Short-Term Effects of β-Amyloid\textsubscript{25-35} Peptide Aggregates on Transmitter Release in Neuromuscular Synapses

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Abstract
The β-amyloid (AB) peptide\textsubscript{25-35} contains the functional domain of the AB precursor protein that is both required for neurotrophic effects in normal neural tissues and is involved in the neurotoxic effects in Alzheimer disease. We demonstrated the presence of the amyloid precursor protein/AB peptide in intramuscular axons, presynaptic motor nerve terminals, terminal and myelinating Schwann cells, and the postsynaptic and subsarclemmal region in the \textit{Levator auris longus} muscle of adult rats by immunocytochemistry. Using intracellular recording, we investigated possible short-term functional effects of the AB fragment (0.1–10 μmol/L) on acetylcholine release in adult and newborn motor end plates. We found no change in evoked, spontaneous transmitter release or resting membrane potential of the muscle cells. A previous block of the presynaptic muscarinic receptor subtypes and a previous block or stimulation of protein kinase C revealed no masked effect of the peptide on the regulation of transmitter release. The aggregated form of AB peptide\textsubscript{25-35}, however, interfered acutely with acetylcholine release (quantal content reduction) when synaptic activity was maintained by electric stimulation. The possible relevance of this inhibition of neurotransmission by AB peptide\textsubscript{25-35} to the pathogenesis of Alzheimer remains to be determined.

Key Words: ACh release, Amyloid precursor protein, β-amyloid peptide\textsubscript{25-35}, Motor nerve terminals, Presynaptic muscarinic receptors, Protein kinase C

INTRODUCTION
Alzheimer disease (AD) is an incurable neurodegenerative condition characterized by a progressive decline in cognitive function. The neuropathologic hallmarks of AD are amyloid plaques, neurofibrillary tangles, and degeneration of basal forebrain cholinergic neurons. Loss of cortical and hippocampal cholinergic function as a result of basal forebrain compromise (1) and amyloid deposits in these regions are consistent features (2). β-amyloid (AB) is a 39– to 43-amino acid peptide initially found within a longer protein, and the amyloid precursor protein (APP) (3) is present in the plaque cores. Amyloid precursor protein is a ubiquitous membrane-bound protein, whereas AB is an internal peptide derived from APP by proteolytic processes (4). It has been suggested that AB possesses deleterious and potentially neurotoxic properties (5). Although its exact roles remain unclear, the full-length APP has been shown to be associated with various biologic activities, including cell growth, neurite outgrowth, and adhesiveness (6), and synaptic plasticity (7). These data underscore the notion that APP contains sequences that are potentially either trophic or deleterious, and that the particular effect may result from differential processing and cleavage of APP. The AB peptide\textsubscript{25-35} (AB\textsubscript{25-35}; Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met) has the functional domain of AB required for both neurotrophic and neurotoxic effects (8, 9).

Many factors influence the processing and metabolism of APP, including those associated with the cholinergic system, that is, M1- and M3-type cholinergic muscarinic receptors (mAChRs) (10), the phosphoinositide second-messenger system, and protein kinase C (PKC) (11). Indeed, many synaptic proteins previously identified in the brain are also concentrated on the neuromuscular synapse, including APP (12). In skeletal muscle, APP is localized at the neuromuscular junctions (NMJs), and there are structural and functional adaptations of the neuromuscular synapse during development in the absence of APP protein, indicating that APP is important for full maturation of the neuromuscular system (13).

In this study, we investigated the precise localization of the APP/AB in the complex tripartite neuromuscular synapse (presynaptic motor nerve terminal, postsynaptic muscle component, and terminal glial Schwann cell) in the rat \textit{Levator auris longus} (LAL) muscle. We also investigated possible short-term functional effects of AB\textsubscript{25-35} in acetylcholine (ACh) release and in interactions with the presynaptic mAChR-PKC–mediated intracellular cascade that modulates transmitter release in the NMJ. We hypothesize that interference or involvement of the AB derivative with normal transmitter release may be relevant to the pathogenesis of AD.

Our major findings are that APP/AB is present in all 3 cellular elements of the NMJ, and that the aggregated form of AB\textsubscript{25-35} acutely interferes with ACh release in a synaptic activity–dependent manner, whereas the nonaggregated form of the peptide does not directly affect ACh release.
MATERIALS AND METHODS

Animals
Experiments were performed on the LAL muscle of neonatal (6 days postnatal) and adult (30 days postnatal) Sprague-Dawley rats (Crlfa, Barcelona, Spain). The rats were cared for in accordance with the guidelines of the European Community’s Council Directive of November 24, 1986 (86/609/EEC) for the humane treatment of laboratory animals. The animals were anesthetized with 2% tribromoethanol (0.15 ml/10 g body weight, i.p.) and killed by exsanguination while they were deeply anesthetized.

Electrophysiologic Recordings
The LAL muscle with its nerve supply was excised and dissected on a Sylgard-coated petri dish containing normal Ringer solution (in mmol/L): NaCl, 137; KCl, 5; CaCl₂, 2; MgSO₄, 1; NaHCO₃, 12; Na₂HPO₄, 1; and glucose, 11, continuously bubbled with 95% O₂/5% CO₂. The preparation was then transferred to a recording chamber of 5 ml for adult muscles and 1 ml for neonatal LAL muscles. Experiments were performed at room temperature (22°C–25°C). The bath temperature was monitored during experiments (23.4°C ± 1.7°C; Digital Thermometer TMP 812; Letica, Barcelona, Spain). End-plate potentials (EPPs) were recorded intracellularly with conventional glass microelectrodes filled with 3 mol/L KCl (resistance, 20–40 MΩ for adult muscles and 40–70 MΩ for the newborn muscles). Recording electrodes were connected to an amplifier (Tecktronics, AMS02), and a distant Ag-AgCl electrode connected to the bath solution via an agar bridge (3.5% agar in 137 mmol/L NaCl) was used as reference. The signals were digitized (DIGIDATA 1322A Interface; Axon Instruments, Inc., Sunnyvale, CA), stored, and computer-analyzed. The software Axoscope 9.0 (Axon Instruments, Inc.) was used for data acquisition and analysis.

In previous studies of muscarinic (14), PKC (15), and protein kinase A (16) drugs, standard sharp-electrode intracellular recording techniques were used to show that miniature end-plate potential (MEPP) amplitudes and postsynaptic resting membrane potentials were unaffected, and, therefore, that all of the drugs act presynaptically under these conditions. We analyzed the dose-response relationships of all the drugs on the MEPPs from adult muscles. Our major goals were to eliminate the postsynaptic effects of the drugs and to use the highest drug concentration that does not change the size of the MEPPs.

To prevent stimulation-induced contractions in both adult and newborn muscles, we used µ-conotoxin GIIB (3 µmol/L) to block muscle fiber action potentials. Small EPPs were visible with this procedure. We were able to determine the number of axonal inputs to each muscle fiber of the newborn animals. The synaptic activity was carefully recorded only in the most superficial fibers of the flat LAL muscle preparation that had been cleaned of connective tissue. In the adult muscles, after a muscle fiber had been impaled, the nerve was continuously stimulated (70 stimuli at 0.5 Hz) using 2 platinum electrodes coupled to a pulse generator (CIBERTEC CS-20) linked to a stimulus isolation unit.

We recorded the last 50 EPPs and used only the results from preparations that had a resting potential lower than −80 mV and which did not deviate by more than 5 mV during the experimental paradigms. The mean amplitude per fiber was calculated and corrected for nonlinear summation (EPPs were usually more than 4 mV, assuming a membrane potential of −80 mV (17). The mean quantal content (m_q) of the evoked response was evaluated by the coefficient of variation method. The membrane potential did not change significantly during the longest exposures to the drugs that modulate PKC activity or mAChRs functionality. We studied a minimum of 15 fibers per muscle and usually a minimum of 5 muscles in each type of experiment.

In newborn animals (6 days postnatal), the nerve was stimulated by a suction electrode. When a neonatal muscle fiber was impaled, the nerve was stimulated with increasing intensity from zero until we observed an EPP. If the size and latency of the EPP remained constant as the stimulus increased, we concluded that the end plate was monoinnervated. In end plates with polynuclear innervation, increasing the stimulus amplitude caused 1 or more axons to be recruited. This produced a stepwise increment in the EPP (18). With dually innervated fibers, a second EPP can appear after the first one when the intensity of the electric stimulus is increased. This compound EPP is built by the recruitment of 2 axons. In this study, we calculated the EPP amplitude of the second axon response by subtracting the first EPP amplitude from the EPP compound. The lowest and highest amplitudes are designated “the small EPP” and “the large EPP,” respectively. Once we had determined the number of axonal inputs per end plate and the single or dual innervation of an NMJ, the nerve was stimulated. At the monoinnervated end plates, a train of 20 stimuli (0.5 Hz) was delivered, and the last 10 EPPs were recorded. At dual junctions, we repeated this procedure for both axonal inputs by changing the stimulus amplitude. We studied a minimum of 15 fibers per muscle and usually a minimum of 5 muscles in each type of experiment. In all cases, the recordings in which the resting membrane potential fell by more than 5 mV during the recording period were rejected. The mean amplitude (mV) per fiber was calculated and corrected for nonlinear summation. To calculate the mean amplitude, the failures were considered as amplitude = 0 mV. To ensure that the recordings were made close to the end plate(s), we only recorded from sites where the EPP had the steepest rise time possible. On Day 6, the mean number of axonal connections per synapse (polynervation index) was 1.63 ± 0.14 (n = 27 muscles; 538 NMJ).

Western Blot
Neonatal and adult LAL muscles, adult brain, and adult spinal cord from 6 adult and 15 neonatal rats were used for Western blotting. After homogenization and extraction of the insoluble material, the samples were centrifuged at 15,000 × g for 15 minutes, and the supernatants were analyzed. Samples (100 µg of protein) were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (19) and transferred to polyvinylidene difluoride membranes (Amersham-Pharmacia, Uppsala, Sweden). The polyvinylidene...
difficulties were incubated with AB rabbit polyclonal (H43, sc-9129; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) raised against amino acids 672–714 of amyloid A4 representing full-length AB protein. This antibody is recommended for the detection of 4- to 46-kDa AB and 100- to 125-kDa amyloid A4. Henceforth, this antibody will be named anti-APP/anti-AB antibody. The antibody was diluted at 1:500 in 4% milk-phosphate-buffered saline (PBS) overnight at 4°C. Horseradish peroxidase–conjugated secondary antibody from Jackson Immunoresearch (Suffolk, UK) was used at a dilution of 1/5000 and revealed by chemiluminescence with an enhanced chemiluminescence kit (Amersham Live Science). To quantify 34-kDa AB expression, we used the densitometry program Phoretix (Non Linear Dynamics, Newcastle-Upon-Tyne, UK) to analyze the density of different bands. Background readings were determined by measuring the optical density outside the sample lanes. After subtracting the background, the means of 3 consecutive readings of protein immunoreactivity at the 34-kDa positions for each sample lane were obtained. Because of intergel variation, only gels that were performed on the same day were used in comparison studies. The results were expressed in average optical density units.

Immunohistochemistry

Levator auris longus muscles were dissected from anesthetized adult rats and fixed with 4% paraformaldehyde in PBS for 30 minutes. After fixation, the muscles were rinsed with PBS and then incubated with 1% bovine serum albumin and 0.1% Triton X-100 in PBS. Triple immunofluorescence and confocal analysis were performed. Whole mounts of LAL were processed to simultaneously detect postsynaptic nicotinic ACh receptors (nAChRs), APP/AB, and syntaxin or S-100. Muscles were incubated overnight with the rabbit antibody against APP/AB (1/50; Santa Cruz) and a mouse anti-syntaxin monoclonal antibody (1:1000; Sigma, St. Louis, MO) or a mouse anti-S-100 antibody (1:10000; Dako, Glostrup, Denmark) in 1% bovine serum albumin. The appropriate 2 secondary antibodies conjugated with Alexa-Fluor 488 or tetrarhodamine isothiocyanate were then added and incubated for 4 hours. Nicotinic ACh receptors were stained with Alexa Fluor 647-α-bungarotoxin (α-BTX). In some cases, we used plastic-embedded semithin sections for high-resolution immunofluorescence analysis of the NMJ molecules (20). All labeled muscles, nerves, and NMJ processed by immunohistochemistry or by plastic-embedded semithin sections were viewed with a confocal laser-scanning microscope (Olympus Fluoview 500; Olympus, Tokyo, Japan). Negative controls included incubation in the absence of primary antibody and incubation with a nonspecific secondary antibody.

Statistical Procedure

In the electrophysiologic experiments, values are expressed as mean ± SEM. We used a 1-way analysis of variance to evaluate differences between groups and the Bonferroni test for multiple comparisons. When differences were evaluated only between 2 groups, we used 2-tailed Welch t-test (for unpaired values and not assuming equal variances). Differences were considered significant at p < 0.05.

RESULTS

Expression of the APP and AB Peptide in the LAL Skeletal Muscle

To determine the presence of the APP and AB in the LAL muscle before analyzing their immunocytochemical localization in the NMJ, the antibody was tested with immunoblots of homogenates of the newborn and adult muscle and adult brain and spinal cord (Fig. 1). In the muscle extracts from newborn and adult animals, anti-APP/AB antibody reacted with 2 immunoreactive bands of 34 (AB) and 99 kDa (amyloid A4). The same bands were observed in the adult brain and spinal cord homogenates.

Localization of the APP/AB by Immunohistochemistry on the LAL Skeletal Muscle NMJ

To determine the presence of APP/AB in the NMJ, we performed a confocal immunofluorescence analysis (Figs. 2–4). Three representative molecules of each cellular component of the NMJ were used: syntaxin as a marker of the axons and nerve terminals, S-100 as a marker of Schwann cells (both teloglia and myelinating), and α-BTX, which links to the postsynaptic AChRs. In the regions of innervation of all the adult muscles, we observed discrete fluorescently labeled areas with the antibody to APP/AB protein. These areas colocalized with Alexa 487-α-BTX-labeled nAChRs and also with anti-syntaxin-labeled motor nerve terminals and anti-S-100-labeled Schwann cells. In some

![FIGURE 1. Detection of amyloid precursor protein (APP)/β-amyloid (AB) in rat tissue lysates. The anti-APP/anti-AB antibody was tested with immunoblot of homogenates (100 µg of protein) of the newborn (lane 2) and adult (lane 1) Levator auris longus muscle, adult brain (positive control, lane 3), and spinal cord (lane 4). Lane 5 is the negative control incubated in the absence of primary antibody. In all cases, the anti-APP/anti-AB antibody reacted with 2 bands of 34 and 99 kDa, and the band position was consistent with the predicted molecular mass of these molecules. Quantitative densitometry showed that during muscle maturation 6 to 30 days postnatal, 34-kDa AB decreases (2.24 ± 0.15-fold). The amounts of AB in adult muscle, in the adult spinal cord, and in the brain are 12.71 ± 1.250, 26.093 ± 3.190, and 33.893 ± 4.567 densitometric units, respectively. This result indicates the higher amount of this protein in the adult brain.](http://jnen.oxfordjournals.org/)

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APP/AB and colocalization pictures of the plates, the following structures were immunoreactive with the APP/AB antibody (Figs. 2–4): 1) the nerve terminal (blue asterisks); 2) the preterminal axon (blue arrow); 3) the extrasynaptic muscle cell (yellow arrow); 4) the postsynaptic end-plate region (pink arrow); 5) the terminal or telogial Schwann cell (green arrow); and 6) the preterminal or myelinogenic Schwann cell (red arrow).

We also examined triple-labeled plastic-embedded semithin cross sections of adult rat LAL muscles (Fig. 4). We previously showed that semithin (0.5–0.7 μm) cross sections from whole-mount multiple immunofluorescent-stained muscles provide a simple and sensitive procedure for analyzing the cellular distribution of molecules at the NMJ. The postsynaptic clusters of nAChRs were identified by labeling with α-B TX (red fluorescence), APP/AB immunoreactivity was identified by green fluorescence and S-100-positive Schwann cells (Fig. 4A), and syntaxin-positive nerve terminals (Fig. 4B) were identified by blue fluorescence. With this procedure, the green axonal elements (Fig. 4A; immunoreactive for the anti-APP/anti-AB antibody) can be seen between the blue Schwann cell and the red arcs of the postsynaptic gutters, with both elements also being immunoreactive for the anti-APP/anti-AB antibody. A clear distinction between the marks of the 3 elements of the NMJ is also observed in Figure 4B, in which the syntaxin is identified instead of the S-100. There was also evidence of some extrajunctional sarcolemma and cytoplasm staining. Therefore, the anti-APP/anti-AB antibody demonstrated distributions consistent with APP/AB concentration at the postsynaptic site of the NMJ, at the Schwann cells, and at the nerve terminal. The distribution of APP/AB at the postsynaptic site of the NMJ indicated that APP/AB was mainly concentrated at the primary gutter region where the nAChRs are located and fully occupy the postsynaptic region. We also observed the APP/AB signal above the location of the axonal terminals, consistent with labeling of Schwann cell processes that cap the nerve terminal. When the distribution of APP/AB was compared with that of S-100 (the Schwann cell marker), the immunoreactivities were largely coincidental.

In summary, APP/AB was present in the 3 cellular elements of the adult neuromuscular synapses: 1) the intra-muscular axons (nerve fibers) and motor nerve terminals; 2) the terminal and myelinating Schwann cells; and 3) the postsynaptic muscle cell area. The subplasmolemmal region of the muscle fibers also shows immunoreactivity.

**AB$_{25,35}$ and Transmitter Release in the Neuromuscular Synapse**

We next investigated a possible functional role for the AB$_{25,35}$ in the NMJ. First, we tried to determine a baseline

**FIGURE 2.** Amyloid precursor protein (APP)/α-amyloid (AB) immunolocalization in neuromuscular junction (NMJ). Muscles were stained with anti-syntaxin (marker of nerve terminals), anti-S-100 (marker of Schwann cells), and α-bungarotoxin (α-BTX), which links to the nicotinic acetylcholine receptor (nAChR) in the postsynaptic membrane. We performed triple labeling with anti-APP/anti-AB, anti-syntaxin, and α-BTX (A, B, C, D). At least 3 muscles were used as negative controls (1 control was incubated in the absence of antibody, and another was incubated with a nonspecific secondary antibody). In the APP/AB and colocalization panels, the following structures are immunoreactive for the APP/AB antibody: 1) the nerve terminal (blue asterisk); 2) the preterminal axon (blue arrow); 3) extrasynaptic muscle cell (yellow arrow); 4) postsynaptic endplate region (pink arrow); 5) terminal or telogial Schwann cell (green arrow); and 6) preterminal or myelinogenic Schwann cell (red arrow). At the postsynaptic level, a finely granulated APP/AB mark was diffuse inside the motor endplate area between the synaptic gutters at the base of the postsynaptic folds (C, D, pink arrows) and extended beyond close to the subsarcolemmal cytoplasm (C, yellow arrow). The colabeling of syntaxin and APP/AB is high. The blue asterisks (B) indicate an APP-/AB-immunoreactive terminal arbor, and high-density APP-/AB-positive spots (C, D) that in the respective colocalization panels correspond to well-delimited areas within AChR-delineated primary gutters. The blue arrow (D) also shows an APP-/AB-immunoreactive preterminal axon that reaches the end plate. The green and red arrows (A) show some faint APP/AB immunoreactivity in an NMJ area outside the borders of syntaxin and nAChR labeling, which indicates the presence of APP/AB also in the Schwann cells (both telogial and preterminal). Scale bar = 10 μm.

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cubation in different AB25-35 concentrations and found that EPPs, and muscle membrane potential after 1 hour of incubation showed a significant postsynaptic modification. The unchanged amplitude (9.5% ± 2.6%), MEPP frequency (% change, 11.4% ± 3.6%), and membrane potential (% change, 1.35% ± 0.12%). The unchanged amplitude of MEPPs indicates that a significant postsynaptic modification of the postsynaptic AChRs by AB25-35 is not likely in these experiments. We also investigated effects of a very high concentration of the peptide (25 μmol/L) on normal evoked neurotransmission (quantal content) and did not observe an effect (% change, 0.64% ± 1.02%; p > 0.05).

We also investigated the existence of a possible action by AB25-35 after exposure of more than 1 hour (Fig. 5B). A subcutaneous injection of AB25-35 (100 μL, 10 μmol/L) was made over the LAL muscle surface, and the muscle was studied 24 hours later, as previously described (21). We found no significant change in the transmitter release parameters considered.

In addition, we investigated the possibility that the system was already AB saturated, so the exogenously applied peptide could not produce an additional effect. We preincubated the LAL muscle for 1 hour with the same antibody used against the APP/AB in the Western immunoblots and immunohistochemistry but at a dose of 10 ng/mL. This concentration is commonly used in blocking experiments with antibodies. Then, we investigated a possible effect of the AB25-35 on transmitter release. We found that neither the antibody incubation by itself nor the subsequent incubation with the peptide modifies the transmitter release (% change, 3.99% ± 4.20%; p > 0.05).

FIGURE 3. Confocal optical sections of the triple labeling of the neuromuscular junctions (NMJs). To further demonstrate the postsynaptic and Schwann cell amyloid precursor protein (APP)/β-amyloid (AB) immunoreactivity, the figure shows 2 nonconsecutive confocal optical sections (A), and the final projection of these and other (4 additional) sections on the z axis of an NMJ in which a triple labeling with anti-APP/anti-AB, anti-S-100, and α-bungarotoxin (α-BTX) has been performed. The pink arrow in optical section 2 indicates a strong postsynaptic immunoreactivity for APP/AB around the sole plate nuclei. Optical section 5 shows APP/AB immunoreactivity around the extrasynaptic myonuclei and subsarcolemmal region (yellow arrow) on a neighboring muscle fiber. This demonstrates the presence of the protein in extrasynaptic sites of the muscle cells. The figure shows a final projection of another NMJ in which triple labeling with anti-APP/anti-AB, anti-syntaxin, and α-BTX has been performed (B). These panels show the preterminal myelinogenic Schwann cell (red arrows) and axon (blue arrow). Scale bar = 10 μm.

FIGURE 4. Semithin (0.5 μm) cross sections of the Levator auris longus (LAL) muscle stained with a triple-immunofluorescence method. The LAL muscles were incubated with primary antibodies (anti-syntaxin, anti-S-100, and anti-amylloid precursor protein [APP]/β-amyloid [AB]) and then incubated with appropriate secondary antibodies conjugated to either Alexa Fluor-488, Alexa Fluor-647, and α-bungarotoxin (α-BTX) conjugated with tetrarhodamine isothiocyanate. After dehydration, the muscles were embedded in Spurr resin in transverse orientation. Sections 0.5- to 0.7-μm thick were cut with a Reichert Ultracut E microtome (Leica Microsystems, Bannockburn, IL). The postsynaptic clusters of nicotinic acetylcholine receptors were identified by labeling with α-BTX (red fluorescence), APP/AB immunoreactivity was identified by the green fluorescence, and S-100-positive Schwann cells (A) and syntaxin-positive nerve terminals (B) were identified by the blue fluorescence. With this procedure (A), a sandwich of the green axonal elements (immunoreactive for the anti-APP/anti-AB antibody) can be seen between the blue Schwann cell and the red arcs of the postsynaptic gutters. Both elements are also immunoreactive for the anti-APP/anti-AB antibody. The antibody against APP/AB therefore demonstrates distributions consistent with APP/AB concentrations at the postsynaptic site of the NMJ, at the Schwann cells, and at the nerve terminal. Scale bar = 10 μm.
We also investigated a possible effect of the AB25-35 on the immature motor end plates of the newborn animal. At birth, almost all synapses are polyinnervated. In previous studies, we found that in the LAL muscles, at 5 to 6 days postnatal, roughly half of the synapses become monoinnervated as in the adult, but the remaining synapses are still polyinnervated (21, 22). We investigated a possible effect of the AB25-35 in singly and dually innervated synapses. Figure 6A shows raw data for the large and small EPP in dual junctions before (left) and after (right) AB25-35 incubation with no change. Figure 6B shows that the EPPs in the monoinnervated synapses and the small and large EPPs in the dually innervated junctions did not change after 1 hour of incubation with AB25-35. The figure also shows that the polyinnervation index was not affected by incubation with AB25-35.

**FIGURE 5.** Neurophysiologic experiments in adult muscles. (A) Concentration-response curve shows miniature end-plate potential (MEPP) frequency, MEPP amplitude, quantal content of the EPPs, and muscle membrane potential plotted against β-amyloid peptide25-35 (AB25-35) concentrations (after 1 h of incubation). β-amyloid peptide25-35 was ineffective in changing the spontaneous and evoked transmitter release parameters (in all cases, p > 0.05). (B) Investigation of possible effect of AB25-35 after exposures of more than 1 hour. The time course includes a 24-hour point for which a subcutaneous injection of AB25-35 (100 µl; 10 µmol/L) over the Levator auris longus muscle surface was performed as previously described (21) and the muscle studied after 1 day. There were no significant changes in transmitter release parameters between 0.5 and 24 hours.

**FIGURE 6.** Neurophysiologic experiments in newborns. We investigated a possible effect of the AB25-35 on the immature motor end plates of the newborn animal. At birth, almost all synapses are polyinnervated. In previous studies, we found that in the LAL muscles, at 5 to 6 days postnatal, roughly half of the synapses become monoinnervated as in the adult, but the remaining synapses are still polyinnervated (21, 22). We investigated a possible effect of the AB25-35 in singly and dually innervated synapses. Figure 6A shows raw data for the large and small EPP in dual junctions before (left) and after (right) AB25-35 incubation with no change. Figure 6B shows that the EPPs in the monoinnervated synapses and the small and large EPPs in the dually innervated junctions did not change after 1 hour of incubation with AB25-35. The figure also shows that the polyinnervation index was not affected by incubation with AB25-35.

**AB25-35, Muscarinic Receptors, and PKC in Transmitter Release**

Figure 7 shows the results of the experiments designed to reveal an effect of AB25-35 on evoked ACh release (quantal content) when the mAChRs-PKC cascade conditions are changed. As the control, we also include for each drug their effect on release before AB25-35. We can see that after PKC block (calphostin C; 10 µmol/L; 1 hour) or stimulation (phorbol ester 12-myristate 13-acetate; 10 nmol/L; 1 hour), AB25-35 does not induce any change in ACh release. Similarly, the selective block of the M1 mAChRs (pirenzepine; 10 µmol/L; 1 hour) or the global block of both M1 and M2 receptors with Atropine (2 µmol/L; 1 hour) does not change the susceptibility of the end-plate neurotransmission to AB25-35 action.

**Aggregated AB25-35 and Synaptic Activity**

We investigate whether the state of amyloid peptide aggregation can play a role in the involvement of this molecule with neurotransmission. β-amyloid peptide25-35 was dissolved in distilled water at a concentration of 15 nmol/5 µl, and the solution was incubated at 37°C for 4 days before use. This procedure is known to produce insoluble precipitates, but we did not find any neurotransmitter release change (Fig. 8). We also tested whether the level of synaptic activity during incubation can influence the physiologic effect of the peptide and the experimental outcome. We stimulated the muscle in the range 1 to 10 Hz for 1 to 2 hours in the presence of 1 to 10 µmol/L AB35-25 and found a nonsignificant maximal difference with respect to the initial
values of approximately 15%. Finally, we stimulated the muscle at 1 Hz for 1 to 2 hours in the presence of the aggregated peptide. We show that the aggregated form of AB25-35 acutely reduces the quantal content when the synaptic activity of the neuromuscular preparation is maintained (Fig. 8).

DISCUSSION

The physiopathology of AD is partly based on degeneration of the basal forebrain cholinergic neurons. The cholinergic neuromuscular synapse is the model from which much of our understanding of the fundamental nature of synaptic transmission has emerged (23). The quantal nature of transmitter release, the dependence of this process on calcium, and the response of the postsynaptic receptor to the released transmitter are concepts that arose as a result of experiments on the NMJ and have been shown to be of great general validity throughout the brain. The NMJ operates as a complex cholinergic mechanism that includes nAChRs for neurotransmission in the postsynaptic membrane and release-regulatory mAChRs (M1 to M4 subtypes) in the presynaptic membrane (14, 24-28). The terminal glial Schwann cells also have mAChRs (27, 29). In previous studies (14, 16, 26, 30), we found that the M1 and M2 mechanisms were altered when PKC, protein kinase A, or the P/Q-type calcium channel were blocked, and that the muscarinic function may be explained by the existence of an M1-mediated PKC-dependent potentiation of release and an M2-mediated protein kinase A–dependent release reduction. All of these molecules have been associated with APP and AB production (1). Therefore, the cholinergic NMJ may be a good model with which to study the molecules involved in AD.

Many brain synaptic proteins are also concentrated on the postsynaptic muscle side of the NMJ, and this is true for APP and AB. Amyloid precursor protein is found in skeletal muscle localized at the base of the postsynaptic folds of the NMJ (12, 31). Here, we extended these observations as the immunocytochemistry images demonstrate the presence of APP/AB immunoreactivity in all cells of the neuromuscular synapse in the adult. No staining was observed in the vascular or connective elements (not shown). By high-resolution immunocytochemistry, we demonstrate for the first time the simultaneous presence of APP/AB immunoreactivity in all cells of the neuromuscular synapse in the adult. No staining was observed in the vascular or connective elements (not shown). By high-resolution immunocytochemistry, we demonstrate for the first time the simultaneous presence of APP/AB in the important synaptic sites of these cells. Strong immunoreactivity was observed in the motor nerve terminals located within the synaptic gutters but also in the intramuscular preterminal axons; staining was also found in the postsynaptic membrane and the end-plate region between the sole plate nuclei and in the extrasynaptic muscle cell in subsarcolemmal and perinuclear sites. Moreover, APP/AB immunoreactivity was observed in the telogial Schwann cells mainly in the subsarcolemmal position but also in the preterminal or myelogenic Schwann cells. The ubiquity of the APP/AB antigenic determinant in the NMJ suggests involvement of...
this molecule in multiple interactions between synaptic cells, perhaps affecting neurotransmission.

The immunoblots show a developmental change in the APP/AB expression in the LAL muscle between 6 and 30 days postnatal and AB decreases. Using immunocytochemistry, Akaaboune et al (31) detected APP in myotube cytoplasm at embryonic Day 16; it becomes progressively concentrated at the NMJ from birth to adulthood. Therefore, the APP family of proteins and their derivatives such as AB may play a role in the development of neuromuscular synapses. Indeed, APP-deficient mice exhibit structural abnormalities accompanied by defective neurotransmitter release and a high incidence of synaptic failure (13). At the developing NMJ in culture, it has been found that the motor nerve-derived trophic factor neuregulin 1 regulates both steady-state APP levels and the metabolism of the cell surface-associated protein (32). In addition, the effect of a secretory form of APP on synaptic transmission was examined using developing neuromuscular synapses in *Xenopus* cell cultures. The frequency of spontaneous postsynaptic currents was reduced by the addition of APP, whereas the amplitude of impulse-evoked postsynaptic currents was increased. Taken together, these data reinforce the potential role of APP as a modulator of synaptic activity during development (33). In fact, it seems that AB25-35 has the functional domain of AB required for both neurotrophic and neurotoxic effects (8, 9).

Our electrophysiologic experiments with AB25-35 showed no effect of the nonaggregated peptide in the evoked ACh release in the strong and weak nerve terminals that compete in polyinnervated junctions during development and in the more mature endings in the monoinnervated synapses. In previous studies, we showed that these nerve terminals have differences in the stoichiometry and coupling to ACh release of the P/Q-, N-, and L-type voltage-dependent calcium channels (22), M1-, M2-, M3-, and M4-type mACHRs (14, 26), and also in the PKC involvement in transmitter release control (16, 30). These molecules have been associated with APP function and AB production. However, no influence of the nonaggregated AB25-35 was observed. This suggests that a possible function of the peptide in the newborn NMJ was not linked to an AB-mediated modulation of ACh release. Moreover, we found no change in polyinnervation index after AB25-35 incubation. This indicates that the peptide did not accelerate or delay the developmentally regulated activity-dependent synaptic disconnection that we previously demonstrated occurs in the same model after disturbing calcium inflow or PKC activity (30, 34).

In several cellular systems, M1 and M3 receptors preferentially couple to pertussis toxin-insensitive G-proteins of the Gq/11 family to stimulate phospholipase C and, therefore, PKC (28, 35–37). Stimulation of M1 and M3 receptors, or direct activation of PKC by phorbol esters in transfected cell lines, greatly increases secreted APP production with concomitant decreases in AB fragment (10). The evidence suggests that deficits in muscarinic receptors or their signaling activity might lead to abnormal APP processing and the deposition of amyloid protein fragments, leading to AD (35). In fact, deficits in muscarinic receptors, alterations in the phosphoinositide signal transduction system and PKC, and a defective release of secreted APP have been demonstrated in AD. In the LAL muscle preparation (a mature complex cholinergic circuit), we therefore modified several points of the mACHR-PKC intracellular cascade to reveal a possible masked effect of AB25-35. We inhibited M1-, M2-type, or both mACHRs (the subtypes that are present in the adult NMJ) and also inhibited or stimulated PKC. However, in none of these experimental situations did the nonaggregated peptide AB25-35 affect evoked ACh release.

In this study, we show that, although APP/AB is present in all 3 cells of the tripartite neuromuscular synapse, when exogenously added, the nonaggregated AB25-35 does not intervene in newborn or adult nerve-muscle transmission. The ubiquitous presence of APP/AB in the synaptic cells may therefore perform functions other than direct neurotransmission control. The effect of the molecules may be exerted through the aggregated form of the peptide. Subtle alterations in synaptic efficacy were recently postulated to result from the action of diffusible AB (38). The presence of APP/AB in all cellular elements of the NMJ can suggest a 3-site complex interaction. Some experimental evidence suggests that APP and their derivatives may participate in the structural stability of synaptic connections because the synaptic location of related molecules plays this role. Examples are the synaptic location (postsynaptic and Schwann cell sites) of protease-activated, G-protein-coupled receptors for thrombin and related proteases (20, 39), their serpin and Kunitz-type protease inhibitors such as protease nexin I, and the Kunitz protease inhibitor-containing secreted forms of APP (40).
Finally, we investigated whether the state of amyloid peptide aggregation can play a role in the involvement of this molecule with neurotransmission, as shown in the brain (41). Insoluble precipitates clearly facilitate the appearance of this molecule with neurotransmission, as shown in the brain peptide aggregation can play a role in the involvement of the neuromuscular preparation is maintained. Therefore, our results preclude doubts regarding the peptide activity and accessibility to the synaptic elements and identify the conditions for short-term amyloid peptide involvement in neurotransmission. The inhibition of neurotransmission by the aggregated form of Aβ25-35 in active synapses is a preliminary result. Further experiments are necessary to investigate their relation to the pathogenesis of AD.

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