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Brief communication

Combination of memantine and vitamin D prevents axon degeneration induced by amyloid-beta and glutamate

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ABSTRACT

The currently available drugs for treatment of Alzheimer's disease are symptomatic and only temporarily slow down the natural history of the disease process. Recently, its has been proposed that the combination of memantine with vitamin D, a neurosteroid hormone, may prevent amyloid-beta and glutamate neurotoxicity. Here, our purpose was to examine the potential protective effects of memantine and vitamin D against amyloid-beta peptide and glutamate toxicity in cortical neuronal cultures. We provide the first evidence that cortical axons degenerate less after exposure to amyloid-beta peptide or glutamate in microfluidic neuronal cultures enriched with memantine plus vitamin D compared to control medium and cultures enriched with only memantine or only vitamin D. The reported synergistic neuroprotective effect of memantine plus vitamin D -the combination originating an effect stronger than the sum- corroborate previous clinical finding that Alzheimer's disease patients using this drug combination have improved cognition. This finding reinforces the pharmacological potential of a new drug combining memantine plus vitamin D for the treatment or the prevention of Alzheimer's disease.

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1. Introduction

Axonopathy, an early step in neuronal death, is a pronounced attribute of Alzheimer's disease (AD) (Luo and O'Leary, 2005; Wirths et al., 2007) and may contribute to the symptoms of AD (Smith et al., 2007). Specifically, decreased axonal density has been reported within extracellular deposits of fibrillogenic amyloid beta (Aβ) peptides, i.e., the initiating etiopathologic lesion in AD (Benes et al., 1991). It has also been found that the exposure of axonal terminals to Aβ-peptides induces axonal damage without first affecting the neuronal cell bodies (Sun et al., 2011) due to the entrance and aggregation of Aβ-peptides into the cell with subsequent impaired axonal transport and oxidative stress (Rosales-Corral et al., 2012). In parallel, axonopathy is also the result of the accumulation of Aβ-peptides in glutamatergic synaptosomes, which leads to an excessive release of glutamate with consequent axonal degeneration in neurons with *N*-methyl-D-aspartate receptors (NMDARs) (Hiruma et al., 2003; Molnár et al., 2004). Memantine, an uncompetitive NMDAR antagonist, partially protects neurons against Aß-peptide (Martinez-Coria et al., 2010, Miguel-Hidalgo et al., 2002) and glutamate toxicity (McShane et al., 2006; Parsons et al., 2007). Its effectiveness on cognition has been shown in randomized placebo-controlled trials in patients with moderate-to-severe AD (McShane et al., 2006). However, cognitive decline is only temporarily delayed and not stopped nor reversed by memantine (McShane et al., 2006), highlighting an only partial neuroprotection.

We recently have proposed that the combination of memantine with vitamin D, a neurosteroid hormone showing calcium-regulating, anti-inflammatory, and antioxidant properties (Annweiler et al., 2010; Brewer et al., 2001; Ibi et al., 2001; Kalueff and Tuohimaa, 2007), could enhance the beneficial action of memantine (Annweiler and Beauchet, 2012). We further found that patients with moderate-to-severe AD who took memantine plus vitamin D for 6 months had a statistically and clinically relevant cognitive gain of 4 points on the Mini-Mental State Examination, although those treated

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with memantine or vitamin D alone had a slight cognitive decline (Annweiler et al., 2012). This result suggests that memantine and vitamin D potentiate each other to protect neurons and improve cognition in AD patients. Here, our purpose was to examine the potential protective effects of memantine plus vitamin D against Aß-peptide and glutamate toxicity in cortical neuronal cultures.

2. Methods

We assessed the impact of Aß-peptide and glutamate on the axons of mouse cortical neurons cultured in microfluidic chips grown in control medium, or pretreated with 1 μM memantine, or 100 nM vitamin D_3, or memantine plus vitamin D_3 (Fig. 1A and B).

2.1. Primary neuronal cultures and treatments

Cerebral cortices were micro-dissected from Swiss mouse embryos (Elevage Janvier, France) at embryonic day 16 (E16). All animals in this study were maintained and used in compliance with the Policy on Ethics approved by the Society for Neuroscience. Cortical neurons were grown in Dulbecco's modified Eagle's medium glutamax (Invitrogen, Carlsbad, CA, USA) + streptomycin/penicillin (Invitrogen) + 10% fetal bovine serum (PAA Laboratories, Velizy-Villacoublay, France) + N2 (Invitrogen) + B27 supplement without antioxidant (Invitrogen). Microfluidic chips were placed in Petri dishes containing water to prevent chambers evaporation of the culture medium and placed in a 5% CO2 and 37 °C incubator. The microfluidic chips compartmentalize cell bodies and axons in fluidically isolated cell culture chambers (Fig. 1A) (Kilinc et al., 2011; Peyrin et al., 2011), enabling the study of stress-induced dying back axonal degeneration similar to that observed in patients with AD (Kilinc et al., 2011). Before the different pharmacologic or disruptive treatments, cortical neuronal cells grown for 10 days in the microfluidic device were examined by phase contrast microscopy to select acceptable cultures based on 3 criteria: cultures presenting a somatic cell death >10%, or <80% of microchannels containing axons less than 600 μm long, or axons with blebs were discarded. Before pharmacologic treatment, the cell culture medium was completely replaced.

Combinations of 1 μM memantine, 100 nM 1,25-dihydroxyvitamin D_3 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) or vehicle alone (0.001% dimethylsulfoxide in phosphate-buffered saline) were added at day 1 after seeding of the neurons (Fig. 1B). The concentrations used were based on previously published in vitro studies on cortical neurons (Obradovic et al., 2006; Xia et al., 2010). In vivo, the concentration of 100 nM 1,25-dihydroxyvitamin D_3 is high enough to exhibit nonosseous effects (Bischoff-Ferrari et al., 2006) and low enough to avoid intoxication (Vieth, 1999). To ensure fluidic isolation, a hydrostatic pressure difference was generated by overpressurizing the nontreated chamber as previously described by Taylor et al. (2005). Therefore, pharmacologic compounds applied in the somatic compartment cannot diffuse into the distal compartment or vice versa.

After neuronal differentiation for 10 days, the stressor (Aß-peptide or glutamate) was added to the culture (Fig. 1B). The following compounds were used at the indicated concentrations: 60 μM Aß 25-35 peptide (Bachem, Bubendorf, Switzerland), 45 μM glutamate (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Immunocytochemistry and quantification of axonal degeneration

Immunostaining was performed directly on the chips by applying reagents to the various compartments. Neurons were immunostained with monoclonal anti-ß-tubulin-III (1:300; Sigma-Alridch), fluorescein isothiocyanate–conjugated monoclonal anti-alpha-tubulin (mouse Immunoglobulin G, 1:500; Sigma-Aldrich). Cells were stained with Hoechst 33342 (4′,6-diamidino-2-phenylindole; 1:25,000; Sigma-Aldrich) to reveal nuclei. Image acquisition was performed using an Axiobserver Z1 (Zeiss) microscope fitted with a cooled CCD camera (CoolsnapHQ2; Roper Scientific, Tucson, AZ, USA). To assess cortical axonal fragmentation after 48 hours, we used a macro developed for the NIH ImageJ software (http://rsbweb.nih.gov/ij/) based on the Otsu thresholding algorithm (Fig. 1C). We quantified a ratio of discrete tubulin staining (reminiscent of fragmented axons) over linear continuous tubulin staining (intact axons).

Tubulin staining presenting as spots with a circularity of 0.9 was considered as fragmented. The total area of such regions was calculated, and this value was normalized by the total axonal area, which was measured from the threshold image (Kilinc et al., 2011). This ratio, termed the fragmentation index (FI), is an indicator of the average axonal fragmentation level and was used for statistical comparisons. Each point was examined in 10 independent experiments for Aß-peptide and 5 independent experiments for glutamate with, for each experiment, 10 triplicate measures on a microfluidic chip. Random locations were selected in the culture chambers, and up to 5 axonal and 3 somatic 40X images were captured per condition. The change in FI (Δ_{FI}) after stressor addition was defined as $\Delta_{FI} = [(\text{FI after stressor} - \text{FI before stressor})/((\text{FI after stressor} + \text{FI before stressor})/2)] \times 100$.

2.3. Statistics

Two-tailed nonparametric Wilcoxon signed-ranks tests were used to compare paired groups and to compare the FI before and after stressor addition within the same group. A $p < 0.05$ was considered significant. All statistics were performed using SPSS (v19.0, IBM Corporation, Chicago, IL, USA).

3. Results

The mean FI was identical in the 4 media after the differentiation phase ($p = 0.728$) (Fig. 2A). In particular, there was no difference between the medium enriched with memantine plus vitamin D (FI = 4.1 ± 1.6%; mean ± SD) and the control medium (FI = 3.9 ± 0.8%, $p = 0.610$).

Both stressors induced an axonal dying back pattern in control medium (blue color in Fig. 2A–C), beginning at 12 hours at the axonal tip and progressing retrograde until the whole axon was fragmented. Forty-eight hours after Aβ-peptide addition, final FI was 10.3 ± 4.7% in control medium ($p = 0.005$). Conversely, FI did not change with memantine plus vitamin D (final FI, 5.2 ± 4.0%; before-and-after $p = 0.332$). Δ_{FI} was 8.0 ± 63.9% with memantine plus vitamin D, which was significantly lower than in the control medium (79.8 ± 37.5%, $p = 0.013$) (Fig. 2C). Memantine-enriched ($p = 0.110$) and vitamin D-enriched media ($p = 0.059$) showed no Δ_{FI} difference with control medium.

Forty-eight hours after glutamate addition, final FI was 13.1 ± 1.9% in control medium ($p = 0.043$). Final FI with memantine plus vitamin D (5.2 ± 0.8%) was lower than that observed in the 3 other groups ($p = 0.043$) (Fig. 2A). Δ_{FI} after glutamate addition was not different between control (100.6 ± 16.1%) and vitamin D-enriched medium ($p = 0.138$), but lower with memantine alone ($p = 0.043$), and particularly with memantine plus vitamin D (32.7 ± 24.0%; $p = 0.043$ compared with control and with memantine alone) (Fig. 2C).

4. Discussion

The combination of memantine with vitamin D improved the cognition of patients with AD in one previous study (Annweiler

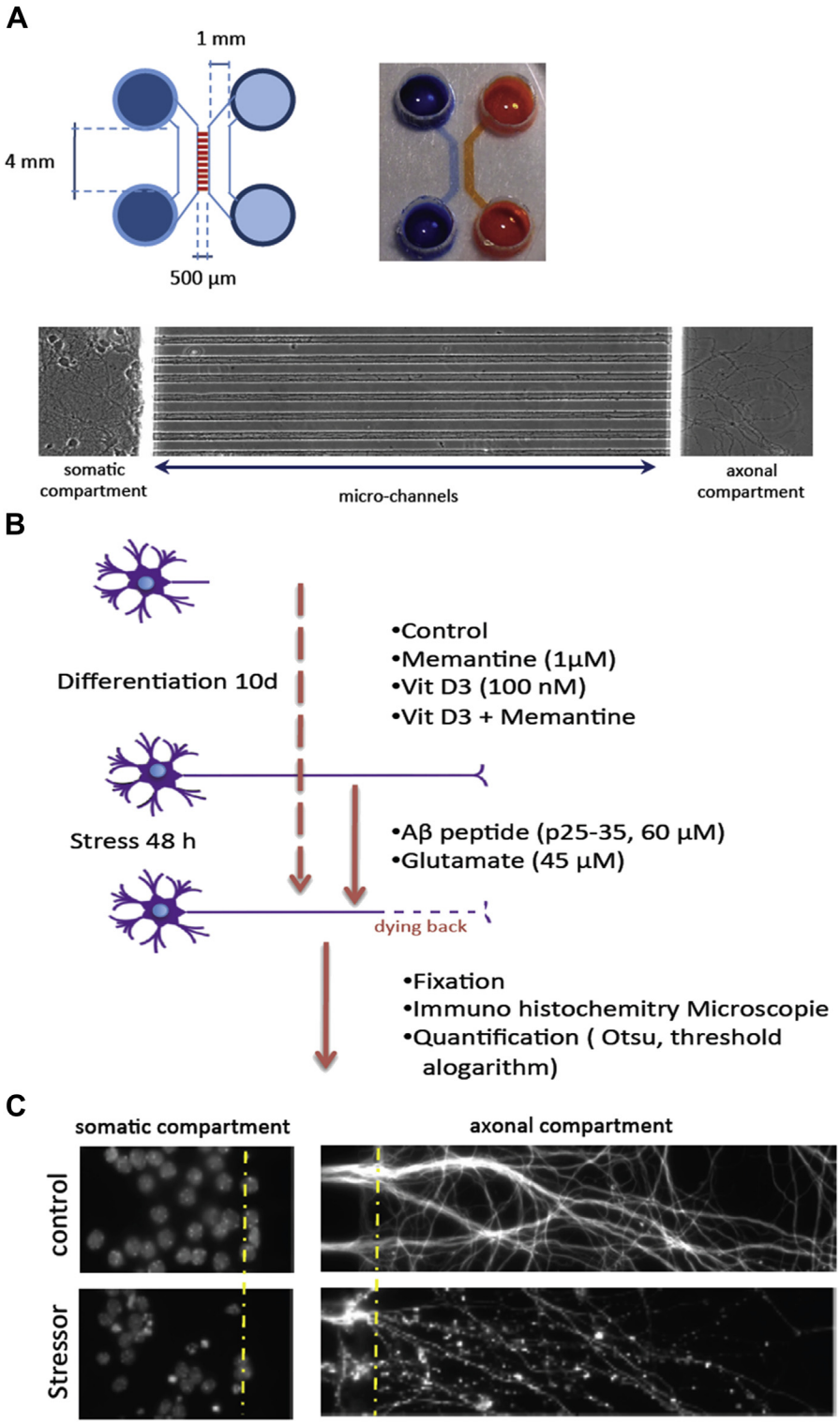

Fig. 1. Methods. (A) (left) Neuron culture chip design; (right) picture of a polydimethylsiloxan microfluidic chip composed of two individual neuronal devices and bonded to a glass cover slip; (bottom) 40× phase image of neurons seeded in somatic compartment at lower density with axons invading microchannels and axonal compartment. (B) Experimental time line. (C) Representative example of axonal degeneration after somatic stress application (cell nuclei are stained with DAPI and axons with β3-tubulin). Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

et al., 2012). Our current results extend this finding by showing that these two compounds combined are not toxic to cortical neurons (Fig. 2A) and protect axons against Aß-peptide and glutamate toxicity (Fig. 2B). Except axonoprotection by memantine against glutamate toxicity, no other protective effect was observed with each compound separately (Fig. 2C), suggesting potentiating benefits of the combination.

Recent findings indicate that increased levels of Aβ-peptide and glutamate have a detrimental impact on neurons via interactions with NMDARs (Hiruma et al., 2003; Martinez-Coria et al., 2010;

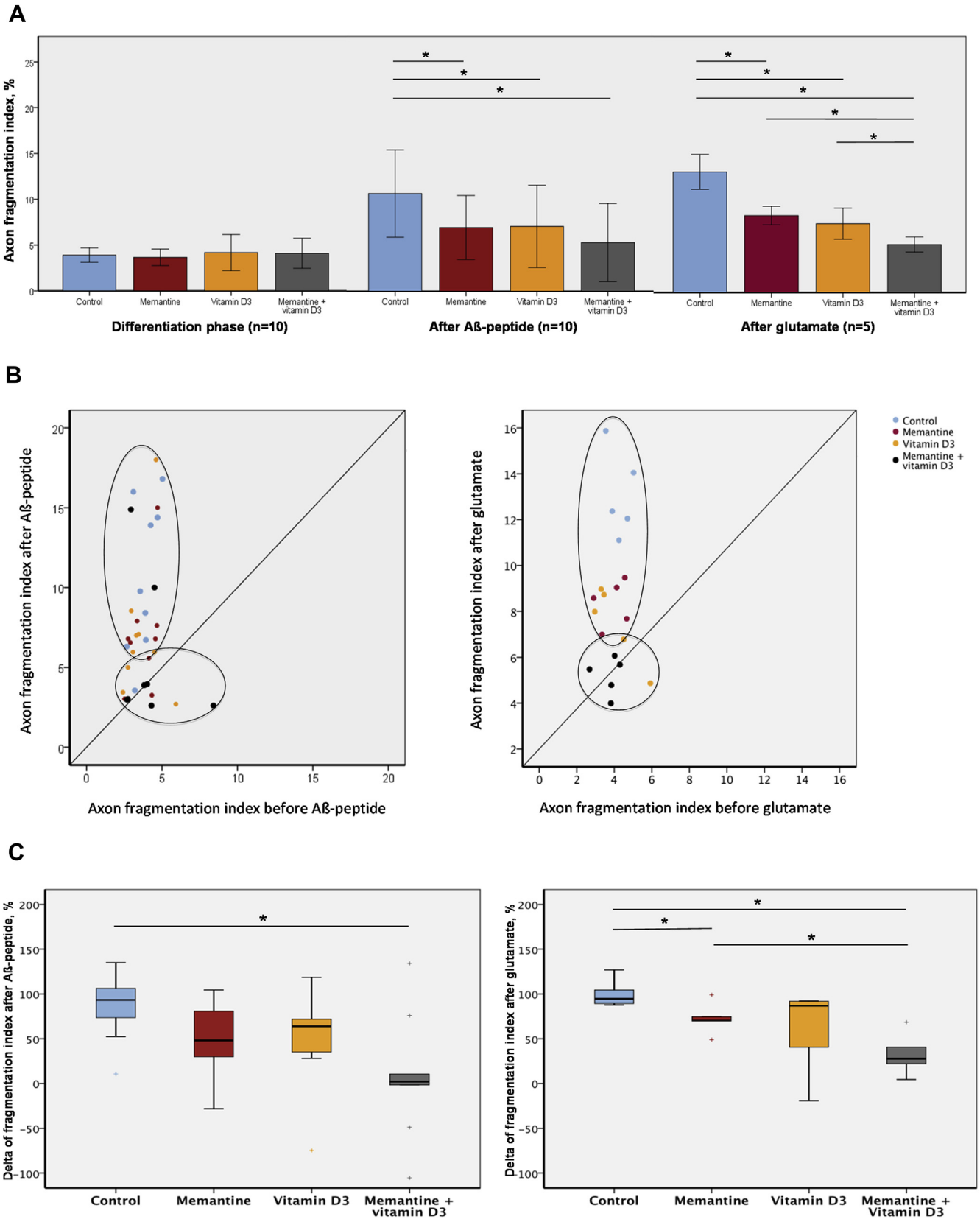

Fig. 2. Axon fragmentation index. (A) Bar plots for mean and SD of fragmentation index. (B) Changes in fragmentation index. Each data point represents the fragmentation index for an experiment before (X-axis value) and after (Y-axis value) stress application. Points lying around the line of identity and on its right (lower encircled area: mainly experiments with memantine plus vitamin D_3) indicate that fragmentation index was broadly identical or lower after stressor; Points to the left of the line (upper encircled area: mainly the 3 other experiments) indicate that fragmentation index was greater after stressor. (C) Box plot for Δ_{FI} after stressor. * $p < 0.05$.

Miguel-Hidalgo et al., 2002; Molnár et al., 2004; Parsons et al., 2007), resulting in a large neuronal calcium entry and subsequent axonal damage and cell necrosis/apoptosis (Parsons et al., 2007). The temporary benefits of memantine in AD are explained by its action as an NMDAR antagonist, which limits calcium influx (McShane et al., 2006; Parsons et al., 2007). Consistently, we found here that Δ_{FI} after glutamate addition was lower with memantine alone compared with control (Fig. 2C). However, the constrained but persistent calcium entry ultimately causes oxidative stress, which justifies additional drugs to prevent initiation of the apoptotic cascade (Annweiler and Beauchet, 2012). Growing evidence suggests that vitamin D, a neurosteroid hormone with receptors on cortical and hippocampal neurons, regulates intraneuronal calcium homeostasis (Brewer et al., 2001), oxidative and inflammatory changes in the hippocampus (Moore et al., 2005), genetic expression of neurotrophins (Brown et al., 2003), and Aβ-peptide clearance (Masoumi et al., 2009; Yu et al., 2011). In line with this evidence, the vitamin D-enriched medium showed a possible trend toward a difference in Δ_{FI} compared with control medium (Fig. 2C), with a p-value of 0.059 and 4 vitamin D data points found within the lower circle of Fig. 2B (less axonal fragmentation). These neuroprotective properties of vitamin D may be complementary and synergistic with memantine, thereby preventing axonal degeneration induced by Aß-peptide and glutamate.

One of the main challenges in the field of AD research during the coming decade lies in finding a curative drug that modifies the neurodegenerative process to prevent AD progression. These data suggest that memantine combined with vitamin D stops axonal degeneration induced by Aß-peptide and glutamate. Beyond its possible utility for slowing down disease progression in patients who already have AD symptoms, our primary hope is that the combination of memantine with vitamin D can be applied to prevent the onset of AD.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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References

Annweiler, C., Beauchet, O., 2012. Possibility of a new anti-Alzheimer's disease pharmaceutical composition combining memantine and vitamin D. Drugs. Aging 29, 81–91.
Annweiler, C., Schott, A.M., Berrut, G., Chauviré, V., Le Gall, D., Inzitari, M., Beauchet, O., 2010. Vitamin D and ageing: neurological issues. Neuropsychobiology 62, 139–150.
Annweiler, C., Herrmann, F.R., Fantino, B., Brugg, B., Beauchet, O., 2012. Effectiveness of the combination of memantine plus vitamin D on cognition in patients with Alzheimer disease: a pre-post pilot study. Cogn. Behav. Neurol. 25, 121–127.
Benes, F.M., Farol, P.A., Majocha, R.E., Marotta, C.A., Bird, E.D., 1991. Evidence for axonal loss in regions occupied by senile plaques in Alzheimer cortex. Neuroscience 42, 651–660.
Bischoff-Ferrari, H.A., Giovannucci, E., Willett, W.C., Dietrich, T., Dawson-Hughes, B., 2006. Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. Am. J. Clin. Nutr. 84, 18–28.
Brewer, L.D., Thibault, V., Chen, K.C., Langub, M.C., Landfield, P.W., Porter, N.M., 2001. Vitamin D hormone confers neuroprotection in parallel with downregulation of L-type calcium channel expression in hippocampal neurons. J. Neurosci. 21, 98–108.
Brown, J., Bianco, J.I., McGrath, J.J., Eyles, D.W., 2003. 1,25-Dihydroxyvitamin D-3 induces nerve growth factor, promotes neurite outgrowth and inhibits mitosis in embryonic rat hippocampal neurons. Neurosci. Lett. 343, 139–143.
Hiruma, H., Katakura, T., Takahashi, S., Ichikawa, T., Kawakami, T., 2003. Glutamate and amyloid beta-protein rapidly inhibit fast axonal transport in cultured rat hippocampal neurons by different mechanisms. J. Neurosci. 23, 8967–8977.
Ibi, M., Sawada, H., Nakanishi, M., Kume, T., Katsuki, H., Kaneko, S., Shimohama, S., Akaike, A., 2001. Protective effects of 1 alpha,25-(OH)(2)D(3) against the neurotoxicity of glutamate and reactive oxygen species in mesencephalic culture. Neuropharmacology 40, 761–771.
Kalueff, A.V., Tuohimaa, P., 2007. Neurosteroid hormone vitamin D and its utility in clinical nutrition. Curr. Opin. Clin. Nutr. Metab. Care 10, 12–19.
Kilinc, D., Peyrin, J.M., Soubeyre, V., Magnifico, S., Saias, L., Viovy, J.L., Brugg, B., 2011. Wallerian-like degeneration of central neurons after synchronized and geometrically registered mass axotomy in a three-compartmental microfluidic chip. Neurotox. Res. 19, 149–161.
Luo, L., O'Leary, D.D., 2005. Axon retraction and degeneration in development and disease. Annu. Rev. Neurosci. 28, 127–156.
Martinez-Coria, H., Green, K.N., Billings, L.M., Kitazawa, M., Albrecht, M., Rammes, G., Parsons, C.G., Gupta, S., Banerjee, P., LaFerla, F.M., 2010. Memantine improves cognition and reduces Alzheimer's-like neuropathology in transgenic mice. Am. J. Pathol. 176, 870–880.
Masoumi, A., Goldenson, B., Ghirmai, S., Avagyan, H., Zaghi, J., Abel, K., Zheng, X., Espinosa-Jeffrey, A., Mahanian, M., Liu, P.T., Hewison, M., Mizwickie, M., Cashman, J., Fiala, M., 2009. 1alpha,25-dihydroxyvitamin D3 interacts with curcuminoids to stimulate amyloid-beta clearance by macrophages of Alzheimer's disease patients. J. Alzheimers. Dis. 17, 703–717.
McShane, R., Areosa Sastre, A., Minakaran, N., 2006. Memantine for dementia. Cochrane. Database. Syst. Rev., CD003154.
Miguel-Hidalgo, J.J., Alvarez, X.A., Cacabelos, R., Quack, G., 2002. Neuroprotection by memantine against neurodegeneration induced by beta-amyloid(1-40). Brain. Res. 958, 210–221.
Molnár, Z., Soós, K., Lengyel, I., Penke, B., Szegedi, V., Budai, D., 2004. Enhancement of NMDA responses by beta-amyloid peptides in the hippocampus in vivo. Neuroreport 15, 1649–1652.
Moore, M.E., Piazza, A., McCartney, Y., Lynch, M.A., 2005. Evidence that vitamin D3 reverses age-related inflammatory changes in the rat hippocampus. Biochem. Soc. Trans. 33, 573–577.
Obradovic, D., Gronemeyer, H., Lutz, B., Rein, T., 2006. Cross-talk of vitamin D and glucocorticoids in hippocampal cells. Neurochem. 96, 500–509.
Parsons, C.G., Stoffler, A., Danysz, W., 2007. Memantine: a NMDA receptor antagonist that improves memory by restoration of homeostasis in the glutamatergic system—too little activation is bad, too much is even worse. Neuropharmacology 53, 699–723.
Peyrin, J.M., Deleglise, B., Saias, L., Vignes, M., Gougis, P., Magnifico, S., Betuing, S., Pietri, M., Caboche, J., Vanhoutte, P., Viovy, J.L., Brugg, B., 2011. Axon diodes for the reconstruction of oriented neuronal networks in microfluidic chambers. Lab. Chip 11, 3663–3673.
Rosales-Corral, S., Acuna-Castroviejo, D., Tan, D.X., López-Armas, G., Cruz-Ramos, J., Munoz, R., Melnikov, V.G., Manchester, L.C., Reiter, R.J., 2012. Accumulation of exogenous amyloid-beta peptide in hippocampal mitochondria causes their dysfunction: a protective role for melatonin. Oxid. Med. Cell. Longev. 2012, 8436–8449.
Smith, K.D., Kallhoff, V., Zheng, H., Pautler, R.G., 2007. In vivo axonal transport rates decrease in a mouse model of Alzheimer's disease. Neuroimage 35, 1401–1408.
Sun, S.W., Campbell, B., Carrick, D., Liang, H.F., 2011. Using DTI for non-invasive evaluation of axonal damage caused by exposure of axon terminals to amyloid beta. Alzheimers Dement. 7, S309–S310.
Taylor, A.M., Blurton-Jones, M., Rhee, S.W., Cribbs, D.H., Cotman, C.W., Jeon, N.L., 2005. A microfluidic culture platform for CNS axonal injury, regeneration and transport. Nat. Methods 2, 599–605.
Vieth, R., 1999. Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety. Am. J. Clin. Nutr. 69, 842–856.
Wirths, O., Weis, J., Kayed, R., Saido, T.C., Bayer, T.A., 2007. Age-dependent axonal degeneration in an Alzheimer mouse model. Neurobiol. Aging 28, 1689–1699.
Yu, J., Gattoni-Celli, M., Zhu, H., Bhat, N.R., Sambamurti, K., Gattoni-Celli, S., Kindy, M.S., 2011. Vitamin D3-enriched diet correlates with a decrease of amyloid plaques in the brain of AbetaPP Transgenic Mice. J. Alzheimers Dis. 25, 295–307.
Xia, P., Chen, H.S., Zhang, D., Lipton, S.A., 2010. Memantine preferentially blocks extrasynaptic over synaptic NMDA receptor currents in hippocampal autapses. J. Neurosci. 30, 11246–11250.