

Nicotinic Acetylcholine Receptors Sensitize a MAPK-linked Toxicity Pathway on Prolonged Exposure to β -Amyloid*

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Background: Signaling pathways involved in the sensitization by target receptors of β -amyloid-triggered neurotoxicity have not been identified.

Results: Exposure to β -amyloid in the presence of nicotinic receptors led to ERK activation followed by JNK activation and increased PHF-tau.

Conclusion: β -amyloid neurotoxicity entails activation of discrete intracellular signaling pathways.

Significance: Activation of select signaling pathways by β -amyloid may be an early event in Alzheimer disease.

Among putative downstream synaptic targets of β -amyloid ($A\beta$) are signaling molecules involved in synaptic function, memory formation and cognition, such as the MAP kinases, MKPs, CaMKII, CREB, Fyn, and Tau. Here, we assessed the activation and interaction of signaling pathways upon prolonged exposure to $A\beta$ in model nerve cells expressing nicotinic acetylcholine receptors (nAChRs). Our goal was to characterize the steps underlying sensitization of the nerve cells to neurotoxicity when $A\beta$ -target receptors are present. Of particular focus was the connection of the activated signaling molecules to oxidative stress. Differentiated neuroblastoma cells expressing mouse $\alpha 4\beta 2$ -nAChRs were exposed to $A\beta_{1-42}$ for intervals from 30 min to 3 days. The cells and cell-derived protein extracts were then probed for activation of signaling pathway molecules (ERK, JNK, CaMKII, CREB, MARCKS, Fyn, tau). Our results show substantial, progressive activation of ERK in response to nanomolar $A\beta$ exposure, starting at the earliest time point. Increased ERK activation was followed by JNK activation as well as an increased expression of PHF-tau, paralleled by increased levels of reactive oxygen species (ROS). The impact of prolonged $A\beta$ on the levels of pERK, pJNK, and ROS was attenuated by MEK-selective and JNK-selective inhibitors. In addition, the MEK inhibitor as well as a JNK inhibitor attenuated $A\beta$ -induced nuclear fragmentation, which followed the changes in ROS levels. These results demonstrate that the presence of nAChRs sensitizes neurons to the neurotoxic action of $A\beta$ through the timed activation of discrete intracellular signaling molecules, suggesting pathways involved in the early stages of Alzheimer disease.

Alzheimer disease (AD)² is a progressive neurodegenerative disorder. It is characterized by various pathological hallmarks including extracellular plaques composed primarily of beta amyloid ($A\beta$), intracellular neurofibrillary tangles formed from hyperphosphorylated tau, synaptic loss, and neurodegeneration in brain regions critical for memory processes. The onset of AD is best correlated with increased levels of $A\beta$ (1). There exists evidence indicating that various different intracellular pathways including MAP kinases (MAPK), Fyn and others can be acutely regulated by oligomeric $A\beta$ (2). The roles for these pathways in AD remain to be determined.

All MAPK pathways (the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 pathways) appear to have been activated in vulnerable neurons by $A\beta$ (2), suggesting involvement in the pathophysiology and pathogenesis of AD. Studies conducted on post-mortem tissues have confirmed that the activation of MAPK occurs at a very early stage in AD (3, 4). In model neuronal systems, ERK and one of its downstream effectors CREB (cyclic AMP-regulatory element binding) were activated by $A\beta$ in a manner dependent on calcium and nAChRs (5, 6). Notably, $A\beta$ activation of the ERK pathway has been linked to caspase activation (7) and the pathological hyper-phosphorylation of tau. Studies have also demonstrated that $A\beta$ can regulate CREB and Ca^{2+} /calmodulin-dependent kinase II (CaMKII) by activating calcineurin and protein phosphatase 1, leading to deficits in long-term potentiation (LTP) in the hippocampus (8). ERK/CREB signaling in the hippocampus normally plays a significant role in determining LTP (9, 10). Hence, contextual and spatial memory formation in mammals (11–14) resulting from alterations in ERK signaling by $A\beta$ may contribute to early memory deficits in AD.

A possible link between the JNK signaling pathway and $A\beta$ -induced oxidative stress has been implicated in neuronal

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² The abbreviations used are: AD, Alzheimer disease; $A\beta$, β -amyloid; CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; CREB, cyclic AMP-regulatory element-binding protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, MAP kinases; MKP, MAPK phosphatase; nAChR, nicotinic acetylcholine receptor; ROS, reactive oxygen species.

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compromise (15–16). Indeed, inhibitors for JNK, as well as ERK1/2, and the upstream regulators MEKs (MAP2Ks) have been widely investigated as potential therapeutic drugs for AD (e.g. 17, 18). Interestingly, MAPK signaling is regulated by a group of MAP kinase phosphatases (MKPs) (19), specifically, the dual-specificity MKPs, which can differentially regulate ERK and JNK (20).

In parallel, Fyn, a member of the Src family of kinases, plays a role in the regulation of $A\beta$ production as well as $A\beta$ -induced synaptic deficits and neurotoxicity, perhaps primarily by inducing tyrosine phosphorylation of tau (21). The relation of this signaling pathway to the MAPK pathway in AD remains unclear.

As we have previously found that the presence of a relatively high-affinity target receptor for $A\beta$ sensitizes nerve cells to $A\beta$ toxicity (22), potential roles for select intracellular pathways involved in this sensitization were evaluated. The study focused on the MAPK pathway, assessing the relationship between receptor-linked, $A\beta$ -induced alterations in signaling and oxidative stress.

Experimental Procedures

Cell Culture and Transfection—Hybrid neuroblastoma NG108–15 cells were used as a model nerve cell system, as they are normally devoid of functional nAChRs (22–24). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% FBS and hypoxanthine/aminopterin/thymidine (HAT selection). Cells were differentiated on poly-L-lysine (plates) or Cell-Tak (coverslips) with 1 mM dibutyryl cyclic AMP in DMEM in the presence of reduced serum (1% FBS) and penicillin-streptomycin-glutamine for 72 h, as described previously (22–24). pcDNA3.1 expression vectors harboring mouse sequences for $\alpha 4$ and $\beta 2$ nAChR subunits (1:4 ratio, respectively) were transfected into the differentiated cells using FuGENE HD, a lipid-based transfection reagent, for 48 h. Mock-transfected NG108–15 cells, exposed only to FuGENE HD and not plasmid DNA, were used as controls (22–24).

Immunoblot Analysis—NG108–15 cells were grown and differentiated on poly-L-lysine-coated 10-cm cell culture plates as previously described. Forty-eight hours after transfection, the cells were treated with 100 nM $A\beta_{1-42}$ for different time-points ranging from 30 min to 3 days in the presence or absence of particular inhibitors. To further distinguish the effects produced by interaction between $A\beta_{1-42}$ and $\alpha 4\beta 2$ nAChRs, mock-transfected cells (untreated or $A\beta$ -treated) were used as controls. The medium was aspirated, and the cells were washed with PBS. After adding 100 μ l of lysis buffer (25 mM HEPES, 200 mM NaCl, 1 mM EDTA, 0.5% digitonin, 0.2% sodium cholate, 0.5% Nonidet P-40, Halt™ Protease Inhibitor Mixture, 0.02% DTT), the cells were scraped gently from the plates sitting on ice and collected in a microcentrifuge tube. The plates were washed with another 100 μ l of lysis buffer for maximum cell recovery. The extracted proteins were collected by centrifugation at 13,000 rpm for 20 min at 4 °C. The pellet was discarded and the supernatant (protein) was collected in a fresh microcentrifuge tube. The protein samples were then diluted with 25 μ l of SDS sample buffer and 10 μ l dithiothreitol to make a total volume of 100 μ l. The samples were heated to 95 °C for 5 min,

cooled immediately on ice, and then centrifuged. Equal amounts of protein were loaded onto 4–20% Tris-HCl gels and the samples resolved by SDS-PAGE. Proteins were then transferred to preactivated PVDF membrane using the Invitrogen iBlot semi-dry blotting system. Membranes were preactivated by incubating with methanol for 30 s, washing with distilled water for 2 min, and adding transfer buffer for 30 min. After transfer, membranes were soaked in PBS for 5 min and incubated with LI-COR blocking buffer for 2 h at room temperature with shaking. The membranes were then separately incubated with various primary antibodies at dilutions of 1:1000 overnight at 4 °C with gentle shaking. The membranes were washed three times for 10 min each with TBS containing 0.1% Tween (TBST). Bound primary antibodies were detected via IR dye-linked LI-COR secondary antibodies (anti-mouse, anti-rabbit, or anti-rat; 1:5000) for 1 h at room temperature followed by washing the membrane three times with TBST for 10 min each. Immunoreactivity for actin or GAPDH was used as a loading control. Immunoreactive bands in the PVDF membrane were visualized using an Odyssey Infrared Imaging system (LI-COR Biosciences). Bands were compared with protein standards.

Immunocytochemistry—Cells were differentiated on Cell-Tak-coated coverslips and transfected as described (22–24). After various treatments, cells were fixed using freshly prepared 4% paraformaldehyde for 40 min and rinsed with PBS for 30 min. To block nonspecific binding, the fixed cells were incubated in TBS containing 5% BSA and 10% normal goat serum for 30 min. Rabbit anti-pERK1/2 and mouse anti-ERK1/2 (1:200) were then added to the cultures and incubated overnight at 4 °C. The cultures were then washed with 10% goat serum in TBS for 30 min, and thereafter, incubated with the fluorescent secondary antibodies (anti-rabbit Alexa-488 and anti-mouse Alexa-633 IgGs, respectively; 1:500) for 30 min at room temperature. The coverslips were finally washed with 10% normal goat serum and TBS, plated onto glass microscope slides and sealed for imaging using a Zeiss LSM 5 Pascal confocal imaging system on a Zeiss Axiovert 200M microscope or a Nikon Cameleon AOTF confocal system.

Reactive Oxygen Species (ROS)/Hoechst Staining—Relative oxidative stress was assessed in live cultures of differentiated cells, transfected as described (22), as changes in the levels of reactive molecular species. Following various treatments, the Image-iT Live Reactive Oxygen Species Detection assay (Life Technologies) was performed in accordance with the manufacturer's protocol. In brief, the cells were incubated with carboxy- H_2DCFDA (component A) at 37 °C for 30 min. During the last 5 min of incubation, 2 μ g/ml of HOECHST stain (component B) was added. The cells were washed twice with HBS and visualized using an Olympus IX71 epifluorescence microscope at excitation/emission of 495/529 nm (ROS) and 350/461 nm (HOECHST), respectively, linked to a Macrofire camera.

$A\beta$ — $A\beta_{1-42}$ was purchased from American Peptide. A stock solution (0.1 mM) of $A\beta_{1-42}$ was prepared by dissolving the solid peptide in double-de-ionized water. The solution was vortexed and sonicated for 15 min to ensure complete solubilization (see Ref. 23). Under these conditions, $A\beta_{1-42}$ is largely present as stable oligomers, as assessed by SDS-PAGE and native gel analysis (6, 23).

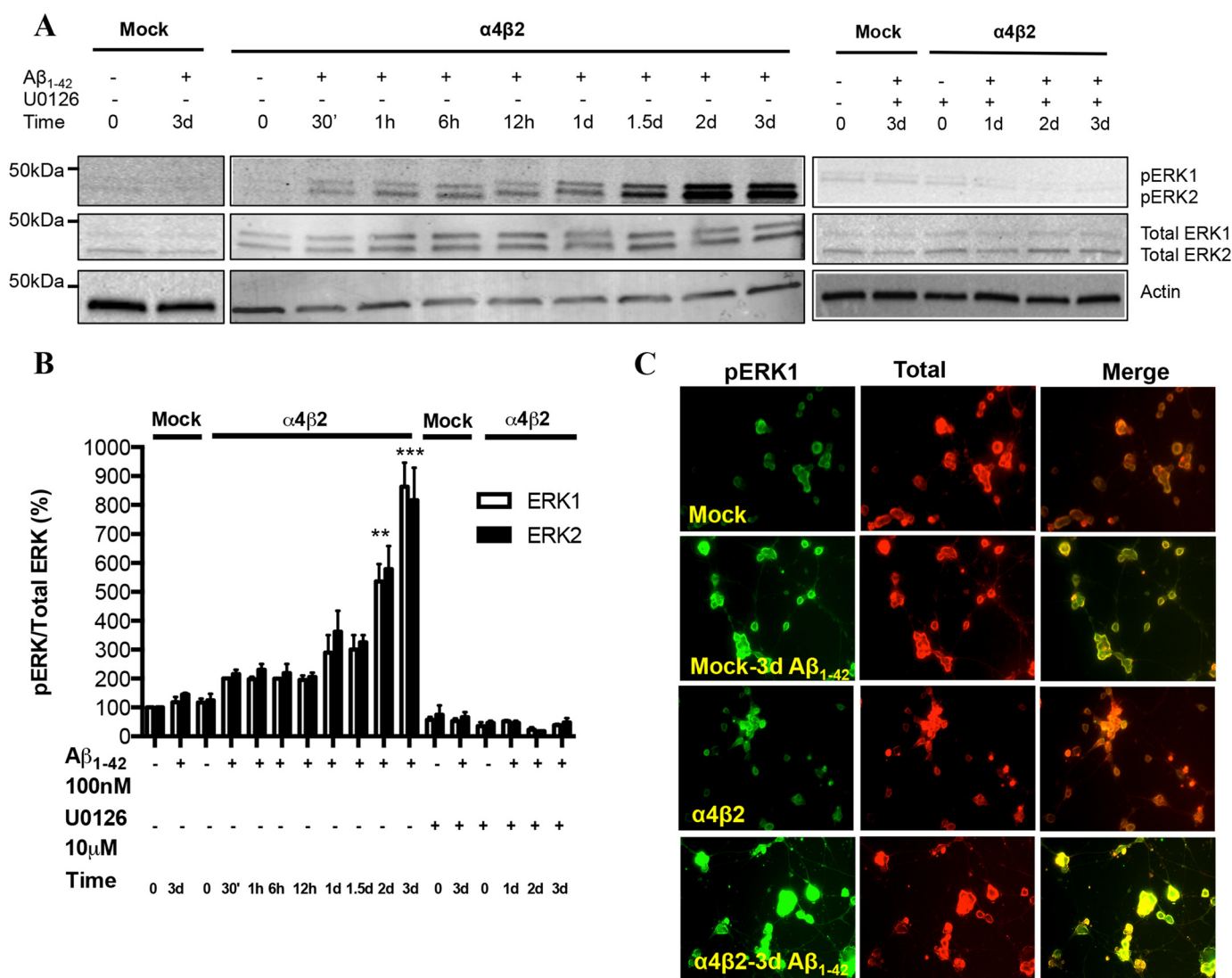


FIGURE 1. Activation of the ERK MAPK pathway in NG108-15 cells expressing $\alpha 4\beta 2$ nAChRs in response to prolonged exposure to A β : time-dependence and sensitivity to a MEK inhibitor. A and B, progressive increases in pERK levels in response to prolonged exposure to 100 nM A β_{1-42} in the absence or presence of U0126, a selective inhibitor of MEK, the upstream regulator of ERK, in cells expressing $\alpha 4\beta 2$ -nAChRs or not (Mock: mock-transfected). pERK levels were compared with total ERK as well as actin (loading control). Data are expressed as % (\pm S.E.) of untreated, mock-transfected controls ($n = 3$ experiments). C, representative images depicting immunostaining of pERK and total ERK in mock- and $\alpha 4\beta 2$ -nAChR-transfected cells treated with A β_{1-42} for 3 days. pERK levels in cells expressing $\alpha 4\beta 2$ -nAChRs and treated with A β were significantly different in comparison to untreated controls.

Chemicals—DMEM was from Mediatech, Inc. FBS and normal goat serum were from GIBCO. Cell-Tak was from BD Biosciences (Bedford, MA). Poly-L-lysine was from Sigma. Image iT Live ROS Detection kit was purchased from Life Technologies. Paraformaldehyde was from Fisher. BSA was purchased from Sigma-Aldrich. Anti-phosphoERK, anti-panERK, anti-phosphoJNK, anti-panJNK, anti-phosphoCREB, and anti-phosphoMARCKS were all from Cell Signaling. Anti-phospho-Fyn was from BD Transduction Labs. Anti-PHF tau (clone AT8), anti-phosphoCaMKII and anti-panCaMKII were from ThermoFisher (Pierce). Anti-MKP7 was from Santa Cruz Biotechnology. Anti-actin was from Sigma; anti-GAPDH was from Abcam. The FITC-labeled and peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse) were from Jackson ImmunoResearch. The Alexa-488 and Alexa-633 secondary antibodies were purchased from Life Technologies. IRDye[®] 680 goat anti-rabbit secondary antibody

were from LI-COR. U0126 was from Cell Signaling, while DJNK-1 was the kind gift of Dr. Tessi Sherrin (University of Hawaii). Both reagents were used at concentrations effecting full inhibition, as based on previous reports (25). All common reagents (e.g. buffers, salts, solvents, detergents, etc.) were either from ThermoFisher or Sigma-Aldrich.

Statistical Analysis—Multiple comparisons were subjected to ANOVA, followed by Bonferroni posthoc tests. A minimum of $p < 0.05$ was used to establish a significant difference. All experiments were replicated at least 3 times.

Results

Differential Activation of MAPK Pathways following Sustained Exposure of Neuronal Cultures Expressing $\alpha 4\beta 2$ -nAChRs to A β —We have previously shown that the presence of $\alpha 4\beta 2$ -nAChRs sensitizes the cells to toxicity induced by prolonged exposure to A β , as measured by increased ROS produc-

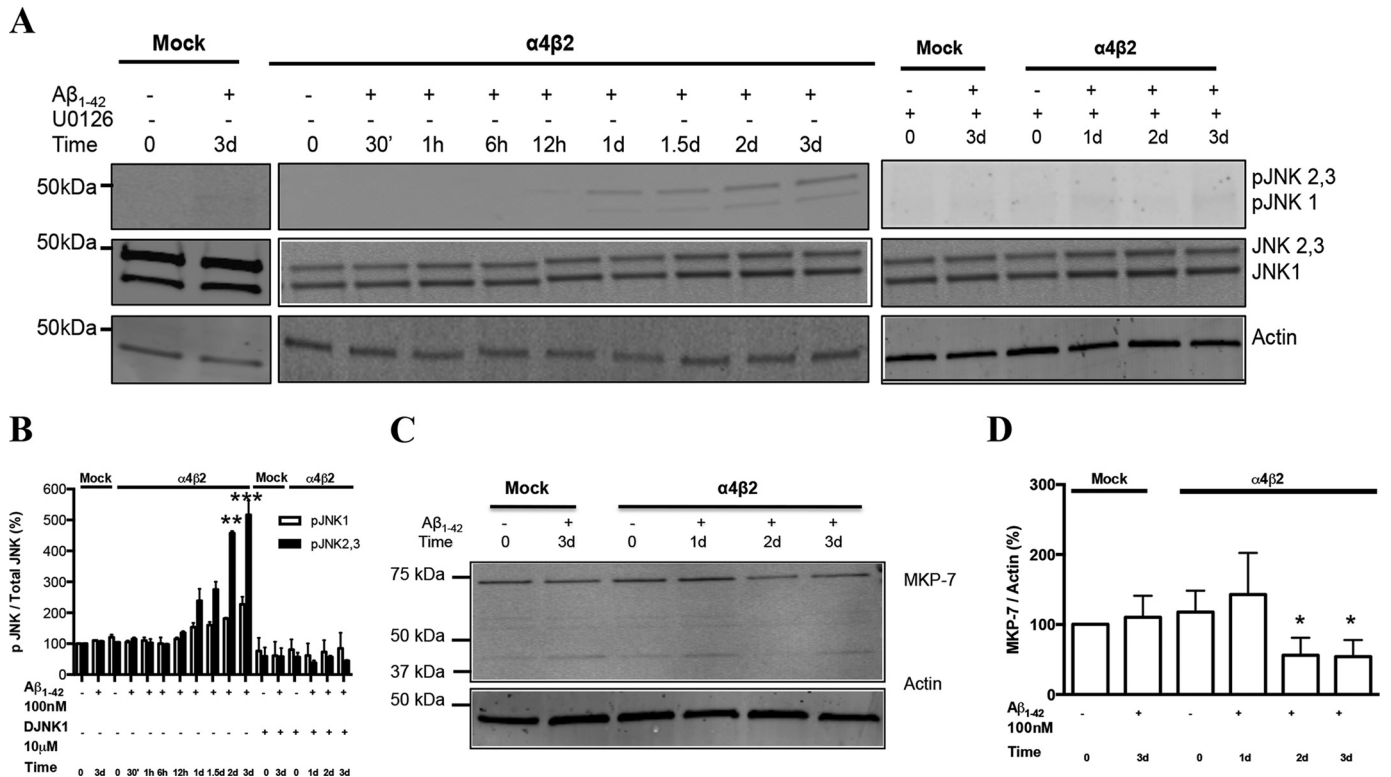


FIGURE 2. Activation of the JNK MAPK pathway in NG108-15 cells expressing $\alpha 4\beta 2$ nAChRs in response to prolonged exposure to $A\beta$: time-dependence and sensitivity to JNK inhibitor. A, progressive increases in the levels of pJNK1 as well as pJNK2,3 in response to prolonged exposure to 100 nM $A\beta_{1-42}$ in the absence or presence of DJNK1, a selective inhibitor of JNK, in cells expressing $\alpha 4\beta 2$ -nAChRs or not (Mock: mock-transfected). B, quantification of averaged immunoblot intensities for pJNKs normalized to total JNK, as compared with actin (loading control). Data are expressed as % (\pm S.E.) of untreated, mock-transfected controls ($n = 3$ experiments). C and D, expression levels of MKP-7 in differentiated NG108-15 cells treated or not with $A\beta_{1-42}$. pJNK1 and pJNK2,3 levels in cells expressing $\alpha 4\beta 2$ -nAChRs and treated with $A\beta$ for 2 and 3 days were significantly different in comparison to controls.

tion, decreased nuclear integrity, and eventual cell death (22). To investigate the initial downstream effects of receptor-coupled sensitization of $A\beta_{1-42}$ toxicity, differentiated NG108-15 cells expressing $\alpha 4\beta 2$ -nAChRs were exposed to fresh, oligomeric $A\beta_{1-42}$ at 100 nM (22) for several time-points from 30 min, 1 h, 6 h, 12 h, 1 d, 1.5 d, 2 to 3 days. Mock-transfected cells treated with $A\beta_{1-42}$ for 0 and 3 days were used as controls. Cell lysates were analyzed via immunoblotting using rabbit anti-phospho-ERK1/2 and mouse anti-ERK1/2 (pan) antibodies. Whereas little if any activation was observed in mock-transfected cells treated with 100 nM $A\beta_{1-42}$ for 3 days, the levels of pERK1 as well as pERK2 were strongly up-regulated in a time-dependent manner in cells transfected with $\alpha 4\beta 2$ -nAChRs, increasing severalfold by 2-3 days, (Fig. 1, A and B; $p < 0.001$ compared with mock-transfected controls). The cells expressing $\alpha 4\beta 2$ -nAChRs had basal levels (t_0) of pERK1/2 comparable to the levels found in mock-transfected cells treated with $A\beta_{1-42}$ for 3 days. Interestingly, an $A\beta$ -induced increase in pERK1 and pERK2 was detectable at 30min out to 12 h for $\alpha 4\beta 2$ -nAChR-transfected cells ($p < 0.05$ compared with baseline), indicating that activation of ERK is a very early event. The increase in pERK induced by $A\beta_{1-42}$ was inhibited upon co-incubation with U0126, a specific MEK1/2 inhibitor (ERK is downstream of MEK1/2), as assessed by both immunoblot analysis (Figs. 1A and B) and immunostaining (Fig. 1C). The selectivity for ERK activation via $\alpha 4\beta 2$ -nAChRs is in accordance with the studies using a transgenic AD mouse model

(Tg2576), which showed an age-dependent increase both in nAChR expression as well as in ERK2 activation in the hippocampus and the cortex (5). A similar effect was observed using organotypic hippocampal slices incubated with $A\beta$ (5).

The phosphorylated forms of JNK, another member of the MAPK family, was found to be significantly up-regulated as a result of $A\beta_{1-42}$ exposure, starting at 1 day and then substantially increasing at 2-3 days. This up-regulation was fully blocked by co-incubation with the JNK peptide inhibitor, DJNK-1 (Fig. 2A). However, the increase in the levels of the neuronal isoforms of pJNK2,3 was dramatically more pronounced than that of pJNK1: over 5-fold as compared around 2-fold, respectively, by 3 days in the presence of the $\alpha 4\beta 2$ -nAChRs (Fig. 2B). The basal levels of pJNK1-3 were nominally higher in $\alpha 4\beta 2$ -nAChR-transfected cells as compared with mock-transfected cells. This up-regulation is consistent with the recent studies demonstrating the neuroprotective effect of inhibiting JNK activity in rodent AD models (26, 27).

As MAPK phosphatases (MKPs) regulate MAPK signaling, we determined the levels of MKP in cells treated with $A\beta$. $A\beta$ -induced up-regulation of ERK and JNK was accompanied by a significant reduction in the levels of MKPs, especially MKP-7, which is JNK-specific (MKP-7, Fig. 2, C and D; MKP-1, not shown), indicating that decreased phosphatase activity may, in part, contribute to the MAPK activation. Under the conditions of the present study, there was no consistent up-

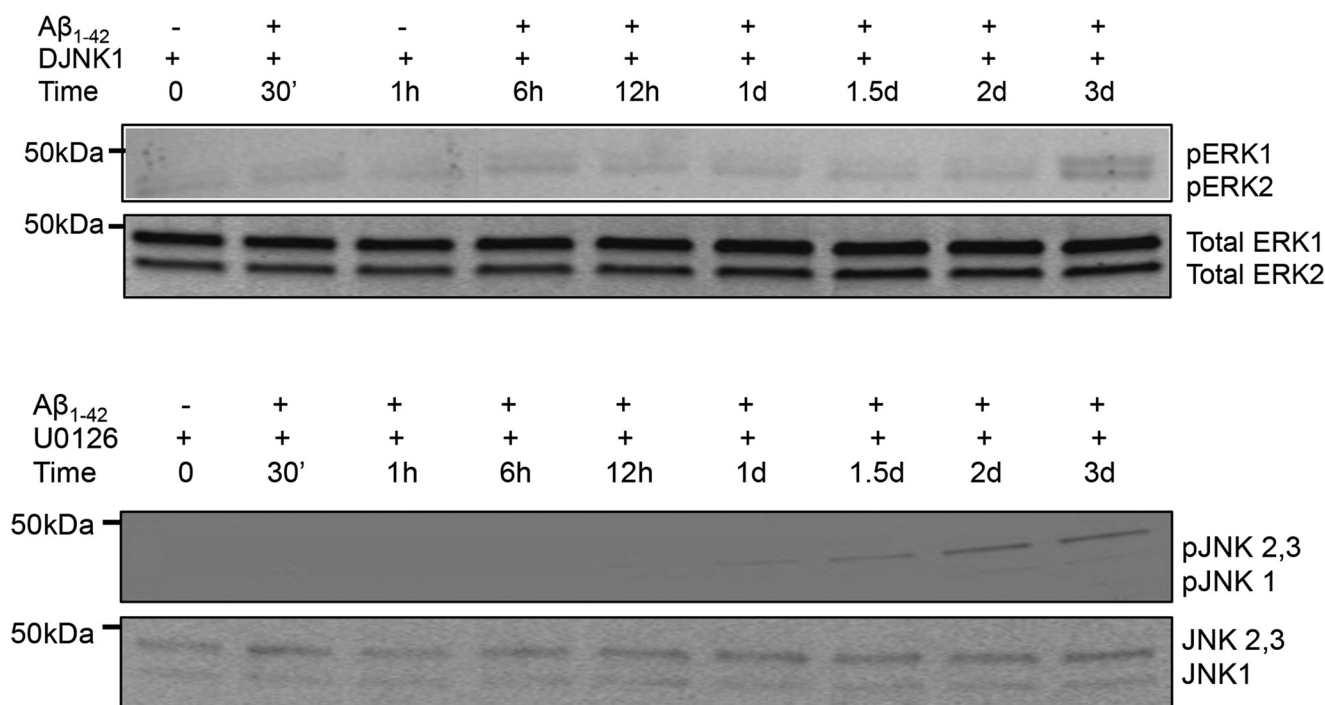
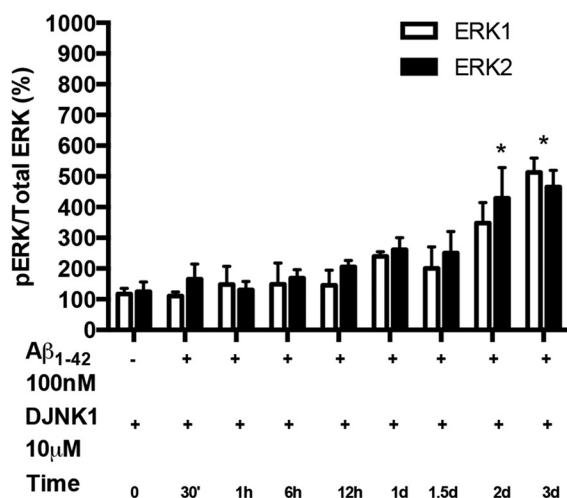
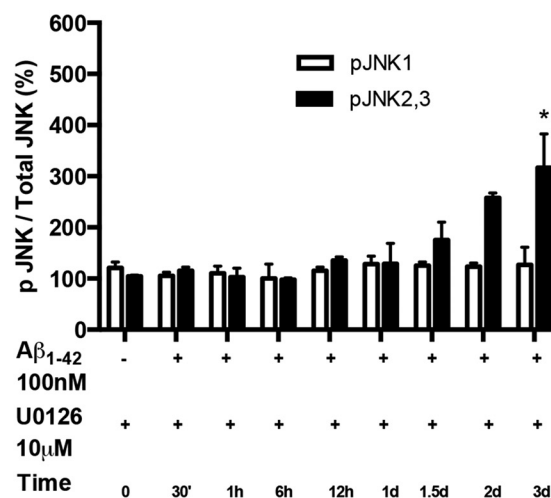
A

B

C


FIGURE 3. Interplay between ERK and JNK pathways activated in cells expressing $\alpha 4\beta 2$ nAChRs in response to prolonged exposure to $A\beta$. *A*, the levels of pERK1/2 were attenuated in response to prolonged exposure to 100 nM $A\beta_{1-42}$ in the presence of DJNK1, a selective inhibitor of JNK, in cells expressing $\alpha 4\beta 2$ -nAChRs. Similarly, levels of pJNK1 as well as pJNK2,3 were reduced significantly on incubation with U0126, MEK inhibitor. *B* and *C* represent the quantification of averaged immunoblot intensities for pERK normalized to total ERK and pJNKs normalized to total JNK, as compared with actin (loading control), respectively. *, $p < 0.05$ compared with controls not treated with $A\beta$.

regulation of p38 MAPK with prolonged exposure of differentiated NG108–15 cells expressing nAChRs (data not shown).

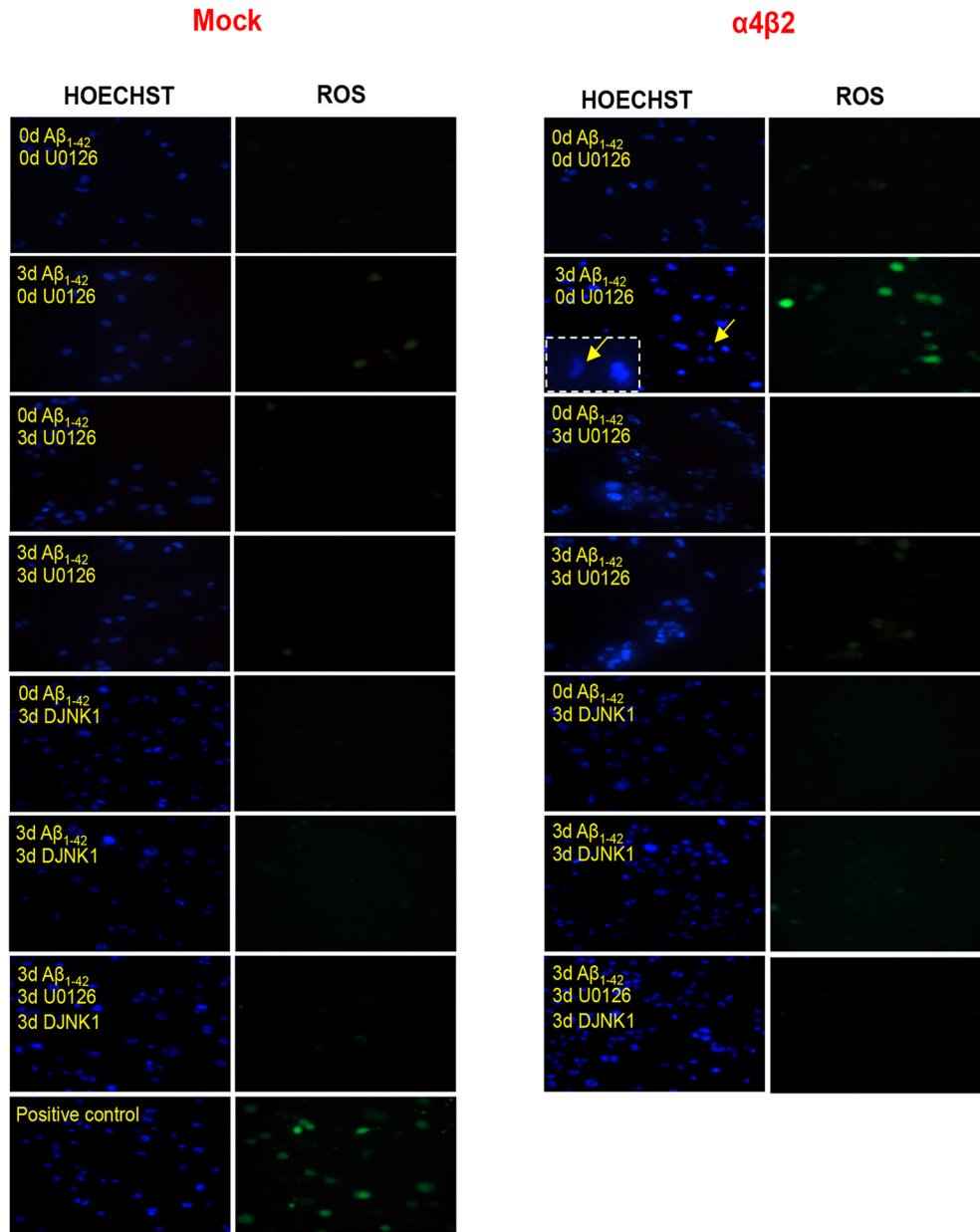
To investigate possible cross-talk between the ERK and JNK pathways, we determined the levels of pERK1/2 against total ERK in cells expressing $\alpha 4\beta 2$ -nAChRs and treated or not with $A\beta_{1-42}$ in the presence of the JNK inhibitor DJNK-1. The levels of pERK1/2 were significantly attenuated (Fig. 3, *A* and *B*), particularly after 1d of treatment. A similar trend was observed for pJNK levels when the $\alpha 4\beta 2$ -nAChR-expressing cells were co-

treated with $A\beta_{1-42}$ and the MEK inhibitor U0126 (Fig. 3, *A* and *C*). The markedly decreased up-regulation suggests a partial interplay between the two pathways.

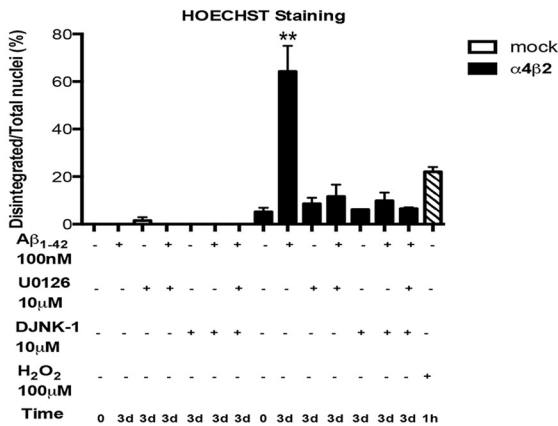
MAPK Pathways in $A\beta$ -induced Oxidative Stress—To test the role of MAPK in the sensitization by $\alpha 4\beta 2$ -nAChRs of $A\beta$ -induced toxicity, specifically oxidative stress, we determined the levels of ROS and nuclear integrity in the cells, untreated (control), or treated with 100 nM $A\beta_{1-42}$ for 3 days in the presence or absence of U0126 (MEK inhibitor) or DJNK-1

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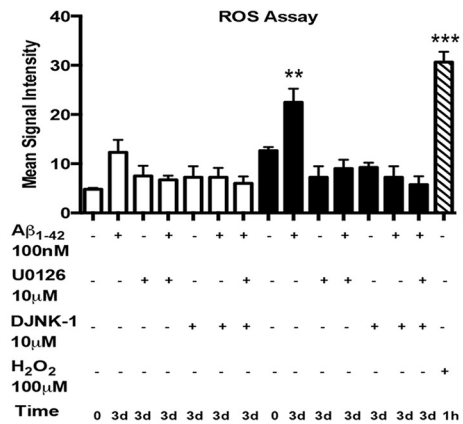
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(JNK inhibitor) or both. Co-treatment with U0126 or DJNK-1 attenuated the number of ROS-positive cells to \sim 15% of the values found in the absence of the inhibitors (Fig. 4, *A* and *B*) over baseline. A similar trend was observed for the number of disintegrated nuclei (Fig. 4, *A* and *C*). Treatment with U0126 or DJNK-1 alone, as separate controls or even co-treatment with both the inhibitors together with $A\beta$, blocked any significant ROS in either mock or $\alpha 4\beta 2$ -nAChR-transfected cells.

Regulation of PHF-tau and pFyn following Sustained Exposure of Neuronal Cultures Expressing $\alpha 4\beta 2$ -nAChRs to $A\beta$ —Mislocalization of hyperphosphorylated tau (and, consequently, PHF-tau) has been widely proposed to be a downstream effector of $A\beta$ toxicity (28, 29). In addition, recent evidence proposes that the pathological mislocalization of tau is correlated with targeting of Fyn to postsynaptic sites at CA1 synapses (30). In accordance with these reports, our studies showed that incubation with $A\beta$ caused a significant up-regulation in the levels of both pFyn and PHF-tau (Fig. 5), which paralleled the time course for MAPK activation. However, the induction of pFyn was nAChR-independent, whereas the extent of the induction of PHF-tau was receptor-dependent. These data suggest that while Fyn may be important if not essential for tau pathology, it is not involved in the nAChR sensitization of $A\beta$ -mediated toxicity.

Altered Levels of pCaMKII and pCREB in Comparison to pMARCKS on Prolonged Incubation of Cells Expressing $\alpha 4\beta 2$ -nAChRs to $A\beta$ —Activation of nAChRs have been shown to trigger, in turn, the calcium-dependent activation of CaMKII/IV, ERK/MAPK, and CREB in several neuronal models (31–35). Based on these studies and our findings showing altered calcium responses in $\alpha 4\beta 2$ -nAChR-transfected cells exposed to $A\beta$ for 3 days (22), we also assessed changes in the levels of CaMKII under same conditions. As expected, the levels of phospho-CaMKII were initially increased at 30 min of $A\beta$ exposure and maintained steady state levels up to 12 h. However, the levels rose up again starting at 1 day, indicating that the induction in pCaMKII by $A\beta$ has two phases, with the later phase delayed as compared with pERK. Overall, the expression of pCaMKII appeared to be modest.

Levels of phospho-CREB, a downstream effector of ERK and CaMKII, were shown to increase at a much later time point around day 2–3 in cells expressing $\alpha 4\beta 2$ -nAChR, following the second phase increase in pCaMKII (Fig. 6C). There was a slight increase in pCREB levels in mock-transfected cells exposed to $A\beta$. Unlike pERK and CaMKII, pCREB was not significantly increased at 30 min.

For comparison, alterations in pMARCKS, identified in a preliminary proteomics screen following $A\beta$ treatment, was examined to assess the involvement of the parallel PKC pathway. There was actually a decrease in pMARCKS in receptor-expressing cells as compared with controls (mock-transfected)

in response to prolonged $A\beta$ exposure (Fig. 7). Interestingly, inhibition of the ERK pathway led to a substantially enhanced but transient up-regulation of pMARCKS (at day 1), most notably in nAChR-expressing cells. This would indicate that the MAPK pathway may be suppressing the PKC-MARCKS pathway upon exposure to $A\beta$.

Discussion

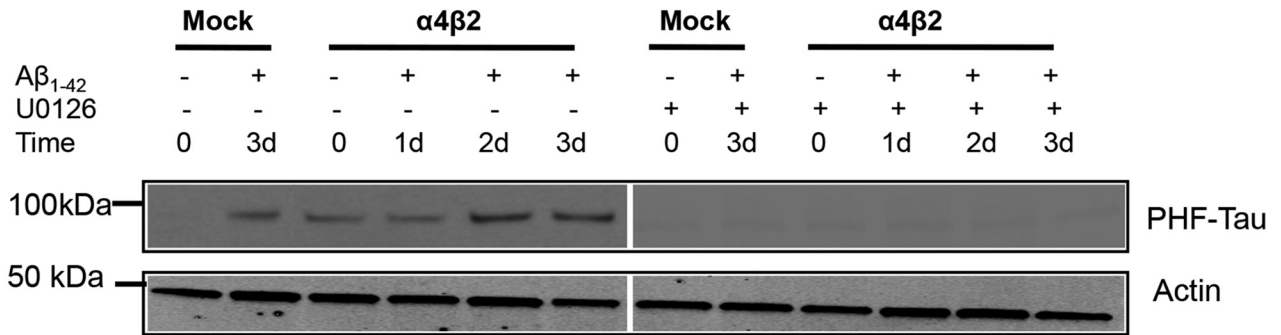
Acute-short-term application of $A\beta$, or often a toxic C-terminal fragment of $A\beta$ ($A\beta_{25-35}$) has been found to activate several signaling pathways (2), including the MAPK cascade (5, 6, 16, 36). Here, we examined the impact of prolonged exposure to $A\beta$ on intracellular signaling in a model cultured nerve cell system (differentiated clonal neuroblastoma cells) selectively expressing $\alpha 4\beta 2$ -nAChRs, as defined high-affinity targets for $A\beta$ (22–24). The advantage of this model neuronal system is that the timeframe for the basic sequence of events in $A\beta$ neurotoxicity, steps previously identified for a wide range of other neural systems, has been clearly delineated (Fig. 8; Ref. 22). In addition, the presence of $\alpha 4\beta 2$ -nAChRs was found to substantially sensitize the cells to $A\beta$ -induced oxidative stress and ultimately apoptosis (22), essentially as a shift in the dose-response such that nanomolar $A\beta$ was now toxic, whereas in the absence of nAChRs micromolar $A\beta$ was required to induce toxicity. How this sensitization engages intracellular signaling pathways was the objective of the present study.

We found substantial ERK activation following nanomolar $A\beta$ treatment of cells expressing $\alpha 4\beta 2$ -nAChRs, as one of the earliest sensitization events connected with oxidative stress leading to frank neurotoxicity (Fig. 8). This is in contrast to the nominal ERK activation on treatment with nanomolar $A\beta$ of cells without nAChRs or in other primary systems (e.g. Ref. 7). CaMKII activation was also an early event, but it did not follow a pronounced, sustained timeline, unlike ERK activation which continued to increase substantially over several days. (There may remain maintained, Ca^{2+} -independent CaMKII activity under these conditions, but the change in the level of pCaMKII was modest.) Activation of the JNK pathway by prolonged $A\beta$ exposure in nerve cells expressing $\alpha 4\beta 2$ -nAChRs, on the other hand, followed ERK activation, correlating with later phases in neurotoxicity. This finding confirms the central role of the JNK pathway in $A\beta$ neurotoxicity, here using a defined, reconstituted neuronal system.

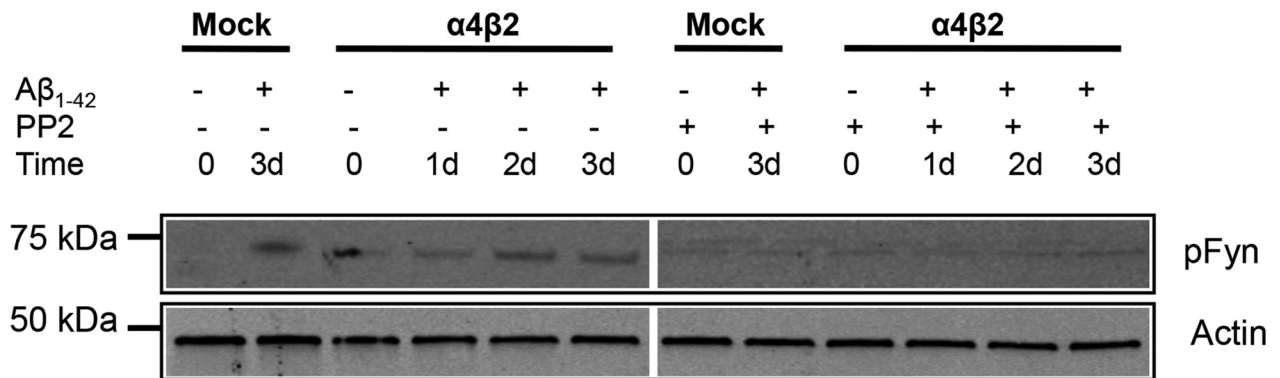
The subsequent oxidative stress induced by chronic $A\beta$ was blocked by co-incubation with selective inhibitors of either the upstream MEK (for ERK) or JNK. As ERK and JNK are known to play important roles in hippocampus-based synaptic plasticity (10), learning (11, 13) and certain types of memory formation (12–14), progressive activation of MAPKs following prolonged $A\beta$ exposure suggests a molecular basis for the disruption of neuronal function in AD. For JNK, this extends to synaptic dys-

FIGURE 4. Effects of ERK and JNK inhibitors on $A\beta$ -induced oxidative stress in the presence of $\alpha 4\beta 2$ nAChRs. *A*, alterations in oxidative stress (as changes in ROS) and nuclear integrity (HOESCHT staining) in differentiated NG108–15 cells expressing high-affinity nAChRs (*right*: $\alpha 4\beta 2$) or not (*left*: Mock) in response to 3-day treatment with 100 nM $A\beta_{1-42}$ in the absence (control) or presence of either 10 μ M U0126 (MEK inhibitor) or DJNK1 (JNK inhibitor) or both. As a positive control, ROS and nuclear integrity were assessed following treatment with 100 μ M hydrogen peroxide. Quantification of HOESCHT (*B*) and ROS (*C*) staining is presented as the means (\pm S.E.) of fluorescent intensity and number (\pm S.E.) of disintegrated nuclei/total nuclei (magnified example in *inset*), respectively, for all cells in a given field (3 experiments). Signals in cells expressing $\alpha 4\beta 2$ -nAChRs and treated with $A\beta$ in presence of inhibitor were not significantly different from control (mock-transfected) cells treated with $A\beta$. **, $p < 0.01$; ***, $p < 0.001$ compared with controls.

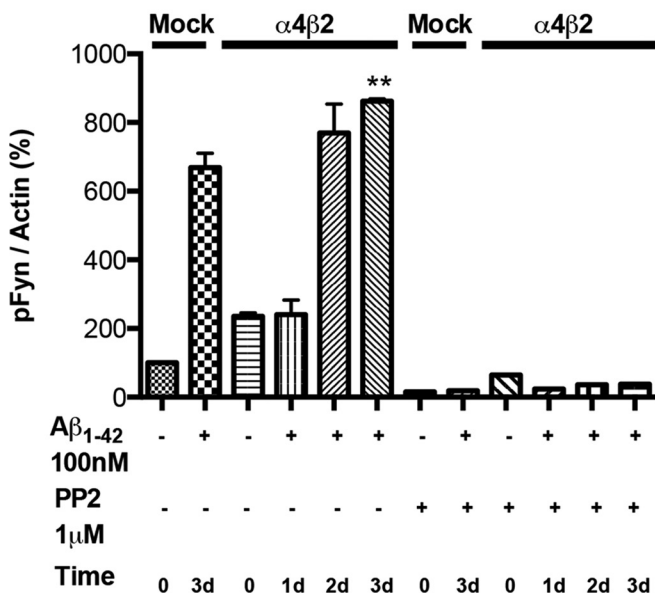
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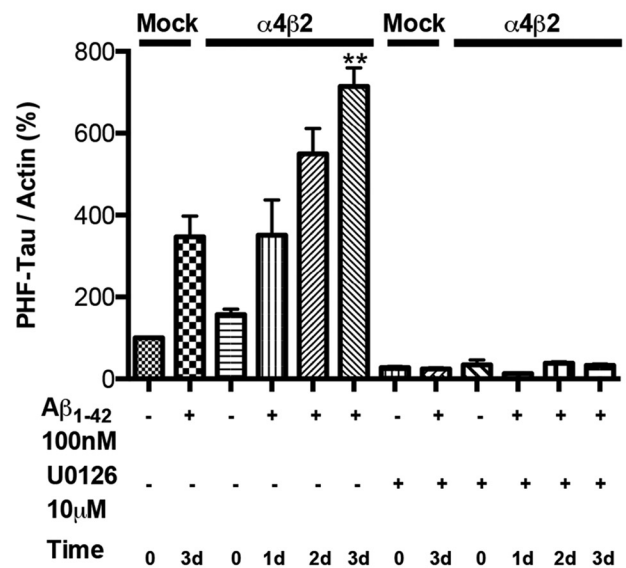


FIGURE 5. Alterations in the levels of PHF-Tau and pFyn in response to prolonged exposure to $A\beta$: receptor-dependence. Progressive increases in PHF-Tau (A) and pFyn (B) in differentiated NG108–15 cells expressing $\alpha 4\beta 2$ -nAChRs or not (Mock: mock-transfected) response to prolonged exposure to 100 nM $A\beta_{1-42}$. Note the significant increases following 3-day treatment with $A\beta$ in the absence of target receptor (Mock). $A\beta$ -induced increases in PHF-Tau were compared in the absence or presence of U0126, a selective inhibitor of MEK, while $A\beta$ -induced increases in pFyn were compared in the absence or presence of PP2, a Src kinase family inhibitor. C and D, quantification of averaged immunoblot intensities, as compared with actin (loading control). Data are expressed as % (\pm S.E.) of untreated, mock-transfected controls ($n = 3$ experiments). **, $p < 0.01$ for PHF-tau or pFyn levels in cells expressing $\alpha 4\beta 2$ -nAChRs and treated with $A\beta$ for 2 and 3 days when compared with untreated control.

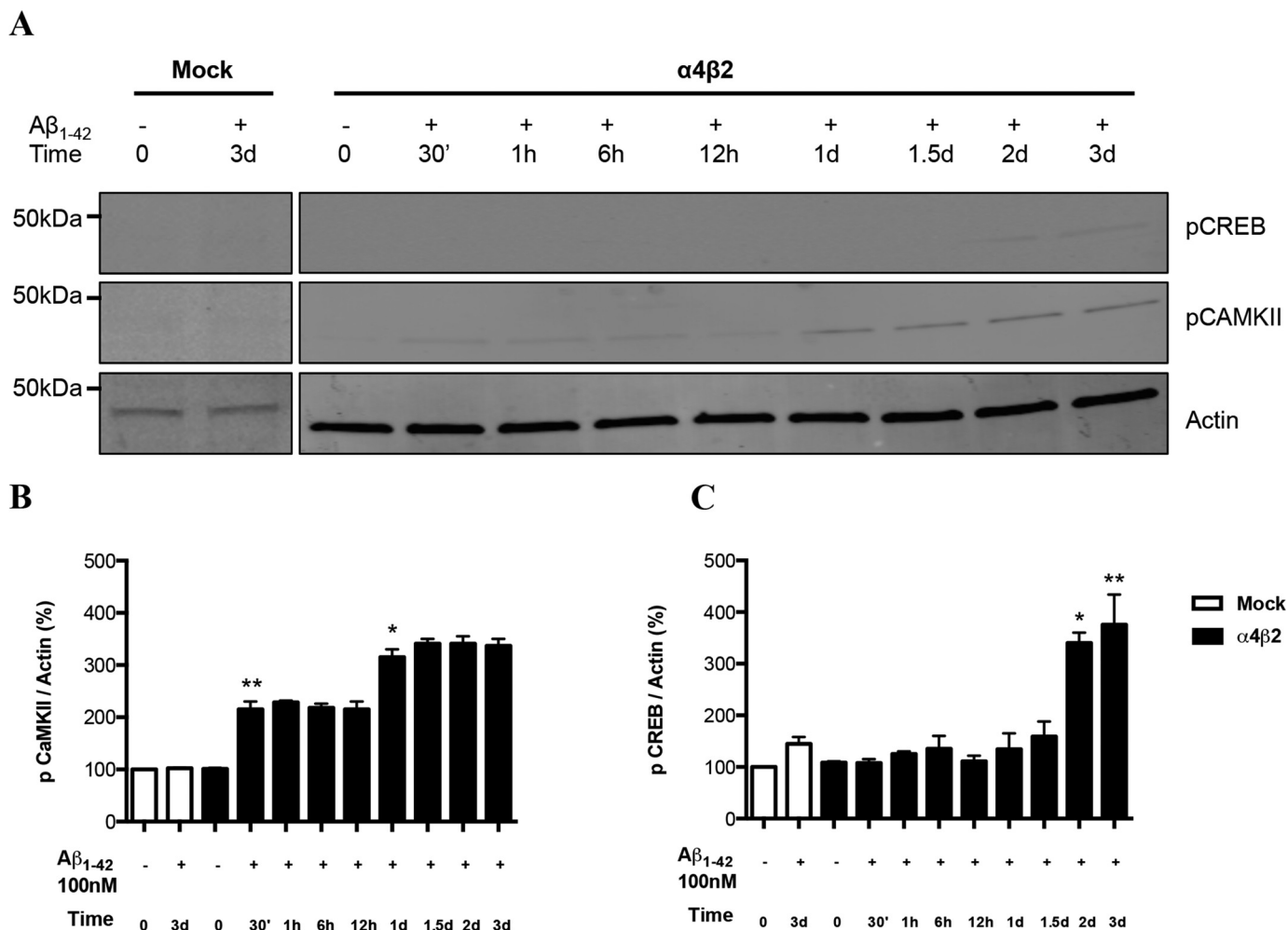


FIGURE 6. Alterations in the levels of pCaMKII and pCREB Fyn in response to prolonged exposure to $A\beta$. *A*, progressive increases in the levels of pCaMKII and pCREB as compared with total CaMKII in differentiated NG108–15 cells expressing $\alpha 4\beta 2$ -nAChRs or not (*Mock*: mock-transfected) response to prolonged exposure to 100 nM $A\beta_{1-42}$. *B* and *C*, quantification of averaged immunoblot intensities, as compared with actin (loading control) for pCaMKII and pCREB, respectively. Data are expressed as % (\pm S.E.) of untreated, mock-transfected controls ($n = 3$ experiments). *, $p < 0.05$ or **, $p < 0.01$ for pCaMKII or pCREB levels in cells expressing $\alpha 4\beta 2$ -nAChRs and treated with $A\beta$ for 2 and 3 days when compared with untreated control.

function (38). Furthermore, this selective toxicity in the presence of $\alpha 4\beta 2$ -nAChRs may contribute to the cholinergic deficit that occurs in Alzheimer disease.

The sensitization by $\alpha 4\beta 2$ -nAChRs appears to be specific. In a preliminary study, it was found that the presence of $\alpha 7$ -nAChRs did not lead to sensitization to $A\beta$ toxicity.³ Moreover, improvement in cognition has been found with activation of $\alpha 7$ -nAChRs (*e.g.* 39), which have separately been shown to play a neuroprotective role against $A\beta$ toxicity (40, 41), possibly involving the PI3K/Akt anti-apoptotic pathway (42). On the other hand, the up-regulation of $\alpha 4\beta 2$ -nAChRs on chronic $A\beta$ treatment may or may not play a role in the sensitization process. Certainly, ERK activation on chronic $A\beta$ treatment well precedes the nAChR up-regulation, which was not significant until day 3 (see Ref. 22). However, to address the role of up-regulation, it would require a means to prevent up-regulation in a controlled and defined way.

While investigating the possible mechanisms underlying the observed changes, it should be noted that MAP kinase path-

ways are implicated in hyperphosphorylation of tau protein. Specifically, both ERK1 and ERK2 are capable of phosphorylating tau at several of the same sites as those found for PHF-tau (43–45). By way of example, it has been demonstrated that microinjection of rat hippocampal primary neurons with purified ERK1 resulted in PHF-like tau hyperphosphorylation associated with compromised microtubule assembly (46). Our findings showing that ERK activation precedes PHF-tau indicate that up-regulation of phospho-ERK might be one of the earliest signals in response to $A\beta$ exposure, followed by formation of PHF-tau.

Besides $A\beta$ and tau, another molecule that has been suggested to be the third member of this toxic triad is Fyn (21, 47). Several lines of evidence suggest that Fyn kinase is involved in the synaptic and behavioral deficits associated with $A\beta$ (48–50). Indeed, mature hippocampal cells from Fyn null ($-/-$) mice are protected against $A\beta$ -mediated toxicity (51). Similarly, reducing or eliminating tau in a mouse model of AD significantly diminished behavioral deficits and improved survival, again strongly implicating tau in the mediation of the toxic effects of $A\beta$ (52). However, we found that Fyn activation was

³ G. Yoshikawa and R. A. Nichols, unpublished results.

MAP Kinase in β -Amyloid Toxicity

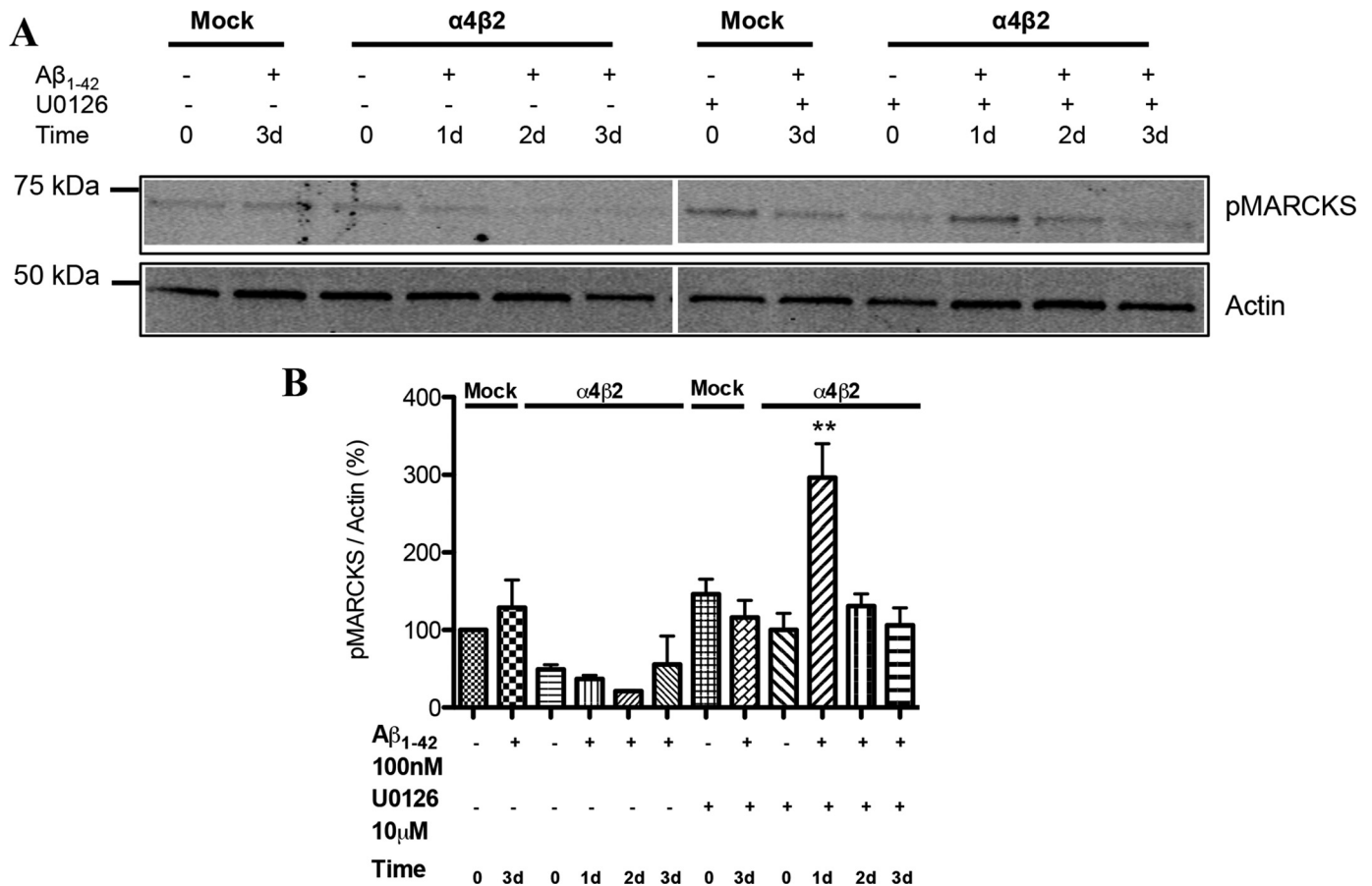


FIGURE 7. Alterations in the levels of pMARCKS in response to prolonged exposure to A β : down-regulation and the impact of ERK inhibition. A, progressive decreases in the levels of pMARCKS in differentiated NG108–15 cells expressing $\alpha 4\beta 2$ -nAChRs or not (Mock: mock-transfected) response to prolonged exposure to 100 nM A β_{1-42} in the absence (control) or presence of 10 μ M U0126 (MEK inhibitor). Progressive decreases in the levels of Western blot micrograph showing the altered levels of pCREB and pCaMKII in mock and $\alpha 4\beta 2$ -nAChR-transfected cells treated with A β for different time-points. GAPDH was used as a loading control. B and C show the quantification of the blots for pCREB and pCaMKII respectively. Data are expressed as % (\pm S.E.) of untreated, mock-transfected controls ($n = 3$ experiments). **, $p < 0.01$ compared with controls.

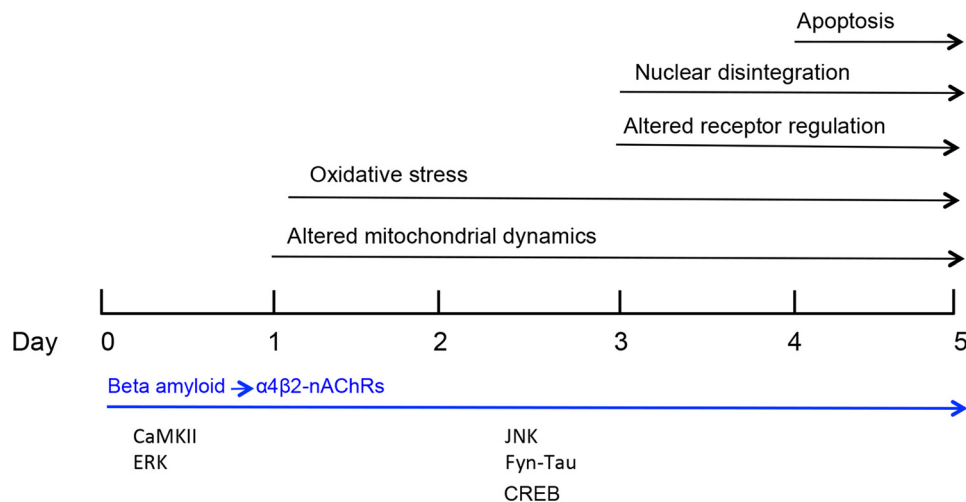


FIGURE 8. Schematic timeline for sensitization of A β -induced neurotoxicity in the presence of high-affinity nAChRs: Steps leading to apoptosis (22) and the key signaling molecules involved.

increased with A β exposure, but not until 2–3 days, and the activation was entirely nAChR-independent. These results are in accordance with receptor-independent cross-talk between Fyn and tau during axonal myelination (53). Thus, while Fyn activation parallels the appearance of PHF-tau, it is likely not

downstream of the ERK activation and represents an independent, converging pathway for A β toxicity. This raises the unique possibility that the signaling sensitized by nAChRs in conjunction with a MAP kinase-independent pathway activated by A β . Recently, it was found that Fyn is linked to A β

signaling through cellular prion (37), which, in turn, regulates surface expression of NMDA receptors. It would be of interest to consider the role of prion, as a separate target for A β , in this scheme.

Based on our findings, we propose a link between receptor targets for A β and A β -mediated neurotoxicity. Taken together, our results provide an insight into how different pathways might converge at a very early stage of Alzheimer disease, particularly in the presence of high-affinity nAChRs.

Author Contributions—K. A. and R. A. N. designed the study and co-wrote the paper. K. A. performed all of the experiments, with assistance by J. C. for several experiments, notably the immunocytochemistry. K. A. and R. A. N. analyzed all of the experiments. All authors analyzed the results and approved the final version of the manuscript.

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