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Ethanol impairs muscarinic receptor-induced neuritogenesis in rat hippocampal slices: Role of astrocytes and extracellular matrix proteins

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ABSTRACT

In an *in vitro* co-culture system of astrocytes and neurons, stimulation of cholinergic muscarinic receptors in astrocytes had been shown to cause neuritogenesis in hippocampal neurons, and this effect was inhibited by ethanol. The present study sought to confirm these earlier findings in a more complex system, *in vitro* rat hippocampal slices in culture. Exposure of hippocampal slices to the cholinergic agonist carbachol (1 mM for 24 h) induced neurite outgrowth in hippocampal pyramidal neurons, which was mediated by activation of muscarinic M3 receptors. Specifically, carbachol induced a >4-fold increase in the length of the longest neurite, and a 4-fold increase in the length of minor neurites and in the number of branches. Co-incubation of carbachol with ethanol (50 mM) resulted in significant inhibition of the effects induced by carbachol on all parameters measured. Neurite outgrowth in CNS neurons is dependent on various permissive factors that are produced and released by glial cells. In hippocampal slices carbachol increased the levels of two extracellular matrix protein, fibronectin and laminin-1, by 1.6-fold, as measured by Western blot. Co-incubation of carbachol with ethanol significantly inhibited these increases. Carbachol-induced increases in levels of extracellular matrix proteins were antagonized by a M3 muscarinic receptor antagonist. Furthermore, function-blocking fibronectin or laminin-1 antibodies antagonized the effect of carbachol on neurite outgrowth. These results indicate that in hippocampal slices stimulation of muscarinic M3 receptors induces neurite outgrowth, which is mediated by fibronectin and laminin-1, two extracellular matrix proteins released by astrocytes. By decreasing fibronectin and laminin levels ethanol prevents carbachol-induced neuritogenesis. These findings highlight the importance of glial–neuronal interactions as important targets in the developmental neurotoxicity of alcohol.

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1. Introduction

Glial cells, particularly astrocytes, significantly influence neuronal development, by providing trophic support for neuronal survival, and by modulating neuronal migration, axonal and dendritic growth, and synaptogenesis [1–4]. Neurite outgrowth is a fundamental event in brain development, and is accomplished by signals from the extracellular space such as extracellular matrix proteins, cell adhesion molecules, and soluble factors that can promote or inhibit the growth of neurites [5]. Astrocyte–neuron interactions play a key role in this process, as astrocytes express and release factors that can promote neurite outgrowth, such as laminin-1, N-cadherin, thrombospondin, fibronectin [5–8], or even inhibit neurite outgrowth (e.g. chondroitin sulfate proteoglycan such as neurocan or brevican; [9]).

Astrocytes express various neurotransmitter receptors whose functions in these cells remain for the most part elusive [10]. Among these, astrocytes also express cholinergic muscarinic receptors, particularly the M2 and M3 subtypes [11]. There is substantial evidence that acetylcholine may influence various aspects of brain development, including proliferation of precursors cells, astrocyte proliferation and maturation, and neuronal differentiation [11–16]. We have recently shown that acetylcholine, by activating muscarinic M3 receptors on astrocytes, increases their ability to foster neuritogenesis in neurons, and that this effect is mediated primarily by extracellular matrix proteins [17]. Rat astrocytes were incubated for 24 h with the cholinergic agonist carbachol, an analogue of acetylcholine which is not hydrolyzed by acetylcholinesterase (AChE), and then co-cultured for an additional 24 h with rat hippocampal

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neurons. Co-culture with carbachol-primed astrocytes increased the length of the longest neurite (identified as the axon by Tau-1 staining), and the length of minor neurites (identified by staining with MAP2) [17]. Stimulation of M3 muscarinic receptors in astrocytes also induced an increased expression and release of fibronectin and laminin-1, which were responsible for the neuritogenic action of carbachol-primed astrocytes [17].

Ethanol is a known human developmental neurotoxicant. In utero exposure to ethanol can cause Fetal Alcohol Spectrum Disorders (FASD), whose most severe manifestation is represented by the Fetal Alcohol Syndrome (FAS), characterized by growth retardation facial dysmorphology, and neurodevelopmental abnormalities, leading to long-lasting cognitive and behavioral deficits [18,19]. The mechanisms involved in the toxic effects of ethanol on the developing nervous system remain elusive; alcohol has been shown to cause apoptotic neuronal death, to inhibit glial cell proliferation, and to alter, through multiple mechanisms, neuronal development. A possible action of ethanol is inhibition of neurite outgrowth, and recent evidence suggests that ethanol may interfere with the ability of astrocytes to induce neurite outgrowth [20,21]. As ethanol had been reported to inhibit the signaling pathways activated by M3 muscarinic receptors in astrocytes [22], it was hypothesized that it may also inhibit carbachol-stimulated, astrocyte-induced neuritogenesis in hippocampal neurons, by inhibiting the release of neuritogenic factors. Indeed, incubation of astrocytes with ethanol, at concentrations as low as 25 mM (corresponding to 0.115 mg/dl), in the presence of carbachol, followed by wash-out, inhibited the effects of carbachol on axonal length and on minor neurite length by inhibiting phospholipase D [23]. By the same mechanism, ethanol also inhibited carbachol-induced increases in fibronectin and laminin-1 in the astrocyte lysate and in the medium [23]. We are not aware of any other study showing an effect of ethanol on fibronectin in the CNS, but one study showed that chronic ethanol exposure followed by ethanol withdrawal decreases the levels of laminin-1 in the hippocampus indicating that the extracellular matrix is indeed a target of ethanol *in vivo* [62].

As these studies were all carried out in astrocyte–neuron co-cultures, aim of the present study was to investigate the effect of a muscarinic agonist and of ethanol on neuritogenesis utilizing a more complex system, the hippocampal slice, in which the regional cytoarchitecture, the appropriate synaptic circuitry, physiology and neurotransmitter receptor distribution of the intact hippocampus are preserved [24]. Results show that carbachol stimulates neuritogenesis in the rat hippocampal slice, that this effect is mainly mediated by fibronectin and laminin-1, which are mostly released by astrocytes, and that this effect is inhibited by ethanol.

2. Materials and methods

2.1. Materials

Time-pregnant Sprague-Dawley rats were purchased from Charles-River (Wilmington, MA). Anti-laminin function blocking antibody was purchased from Biomedical Technologies (Stoughton, MA), and anti-fibronectin function blocking antibody was purchased from Dako (High Wycombe, UK). Millicell-CM cell culture inserts were from Millipore (Bedford, MA), and coverglass and slides were from Fisher Scientific (Federal Way, WA). Minimum essential medium, gentamycin, horse serum, and Hank's balanced salt solution (HBSS) were from Invitrogen (Carlsbad, CA). Cell lysis buffer was purchased from Cell Signaling (Danvers, MA). The commercial kit for plasmid DNA was from Quiagen (Valencia, CA). Gold particles, cartridge tubing, polyvinylpyrrolidone, cartridge holder, tubing prep station, and tubing cutter were from Biorad (Hercules, CA). Ethanol was from EMD Chemical Inc. (Gibbston, NJ), while the bicinchoninic acid assay kit for protein determination was purchased from Thermo Scientific (Rockford, IL). All other chemicals and antibodies were from Sigma–Aldrich (St. Louis, MO).

2.2. Preparation of rat hippocampal slices

Hippocampal slices were prepared from postnatal day 5 Sprague-Dawley rats of either gender, as previously described [25,26]. The brain was removed and placed into pre-cooled Hanks' balanced salt solution (HBSS). Hippocampi were isolated and placed on a Teflon stage of a McIlwain-type tissue chopper, aligned perpendicularly to the blade to preserve the structure. Slices, 400 μm thick were rapidly cut (2/s), and transferred to a Petri dish. Utilizing a dissecting microscope, slices were gently separated, and selected individually to ensure the integrity of the structure; damaged slices were discarded. Slices were transferred onto a Millipore insertion in a multi-well plate. Four slices were placed in each well, few millimeters apart from each other. Excess medium was removed, and replaced with complete medium (minimum essential medium containing 20% horse serum). Plates were placed into an incubator equilibrated with 5% CO_2 in air at a temperature of 37 °C. Medium was replaced the day after the preparation, and 2 h before transfection.

2.3. Transfection

Transfection of hippocampal slices was done using the Helios Gene Gun (Biorad). Green fluorescence protein (GFP) was expressed in cultured hippocampal slices using the cytomegalovirus (CMV) promoter. CMV-GFP plasmid DNA was prepared using a commercial kit (Quiagen). Gene gun cartridges were prepared according to the manufacturer's protocol with slight modifications (Biorad). To prepare the bullets, gold particles and plasmid DNA were mixed with spermidine, precipitated with $CaCl_2$, washed with 100% ethanol, and resuspended in ethanol. This solution was precipitated onto the internal wall of the plastic tube. Slices were bombarded with one cartridge per insert under helium pressure through a nylon mesh that served to reduce the mechanical effects of the air pressure. After transfection, slices were cultured for 72 h before treatments.

2.4. Treatment of slices

Slices were treated for 24 h with carbachol (1 mM) in the absence or presence of ethanol (50 mM). In order to reduce ethanol evaporation, cultures were placed in sealed chambers (Billups-Rothenberg Inc., Del Mar, CA), together with a reservoir tray containing the same concentration of ethanol as in the culture medium [27]. When antagonists were used, these were added 1 h before carbachol. Function-blocking laminin-1 or fibronectin antibodies (10 μg/ml) were added together with carbachol.

2.5. Morphological analysis

At the end of treatments, slices were washed three times with HBSS and dipped for 30 min into a 4% paraformaldehyde solution. Fixed slices were rinsed with PBS (0.1 M) for 10 min at room temperature, and placed on a glass slide and mounted with antifade solution with a coverglass. The whole mount was then glued with nail polish. Neurons were imaged on a confocal laser scanning microscope (Fluoview 1000, Olympus, Center Valley, PA), with an Olympus 20× objective. Successful expression was confirmed using epifluorescence. Neurites were imaged using the green channel with 0.35 um steps in the *z* axis. Each optical plane was averaged twice (Kalman filter). Image stacks were

deconvolved using Huygens Essential version 11.0 (Scientific Volume Imaging, Hilversum, The Netherlands) for better visibility of the neurites and the fine structures of the cells. Image analysis was done using Image J version 1.4 with the simple neurite tracer plug-in, selecting Hessian-based analysis option. The analysis was carried out blind; neurons whose processes were intermingled with those of neighboring cells were excluded from the analysis. Neurite length was measured from the point of emergence at the cell body to the tip of each segment as described by Guizzetti et al. [17]. In each experiment, at least four slices per treatment were analyzed. Only pyramidal neurons (*i.e.* neurons with an oval or pyramidal cell body and a multipolar morphology) were selected for analysis, and a total of 20 neurons per treatment were analyzed. For each neuron, the following parameters were calculated: length of the longest neurite, length of minor neurites, number of neurites/cell, and number of branches/cell (defined as bifurcations in which a parent neurite splits into two daughters; [28]).

2.6. Western blot analysis

Measurements of fibronectin and laminin protein levels in hippocampal slice lysate were performed by Western blot. At the end of treatment, slices were collected in 250 μl of lysis buffer (1% Triton X-100, 50 mM Tris buffer, pH 7.5, and a protease inhibitor EDTA-free mixture), sonicated, and centrifuged at 16,000 × *g*. The supernatant was collected, and proteins were quantified by the bicinchoninic acid method, separated by electrophoresis and transferred to PVDF membranes. Fibronectin and laminin-1 were detected using specific antibodies. Membranes from cell lysate proteins were re-probed for beta-actin used as loading control. Optical density of each band was quantified by the ImageJ software (National Institutes of Health).

2.7. Statistical analysis

Results were analyzed for statistical significance by repeated measures ANOVA followed by the Bonferroni post hoc test, using the Prism 5 software (GraphPad Software Inc., LA Jolla, CA). Data are presented as means ± standard deviation (SD).

3. Results

Exposure of rat hippocampal slices to the cholinergic agonist carbachol stimulated neurite outgrowth. Fig. 1 shows that carbachol increased the length of the longest neurite (presumably the axon, as indicated by staining with Tau-1 in an earlier study; [17]) by about 4-fold. Length of minor neurites and the number of branches per cell were also increased by about 4-fold by carbachol. In contrast, carbachol had no effects on the number of neurites per cell. Ethanol (50 mM) when present alone, had no significant effect on any of these parameters of neuritogenesis; however, it almost completely antagonized the effects of carbachol (Fig. 1). These findings confirm those previously obtained in co-cultures of astrocytes and neurons [17,23].

We had previously found that carbachol could exert a dual effect on hippocampal neuron neuritogenesis: one indirect, mediated by activation of M3 muscarinic receptors on astrocytes, and the other direct, mediated by M1 muscarinic receptors on neurons [17,29]. As in the hippocampal slice carbachol would be expected to activate both receptors on both cell types, we sought to determine which of the two muscarinic receptors, and hence which cell type, may be primarily involved in carbachol-induced neuritogenesis. Fig. 2 shows the results obtained with antagonists of the four major types of muscarinic receptors. As expected, methoctramine and tropicamide, antagonists of muscarinic M2 and M4 receptors, respectively, had no effect on carbachol-induced increases in length of the longest neurite, length of minor neurites, and number of branches per cell. In contrast, the M3 muscarinic receptor antagonist 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine) completely inhibited the effect of carbachol. The M1 muscarinic antagonist pirenzepine caused a small, not statistically significant inhibition of carbachol's effects (Fig. 2).

We had previously shown in astrocyte–neuron co-cultures that activation of muscarinic M3 receptors on astrocytes promotes neuritogenesis by increasing the expression and release of the extracellular matrix proteins laminin and fibronectin [17]. We thus investigated whether carbachol would alter the levels of fibronectin and laminin-1 in the hippocampal slice. As shown in Fig. 3, carbachol increased protein levels of both fibronectin and laminin-1 (by about 60%), and such effect was antagonized by ethanol (50 mM), which alone had no effect on the levels of these two extracellular matrix proteins. The effects of carbachol on fibronectin and laminin-1 levels were blocked by 4-DAMP but not by pirenzepine (Fig. 4), indicating that they are due to activation of M3 muscarinic receptors. To investigate the role of these two extracellular matrix proteins in carbachol-induced neuritogenesis, hippocampal slices were treated with fibronectin and laminin-1 function-blocking antibodies; both antibodies antagonized the effect of carbachol on neuritogenesis (Fig. 5). This latter finding lends supports to the notion that astrocyte-derived fibronectin and laminin-1 play a most relevant role in carbachol-induced neuritogenesis in the hippocampus.

4. Discussion

There is substantial evidence that acetylcholine may influence various aspects of brain development [11–16,30,31]. Components of the cholinergic system, including choline acetyltransferase and acetylcholine receptors, are present early in development, long before the appearance of synapses [12]. Furthermore, acetylcholine levels are high in the neonatal rat brain (80–90% of adult values), and muscarinic receptor-activated signal transduction systems (notably phospholipases C and D) are greatly enhanced in the neonatal rat [32,33]. Acetylcholine is believed to have non-transmitter effects during development, as it can regulate morphogenic cell movements during gastrulation, glial cell proliferation, and neuronal differentiation and survival [15,31,34,35]. Developing neurons may fire action potentials and trigger acetylcholine secretion from the axonal growth cone while the axon is still growing, and neuron-released acetylcholine may contribute to further axonal growth and formation of synaptic contacts [36]. Furthermore, astrocytes may themselves release acetylcholine, which may act in an autocrine fashion [37].

Utilizing a co-culture system of astrocytes and neurons, we had previously found that stimulation of muscarinic M3 receptors on astrocytes would elicit an increased synthesis and release of extracellular matrix proteins (fibronectin and laminin-1) that would act as neuritogenic factors on hippocampal neurons [17]. In addition to this astrocyte-mediated effect, carbachol has also been shown to increase neurite outgrowth through a direct action on hippocampal neurons; such direct effect was mediated by M1 muscarinic receptors, and was limited to an increase in axonal length, with no changes in the length of minor neurites [29].

The hippocampal slice provides a mean to assess the effect of muscarinic receptor stimulation in a brain preparation in which the normal hippocampal cytoarchitecture is maintained, and is thus most similar to the *in vivo* situation [24]. Acetylcholine and cholinergic neurons are widely distributed in the brain. In the hippocampus, the main cholinergic inputs are from projections originating in the medial septum and the diagonal band of Broca, with the septo-hippocampal projection believed to be most relevant for hippocampal function [38,39]. Furthermore, in

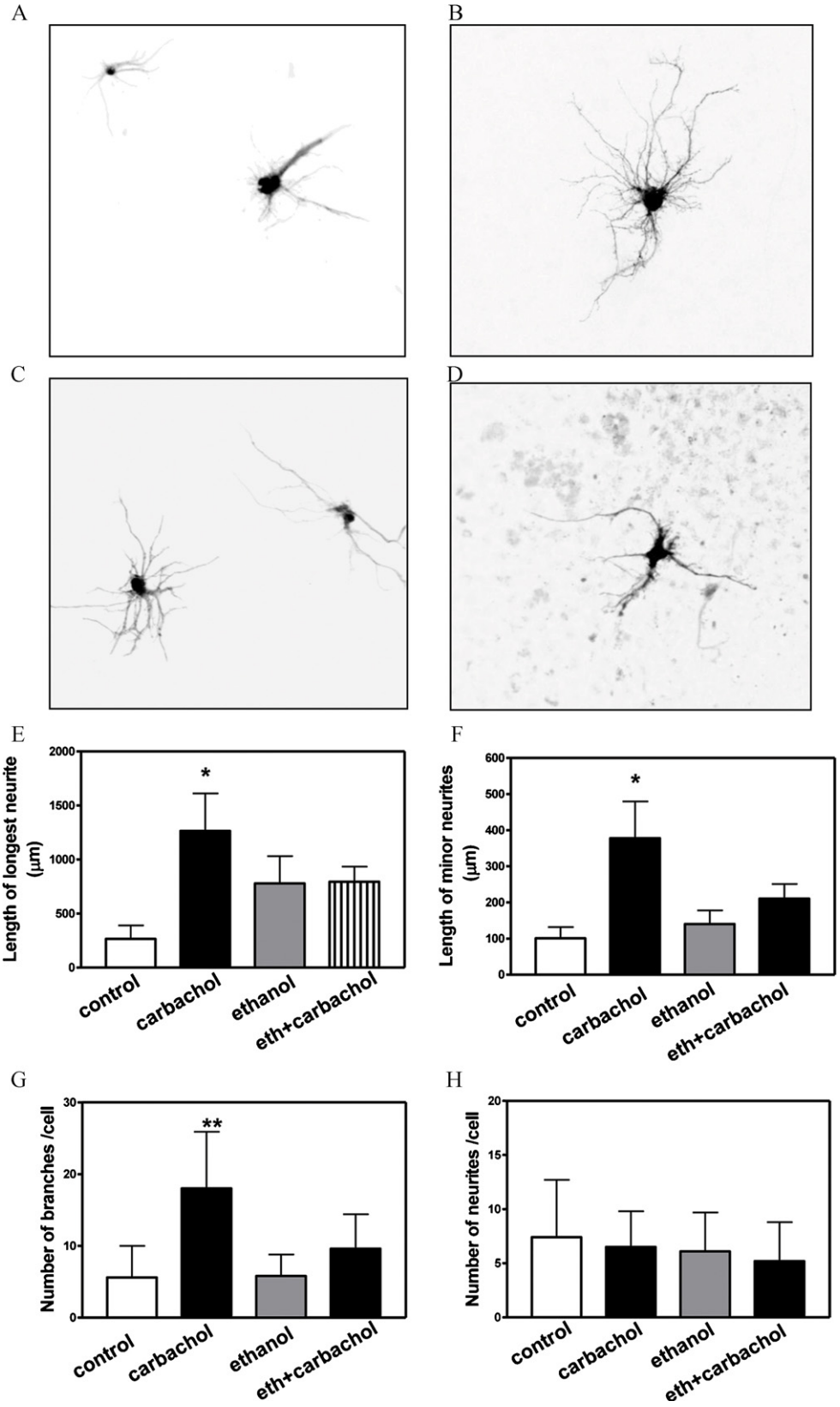

Fig. 1. Effect of carbachol and ethanol on neurite outgrowth in rat hippocampal slices. Organotypic hippocampal slices were prepared from PND 5 Sprague-Dawley rats. After four days in culture, slices were transfected with a gene gun, and cultured for 72 h before treatments for 24 h with 1 mM carbachol and/or 50 mM ethanol. Slices were then fixed in 4% paraformaldehyde, mounted and imaged using an Olympus FV-1000 confocal microscope. A–D show representative fields of hippocampal neurons: (A) control; (B) 1 mM carbachol; (C) 50 mM ethanol; (D) 1 mM carbachol and 50 mM ethanol. E–H show the quantification of morphometric analysis. E, length of the longest neurite; F, length of minor neurites; G, number of branches/cell; H, number of neurites/cell. Results represent the mean (±SD) of a total of 60 cells per treatment from three separate experiments. Significantly different from control, $^{*}p < 0.05$; $^{**}p < 0.01$.

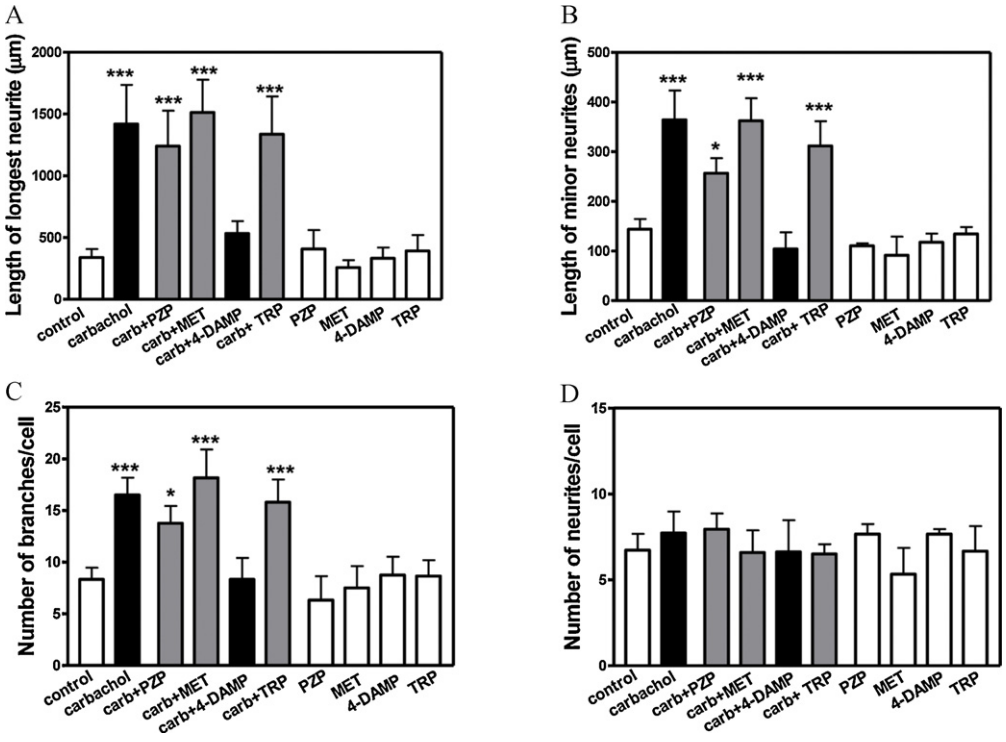

Fig. 2. Effect of muscarinic antagonists on carbachol-induced neurite outgrowth in rat hippocampal slices. Hippocampal slices were treated for 24 h with carbachol (1 mM) in the absence or presence of the M_1 muscarinic receptor antagonist pirenzepine (PZP), the M_2 antagonist methoctramine (MET), the M_3 antagonist 4-DAMP, or the M_4 antagonist tropicamide (TRP), all at 10 μM. A, length of the longest neurite; B, length of minor neurites; C, number of branches/cell; D, number of neurites/cell. Results represent the mean (±SD) of 60 cells per treatment from three separate experiments. Significantly different from control, $^{*}p < 0.05$; $^{***}p < 0.001$.

addition to extrinsically innervated neurons, cholinergic interneurons also exist in the hippocampus [40]. In the present study we found that in hippocampal slices cholinergic stimulation increases the length of the longest neurite ad of minor neurites, as well as the number of branches per cell. Among the subtype-specific muscarinic receptor antagonist tested, only a muscarinic M3 receptor antagonist blocked the effect of carbachol on neuritogenesis, while a M1 antagonist had a small, not statistically significant inhibitory effect, and muscarinic M2 and M4 antagonists were ineffective. All five types of muscarinic receptors are expressed in the hippocampus, with a predominance of M1 and M3 receptors [41,42]. We had previously shown that carbachol could stimulate neuritogenesis indirectly, by activating muscarinic M3 receptors on astrocytes, thereby increasing the expression and release of neuritogenic extracellular matrix proteins [17], or directly, by activating muscarinic M1 receptors on hippocampal neurons [29]. The ability of 4-DAMP to antagonize the neuritogenic effect of carbachol would suggest that this action is primarily due to activation of M3

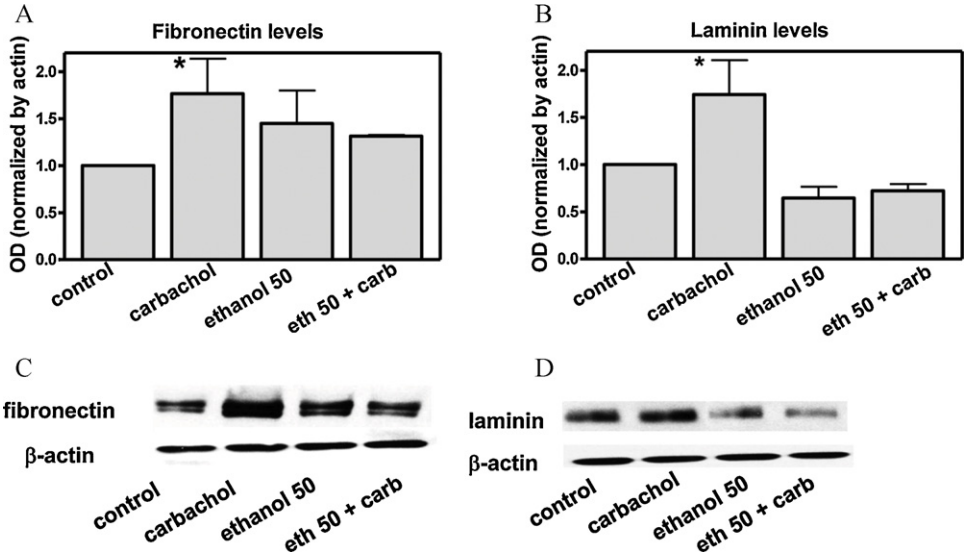

Fig. 3. Effect carbachol and ethanol on fibronectin and laminin-1 levels in rat hippocampal slices. Western blot analysis was carried out in tissue lysate of hippocampal slices treated for 24 h with 1 mM carbachol in the presence or absence of 50 mM ethanol. After protein transfer, membranes were labeled with a fibronectin, laminin-1, or β-actin antibody. (A and B) Densitometric analysis of levels of fibronectin and laminin normalized to β-actin; (C and D) representative blots. Results represent the mean (±SD) of three separate experiments. Significantly different from control, $^{*}p < 0.05$.

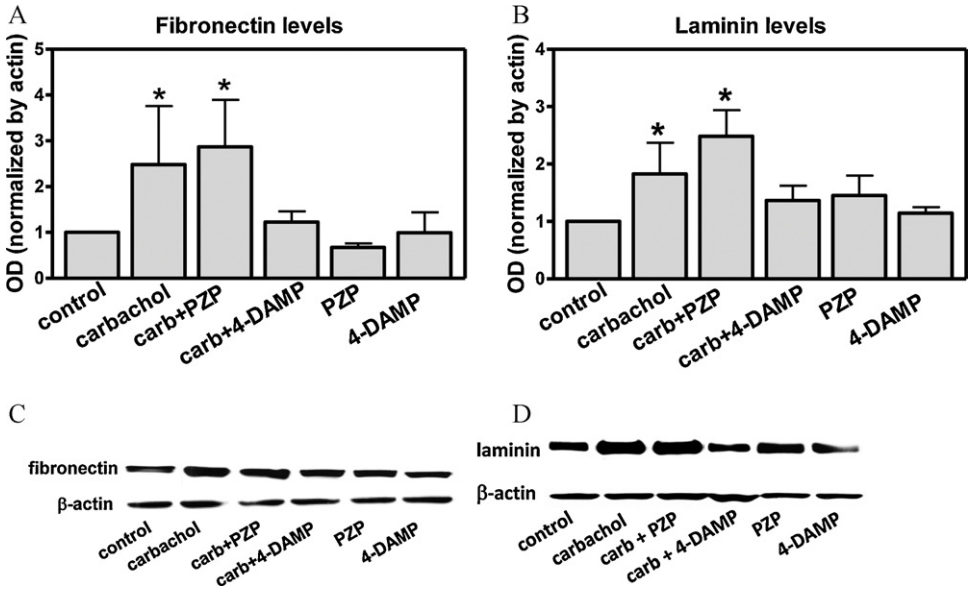

Fig. 4. Effect of muscarinic antagonists on carbachol-induced increase of fibronectin or laminin-1 in rat hippocampal slices. Western blot analysis was carried out in tissue lysate of hippocampal slices treated for 24 h with 1 mM carbachol in the presence or absence of the M_1 muscarinic receptor antagonist pirenzepine (PZP), or the M_3 antagonist 4-DAMP. After protein transfer, membranes were labeled with a fibronectin, laminin, or β-actin antibody. (A and B) Densitometric analysis of levels of fibronectin and laminin normalized to β-actin; (C and D) representative blots. Results represent the mean (±SD) of three separate experiments. Significantly different from control, $^{*}p < 0.05$.

muscarinic receptors, presumably located on astrocytes. Further evidence is provided by the finding that carbachol causes an increase in the hippocampal levels of fibronectin and laminin-1 (synthesized and released by astrocytes), which is antagonized by the M3 receptor antagonist 4-DAMP. Additionally, function-blocking fibronectin and laminin-1 antibodies antagonize the effect of carbachol on neuritogenesis. Altogether these findings suggest that the primary mechanism involved in the neuritogenic action of carbachol in the hippocampal slice is the activation of M3 muscarinic receptors, presumably located in astrocytes, followed by increased expression

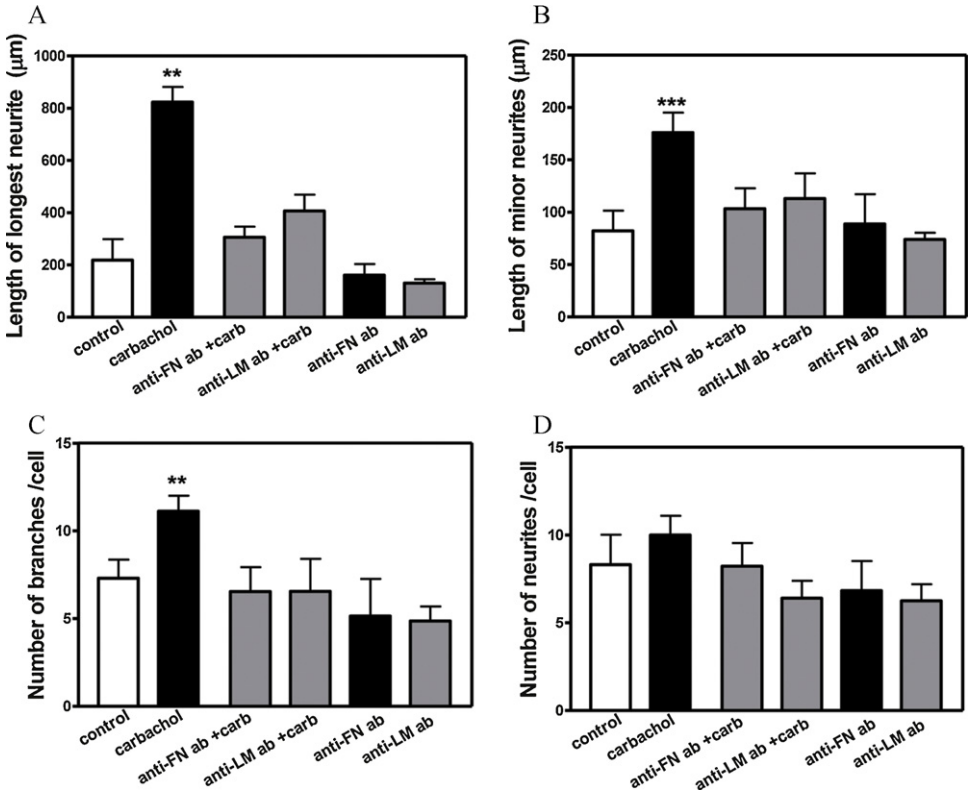

Fig. 5. Effect of fibronectin and laminin-1 function-blocking antibodies on neurite outgrowth increase induced by carbachol in rat hippocampal slices. Hippocampal slices were treated for 24 h with or without 1 mM carbachol and function-blocking anti-fibronectin (FN) or anti-laminin (LM) antibodies. (A) Length of the longest neurite; (B) length of minor neurites; (C) number of branches/cell; (D) number of neurites/cell. Results represent the mean (±SD) of 60 cells per treatment from three separate experiments. Significantly different from control, $^{*}p < 0.05$; $^{***}p < 0.001$.

and release of fibronectin and laminin-1, which in turn exert their neuritogenic action on hippocampal pyramidal neurons. The results are mostly in agreement with those reported in astrocyte–neuron co-cultures [17]. A minor difference between the two systems is that the anti-laminin antibody blocked only carbachol's effect on minor neurite length in co-cultures [17], while it also antagonized the increase in the longest neurite in the hippocampal slice (Fig. 2).

An important objective of this project was that of identifying and characterizing novel modes of action of ethanol, involved in its developmental neurotoxicity. Pre- and post-natal exposure to ethanol have been shown to affect the hippocampus, as evidenced by morphological and biochemical alterations found in this brain region, as well as by changes in hippocampus-mediated behaviors [43–49]. With regard to the effects of ethanol on neuritogenesis, contradictory results have been reported, with some *in vitro* studies indicating that ethanol increases the elongation of neurites, and others showing an inhibitory effect [47,50–53]. We had previously shown that ethanol (25, 50 or 100 mM) inhibited carbachol-induced, astrocyte-mediated neuritogenesis in hippocampal neurons in co-culture, as well as carbachol-induced expression and release of fibronectin and laminin-1 in astrocytes [23]. The same effects were also found in the present study in hippocampal slices. We chose an intermediate concentration of ethanol for these experiments (50 mM), as it was shown to profoundly antagonize the effect of carbachol in the co-culture experiments [23]. As shown in Fig. 1, in the hippocampal slice, 50 mM ethanol almost completely inhibited the effect of carbachol on neurite outgrowth (Fig. 1). In contrast, ethanol had been found to inhibit the direct action of carbachol on neurons (mediated, as said, by M1 muscarinic receptors) only at higher concentrations (75 mM; [54]). Upon *in vivo* exposure effects on the hippocampus have been reported at blood alcohol concentrations of 40–60 mM [43,46]. Furthermore, at the same 50 mM concentration, ethanol also inhibited carbachol-induced decreases in fibronectin and laminin-1, which are produced and released by astrocytes [5,7,8]. These findings suggest that the effects of ethanol in the hippocampal slice are primarily due to inhibition of muscarinic receptor signal transduction in astrocytes. Ethanol can compete with water as a substrate for phospholipase D, whereby production of phosphatidic acid is inhibited and phosphatidylethanol is formed instead by a transphosphatidylation reaction [55,56]. In astrocyte–neuron co-cultures, the inhibitory effect of ethanol on neuritogenesis was shown to be due to inhibition of phospholipase D activated by M3 muscarinic receptors in astrocytes [23], leading to decreased release of fibronectin and laminin, and a similar mechanism is most likely to be involved also in hippocampal slices.

Our results in co-cultures [17,23], and in the hippocampal slice (the present study) suggest that the contradictory results reported on the *in vitro* effects of ethanol on neuritogenesis [7,50–53] may reflect the lack of consideration for astrocyte–neuron interactions, as also indicated by other more recent findings [20,21]. Indeed, perinatal exposure of mice to ethanol results in shorter and less branched denditric arbors in hippocampal pyramidal neurons [57], and in FAS children the size of the corpus callosum (an area consisting mostly of axonal projection) has been shown to be decreased [58–60].

In summary, the present results indicate that cholinergic stimulation of muscarinic M3 receptors in the hippocampus increases the expression of extracellular matrix proteins which in turn stimulate neuritogenesis in hippocampal pyramidal neurons; we also show that this series of events in inhibited by ethanol. These results thus confirm observations after *in vivo* developmental exposure to ethanol [57], and suggest that the hippocampal slice may represent a relevant *in vitro* model system to study the effects of ethanol on developmental processes involving astrocyte–neuron interactions. Also of much interest is the role of the cholinergic system in hippocampal neuritogenesis, as this may provide biological plausibility for the observed beneficial effects of choline, a precursor of both acetylcholine and phosphatidylcholine, on hippocampus-mediated behaviors [61].

Acknowledgments

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