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A β Oligomers-Induced Toxicity is Attenuated in Cells Cultured with NbActiv4™ Medium

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Abstract

Pathogenic A β -derived diffusible ligands (ADDLs) bind to post-synaptic targets, induce excessive reactive oxygen species (ROS) and stimulate tau hyperphosphorylation in cultured neurons. Recently, NbActiv4™ medium was reported to increase neuron synapse densities in cultured hippocampal neurons. We aimed to investigate the effect of this novel medium on ADDL-induced toxicity. We found that ADDL-induced ROS was attenuated in cells cultured with NbActiv4™. ADDL binding assay was performed in neurons cultured by different feeding conditions with NbActiv4™. Feeding cells with 30 % medium once a week, ADDL binding sites were abundant at days in vitro (DIV) 18. However, changing 50 % medium once a week decreased ADDL binding about 80 %. NbActiv4™ produced about 40 % more glial fibrillary acidic protein (GFAP) positive astrocytes than the widely used hippocampal culture medium, neurobasal supplemented with B27 (neurobasal/B27). Astrocytes are reported to produce kinds of trophic factors including insulin-like growth factor 1 (IGF-1). Consistently, when cultured with NbActiv4™, neurons were sensitive to inhibitors of insulin/IGF-1 signaling in response to ADDL attack. Overall, this study supports the important role of astrocytes in neuroprotection and indicates that targeting astrocytes dysfunction may lead to new therapeutic strategies for Alzheimer's disease.

Keywords

ADDLs; NbActiv4™; Astrocytes; Insulin signaling; Neuroprotection

Introduction

Alzheimer's disease (AD), the most prevalent cause of progressive dementia, is characterized by severe neurodegeneration associated with various pathological markers, including neuritic amyloid plaques, neurofibrillary tangles and cerebrovascular amyloidosis

(Wurtman 1985). Mounting evidence suggests that pathogenic A β -derived diffusible ligands (ADDLs) rather than A β plaques are the cause of AD (Lambert et al. 1998; Gong et al. 2003; Klein 2006). In transgenic (Tg) AD mouse models, onset of cognitive impairment was found to correlate with elevated ADDL levels (Tomiyama et al. 2010; Lesne et al. 2006). At the intracellular level, ADDLs stimulate tau hyperphosphorylation at AD-specific epitopes and trigger excessive formation of reactive oxygen species (ROS) (De Felice et al. 2007, 2008). ADDLs have been shown to target at synapses (Lacor et al. 2004). On mature hippocampal neurons, ADDLs cause a rapid and substantial loss of receptors, including N-methyl D-aspartate (NMDA) receptors and insulin receptor (IR) specifically on dendrites bound by ADDLs (Lacor et al. 2007; Zhao et al. 2008). Longer exposure to ADDLs results in synaptic deterioration, which is accompanied by large loss of drebrin, an F-actin binding protein typically localized to spines (Lacor et al. 2007).

The human brain contains 100 billion neurons and about ten times more glial cells. In the central nervous system (CNS), astrocytes are the major type of glia, characterized by complex morphology with elaborate processes, which cover neuronal membranes. It was estimated that the processes of a single astrocyte support and modulate the function of roughly 2 million synapses in human brain (Oberheim et al. 2006). Not only hold neurons in place but also astrocytes play a key role in neuronal survival and function. Abnormalities in astrocytes have been reported to be associated with several neurodegenerative disorders including AD (Seth and Koul 2008). A significant reduction in surface complexity was found in astrocytes from the postmortem brains of dementia (Senitz et al. 1995). In the 3xTg-AD mouse model, reduction in GFAP expression was found to progress with age and become severe at the age of 12 months (Rodriguez et al. 2009). Furthermore, the first sign of atrophy appeared even before the formation of both plaques and tangles, which suggests that soluble A β oligomers might contribute to the atrophy. Astrocytes produce various trophic factors, including brain-derived neurotrophic factors, glial-derived neurotrophic factor, neurotrophins and IGF- I (De Keyser et al. 2008). Although in mixed astroglial-neuronal cultures it was reported that A β ₄₂ peptide has different effects on astrocytes and neurons (Abramov et al. 2003), there is limited study on the role of astrocytes in protecting neuron from ADDL attack.

Recently, a novel medium, NbActiv4^{TM}, was developed. In addition to an increase in neuron synapse densities and network spike rates in cultured hippocampal neurons, it generates higher amounts of astrocytes (Brewer et al. 2008, 2009). Taking advantage of this new medium, we studied ADDL-induced toxicity in the mixed astroglial-neuronal culture and found that ADDL-induced toxicity was attenuated. Because membrane binding has been shown to be required for A β toxicity (De Felice et al. 2007; Simakova and Arispe 2007), we compared the ability of ADDL binding to neurons under different feeding conditions and found that developmental changes induced by enriched medium decreased ADDL binding about 80 %. In the neuronal culture, insulin has been shown to decrease ADDL binding approximately 60 % (De Felice et al. 2009). On the contrary, we found that under the mixed astroglial-neuronal culture generated by NbActiv4^{TM} medium, insulin failed to decrease ADDL binding to neurons. Instead, we found neurons are sensitive to inhibitors of insulin/IGF-1 signaling in response to ADDL attack.

Methods

ADDLs Preparation

A β ₁₋₄₂ from California peptide (Napa, CA) was used to prepare synthetic ADDLs according to published protocols (Lambert et al. 2001). Briefly A β ₁₋₄₂ was dissolved in hexafluoro-2-propanol (HFIP) to 1 mM. The peptide was incubated at room temperature (RT) for at least 1 h to establish monomerization and randomization of the structure. After solvent evaporation, the aliquot was stored as a dried film at -80 °C. The film was resuspended in anhydrous dimethyl sulfoxide (DMSO) to make a 5-mM solution. After thoroughly vortexing, the solution was further diluted to a final concentration of 100 μ M with ice-cold phenol red-free F12 medium (bioSource International, Rockville, MD). After incubation at 4 °C overnight, the solution was centrifuged at 14,000 $\times g$ for 10 min to remove insoluble aggregates. The supernatant that contains fibril-free solutions of A β oligomers was collected and stored in 4 °C. Protein concentration was determined using Coomassie Plus protein assay kit (Pierce, IL). Due to the heterogeneity of A β aggregation states, molar concentration of ADDLs refers to the molar concentration of starting A β ₁₋₄₂ peptide (Generally yield is about 50–70 % per se).

Cell Culture

Primary hippocampal neurons were isolated from E18 rat embryos and plated at a density of 100 (for ADDL binding) or 500 cells (for western blot) per mm² on a 0.002 % ploy-L-lysine-coated coverslips. One hour after plating and incubation, the medium was aspirated. Culture medium either neurobasal/B27 with 0.5-mM glutamine, 50 units penicillin and 0.05 μ g/ml streptomycin or NbActiv4^{TM} (BrainBits, IL) was added. Unless stated otherwise, 50 % medium change was performed twice a week.

Measurement of ROS Formation

CM-H₂DCFDA (Invitrogen, CA), a fluorescent probe that is sensitive to superoxide anion formation, was used to measure ROS formation in live neurons. Hippocampal neurons were cultured either in neurobasal/B27 or in NbActiv4^{TM} medium for 20 days. Cultures were incubated with vehicle or 500-nM ADDLs for 4 h at 37 °C. About 2- μ M CM-H₂DCFDA was added and incubated for 40 min. Then, cells were rinsed three times with warm phosphate-buffered saline (PBS) and twice with phenol red-free neurobasal medium. Cells were immediately visualized under the Nikon Eclipse TE 2000-U fluorescence microscope, and images were digitally acquired by using MetaMorph software. DCF fluorescence data were analyzed using Image J software. Appropriate threshold was applied to eliminate background signal in the image (De Felice et al. 2007). Results from the analysis of five images acquired in each experimental condition carried out in duplicate (ten images, about 400 cells analyzed in total).

ADDL Binding Assay

Primary cultures of E18 hippocampal neurons were grown for 18 days. Agonist or antagonist of insulin/IGF-1 signaling was added 30 min before ADDLs application. After 30-min incubation with 100-nM synthetic ADDLs, neurons were rinsed by neurobasal

medium twice and were fixed with 3.7 % formaldehyde (in PBS buffer) and the neurobasal medium (1:1) for 10 min followed by the removal of the entire fix: medium solution and replacement with 3.7 % formaldehyde for 10 min. Cells were rinsed three times with PBS, pH 7.4, incubated with PBS: 10 % normal goat serum (NGS) for 90 min at RT. Then, the cells were immunolabeled with the ADDL-selective NU4 antibody (1 mg/l final concentration in PBS, 10 % NGS) (Lambert et al. 2007) at 4 °C. After overnight incubation, cells were rinsed three times with PBS and incubated at RT with Alexa Fluor 488 anti-mouse IgG secondary antibody (1:1,000; Invitrogen) in PBS, 1 % NGS. After 3-h incubation, cells were rinsed five times with PBS, and coverslips were mounted with Prolong Gold mounting medium (Invitrogen, CA). Cells were visualized under the Nikon microscope. Quantification was performed by Image J and analyzed by Student's paired *t* test.

GFAP and MAP2 Staining

At DIV21, immunocytochemistry was performed as described (De Felice et al. 2007; Lacor et al. 2007). Cells were permeabilized with 0.1 % Triton X-100 in 10 % NGS and PBS for 1 h at RT. Then, cells were double stained with anti-GFAP (1:2,000, Promega, WI) and anti-MAP2 (1:3,000, Sigma, MO) antibodies at 4 °C overnight. Then, cells were washed with PBS, incubated for 3 h at RT with Alexa Fluor 488 anti-rabbit IgG (1:1,000; Invitrogen, CA) and Alexa Fluor 555 anti-mouse IgG (1:1,000; Invitrogen, CA). The cell nuclei were labeled with 4',6'-diamidino-2-phenylindole (DAPI) in Prolong Gold mounting medium.

FAM-ADDLs Preparation

FAM-ADDLs (Alexa Fluor488-labeled ADDL) were prepared as a mixture of labeled and unlabeled A β ₁₋₄₂. A β ₁₋₄₂ peptide (California Peptide, CA), or FAM-A β ₁₋₄₂ (Ana-Spec, CA) was dissolved in cold HFIP and stored as a film at -80 °C. FAM-A β ₁₋₄₂ and A β ₁₋₄₂ HFIP films were solubilized in DMSO and mixed at a ratio of 1:4 to a final concentration of 5 mM. Then, following the above ADDL preparation protocol, cold F12 was added and incubated at 4 °C overnight. The solution was centrifuged for 10 min and supernatant was kept as FAM-ADDLs. MicroconYM-50 centrifugal filter (Millipore, Bedford, MA) was used to prepare FAM-LMW (lower than 50 kDa). According to the protocol provided by manufacturer, briefly the filter was washed by ddH₂O and F12 buffer. Then, 500- μ l ADDLs was added to the filter and centrifuge at 6,000 $\times$ g for 8–10 min at 4 °C. LMW species in the collection tube were transferred into a clean tube for storage. The preparation was kept in the dark at 4 °C.

FAM-ADDL Binding Assay

Hippocampal neurons were cultured with NbActiv4^{TM} medium for 20 days. The cells were incubated with 500-nM FAM-ADDLs for 45 min in the conditioned medium. Cultures were washed twice with neurobasal medium and fixed slowly (fixed with 3.7 % formaldehyde and the neurobasal medium at ratio 1:1 for 10 min followed by the removal of the entire solution and replacement with 3.7 % formaldehyde for 10 min). Then, cultures were rinsed 3–5 times with PBS. Coverslips were mounted using Prolong anti-fade reagent with DAPI (Invitrogen, CA) and allowed to dry in the dark.

Results

ADDLs-Induced Excessive ROS Formation is Attenuated in Cells Cultured with NbActiv4™ Medium

ROS are the radical forms of oxygen that arise as by-products of mitochondrial respiration and enzymatic oxidases. They are capable of acting as signaling molecules, but cause damage to cellular proteins, lipids and nucleic acids as well (Houstis, Rosen et al. 2006). In hippocampal neuronal cultures, ADDLs have been shown to stimulate excessive formation of ROS through an NMDA receptors dependent mechanism (De Felice et al. 2007). Neurobasal/B27 is a widely used medium for the hippocampal neuronal culture. NbActiv4™ has been developed to improve cultured networks with an increased density of synapses and transmitter receptors that produces an eightfold increase in spontaneous spike activity (Brewer et al. 2008, 2009). We performed a side by side comparison to measure ADDL-induced ROS formation in the cells cultured with these two media (Fig. 1a–d). We found that cultured with neurobasal/B27, neurons showed a 14-fold increase in dichlorodihydrofluorescein (DCF) fluorescence after 500-nM ADDLs treatment. Whereas, ADDL increased ROS production were only 2.5-fold in cells cultured with NbActiv4™ medium (Fig. 1e). Vehicle-treated neurons exhibited basal fluorescence levels corresponding to the physiological endogenous production of ROS. Consistently, the basal level of ROS in cells cultured with NbActiv4™ is about one third as much as the basal ROS level in cells cultured with neurobasal/B27 (Fig. 1e).

Higher Percentage Astrocytes in Cells Cultured with NbActiv4™

As NbActiv4™ medium was reported to increase astroglia differentiation (Brewer et al. 2008, 2009), we examined the percentage of astrocytes in cultures. Astrocytes are classically identified by the expression of GFAP (Eng et al. 2000). Microtubule-associated protein 2 (MAP2) is widely used as a neuronal marker (Sanchez et al. 1995). Co-immunostaining with antibodies against GFAP and MAP2 was performed (Fig. 2). We found that the relative ratio of MAP2-positive cells to GFAP-positive cells was about nine to one in cells cultured with neurobasal/B27, whereas in cells cultured with NbActiv4™, the relative ratio was about one to one. There was about 40 % increase of astrocytes in cells cultured with NbActiv4™ (Fig. 2c).

Cultured with NbActiv4™ Medium, Different Feeding Procedures have Distinct Effects on ADDL Binding to Neurons

Gong and colleagues found that oligomers that were extracted from AD brain and synthetic ADDLs showed identical dendritic punctate binding to mature hippocampal neurons (Gong et al. 2003; Lacor et al. 2004). De Felice et al. (2007) demonstrated that increased ROS production took place in the vicinity of synaptic ADDL binding sites, which suggests that ADDL binding is required for ROS induction. Based on the early association of oligomers with dendritic arbors, Klein et al. (2008) proposed that an attack on synapses by ADDLs may be the key reason why AD is memory specific. According to this hypothesis, ADDL binding assay was established to screen drugs that may help improve memory and protect hippocampal neurons from ADDL attack.

To optimize culture conditions for ADDL binding assay, different feeding methods with NbActiv4^{TM} medium were tested. When fed the cell with 30 % fresh medium at DIV7 and DIV14, ADDL binding sites were abundant at DIV18. It was defined as the established condition. Enriched condition was defined as 50 % medium changing at DIV7 and DIV14. Quantification data showed that under the enriched condition, ADDL binding decreased about 80 % (Fig. 3a). Percentage of astrocytes was compared under different culture conditions. In cells fed with enriched medium, the ratio of neurons to astrocytes was similar to those cultured under the established condition (Fig. 2c).

Comparing to neurobasal/B27, NbActiv4^{TM} contains additional creatine, cholesterol and estrogen (Brewer et al. 2008). We found that changing 50 % NbActiv4^{TM} medium 4 h before ADDL application did not affect ADDL binding, which suggests that, in a short term, additional components do not interfere with ADDL binding. Changing enriched medium 48 h before ADDL application decreased ADDL binding about 40 % as detected by NU4 immunoreactivity (Fig. 3b). Taken together, the findings suggest that significant reduction in ADDL binding is due to developmental changes in cells cultured with the enriched medium, in harmony with the idea that the abundance for ADDL binding sites can be regulated.

Inhibitors of PI3 Kinase and MAP Kinase Strongly Affect ADDL Binding

Using the neuronal culture, De Felice et al. (2009) and Zhao et al. (2009) have found that application of 1- μ M insulin or IGF-1 significantly decreases ADDL binding to neurons, whereas 5- μ M AG1024, an IR and IGF-1R tyrosine kinase inhibitor, significantly increases ADDL binding. So far, the next question we asked, under the mixed astroglial-neuronal culture condition generated by NbActiv4^{TM} medium, what about the effect of insulin, IGF-1 or inhibitors of insulin signaling on ADDL binding to neurons? We found that 30-min pretreatment with 1- μ M insulin or 0.1- μ M IGF-1 (data not shown) did not have strong effect on ADDL binding to neurons. PI3 Kinase and MAP kinase are two major downstream targets of insulin/IGF-1 signaling. Under the same culture condition, LY 294002, an inhibitor of PI3 kinase, strongly decreased ADDL binding and the effect was dose dependent (Fig. 4e). Similarly, U0126, an inhibitor of MAP kinase was found to decrease ADDL binding about 90 % as detected by NU4 immunoreactivity (Fig. 4f).

AG1024, a Receptor Tyrosine Kinase Inhibitor, Significantly Increases ADDL Binding to Hippocampal Neurons

We examined if blockage of receptor tyrosine kinase activity has any effect on ADDL binding. AG1024 was identified as an IR and IGF-1R tyrosine kinase inhibitor in 1997 (Parrizas et al. 1997). We found that 30-min preincubation with AG1024 increased about 100 % ADDL binding in NU4 immunoactivity. In the study of synaptic receptors, high threshold for bright densities was utilized to implicate the synaptic receptor clustering and a lower density for less clustered receptors (Elmariah et al. 2004; Brewer et al. 2008). We applied this concept into quantification and found that AG1024 shows the similar robust effect in both total (including bright and dull) and bright immunoreactivities (Fig. 5b). ADDLs comprise globular oligomers ranging from SDS stable dimers to 24mers (Chromy et al. 2003; Klein 2006) and only high molecular weight (HMW) species were reported to contribute to the punctate binding on neurons (Gong et al. 2003; Lacor et al. 2007). It is

possible that AG1024 facilitates extracellular aggregation of LMW oligomers in the ADDL preparation. Thereby, 30-min preincubation with AG1024 generated more HMW species in culture and the stronger immunoreactivity was observed. Thus, we utilized fluorophore-labeled A β ₄₂ to test this hypothesis.

AG1024 Does not have Strong Effect on Aggregation of FAM-LMW Oligomers

We prepared Alexa Fluor488-labeled ADDLs (FAM-ADDLs) and characterized the preparation using western blot and neuronal cell binding assay. We found that they are similar to unlabeled ADDLs in both the structural morphology and binding behavior (data not shown). On hippocampal neurons, FAM-ADDLs showed the similar dendritic punctate binding pattern. Then, we utilized 50-kDa cutoff filters to isolate different molecular weight-sized fractions. The species that passed through the filter were defined as “fluor-labeled low molecular weight (FAM-LMW) oligomers.” After incubating FAM-LMW oligomers with the hippocampal culture for 1 h, few fluorophore aggregates were observed in the absence and presence of AG1024 (Fig. 5c). After 24-h incubation, fluorophore aggregation was readily detectable. Some of them looked like synaptic punctuate labeling, which suggests that FAM-LMW oligomers may develop punctuate binding after long-term incubation with cultures. However, there was no significant difference in the absence and presence of AG1024 (data not shown).

Discussion

Taking advantage of the novel medium, NbActiv4™, we examined ADDL-induced toxicity in mixed astroglial-neuronal cultures. We found that ADDL-induced toxicity was attenuated in the presence of higher percentage astrocytes, which supports the important role of astrocytes in protecting neurons. ADDL binding sites were proposed to be cell specific and temporally regulated (Shughrue 2008). Consistently, we found that developmental changes induced by enriched medium significantly decreased ADDL binding to neurons. Under the established ADDL binding condition, we found that inhibitors of PI3 Kinase and MAP kinase significantly decreased ADDL binding to neurons, which gives clues to potential targets for memory improvement.

Mitochondrial deficiency was reported in AD by several groups. Studies in cell lines have shown that proapoptotic proteins, Bad and Bax eliminate the mitochondrial membrane potential and facilitate the release of *cytochrome c*. On the contrary, Bcl-2 and Bcl-X_L function to conserve the membrane potential and block the release of *cytochrome c*. It is well established that Akt phosphorylates Bad and obviates its inhibitory effects on Bcl-X_L, by blocking Bax associated channel formation ultimately inhibiting the release of *cytochrome c* on the mitochondrial membrane (Kennedy et al. 1999; Rusinol et al. 2004). On hippocampal neurons, ADDLs impair mitochondria function indicating by induction of excessive ROS formation (Parker et al. 1990; Parker and Parks 1995; Chen and Yan 2007). We found that ADDL inhibit Akt activity (Zhou and Klein unpublished data). Through inhibition of Akt, ADDL might exert its toxic effect on membrane protein dysfunction at mitochondria. This could be a potential pathway to explain mitochondria abnormality in AD patients. In the neuronal culture, Zhao et al. (2009) found that both insulin and IGF-1 reduce

ADDL binding, protect synapses from ADDL synaptotoxic effects. Furthermore, ADDL-induced ROS was completely prevented by preincubation with insulin (De Felice et al. 2009). We have found that 1- μ M insulin completely reversed ADDL inhibition on Akt phosphorylation (data not shown). NbActiv4^{TM} medium produces mixed cultures of 50 % hippocampal neurons and 50 % astrocytes, which secrete various trophic factors including IGF-1. Thus, it is reasonable to observe that ADDL-induced toxicity (elevated ROS formation and inhibited Akt phosphorylation) was attenuated in the mixed culture generated by NbActiv4^{TM}.

Abramov et al. (2003) applied aggregated A β to mixed cultures prepared from the hippocampus and found that A β increased the rate of ROS generation in astrocytes but not in neurons, a response that was calcium dependent. The inconsistency with our finding is most likely due to the difference in oligomer species, dose of A β peptide or culture media. ADDLs do not bind to astrocytes (Shughrue 2008), which eliminates the possibility of attacking astrocytes through receptors on the membrane. Furthermore, membrane cholesterol content has been shown to be inversely related to the pore-forming activity of A β peptide (Abramov et al. 2004). Comparing to neurobasal/B27, NbActiv4^{TM} contains additional cholesterol, estrogen and creatine (Brewer et al. 2008). It is possible that cultured with NbActiv4^{TM}, the cholesterol level in the plasma membrane of astrocytes is elevated, which helps keep the integrity of membrane during the attack from A β oligomers. Estrogen and creatine have been reported to exert direct or indirect effects on mitochondrial function and protect neurons against both acute brain injury and chronic neurodegeneration (Simpkins et al. 2010; Adhihetty and Beal 2008; Brinton 2008). Carbonaro et al. (2009) reported that increased estrogen receptor- α (ER α) expression in astrocytes may elicit neuroprotective effects. It is also plausible that long-term incubation with enriched NbActiv4^{TM} boosts mitochondrial function and increases expression of ER α and protects neurons from ADDL attack.

It was reported that a deficient secretion of trophic factors by nonneuronal cells in AD affected neuronal function (Kessler 1987). On brain slices, astrocytes have been shown to release glutamate or γ -aminobutyric acid (GABA) neurotransmitters and modulate neuronal function (Hamilton and Attwell 2010). NbActiv4^{TM} was optimized to produce an eightfold increase in spontaneous spike activity (Brewer et al. 2008). In addition to the finding that enriched medium with NbActiv4^{TM} strongly decreased ADDL binding, we found that memantine, an NMDA channel blocker cannot restore the decrease of ADDL binding by enriched medium (data not shown). The effect of other channel blockers (e.g., α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate AMPA or GABA channel blockers) on ADDL binding could be tested to explore the mechanism underneath. When cultured with NbActiv4^{TM} medium, expression of IR was increased in neurons (De Felice personal communication), it is likely that activation of IR regulates binding sites under enriched conditions. Anti-insulin or IGF-1 antibodies could be applied to test this hypothesis.

It is interesting to observe that the inhibitor of IR/IGF-1R kinase had different effect from inhibitors of intracellular signaling. AG1024 has been shown a tendency to lower IC₅₀ for the IGF-1R than the IR (Parrizas et al. 1997). The inconsistency from inhibitors of insulin signaling (from the receptor to the intracellular signaling) may be resulted from distinct roles

of IR and IGF-1R in ADDL-induced toxicity. Further application of selective inhibitors (e.g., picropodophyllin, a selective inhibitor of IGF-1 R) or genetic manipulation of IR is needed to identify the difference. AG1024 significantly increases ADDL binding to hippocampal neurons. For, in a short term, its effect on accumulation of LMW species has been eliminated, the appealing explanation is that AG1024 increases ADDL receptors on the synaptic membrane. There are two possibilities. First, AG1024 stimulates more ADDL receptors insertion into synaptic membrane. Second, AG1024 prevents ADDL receptors from undergoing endocytosis and stabilizes ADDL receptor complex by clustering them. More detailed investigations (e.g., AG1024 effect on ADDL receptors in synaptosome or experimental evidence to prove that AG1024 stabilize IR or other receptors) could be performed in the future.

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Fig. 1. ADDL-induced ROS formation is attenuated in the cells cultured with NbActiv4^{TM} medium. Primary cultures of E18 hippocampal neurons (DIV20) were maintained for 4 h at 37 °C in the presence of vehicle (**a, b**), or 500-nM ADDLs (**c, d**). Cells were loaded with CMH₂DCFDA as described in methods. **a–d** Representative images from DCF fluorescence in cells cultured with neurobasal/B27 (**a, c**) versus NbActiv4^{TM} (**b, d**). Identical conditions and exposure times for image acquisition were employed for all experimental conditions. **f** Quantification data from DCF fluorescence were obtained from two different culture sets and five independent experiments in each set (about 200 cells analyzed/experimental condition in each experiment). Immunofluorescence intensities were quantified by NIH Image J software

Fig. 2.

NbActiv4^{TM} medium produces more astrocytes in culture. At DIV21, coimmunostaining with anti-GFAP (*green*) and anti-MAP2 (*red*) was performed in cells cultured with neurobasal/B27 (**a, b**) and NbActiv4^{TM} medium (**c, d**). **e** Quantification data were obtained from neuron and astrocyte counts in two independent experiments. Ten fields of 0.145 mm² were analyzed per experimental condition per culture. 50% medium changing at DIV7 and DIV14 is denoted as “50%”. Feeding the cells with 30% medium at DIV7 and DIV14 is denoted as “30%”

Fig. 3.

Enriched medium significantly decreases ADDL binding. ADDL binding was detected using NU4 antibody in the primary cultures of E-18 hippocampal neurons at DIV18. Neurons treated with vehicle and 100-nM ADDLs. **a, b** Integrated ADDL immunofluorescence intensities from three independent experiments (10–20 images analyzed per experimental condition). **A** stands for the comparison of ADDL binding in the established condition (add 30 % medium per week) versus enriched condition (change 50 % medium per week). **B** represents ADDL binding at different incubation time with 50 % fresh medium changing. Immunofluorescence intensities were quantified by NIH image J, normalized with the established group, and analyzed by student's *t* test. Asterisk indicates statistically significant (***) $p < 0.001$

Fig. 4.

Inhibition of PI3 kinase and MAP kinase strongly decreases ADDL binding to cells cultured with NbActiv4^{TM} medium. NU-4 antibody was used to detect dendritic ADDL binding. **a–d** Representative images from hippocampal neurons (DIV18) treated with VEH (**a**), ADDLs (**b**), 5-μM LY294002 + ADDLs (**c**) and 5-μM U0126 + ADDLs (**d**). **e, f** Integrated ADDL immunofluorescence from three independent experiments. LY294002 significantly decreased ADDL binding (**e**). U0126 had the similar effect (**f**)

Fig. 5.

AG1024 significantly increases ADDL binding to hippocampal neurons. Representative images from hippocampal neurons (DIV18) treated with 100-nM ADDLs (indicated as *control*) or 1-μM AG1024 + 100-nM ADDLs (indicated as *AG1024*). ADDL binding was detected using the NU4 antibody. **b** Integrated ADDL immunofluorescence from three experiments using independent neuronal cultures (20 images analyzed per experimental condition). Immunofluorescence intensities were quantified by NIH Image J and analyzed by student's *t* test. **c** AG1024 does not have strong effect on aggregation of FAM-LMW oligomers. The 100-nM FAM-LMW oligomers were incubated with hippocampal cultures at DIV19 for the time indicated (30 min or 1 h). The 1.5-μM AG1024 (indicated as *AG*) or DMSO was added 30 min before FAM-LMW oligomers application. After 1-h incubation with FAM-LMW oligomers, few synaptic puncta labeling was observed. Furthermore, there is no significant difference between AG1024-treated and untreated cultures