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Defining Presynaptic Nicotinic Receptors Regulated by Beta Amyloid in Mouse Cortex and Hippocampus with Receptor Null Mutants

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Abstract

Disruption of neuronal signaling by soluble β-amyloid has been implicated in deficits in short-term recall in the early stages of Alzheimer's disease. One potential target for β-amyloid is the synapse, with evidence for differential interaction with both pre- and postsynaptic elements. Our previous work revealed an agonist-like action of pM-nM soluble β-amyloid on isolated presynaptic terminals to increase $[Ca^{2+}]i$, with apparent involvement of presynaptic nicotinic receptors. To directly establish the role of nicotinic receptors in presynaptic Ca^{2+} regulation, we investigated the presynaptic action of β-amyloid on terminals isolated from mice harboring either β2 or α7 nicotinic receptor null mutants (knockouts). Average presynaptic responses to β-amyloid in hippocampal terminals of α7 knockout mice were unchanged, whereas responses in hippocampal terminals from β2 knockout mice were strongly attenuated. In contrast, presynaptic responses to soluble β-amyloid were strongly attenuated in cortical terminals from α7 knockout mice, but were moderately attenuated in cortical terminals from β2 knockout mice. The latter responses, having distinct kinetics, were completely blocked by α-bungarotoxin. The use of receptor null mutants thus permitted direct demonstration of the involvement of specific nicotinic receptors in presynaptic Ca^{2+} regulation by soluble β-amyloid, and also indicated differential neuromodulation by β-amyloid of synapses in hippocampus and cortex.

Keywords

Calcium regulation; Presynaptic terminal; Nicotinic Receptors

Introduction

The physiological role for β-amyloid (Aβ) is unknown. It is present in the adult mammalian brain in the absence of cognitive impairment, being produced and cleared at remarkable rates (Bateman *et al*. 2006). There is also evidence that the level of soluble β-amyloid in the brain is regulated by nerve activity (Kamenetz *et al*. 2003; Cirrito *et al*. 2005). Finally, there is evidence that β-amyloid is released from nerve endings, based on the finding that β-

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amyloid pathology is sharply reduced in terminal fields of lesioned pathways in APP transgenic mice (Lazarov *et al*. 2002; Sheng *et al*. 2002). Thus, there is abundant β-amyloid at or near select synapses in the brain.

Aβ refers to a collection of 38−43 amino acid peptides derived from the amyloid precursor protein by successive rounds of proteolytic cleavage (see Walsh and Selkoe 2007), with $A\beta_{1-40}$ and $A\beta_{1-42}$ being the predominant species found in the brains of Alzheimer's patients. A significant portion of the β-amyloid remains soluble, mainly as small oligomers, over the course of Alzheimer's disease, with the absolute amyloid burden correlating best with symptoms (McLean *et al*. 1999). However, as it accumulates, the Aβ assembles into fibrils, which is the primary form found in neuritic plaques. It is likely that Aβ has multiple targets and consequently actions, particularly across the various molecular forms.

In searching for candidate targets, Aβ was reported to bind with relatively high affinity to nicotinic acetylcholine receptors (nAChRs), in particular those containing the α7 subunit (Wang *et al*. 2000). Later, Aβ was found to have both antagonist (Pettit *et al*. 2001; Liu *et al*. 2001; Grassi *et al*. 2003; Wu *et al*. 2004) and agonist (Dineley *et al*. 2001; Dougherty *et al*. 2003; Fu and Jhamandas 2003; Puzzo *et al*. 2008) actions, depending on the preparation. Specifically, we have shown that application of low concentrations (pM-nM) of soluble $A\beta_{1-42}$ to isolated presynaptic terminals from rat cortex or hippocampus evoked increases in $[Ca^{2+}]i$ that were antagonized, at least in part, by classical nAChR antagonists, though the antagonism depended upon the Aβ concentration (Dougherty *et al*. 2003). In addition, prior activation of presynaptic nAChRs with nicotine led to an occlusion of subsequent responses to soluble $A\beta_{1-42}$. These results implicated presynaptic AChRs as a target for Aβ. However, the possibility remains that the effect of Aβ on nAChRs is indirect. Here, we employ preparations from mice harboring null mutations for either the α7 subunit or the β2 subunit of the two major nAChR subtypes present in brain, namely the α-bungarotoxin-sensitive and high affinity subtypes, respectively (McGehee and Role 1995; Role and Berg 1996; Zoli *et al*. 1998; Nashmi and Lester 2006) in order to assess their roles in the presynaptic agonist action of Aβ.

EXPERIMENTAL PROCEDURES

Purification of isolated presynaptic nerve terminals

Intact isolated presynaptic nerve terminals (synaptosomes) were purified as described previously (Nayak *et al*. 2001). In brief, hippocampi or cerebral cortices from adult C57Bl/6J mice (Jackson Labs, Bar Harbor, ME) were dissected in ice-cold 0.32 M sucrose. The tissue was rapidly homogenized in ice-cold 0.32 M sucrose with a glass-Teflon tissue grinder. Synaptosomes were isolated using the Percoll step gradient method (Dunkley *et al*. 1986). The purified synaptosomes were washed with oxygenated HEPES-buffered saline (HBS, pH 7.4) containing 142 mM NaCl, 2.4 mM KCl, 1.2 mM K_2HPO_4, 1 mM $MgCl_2$, 5 mM D-glucose, and 10 mM HEPES, containing 1mM Ca^{2+}. The protocol used for this study was approved by the Drexel University College of Medicine Institutional Animal Care and Use Committee.

Animals

Adult wild-type C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Adult mice harboring a null mutation of β2 subunit of the nAChR were from an established colony of heterozygous breeders, originally obtained from Dr. Marina Picciotto (Yale University). Adult mice harboring a null mutation of the β7 subunit of the nAChR were from an established colony of heterozygous breeders, originally obtained from Dr. Michael Marks (University of Colorado). Homozygous null mutants were identified via

genotyping. Both transgenic lines were maintained on a C57Bl/6J background. All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility.

Measurement of relative Ca^{2+} levels

Fluo-4 was loaded into the purified synaptosomes suspended in HBS containing 1 mM Ca^{2+}, using the acetoxymethyl ester derivatives (AM) of the dye at 5μM final concentration, for 60 min at 37°C as previously described (Nayak *et al*. 2001). The dye-loaded synaptosomes were then washed by centrifugation and resuspended in HBS. The preparations were plated onto coverslips coated with Cell-Tak and then inserted into a rapid-exchange Warner perfusion system mounted on a Nikon Diaphot microscope attached to a Nikon PCM 2000 laser-scanning confocal imaging system. Fluorescent images were recorded in response to excitation at 488 nm. During the confocal imaging, the preparations were under constant perfusion at 3−5 ml/min with HBS. Images were typically collected at 2-s or 4-s intervals, with the first 5 consecutive images collected as a baseline. Each experiment corresponds to sequential images collected using a single preparation subjected to various conditions and/or reagents.

Data analysis

The quantification of fluorescence intensities associated with individual synaptosomes recorded in digitized images was calculated using MetaMorph (Molecular Devices, Downingtown, PA, USA) and corrected for photobleaching based on the baseline images (typically <3%). Individual synaptosomes are identified by their size (0.5−2μm) and correspondent positive immunostaining for presynaptic markers (Nichols and Mollard 1996; Rondé and Nichols 1998; Díaz-Hernández *et al*. 2002; Wu *et al*. 2006), and are well resolved in confocal imaging, where only dye-loaded structures are immunopositive for the presynaptic marker (Rondé and Nichols 1998). Analysis of synaptosome-associated fluorescent intensities was performed by an observer blind to the experimental conditions. Response to depolarization evoked by elevated extracellular K^+ was used as a criterion for synaptosomal viability for each preparation. Data are presented as normalized responses (F/F_0, where F_0 is the fluorescence intensity associated with a given structure at t_0). All experiments were independently replicated at least 3 times. Sample number (*n*) refers to the number of individual synaptosomes included in the averaged (pooled) results.

Materials

Fluo-4/AM was purchased from Molecular Probes (Eugene, OR, USA). The adhesive matrix Cell-Tak was from BD Sciences (Bedford, MA, USA). Percoll was originally from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Ultrapure sucrose was from ICN Biomedicals (Aurora, OH, USA). HEPES (ULTROL grade) and α-bungarotoxin were from Calbiochem (San Diego, CA, USA). Nicotine was from Sigma (St. Louis, MO, USA). $Aβ_{1–42}$ and "$Aβ_{42–1}$" were from Bachem Bioscience (King of Prussia, PA, USA). The amyloid peptides were solubilized by vigorous vortexing in HBS prior to use at pM to nM concentration (Dougherty *et al*. 2003). Under these conditions, the Aβ largely consists of small oligomers (Bell *et al*. 2004). All other chemicals were of the highest reagent grade.

RESULTS

Nicotine induced significant increases in relative $[Ca^{2+}]i$ in individual isolated nerve terminals (synaptosomes) from mouse hippocampus (Fig.1) in a manner very similar to that found using rat preparations (Nayak *et al*. 2001). In particular, the Ca^{2+} responses in hippocampal terminals to nicotine were relatively sustained, inactivating after 1−2 min. Such prolonged presynaptic Ca^{2+} responses are consistent with sustained changes in

synaptic spontaneous release frequency in response to nicotine found in several previous studies (eg. McGehee *et al*. 1995; Gray *et al*. 1996). Likewise, nM concentrations of $A\beta_{1-42}$ induced increases in relative $[Ca^{2+}]i$ in hippocampal synaptosomes that were mainly sustained, with occasional transient responses observed (Fig. 2, top). No effect of the control peptide $A\beta_{42-1}$ was observed (not shown). The responses were unaffected in preparations from α7 nAChR subunit null mutant mice (Fig. 2A), were only partially affected by α-bungarotoxin (BgTx), a highly selective α7 antagonist (Fig. 2B), and were largely blocked by dihydro-β-erythroidine (DHBE; Fig. 2C), a nicotinic antagonist with some degree of selectivity for α4 containing nAChRs and no activity at α7 nAChRs (Luetje *et al*. 1990). These results parallel previous evidence showing that presynaptic responses to nicotine in hippocampal synaptosomes are sensitive to DHBE but not methyllycaconitine (eg. Wilkie *et al*. 1996). Finally, the responses were lost in preparations from β2 nAChR subunit null mutant mice (Fig. 2D). As previously shown for rat preparations (Dougherty *et al*. 2003), prior stimulation with Aβ attenuated subsequent responses to nicotine, but only following robust responses to Aβ (Fig. 2E).

In contrast, Ca^{2+} responses to nM concentrations of $A\beta_{1-42}$, in cortical synaptosomes were relatively transient but quite robust, with a subset displaying more sustained time-courses, but not sustained to the extent observed for hippocampal presynaptic responses (Fig. 3, top). The proportion of responses with more sustained time-courses was larger in preparations stimulated with 100pM $A\beta_{1-42}$, whereas only sustained, small amplitude responses were evident on stimulation with 10pM $A\beta_{1-42}$. This difference was previously observed for responses to nicotine in striatal synaptosomes (Nayak *et al*. 2001) and cortical synaptosomes (Dougherty *et al*. 2008). The differences in kinetics of responses to various concentrations of Aβ likely indicate a dose-dependency of inactivation (Dani and Bertrand 2007), with higher concentration resulting in higher rates of inactivation. The Aβ-induced responses were nearly eliminated in preparations from α7 nAChR subunit null mutant mice, using either 100nM or 100pM $A\beta_{1-42}$. In cortical synaptosome preparations from β2 nAChR subunit null mutant mice, Ca^{2+} responses to Aβ were significantly attenuated and, more importantly, had more sustained time-courses (Fig. 3B). The smaller Ca^{2+} responses to Aβ in cortical synaptosomes from β2 nAChR subunit null mutant mice were sensitive to α-BgTx (Fig. 3C), indicating that they involve an α7 containing nAChR.

DISCUSSION

Soluble $A\beta_{1-42}$ at pM to nM concentration, largely in oligomeric form (Bell *et al*, 2004), was previously found to evoke increases in presynaptic $[Ca^{2+}]i$ in individual terminals from rat brain in a nAChR antagonist-sensitive manner (Dougherty *et al*. 2003). However, block of Aβ-induced presynaptic Ca^{2+} responses by nicotinic antagonist was partial and hence the present study was performed to more directly address the role of nAChRs. To identify the basic subtypes of nAChR possibly involved in the presynaptic Ca^{2+} responses to acute Aβ, synaptosomal preparations from mice harboring null mutations either for the α7 nAChR subunit or the β2 nAChR subunit were used.

Aβ-induced Ca^{2+} responses in hippocampal synaptosomes appeared to largely involve β2* nAChRs, though some expression of presynaptic α7* nAChRs is suggested by the findings. Presynaptic α7* nAChRs have been noted in rat hippocampal terminals (Fabian-Fine *et al*. 2001). That they did not appear to be prominent among Ca^{2+} responses in mouse hippocampal synaptosomes may reflect a species difference in their relative localization on the nerve terminal (preterminal vs. presynaptic) or, as noted previously, their coupling to the pathways/sources for synaptosomal Ca^{2+}.

In contrast, Aβ-induced Ca^{2+} responses and nicotine-induced Ca^{2+} responses (Dougherty *et al*. 2008) in cortical synaptosomes appeared to largely involve α7* nAChRs, but the results using Aβ also indicated the possible presence of β2 containing receptors. By combining the use of a highly selective α7 nAChR antagonist, namely α-bungarotoxin, with synaptosomes prepared from cortices of β2 subunit null mutants, the results suggest the presence of α7 homomeric channels together with a possible β2* containing nAChR. However, the apparent α7 homomeric nAChR-coupled responses induced in cortical synaptosomes from β2 subunit null mutant mice to nM Aβ were smaller in amplitude, similar to what was observed to pM Aβ applied to control cortical preparations. There are several possible factors contributing to this difference. A very likely possibility is that α7β2* containing nAChRs, demonstrated via *in vitro* expression (Khiroug *et al*. 2002) and recently in primary neurons (Liu *et al*. 2009), are present. It may be that the presence of β2 containing nAChRs influences the kinetics and/or dose-response characteristics of the α7 homomeric nAChRs-coupled responses, or they may affect the coupling of the α7 nAChRs to changes in synaptosomal Ca^{2+}. Finally, the presynaptic expression of other subunits or regulators may have been altered in the β2 subunit null mutants, which, in turn, may have altered the responses characteristics of the α7 nAChRs. There also appears to be presynaptic nAChRs in both hippocampus and cortex that are unaffected by Aβ, and likewise a small subset of responses to Aβ that occur independently of nAChRs. Together, these results are consistent with previous findings indicating that acute application of soluble Aβ can activate, in an agonist-like manner, distinct subtypes of nAChR on presynaptic nerve terminals in mouse brain, but not all nAChR subtypes. The site(s) on the nAChRs or the nAChR complex with which Aβ interacts is under study (Nichols *et al*. 2008). Moreover, there remains a possibility that the interaction also involves membrane elements (Small *et al*. 2007; Nichols *et al*. 2008).

One important question that may be posed in view of the present study is whether acute agonist-like actions of Aβ reflect a possible physiological effect or a potential pathological action (Wilquet and De Strooper 2004; Pearson and Peers 2006). Interestingly, previous findings indicated that nerve terminal activity and/or presynaptic nAChR activation (by nicotine) strongly attenuated the agonist-like action of soluble Aβ on presynaptic Ca^{2+}, but that this attenuation could be overcome with increasing levels of Aβ (Dougherty *et al*. 2003). However, at higher levels (≥μM) of Aβ, the potential for non-selective membrane effects of the soluble peptide, probably as an oligomer, may arise (Arispe *et al*. 2007: Small *et al*. 2007). In addition, fibrillar species of Aβ will form over time and very likely have completely different targets and, consequently, different effects. Thus, it is proposed that at relatively low concentrations (pM to low nM) of Aβ, the acute effects are neuromodulatory, involving to some degree nAChRs at presynaptic sites, as well as postsynaptic sites (Pettit *et al*. 2001; Liu *et al*. 2001), and perhaps metabotropic glutamate receptors (see Chin *et al*. 2007). In contrast, as the concentrations of Aβ rise over the course of Alzheimer's disease, pathological actions commence, involving other targets and effects.

A physiological action of Aβ has been suggested by studies wherein APP processing or direct application of Aβ or Aβ fragments leads to alterations in synaptic transmission (Kamenetz *et al*. 2003; Ashenafi *et al*. 2005; Hsieh *et al*. 2006; Santos-Torres *et al*. 2007; Ting *et al*. 2007). Aβ-induced alterations in presynaptic Ca^{2+} will likely alter synaptic function, and both positive and negative synaptic effects have been noted. (Chin *et al*. 2007; Wu *et al*. 2007; Trabace *et al*. 2007). A recent study has demonstrated that the synaptic effects of Aβ depend entirely on concentration (Puzzo et al. 2008). Application of picomolar Aβ was found to markedly increase long-term potentiation (LTP) in a manner dependent on presynaptic α7 nAChRs, whereas application high nanomolar Aβ inhibited LTP independent of nAChRs. Picomolar level corresponds to what is typically observed for Aβ in normal, adult brain (Schmidt *et al*. 2005), while high nanomolar concentrations and above arise over the course of Alzheimer's disease. This stimulatory effect of picomolar Aβ is quite consistent

with our findings and strongly suggests that Aβ may function as a neuromodulator at select presynaptic sites in normal, intact brain. Its exact physiological role in these circuits remains to be elucidated. Use of APP null mutant mice (Kamenetz *et al*. 2003) or selective, reversible blockers of Aβ production, using γ-secretase inhibitors, for example, or inhibitors of Aβ release would be particularly useful in addressing the physiological role of Aβ in synaptic function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BgTx	α-bungarotoxin
DHBE	dihydro-β-erythroidine
HBS	HEPES-buffered saline
VGCC	voltage-gated calcium channel
Aβ	beta amyloid
nAChR	nicotinic acetylcholine receptors

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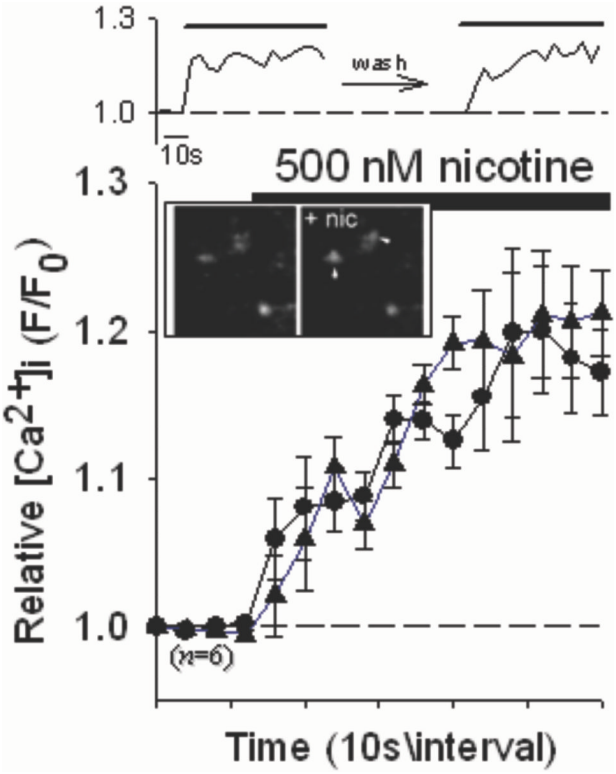

Fig. 1.

Nicotine-induced increases in $[Ca^{2+}]i$ in individual hippocampal synaptosomes. Synaptosomes purified from mouse hippocampus were loaded with Fluo-4 and imaged via confocal microscopy while under perfusion. Top sequence shows representative time courses of successive responses in an individual synaptosome from wild-type C57Bl/6J mice to stimulation with 500nM nicotine (Nayak et al. 2001), with an intervening 10min wash with HBS (circles – before wash; triangles – after wash). Inset: magnified micrographs of sequential confocal images of synaptosomes (~1μm) before and after stimulation with nicotine (+nic). Graph shows averaged data of successive responses in individual synaptosomes to 500nM nicotine. Relative $[Ca^{2+}]i$ is expressed as F/F_0, where F_0 represents the fluorescent intensity of the individual synaptosome at t_0. Error bars are s.e.m.

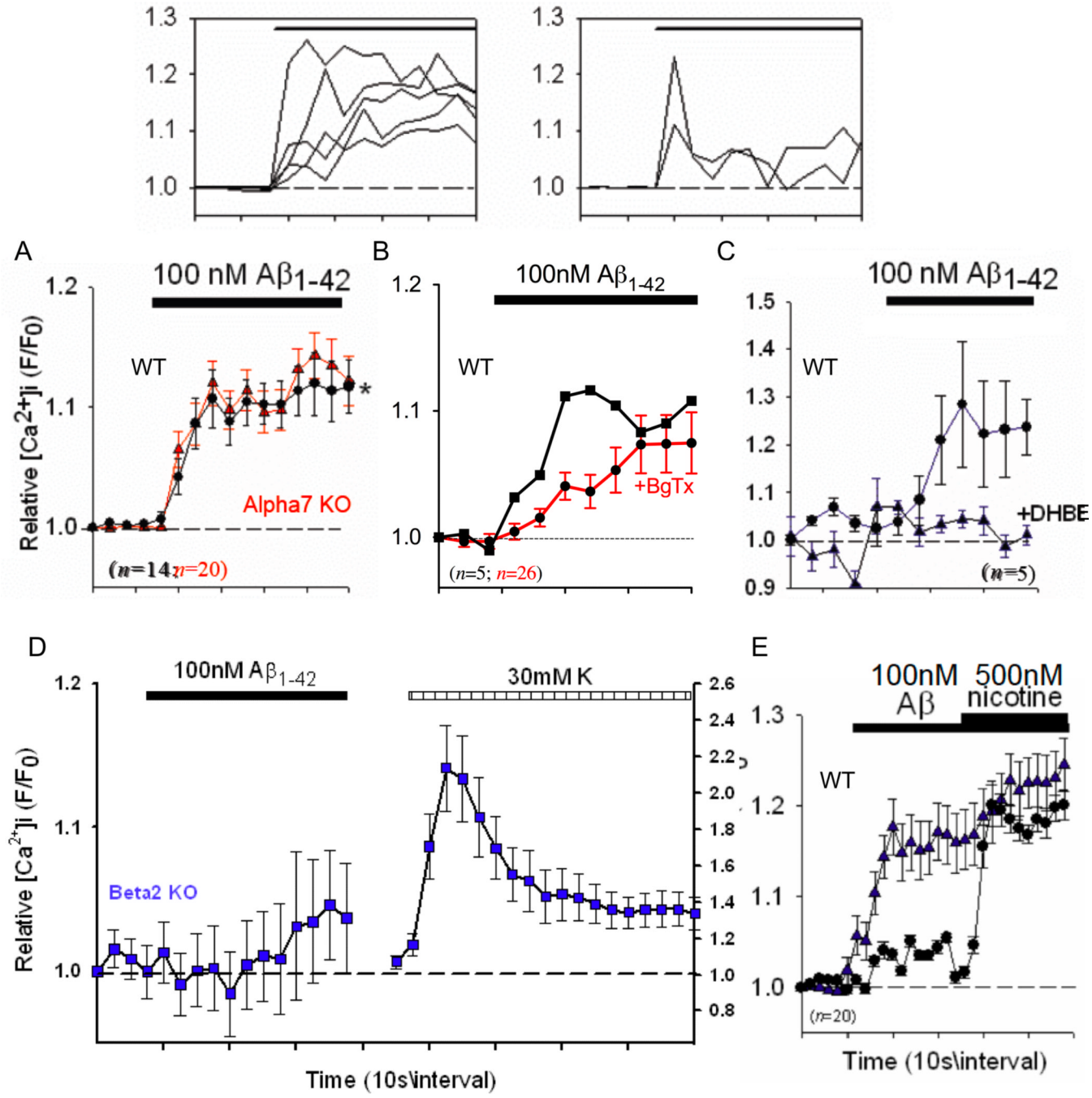

Fig. 2.

Aβ-induced responses in hippocampal synaptosomes from wild-type and nAChR null mutant mice. Top: compilation of individual Ca^{2+} responses in wild-type hippocampal synaptosomes to 100nM $Aβ_{1–42}$, separated into sustained responses, which represent the majority, and transient responses, which were rare (2−4%). (A) Averaged responses to 100nM $Aβ_{1–42}$ in individual hippocampal synaptosomes from wild-type mice (WT, black curve) compared to preparations from mice harboring a null mutation for the α7 nAChR (Alpha7 KO, red curve). (B) Averaged responses for a wild-type preparation (WT) treated with α-bungarotoxin (+BgTX, red curve). (C) Averaged responses to 100nM $Aβ_{1–42}$ in individual wild-type (WT) hippocampal synaptosomes pretreated or not (control) with 1μM

dihydro-β-erythroidine (+DHBE). Similar results were obtained for DHBE with 100pM $A\beta_{1-42}$.. (D) Averaged responses to 100nM $A\beta_{1-42}$ in individual hippocampal synaptosomes from mice harboring a null mutation for the β2 nAChR (Beta2 KO), followed by stimulation with 30mM K^+ to assess synaptosomal viability (right scale). *n*=13 (E) Averaged responses to 100nM $A\beta_{1-42}$ in individual wild-type (WT) hippocampal synaptosomes, followed by stimulation with 500nM nicotine. Responses were separated into a subset that robustly responded to Aβ (majority; triangles) and a subset that weakly responded to Aβ (small minority: 5−10%; circles). Relative $[Ca^{2+}]i$ is expressed as F/F_0, where F_0 represents the fluorescent intensity of the individual synaptosome at t_0. Error bars are s.e.m.

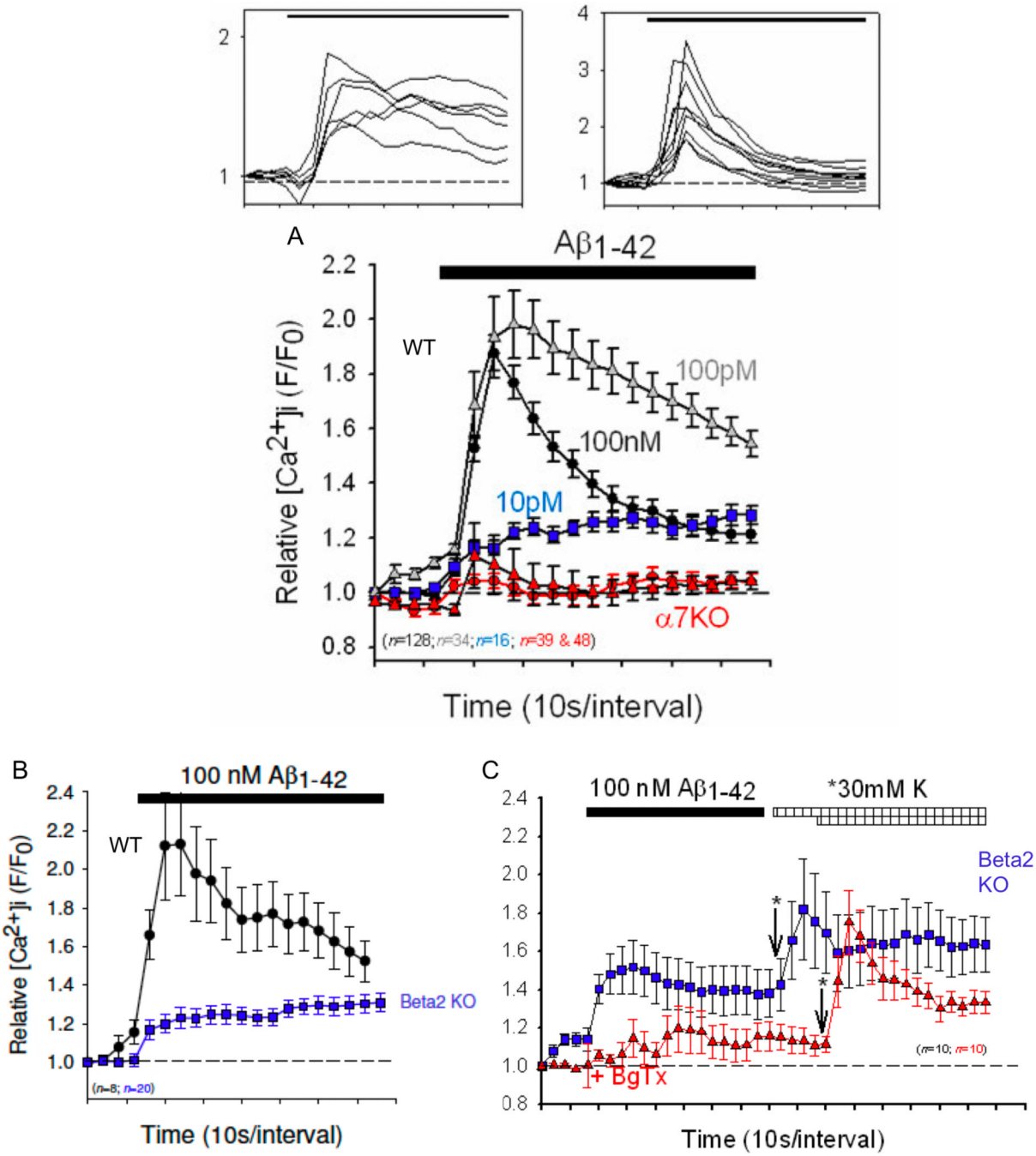

Fig. 3.

$A\beta_{1-42}$-induced increases in $[Ca^{2+}]i$ in individual cortical synaptosomes from wild-type, α7 nAChR-null and β2 nAChR-null mice. Top: compilation of individual Ca^{2+} responses to 100nM $A\beta_{1-42}$, separated into sustained responses, which were the minority (<40%), and transient responses, which represent the majority. (A) Averaged responses to 10pM (blue squares), 100pM (gray triangles) or 100nM (black circles) $A\beta_{1-42}$ in individual synaptosomes from wild-type (WT) mice compared preparations from mice harboring a null mutation for the α7 nAChR (Alpha7 KO, red symbols; triangles: 100pM; circles: 100nM). (B) Averaged responses to 100nM $A\beta_{1-42}$ in individual synaptosomes from wild-type mice (WT) compared to preparations from mice harboring a null mutation for the β2 nAChR

(Beta2 KO, blue symbols). (C) Averaged responses to 100nM $A\beta_{1-42}$ in individual synaptosomes from preparations from mice harboring a null mutation for the β2 nAChR (Beta2 KO), treated or not (control, blue symbols) with 50nM α-bungarotoxin (BgTx, red symbols) for 30min, followed by stimulation with 30mM K^+, where indicated (arrows; asterisks highlight the difference start times for K^+). There was no significant difference in the responses to K^+-depolarization in the absence or presence of BgTx. For all panels, relative $[Ca^{2+}]i$ is expressed as F/F_0, where F_0 represents the fluorescent intensity of the individual synaptosome at t_0. Error bars are s.e.m.