. ASN (10 μM), NAC (10 μM) or other investigated compounds were subsequently added to the appropriate tube and pre-incubation was prolonged for the other 30 min at 37 °C. Pre-incubations were performed in the presence and absence of nomifensine (10 μM), a specific DAT inhibitor, in order to check the specific DA uptake. The assay was initiated by addition of [^{3}H]DA to synaptosomes (60–100 μg protein/assay). After 40-s, 2-min and 5-min of DA uptake at 37 °C the reaction was stopped by addition of 3 ml of ice-cold Krebs–Ringer buffer, followed by vacuum filtration through Whatman GF/C filters. Each tube was rinsed 2× 3 ml of ice-cold Krebs–Ringer buffer. The filters were placed in scintillation vials containing 7 ml of scintillator and stored at room temperature overnight. Radioactivity was determined by liquid scintillation spectrometry (Wallac 1409, LKB). Specific uptake was calculated by subtracting non-specific uptake, determined in the presence of nomifensine, from total uptake. In each experiment, assays were performed in duplicate.

2.6. Determination of oxidative stress

Intrasynaptosomal generation of ROS was measured using fluorogenic probe, 2′7′-dichlorofluorescin diacetate (also known as 2′7′-didichlorodihydrofluorescein diacetate; DCFH-DA). DCFH-DA is intracellularly/intrasynaptosomal deacetylated to 2′7′-dichlorofluorescin (DCFH) and then oxidized by hydrogen peroxide to a fluorescent compound, 2′7′-dichlorofluorescein (DCF). Freshly isolated synaptosomes suspended in Locke's buffer, pH 7.2 (NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2$, 2.3 mM; $MgCl_2$, 1.0 mM; $NaHCO_3$, 3.6 mM; Hepes, 5 mM; glucose, 5 mM) were mixed with DCFH-DA at 15 μM concentration and incubated in a water shaking bath in the presence of ASN and NAC at 10 or 25 μM $FeCl_2$ in the dark for 30 min at 37 °C under carbogen. The concentration of DCF was measured by a fluorescence spectrophotometer (ELS 50B, Perkin-Elmer) with excitation at 488 nm and emission at 530 nm.

2.7. Lipid peroxidation assay

Lipid peroxidation was evaluated by determining the malondialdehyde using thiobarbituric acid assay (Asakawa and Matsushita, 1980). Synaptosomes suspended in Krebs–Ringer buffer, pH 7.4 at protein concentrations of approximately 1.0 mg/ml were incubated for 30 min at 37 °C with 10 μM SNP or $FeCl_2$ (25 μM)/ascorbic acid (250 μM). After incubation, 1 ml of 30% trichloroacetic acid (TCA), 0.1 ml of 5 M HCl and 1 ml of 0.75% thiobarbituric acid were added to the vials. The tubes were capped, the mixtures were heated at 100 °C for 15 min in a boiling water bath, and then centrifuged at 1000 × *g* for 10 min. Thiobarbituric acid reactive substances (TBARS) were measured in supernatant at 535 nm.

2.8. Nitric oxide synthase assay

Nitric oxide synthase activity was assayed in striatal cytosol as described by Strosznajder and Chalimoniuk (1996). Cytosol (about 350 μg/150 μl) was pre-incubated for 30 min at 37 °C in the presence and absence (control) of 10 μM ASN. Then, 150 μl of assay mixture containing 50 mM Tris–HCl, pH 7.4, 100 μM arginine L-[^{14}C (U)] 0.1 μCi (sp. act. 360 mCi/mmol), 2 mM $CaCl_2$, 1 μM calmodulin, 15 μM FAD, 10 μM tetrahydrobiopterin (BH_4), 1 mM NADPH, 1 mM EDTA and 1 mM DTT were added to each vial. The mixture was incubated for 20 min at 37 °C. Reaction was terminated by addition of 1ml buffer containing 100 mM Tris–HCl, pH 5.5 with 10 mM EDTA. Then the mixture (500 μl) was passed through 0.5 ml of Dowex™ 50WX-8 columns (Na^+ form) and [^{14}C]L-citrulline was eluted with H_2O (2× 1 ml). Radioactivity of eluted [^{14}C]L-citrulline was determined using Bray's solution in LKB Wallac 1409 counter.

2.9. Western blot analysis

Aliquots containing equal amounts of protein from cytosol or PM were diluted 1:1 in 2× electrophoresis sample buffer (2× SB) and incubated at 95 °C for 5 min. Then, proteins were loaded onto 15% polyacrylamide denaturing gels (40 μg per lane) and subjected to electrophoresis (applied constant voltage, 100 V). After protein separation, each gel was placed on a nitrocellulose membrane (Amersham, Hybond-Extra C) in transfer buffer, and the proteins were transferred to the membrane by the application of 100 V for 2 h. After the transfer was completed, equal protein loading and protein transfer was confirmed by Ponceau S staining. In order to block non-specific binding, the membrane was incubated at room temperature (RT) for 1 h in 5% dried skimmed milk and 0.3% Tween 20 in phosphate-buffered saline (PBS), pH 7.4. Incubation with anti-synuclein α antibody (Sigma, St. Louis, MO, USA, 1:1000 dilution) in PBS with 0.1% Tween 20 and 1% bovine serum albumin (BSA) was performed overnight at 4 °C. Then blots were washed three times for 15 min at RT in PBS with 0.1% Tween 20. The next membranes were incubated with horseradish peroxidase (HRP) conjugated anti-rabbit IgG secondary antibody diluted 1:8000 (Sigma, St. Louis, MO, USA) for 1 h at RT. After that blots were washed as described above and bound antibodies visualized by enhanced chemiluminescence (Amersham, UK). Incubation with anti-β actin antibody confirmed additionally equal protein loading. Quantification of immunoblots was performed with the NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

2.10. Statistical analysis

Statistical analyses between two groups were conducted using a two-tailed, paired Student's *t*-test. Analyses among multigroup data were conducted using one-way analysis of variance (ANOVA), followed by a Newman–Keuls post hoc test. Differences among groups were considered significant if the probability of error was less than 5%. The data represent mean ± S.E.M.

3. Results

DA (400 nM) uptake was measured into striatal synaptosomes from 4- and 24-month-old rats. In aged animals DAT activity was suppressed by 40–50% comparing to adult (Fig. 1). ASN immunoreactivity decreased by 30% in PM from aged striatum (Table 1). Results presented in Fig. 2 demonstrated that ASN and NAC peptide at 10 μM inhibited DAT activity in striatal synaptosomes by about 30% at each incubation time point. The question arises through what kind of processes ASN and NAC peptide could be responsible for DAT alteration. The free radicals mechanism was evaluated. Our data indicated that ASN and NAC peptide at 10 μM concentration activated free radicals generation in rat striatal synaptosomes by 20 and 80%, respectively, comparing to control. The effect of NAC was similar to $FeCl_2$ (Fig. 3). Moreover, ASN enhanced striatal cytosolic NOS activity by 20% (Fig. 4). NO donor SNP (10 μM) and $FeCl_2$ (25 μM) in the presence of ascorbic acid (250 μM) caused significant inhibition of DAT activity by 28 and 41%, respectively (Fig. 5), and increased membrane lipid peroxidation (Fig. 5). Inhibition of NOS by Nω-nitro-L-arginine (NNLA, 100 μM) prevented ASN-evoked DAT down-regulation (Fig. 6). Antioxidants used in this study, Trolox and 4-hydroxy-Tempo that imitates superoxide dismutase had no effect on DAT function altered by ASN and NAC (data not shown).

Table 1
ASN immunoreactivity in striatal PM and cytosol from adult and aged animals

	ASN immunoreactivity [a.u.]	
	Adult	Aged
PM	9172 ± 1533	6475 ± 1205*
Cytosol	3260 ± 440	2654 ± 387

Data represent the mean value ± S.E.M. for five separate experiments.
* $p < 0.05$ by unpaired *t*-test between adult and aged animals.

4. Discussion

Our present results showed that in aged striatum, in which ASN protein level is reduced in plasma membranes also DA uptake significantly decreased. Earlier work measuring DA uptake and DAT expression in aged rat striatum reported a marked loss of both DA uptake and DAT level in plasma membranes (Salvatore et al., 2003). Based on our results showing decrease of ASN protein level in aged striatum and in the light of data presenting important chaperoning and regulatory role of ASN in DAT function we proposed that

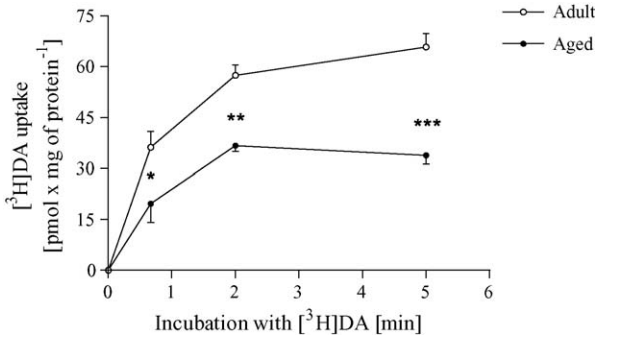

Fig. 1. Dopamine uptake into synaptosomes is decreased in aged striatum comparing to adult. [^{3}H]DA uptake into striatal synaptosomes prepared from 4- and 24-month-old Wistar rats were measured during 40 s, 2 min and 5 min of incubation with DA at 37 °C. Synaptosomes were incubated with or without 10 μM nomifensine prior to the addition of DA to calculate specific [^{3}H]DA uptake. Data represent the mean value ± S.E.M. for four to five separate experiments, each carried out in duplicate. Significance of data was determined with one-way ANOVA followed by Newman–Keuls test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

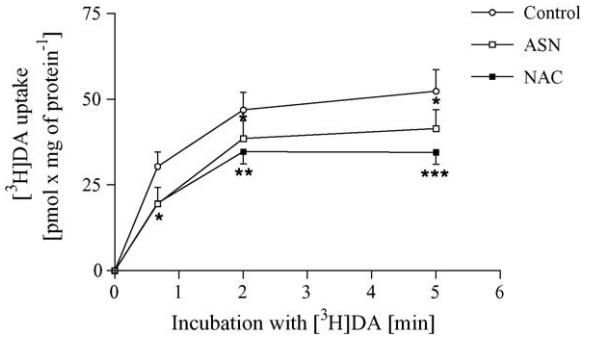

Fig. 2. ASN inhibited specific [^{3}H]DA uptake into rat striatal synaptosomes. Synaptosomes were stabilized for 30 min at 37 °C in Krebs–Ringer buffer without $CaCl_2$. Then were pre-incubated for 30 min with ASN and NAC at 10 μM in the presence of $CaCl_2$ (2 mM). Specific [^{3}H]DA uptake was assayed as described in Section 2. Data represent the mean value ± S.E.M. for three to four separate experiments, each carried out in duplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by one-way ANOVA followed by Newman–Keuls test.

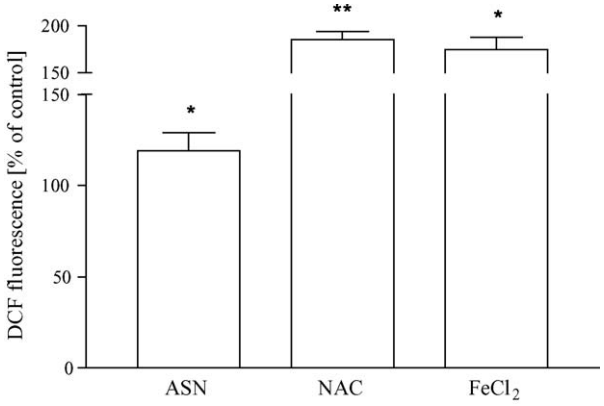

Fig. 3. ASN and NAC evoked intrasynaptosomal generation of ROS. ROS generation was measured in synaptosomes using DCFH-DA after 30-min incubation in the presence of ASN and NAC at 10 μM. $FeCl_2$ at 25 mM was used as a positive control. Data represent the mean value ± S.E.M. for three to four separate experiments, each carried out in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ by paired *t*-test. Control value of DCF fluorescence in synaptosomes evaluated 457.80 ± 18.32 a.u.

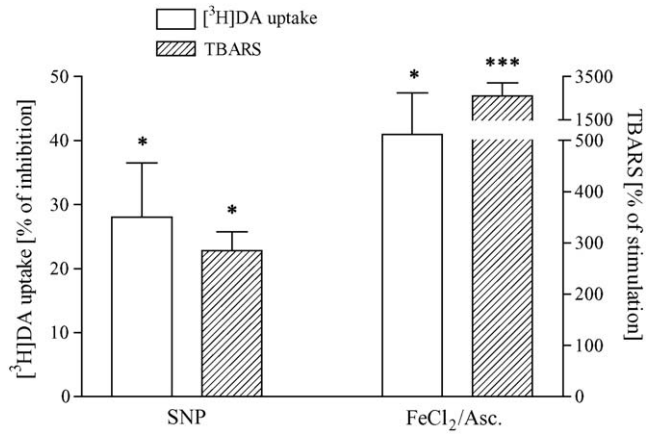

Fig. 5. Effect of NO donor, sodium nitroprusside (SNP) and $FeCl_2$/Asc on DAT activity and lipid peroxidation. Synaptosomes were stabilized for 30 min at 37 °C in Krebs–Ringer buffer without $CaCl_2$. Then were pre-incubated for 30 min with SNP at 10 μM or $FeCl_2$ at 25 μM together with ascorbic acid at 250 μM. The DA uptake was measured after 2 min of incubation with [^{3}H]DA. Data represent the mean value ± S.E.M. for three separate experiments, each carried out in duplicate. $^{*}p < 0.05$ by paired *t*-test. TBARS were assayed as described in Section 2. Data represent the mean value ± S.E.M. for four separate experiments, each carried out in triplicate. $^{*}p < 0.05$, $^{***}p < 0.001$ by one-way ANOVA followed by Newman–Keuls test.

loss of ASN could be responsible for DAT dysfunction. Moreover, this data showed that ASN at high toxic concentration applied extracellularly damaged DAT function in rat striatal synaptosomes. Our data corresponded in some aspects with the observations of Seo et al. (2002). He indicated that ASN at high concentration had a significant cytotoxic effect on PC12 cells by decreasing Bcl-xL expression and increasing bax levels followed by cytochrome *c* release and caspase activation. Furthermore, it was presented that extracellular application of ASN caused a significant decrease of primary and immortalized neuronal cells viability via Rab5A-dependent endocytosis (El-Agnaf et al., 1998; Sung et al., 2001).

The role of ASN in dopaminergic neurotransmission under physiological and pathological conditions is now an important scientific approach. Sidhu et al., 2004 suggested that ASN might by an adaptor protein or a chaperone, which can regulate nearly every step of DA turnover. Previous data revealed that non-aggregated ASN modulate DA storage and biosynthesis through inhibition of tyrosine hydroxylase (Perez et al., 2002). On the other hand, it has been proposed that ASN protofibrils may form integral pores in vesicular membranes, leading to DA leakage and to increased level of cytosolic DA (Goldberg and Lansbury, 2000; Volles and Lansbury, 2002; Lashuel et al., 2002a,b). The role of ASN in dopaminergic neurotransmission is till now not clear. Wersinger et al. (2003) presented down-regulation of DAT activity in Ltk$^-$, COS-7 and HEK293 cells and suggested a protective role of ASN. The last data suggested that reactive oxygen and nitrogen species could modulate DAT function in rat striatum (Lonart and Johnson, 1994; Morel et al., 1998; Park et al., 2002; Kiss et al., 2004). Barrier et al. (2003)

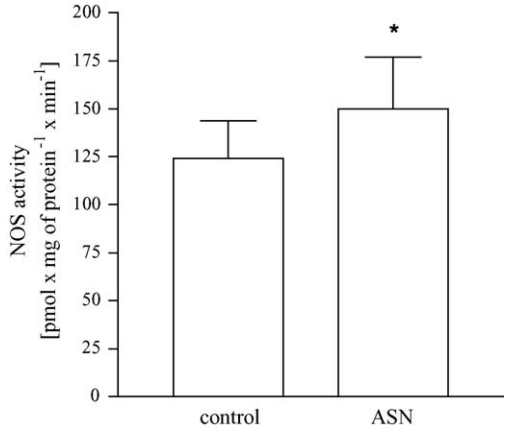

Fig. 4. ASN stimulated NOS activity in rat striatal cytosolic fraction. NOS activity was measured in striatal cytosol after 15-min pre-incubation in the absence (control) and presence of ASN at 10 μM concentration as described in Section 2. Data represent the mean value ± S.E.M. for three separate experiments, each carried out in triplicate. $^{*}p < 0.05$, by paired *t*-test.

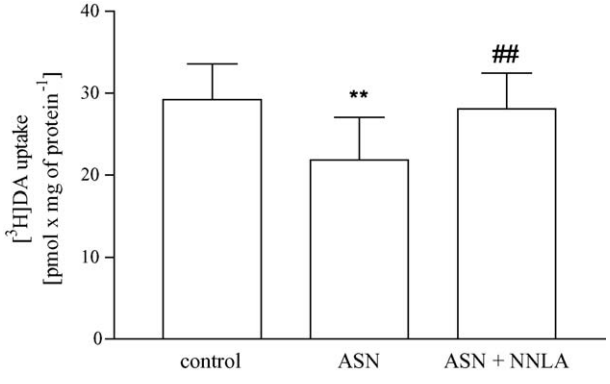

Fig. 6. NNLA prevented DAT inhibition evoked by ASN. Synaptosomes were stabilized for 30 min at 37 °C in Krebs–Ringer buffer without $CaCl_2$. Then, were pre-incubated for 30 min at 37 °C in the presence of 2 mM $CaCl_2$ with ASN at 10 μM or ASN + NNLA at 100 μM or without treatment (control). The DA uptake was measured after 2 min of incubation with [^{3}H]DA. Data represent the mean value ± S.E.M. for five separate experiments, each carried out in duplicate. $^{**}p < 0.01$ compared to control, $^{\#\#}p < 0.01$ compared to ASN, by one-way ANOVA followed by Newman–Keuls test.

indicated that DAT inhibition is only partially related to oxidative stress. Data presented in this study showed that in spite of significant ROS generation by ASN, the potent antioxidants: Trolox and 4-hydroxy-Tempo did not prevented DAT inhibition evoked by this protein. In the light of these data the role of free radicals in DAT modulation is unclear. In this study, we showed for the first time that ASN enhanced NOS activity and that NO may be involved in DAT down-regulation. Kiss et al. (2004) observed significant enhancement of DA uptake in rat striatal slices by NOS inhibitor L-NAME. However, impairment of the neuronal DAT activity in MPP^+ treated rat was not prevented by treatments with NOS inhibitors (Barc et al., 2001). Our data demonstrated that NNLA prevented ASN evoked DA uptake inhibition. Depending on the redox state, NO can occur in free radical form ($NO^\bullet$), as nitrosonium cation (NO^+), or as a nitroxyl anion (NO^-). Moreover, $NO^\bullet$ can react with superoxide to form peroxynitrite, a highly toxic compound. However, ineffectiveness of 4-hydroxy-Tempo treatment suggests that in our experimental conditions superoxide is not involved in DAT inhibition. In conclusion, our data showed decreased DAT activity in the aged brain related with lower ASN level in plasma membranes. Moreover, these results indicated that extracellular ASN affected DAT function probably by NO induced transporter alteration.

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α-Synuclein enhances secretion and toxicity of amyloid beta peptides in PC12 cells

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ABSTRACT

α-Synuclein is the fundamental component of Lewy bodies which occur in the brain of 60% of sporadic and familial Alzheimer's disease patients. Moreover, a proteolytic fragment of α-synuclein, the so-called non-amyloid component of Alzheimer's disease amyloid, was found to be an integral part of Alzheimer's dementia related plaques. However, the role of α-synuclein in pathomechanism of Alzheimer's disease remains elusive. In particular, the relationship between α-synuclein and amyloid beta is unknown. In the present study we showed the involvement of α-synuclein in amyloid beta secretion and in the mechanism of amyloid beta evoked mitochondria dysfunction and cell death. Rat pheochromocytoma PC12 cells transfected with amyloid beta precursor protein bearing Swedish double mutation (APPsw) and control PC12 cells transfected with empty vector were used in this study. α-Synuclein (10 μM) was found to increase by twofold amyloid beta secretion from control and APPsw PC12 cells. Moreover, α-synuclein decreased the viability of PC12 cells by about 50% and potentiated amyloid beta toxicity leading to mitochondrial dysfunction and caspase-dependent programmed cell death. Inhibitor of caspase-3 (Z-DEVD-FMK, 100 μM), and a mitochondrial permeability transition pore blocker, cyclosporine A (2 μM) protected PC12 cells against α-synuclein or amyloid beta evoked cell death. In contrast Z-DEVD-FMK and cyclosporine A were ineffective in APPsw cells containing elevated amount of amyloid beta treated with α-synuclein. It was found that the inhibition of neuronal and inducible nitric oxide synthase reversed the toxic effect of α-synuclein in control but not in APPsw cells. Our results indicate that α-synuclein enhances the release and toxicity of amyloid beta leading to nitric oxide mediated irreversible mitochondria dysfunction and caspase-dependent programmed cell death.

1. Introduction

The pathogenesis of Alzheimer's disease (AD), a progressive neurodegenerative disorder of the elderly and the most common form of dementia, is till now not fully elucidated and there is no efficient method for its treatment. The most frequent sporadic forms of AD are associated with an oligomerisation and abnormal accumulation of amyloid beta (Aβ) peptides into senile plaques (Selkoe, 2000; Marks and Berg, 2008; Meyer-Luehmann et al., 2008; Ye et al., 2008). Aβ is formed by proteolytic processing of amyloid precursor protein (APP) by β- and γ-secretase (Marks and Berg, 2008). These enzymes generate the Aβ peptide and carboxyl terminal fragments (CTF) of APP, which have been implicated in the pathogenesis of AD (Suh and Checler, 2002; Walsh et al., 2005, Cacquevel et al., 2008). Extracellular Aβ peptides are highly cytotoxic to neuronal cells by activating a variety of cell signaling pathways, such as mitogen-activated protein (MAP) kinase cascades (Dineley et al., 2001), by inducing the dephosphorylation/inactivation of Akt (Nassif et al., 2007) and by the activation of NMDA receptors and disruption in calcium homeostasis (Domingues et al., 2007). Besides the presence of amyloid β-peptides, the major component of senile plaques corresponds to a component now referred to as NAC (non-amyloid component of Alzheimer's disease plaques; Tanaka et al., 2002). The molecular cloning of NAC indicated that it derives from the proteolytic cleavage of a precursor protein (NACP) of 19 kDa now known as α-synuclein (ASN; Ueda et al., 1993). ASN is a small protein recognized in various cell types of the central nervous system, and is especially abundant in presynaptic terminals of neocortex, hippocampus, dentate gyrus, olfactory bulb, thalamus, cerebellum and striatum (Sidhu et al., 2004; Adamczyk et al., 2005).

It is postulated that in physiological conditions, in nanomolar concentration, ASN plays an important role in synaptic plasticity, regulation of vesicle transport, dopaminergic neurotransmission

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and also acts as a chaperon protein (Sidhu et al., 2004). It can also protect neurons against oxidative stress and inhibit apoptosis. Its protective effect is suggested to be mediated by inactivation of JNK signaling pathway (Hashimoto et al., 2002), by activation of PI3/Akt kinase pathway (Seo et al., 2002) and or by inhibition of p53 mediated apoptosis (Alves Da Costa et al., 2002). However, under some pathogenic conditions, oxidative and genotoxic stress, which lead to overexpression and mutations, ASN changes its native conformation and tends to form oligomers, which are highly toxic and induce cell death (Amer et al., 2006). The progressive aggregation of this protein leads to the formation of insoluble inclusions called Lewy bodies that can mechanically damage the cell structure. Nowadays it is obvious that over 60% of AD cases is accompanied by Lewy body formation (Mikolaenko et al., 2005; Jellinger, 2004). Lewy bodies and associated Lewy neurites are also pathological hallmarks of other neurodegenerative diseases such as Parkinson's disease (PD) and dementia with Lewy bodies (Braak et al., 2003; Pletnikova et al., 2005). Abnormal ASN aggregates were also found in other neuronal and glial inclusions, such as Lewy body-like glial cytoplasmic inclusions of multiple system atrophy (MSA; Parkkinen et al., 2007; Ishizawa et al., 2008).

While the role of ASN in the pathogenesis of AD remains unclear, indirect evidence suggests that this protein may interact with Aβ and increase its toxicity. Full-length ASN is present in blood and CSF of normal subjects suggesting that this protein is released by neurons in the extracellular space as a part of its normal cellular processing (El-Agnaf et al., 2006). A recent study showed that membrane-bound ASN interacts with extracellular Aβ and that the NAC fragment is liberated from ASN (Mandal et al., 2006). Other data suggest that ASN liberated from presynaptic terminals during the process of neurodegeneration is cleaved to NAC by extracellular metaloproteinases (Sung et al., 2005).

The aim of this study was to investigate the role of extracellular ASN in Aβ secretion and in molecular mechanisms of its toxicity. Moreover, the protective effect of selected specific inhibitors against cytotoxicity of ASN and Aβ was evaluated.

2. Experimental procedures

2.1. Preparation of soluble and aggregated α-synuclein

ASN protein was dissolved in PBS (phosphate-buffered saline pH 7.4) at a concentration of 100 μM, and directly used for experiments or the resulting solution was aggregated for 14 days at 37 °C.

2.2. PC12 cell culture

The studies were carried out using PC12 (pheochromocytoma) cells transfected with DNA constructs harboring human mutant APP gene (APPsw, K670M/N671L) according to Keil et al. (2004). Transfected PC12 cells bearing the Swedish double mutation in the APPsw, and control PC12 cells (transfected with empty vector) were cultured as described previously by Chalimoniuk et al. (2007) in 75-cm^2 flasks in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5% heat-inactivated horse serum (HS), 0.5% penicillin/streptomycin (50 U/ml), 400 μg/ml G418 and 2 mM glutamine. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. Cells were subcultured about twice a week. For experiment, confluent cells were subcultured into polyethylenimine-coated 35-mm^2 dishes or 24-well plates. Cells were used for experiments at 75–90% confluence or 1 day after being plated in the 24-well plate. Prior to treatment, cells were replenished with 2% FBS medium.

2.3. PC12 cell treatment

Cells were treated with soluble and aggregated ASN in concentrations of 1 μM, 5 μM and 10 μM for 24 and 48 h at 37 °C. Moreover, the cells were treated with inhibitors of protein kinases (staurosporine, STS, 1 μM), caspase-3 (Z-DVD-FMK, 100 μM), mitochondrial permeability transition pore (cyclosporine A, CsA, 2 μM), Ca^{2+}-dependent nitric oxide synthase (NOS; *N*-nitro-L-arginine, NNLA, 100 μM), neuronal NOS ((4*S*)-*N*-(4-amino-5(aminoethyl)aminopentyl)-*N*′-nitroguanidine, NAAN, 50 μM) or inducible NOS (1400 W, 100 μM), in the absence or presence of soluble ASN (10 μM) as described in legend to figures.

2.4. Determination of Aβ1–40

Cells were plated at equal density in 6-well plates. After reaching confluence, 2 ml of conditioned media was collected after 48 h of treatment. Media were centrifuged to remove cell fragments, and aliquots were then used to determine the Aβ1–40 level. For determination of secreted Aβ1–40, a specific sandwich enzyme-linked immunosorbent assay (ELISA) employing monoclonal antibodies was used. The ELISA was performed according to the instructions given in the Aβ-ELISA kit by Genetics Company. The assay principle is that of a standard sandwich ELISA, which utilizes a site-specific rabbit anti-human N-terminal capture, a cleavage site-specific rabbit anti-human Aβ1–40 terminal detection antibody, and anti-rabbit IgG peroxidase-conjugated secondary antibody.

2.5. MTT analysis

Mitochondria function and cellular viability was evaluated using 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Medium with 2% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin, containing investigated substances were added to the cells for 48 h. MTT was added to all wells and allowed to MTT reduction to formazan during incubation at 37 °C for 3 h, followed by cell lysis and spectrophotometric measurement at 595 nm.

2.6. Determination of apoptosis

Apoptosis was determined by Hoechst 33342 fluorescent staining. The cells were examined under a fluorescence microscope (Olympus BX51, Japan) and photographed with a digital camera (Olympus DP70, Japan). Cells with typical apoptotic nuclear morphology (nuclear shrinkage, condensation) were identified and counted. The results were expressed as the percentages of apoptotic cells in whole cell population.

2.7. Determination of free radicals using 2′,7-dichlorofluorescein (DCF)

The DCF fluorescence assay detects the level of hydrogen peroxide and other free radicals (ROS) in cells. Free radicals were determined based on reactive oxygen species-mediated conversion of fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA) into fluorescent DCF (Royall and Ischiropoulos, 1993; Adamczyk et al., 2006). PC12 cells were treated with 10 μM ASN for 4 h and then loaded with 10 μM CM-H2DCF-DA in DMSO by incubating for 50 min at 37 °C in Hank's buffer without Phenol Red (Sigma, H8264). DMSO was used at a final concentration of 0.05% and at this concentration had no effect on free radical levels. Cells were washed three times in Hank's buffer. Fluorescence of DCF was measured using a Perkin Elmer LS 50B spectrofluorometer with excitation and emission wavelengths at 488 and 535 nm, respectively. DCF fluorescence is reported in auxiliary units.

2.8. Determination of caspase-3 activity

Caspase-3 (EC 3.4.22.56) activity was determinated by using colorimetric assay kit; Sigma, St. Louis, USA. Cells were cultured at 3×10^6 cells/well, harvested with lysis buffer (caspase colorimetric assay kits; Sigma), incubated during 20 min at 4 °C, disrupted by multiple passes through a 26-gauge needle and centrifuged at 14,000 × *g* for 15 min. The activity of caspase-3 was measured in 10 μl of supernatant using 20 μM of the synthetic caspase-3 substrate Ac-DEVD-AMC in reaction buffer in a final volume of 200 μl and incubated at 37 °C during 4 h. The concentration of AMC as the product from enzymatic converting of Ac-DEVD-AMC by caspase-3 was measured at the excitation and emission wavelengths at 360 and 460 nm, respectively. Caspase-3 activity was estimated as nanomolar AMC/min/mg of protein.

2.9. Statistical analysis

The results were expressed as mean values ± S.E.M. Differences between means were analyzed using one-way or two-way (type of cells and treatment) ANOVA followed by the Newman–Keuls test when appropriate. $p < 0.05$ was considered statistically significant. The statistical analyses were performed by using Statistica Version 4.0 Software (Kraków, Poland).

3. Results

To study the role of ASN in amyloid beta secretion and toxicity, we used PC12 cells stably transfected with APP bearing the Swedish double mutation (APPsw), which liberate five times more Aβ as compared to PC12 control cells (Fig. 1, see also Chalimoniuk et al., 2007). We observed about twofold increase of Aβ release by 10 μM soluble ASN treatment during 48 h in both control and APPsw cells (Fig. 1). MTT test revealed that soluble form of ASN at

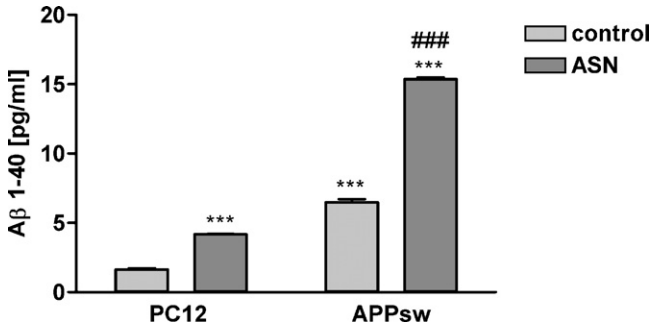

Fig. 1. ASN evokes Aβ1–40 secretion by APP transfected and control PC12 cells treated with ASN. Cells were plated at equal density in 6-well plates. After reaching confluence, cells were treated with 10 μM ASN for 48 h and then 2 ml of conditioned media was collected. Aβ1–40 level was determined by ELISA kit. Data represent the mean value ± S.E.M. for three separate experiments. $^{***}p < 0.001$ *versus* control (non-treated) PC12 cells, $^{###}p < 0.001$ *versus* control (non-treated) APPsw PC12 cells by two-way ANOVA followed by Newman–Keuls test.

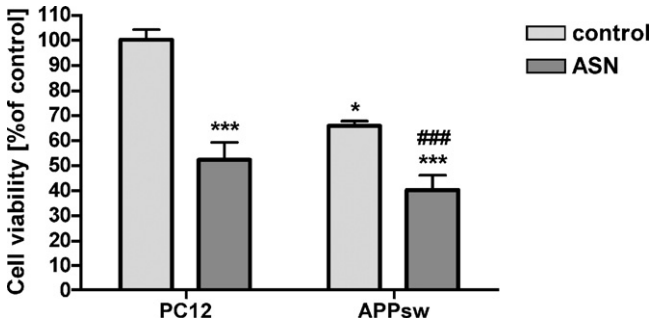

Fig. 3. ASN increased cytotoxic effect of endogenous Aβ in PC12 APPsw cells. The cell viability was spectrophotometrically determined in PC12 control and PC12 APPsw cells by MTT assay after 48 h incubation with 10 μM ASN in culture medium containing 2% fetal bovine serum. Data are expressed in percent of control and represent the mean ± S.E.M. for four separate experiments. $^{*}p < 0.05$; $^{***}p < 0.001$ *versus* control (non-treated) PC12, $^{###}p < 0.001$ *versus* control (non-treated) APPsw by two-way ANOVA followed by Newman–Keuls test.

10 μM concentration reduced the PC12 control cells viability by about 50% (Fig. 2). Aggregated form of ASN had no effect in any investigated concentration (Fig. 2). Overproduction of endogenously liberated Aβ significantly decreased APPsw cells viability as compared to control PC12 cells (MTT test). This negative effect of Aβ was significantly enhanced by ASN treatment (Fig. 3). Subsequently we investigated the mechanism involved in the ASN toxicity. Morphological examination of cell nuclei, stained with DNA-binding fluorochrome Hoechst 33342, showed that PC12 control and APPsw cells exposed to 10 μM ASN presented typical apoptotic morphology, including condensation of chromatin and nuclear fragmentation (Fig. 4B and D). APPsw cells showed increased apoptotic cell death when treated with exogenous ASN as compared to PC12 control cells (Fig. 4A and C). Then the concentration of free radicals in ASN treated cells was assessed. The results of Fig. 5 showed that ASN activated free radicals generation in PC12 cells by 20%, but had no additional effect on free radicals level already enhanced by endogenous Aβ in APPsw cells. Then the role of NO in cell damage caused by ASN was analyzed. The non-selective inhibitor of NOS, NNLA at 100 μM, and the specific inhibitors of NOS isoforms: nNOS inhibitor NAAN (50 μM) and iNOS inhibitor, 1400 W (100 μM) protected significantly PC12 control cells against ASN toxicity (Fig. 6A), but these inhibitors were ineffective in APPsw cells (Fig. 6B). Cyclosporine A (2 μM), an inhibitor of mitochondrial permeability transition pore partly reversed the cytotoxic effect of ASN in control cells but not in APPsw cells (Fig. 7). However, CsA alone increased the viability of APPsw cells (Fig. 7). In following experiments the activity of caspase-3 (EC 3.4.22.56), the executor enzyme in death signaling evoked by mitochondria failure, was analyzed. In both cells lines ASN enhanced caspase-3 activity by about 100% (Fig. 8). Inhibitor of caspase-3, Z-DEVD-FMK (100 μM) increased the viability of PC12 control cells treated with ASN and APPsw cells non-treated with ASN (Fig. 9). However, this inhibitor had no protective effect in APPsw cells treated with ASN (Fig. 9).

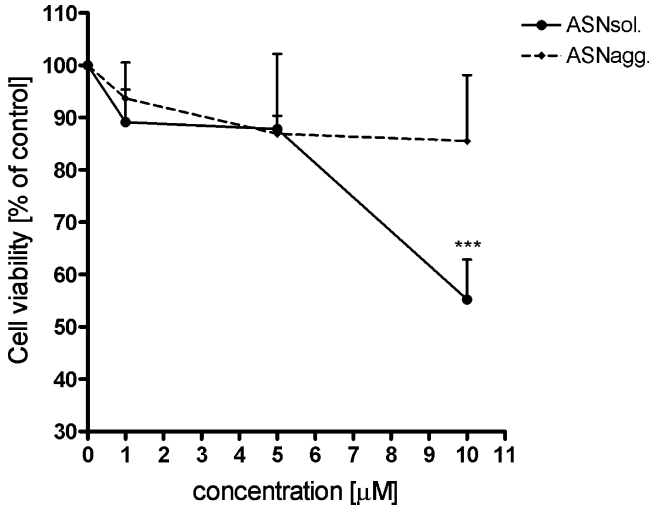

Fig. 2. Concentration-dependent effect of soluble and aggregated ASN on cell viability in PC12 control cells. PC12 control cells viability was spectrophotometrically determined by MTT assay after 48 h incubation with different concentrations of investigated agents in culture medium with 2% serum. Data represent the mean value ± S.E.M. for five separate experiments. $^{***}p < 0.001$ *versus* control (non-treated) PC12 cells by one-way ANOVA followed by Newman–Keuls test.

4. Discussion

The interaction of ASN and Aβ in neurodegenerative processes has become a matter of increasing interest. The major finding of the present study is that extracellularly applied ASN increases the release of Aβ peptides and enhances its toxicity, leading to mitochondrial dysfunction and PC12 cell death. PC12 cells are till now widely used for the investigation of Aβ processing (Yang et al., 2007) and the molecular mechanisms of Aβ and ASN toxicity (Qian et al., 2008; Girigoswami et al., 2008; Hu et al., 2008) and also for cytotoxic effects of various stressors (Piga et al., 2007). This cell line was selected for our study on the role of exogenous ASN in Aβ toxicity. Our data are thus consistent with previous studies showing that ASN interact with Aβ peptides (Mandal et al., 2006) and accelerate their aggregation (Yoshimoto et al., 1995), and extend the previous findings into defining the intracellular effect.

There is an increasing body of evidence that ASN might exert pathogenic functions in the extracellular space (Albani et al., 2004; Du et al., 2003; Seo et al., 2002; Sung et al., 2001). ASN and its oligomers have been detected in human cerebrospinal fluids and blood plasma (Borghi et al., 2000; El-Agnaf et al., 2006; Lee et al., 2006; Tokuda et al., 2006), which has emphasized concerns about the extracellular ASN forms. Moreover, our previous in vitro studies show that ASN is released from synaptoneurosomes during the oxidative stress conditions (Adamczyk et al., 2007). Excessive amount of ASN is released from the degenerated neurons and spread its cytotoxic effect to the neighboring cells (El-Agnaf et al., 1998; Sung et al., 2001). Extracellular ASN was shown to cause up-regulation of Bax expression, decrease in Bcl-xL level, mitochondria alteration and subsequent release of cytochrome *c*. The consequence of these events is activation of caspases cascade and

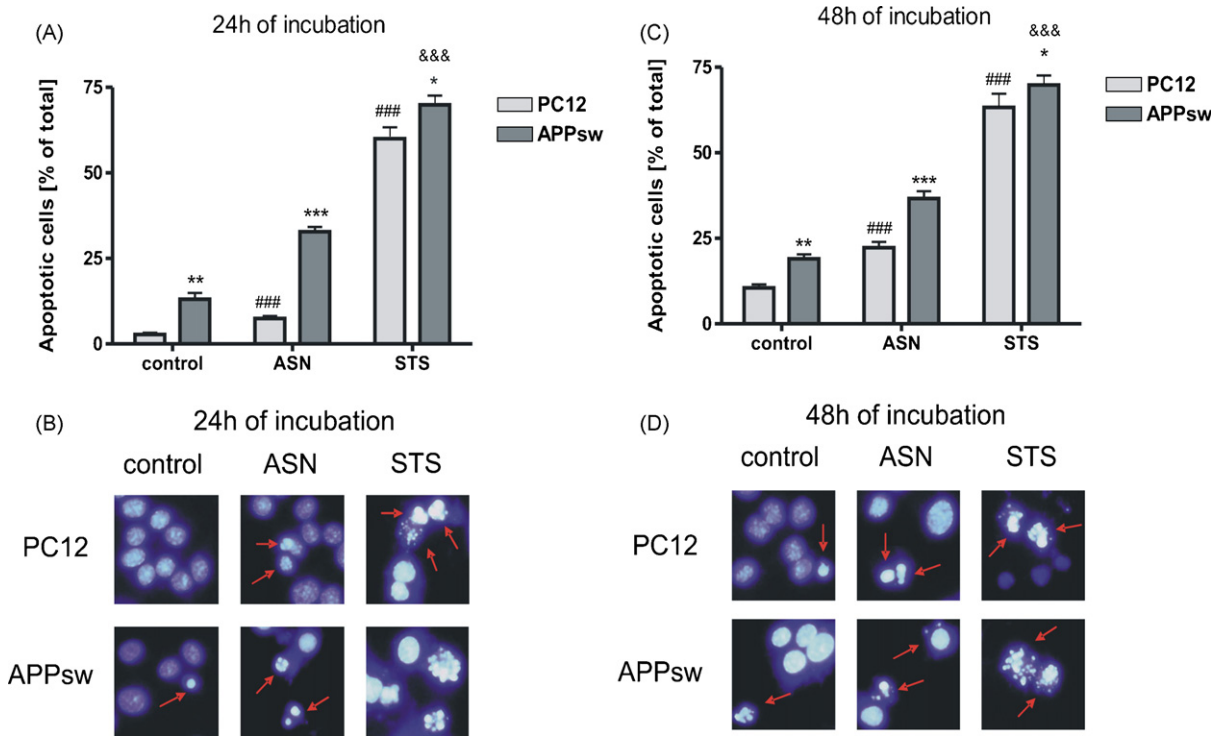

Fig. 4. Time-dependent apoptosis induced by ASN in PC12 control and PC12 APPsw cells. After 24 h and 48 h exposure to the 10 μM ASN and 1 μM STS nuclei were visualized with Hoechst 33342 and the number of live and apoptotic cells was counted. STS was used as a positive control. Apoptosis is expressed as the number of apoptotic cells on the coverslips 24 h (A) and 48 h (C) after treatment. The arrows indicate a nuclei with typical apoptotic features after 24 h (B) and 48 h (D) of treatment. Data represent the mean value ± S.E.M. for five separate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ APPsw *versus* PC12, $^{\#\#\#}p < 0.001$ *versus* control (non-treated) PC12 cells, $^{\&\&\&}p < 0.001$ *versus* control (non-treated) APPsw cells by two-way ANOVA followed by Newman–Keuls test.

cell death (Seo et al., 2002). Other studies showed that ASN applied extracellularly alters dopaminergic neurotransmission (Adamczyk et al., 2006) and enhances Ca^{2+} influx by voltage-dependent calcium channels, which causes the alteration in Ca^{2+} regulated processes and calcium signalling pathway (Adamczyk and Strosznajder, 2006). In the present study exogenous ASN induced mitochondria failure and activated caspase-dependent programmed cell death. Extracellular ASN aggregates also activate microglia, resulting in the release of reactive oxygen species, which causes the death of dopaminergic neurons (Zhang et al., 2005). Interestingly many previous data emphasize the important role of non-aggregated as well as aggregated ASN in the cell pathology and in neurodegenerative diseases (Hashimoto and Masliah, 1999; Mandal et al., 2006). In this study we showed that aggregated ASN had no effect on PC12 cells viability. This is consistent with recent biochemical studies that have focused more attention on the oligomeric and protofibril forms of abnormally accumulated proteins than on intracytoplasmic inclusions as the causes of neurodegeneration (Chen and Feany, 2005; Shults, 2006). In support of this idea, the study of Lee et al. (2008) demonstrated that extracellular ASN aggregates enter neuronal cells via receptor mediated endocytosis and are rapidly degraded in lysosomes. In contrast, monomeric ASN can freely diffuse the plasma membrane and appear to be only minimally degraded within the cells.

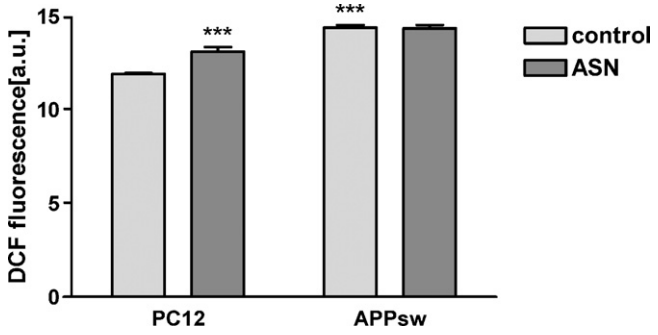

Fig. 5. ASN evokes intracellular generation of ROS. ROS generation was measured in PC12 control and APPsw cells using DCFH-DA after 4 h of incubation in the presence of ASN at 10 μM. Data represent the mean value ± S.E.M. for four separate experiments. $^{***}p < 0.001$ *versus* control (non-treated) PC12 cells, by two-way ANOVA followed by Newman–Keuls test.

In the recent years, there has been an increased interest in the role of the ASN and Aβ interaction and the contribution of ASN in Aβ toxicity. Different groups reported that more than 60% of the sporadic AD patients harbored ASN-positive Lewy bodies in multiple brain regions (Mikolaenko et al., 2005; Jellinger, 2004) and in these patients a faster progression of extrapyramidal signs was shown (Lopez et al., 2000). In addition, recent evidence indicates that ASN is often present in dystrophic neurites in senile plaques in the cortex of AD cases without significant cortical Lewy pathology. There is a growing body of evidence that Aβ enhances ASN oligomerisation, accumulation and toxicity (Masliah et al., 2001). Postmortem studies suggest that the existence of AD pathology accelerates Lewy pathology (Wakisaka et al., 2003), and the presence of Aβ deposits was associated with an increased number of Lewy bodies and Lewy neuritis in the cerebral cortex (Pletnikova et al., 2005). A biochemical analysis of AD and DLB brains also showed the relationship between Aβ and ASN accumulation (Deramecourt et al., 2006). Moreover, in doubly transgenic mice expressing both mutant APP and presenilin-1-accelerated accumulation of phosphorylated ASN was shown (Kurata et al., 2007). Our results extend this idea by showing that not only Aβ enhances α-synucleinopathy but also ASN aberrantly released from damaged presynaptic terminals may promote the liberation of, and interact with Aβ protein that results in more

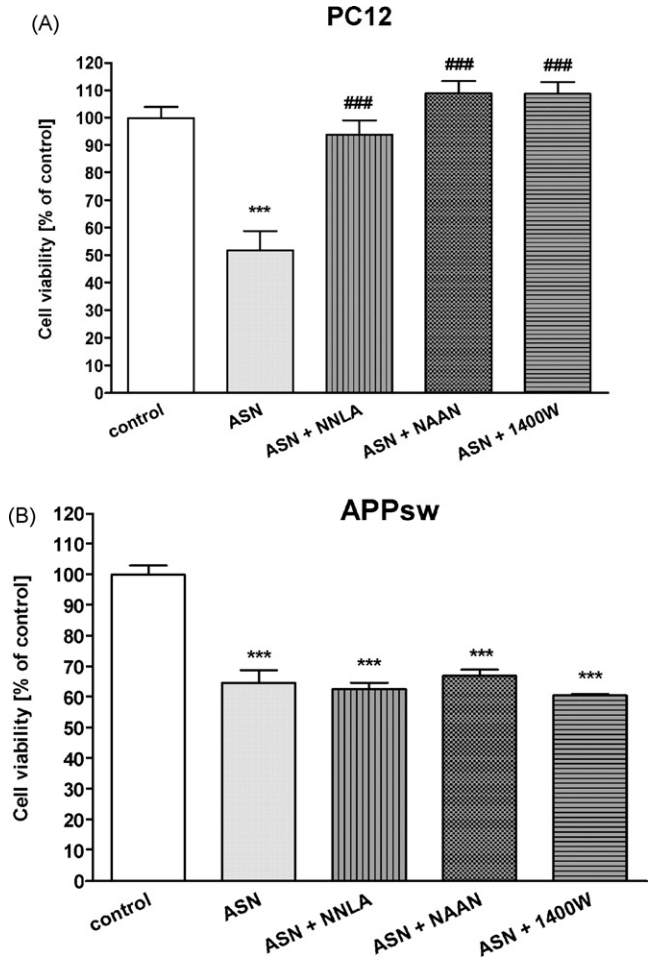

Fig. 6. Nitric oxide synthase inhibitors prevented cell death in PC12 control cells (A), but not in APPsw (B) cells. The cell viability was spectrophotometrically determined by MTT assay after 48 h incubation with inhibitor of Ca^{2+}-dependent NOS (100 μM NNLA), neuronal isoform of NOS (50 μM NAAN) and inducible isoform of NOS (100 μM, 1400 W) in the presence of ASN at 10 μM. Data are expressed in percent of control and represent the mean value ± S.E.M for four separate experiments. $^{***}p < 0.001$ *versus* control (non-treated) cells; $^{\#\#}p < 0.001$ *versus* ASN treated cells by one-way ANOVA followed by Newman–Keuls test.

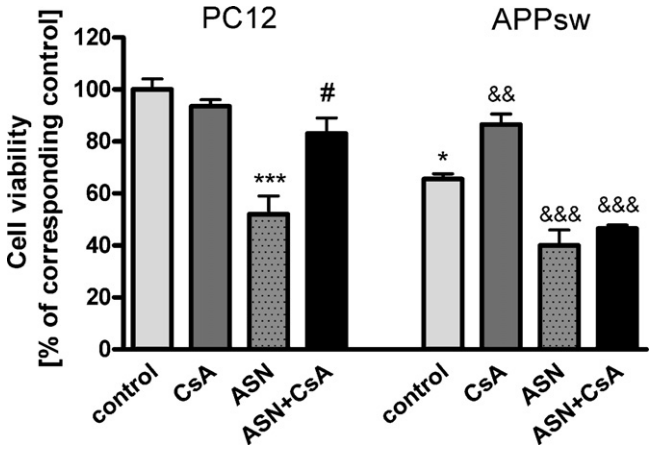

Fig. 7. Effect of mitochondrial permeability transition pore (MPTP) blocking on PC12 control and APPsw cells viability. MTT assay was performed in PC12 control and APPsw cells after 48 h incubation with 2 μM cyclosporine in the presence or absence of 10 μM ASN in culture medium containing 2% fetal bovine serum. Data are expressed in percent of control and represent the mean value ± S.E.M. for four separate experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ *versus* control (non-treated) PC12, $^{\&\&\&}p < 0.001$ *versus* control (non-treated) APPsw, $^{\#}p < 0.05$ *versus* ASN treated PC12 by two-way ANOVA followed by Newman–Keuls test.

significant cell death. It is difficult to say straightforward what type of cell death occurred after ASN treatment. We observed that only half of the dead cells exposed morphological changes typical for apoptosis. It is possible that the cells may enter different types of programmed cell death, but they can also undergo necrosis. The previous data show that both ASN and Aβ peptides induce apoptotic cell death (Du et al., 2003; El-Agnaf et al., 1998; Seo et al., 2002; Sanz-Blasco et al., 2008), necrosis and other controlled cell death (Domingues et al., 2007; Büttner et al., 2008). Abnormal oligomerisation and accumulation of ASN may affect the metabolism of Aβ or APP protein, leading to enhanced secretion, accumulation and toxicity of Aβ. Moreover, extracellular ASN could exert its cytotoxic effect not only by the enhancement of Aβ secretion but also by its independent signals. Our data showed that the lower viability of ASN treated control PC12 cells than non-treated APPsw cells is the effect of action of Aβ liberated by ASN and ASN itself.

Since Aβ protein is normally secreted from cells, extracellular ASN may enhance the liberation and interact with Aβ protein. In this way ASN may participate in suppressing clearance of Aβ protein. We suggest that the observed higher toxicity of Aβ in the presence of ASN resulted from Aβ overproduction and from the overlapping effect of these two proteins that may cause nerve endings injury, loss of synapses and cognitive alterations (Iwai, 2000; Obi et al., 2007).

The mechanism through which ASN might accelerate Aβ release and toxicity is not completely clear. It was earlier indicated that oligomerisation of both ASN and Aβ causes free radicals formation (Tabner et al., 2002; Paik et al., 1999; Huang et al., 1999a,b; Adamczyk et al., 2006). Also enhanced level of intracellular Aβ led to overproduction of NO, mitochondria alteration (Keil et al., 2004; Chalimoniuk et al., 2007) and increased apoptosis (Lüth and Arendt, 1997; Lüth et al., 2001; Lahiri et al., 2003). In this study we also demonstrated that overproduction of Aβ in APPsw cells resulted in the enhancement of programmed cell death. Our previous data showed that extracellular ASN enhanced nitric oxide synthase activity (Adamczyk et al., 2006). Results obtained in control PC12 cells, demonstrate a significant role of constitutive and inducible NOS in mitochondria dysfunction and cell death evoked by ASN. However the inhibition of NOS in APPsw transfected cells, in contrast to control cells, did not prevent cell death evoked by ASN. This emphasize the role of ASN-Aβ

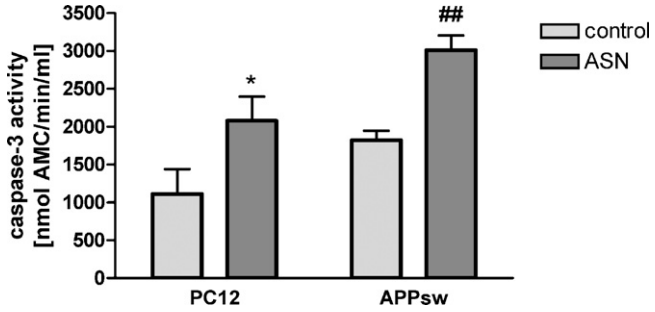

Fig. 8. ASN stimulated caspase-3 activity in PC12 control and APPsw cells. PC12 cells were incubated with 10 μM ASN for 8 h, lysed, centrifuged at 14,000 × *g* for 15 min and supernatant was assayed for caspase-3 activity, measuring the release of AMC from the fluorogenic caspase-3 substrate DEVD-AMC. Data are expressed as nanomolar of released AMC/min/mg of protein and represent the mean value ± S.E.M. for three separate experiments. $^{*}p < 0.05$ *versus* control (non-treated) PC12 cells; $^{\#}p < 0.05$ *versus* control (non-treated) APPsw cells by two-way ANOVA followed by Newman–Keuls test.

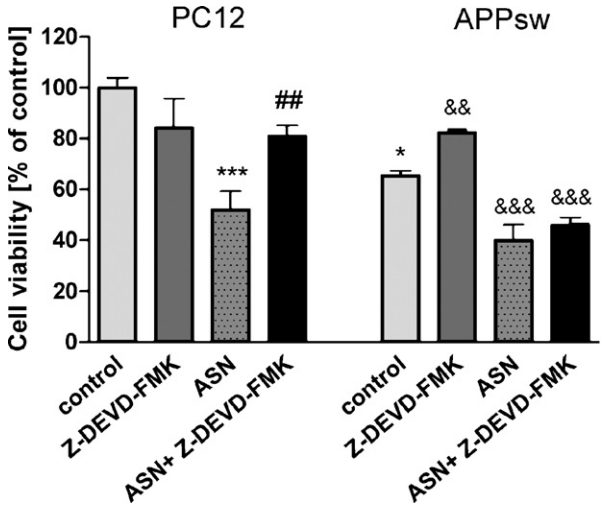

Fig. 9. Effect of caspase-3 inhibition on viability of PC12 control and APPsw cells. MTT assay was performed in PC12 control and APPsw cells after 48 h incubation with 100 μM Z-DEVD-FMK in the presence or absence of 10 μM ASN in culture medium containing 2% fetal bovine serum. Data are expressed in percent of control and represent the mean value ± S.E.M. for four separate experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ *versus* control (non-treated) PC12, $^{\&\&\&}p < 0.001$ *versus* control (non-treated) APPsw, $^{\#\#}p < 0.01$ *versus* ASN treated PC12 by two-way ANOVA followed by Newman–Keuls test.

interaction in execution of cell death under conditions when APP is overexpressed.

Finally, in the present study, we showed that ASN enhances mitochondria failure and caspase-3 activity evoked by Aβ in APPsw cells. Our data demonstrated that the toxicity of ASN and Aβ could be partially reversed by CsA treatment in the case when each agent acted alone, but not in the case when these peptides acted together. The lack of protective effect of CsA indicated that mitochondria dysfunction caused by ASN and Aβ leads to an irreversible opening of the mitochondrial permeability transition pore (MPTP). While many of the phenomena associated with pore opening appear to be prevented by CsA; it has been reported that peroxynitrite ($ONOO^-$) induces mitochondrial swelling (Gadelha et al., 1997) and causes an increase in state 4 respiration (oxygen consumption in the absence of ADP) that is insensitive to CsA (Brookes et al., 1998). This is further consistent with our hypothesis that increased oxidative index in APPsw cells leads to rapid formation of peroxynitrite after ASN treatment. Additionally we demonstrated that the treatment with specific inhibitor of caspase-3 alone improved the cell viability reduced by the Aβ in APPsw cells but also protected PC12 control cells against ASN toxicity. However, in the case of PC12 cells over-expressing mutant APP treated with ASN specific inhibition of caspase-3 was not effective. We suggest that the mitochondria alteration evoked by the both toxic proteins acting together was too strong to rescue the cells.

In conclusion, the present study has shown that extracellular ASN enhanced the release and toxicity of Aβ peptides leading to NO mediated irreversible mitochondria dysfunction and caspase-dependent programmed cell death. This provides additional evidence in favor of the role of the interaction between Aβ and ASN in AD pathology. The results indicate that the relationship between ASN and Aβ should be taken into consideration in understanding of molecular mechanism of neurodegenerative diseases and in creation of more effective therapeutic strategy.

Acknowledgements

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