Nicotinic Receptors Differentially Regulate \(N\)-Methyl-D-aspartate Damage in Acute Hippocampal Slices

P. A. FERCHMIN, DINELY PEREZ, VESNA A. ETEROVIC, and JEAN DE VELLIS

Department of Biochemistry, Universidad Central del Caribe, Bayamon, Puerto Rico (P.A.F., D.P., V.A.E.); and Mental Retardation Research Center, University of California-Los Angeles, Los Angeles, California (J.d.V.)

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ABSTRACT

Although in neuronal cultures nicotine was reported to prevent early and delayed excitotoxic death, no studies with nicotinic drugs have been done with acute hippocampal slices. We investigated the effect of nicotine and methyllycaconitine (MLA) on the toxicity of \(N\)-methyl-\(D\)-aspartate (NMDA) in the CA1 area of hippocampal slices. The excitotoxic effect of NMDA was assessed as decreased recovery of the capability to produce synaptically evoked population spikes (PSs). Application of nicotine or MLA before NMDA application increased the recovery of PSs. This electrophysiological recovery was used as a measure of the early neuroprotective events. The neuroprotection conferred by both nicotine and MLA was inhibited by dihydro-beta-erythroidine, showing mediation of neuroprotection by \(\alpha4\beta2\) neuronal nicotinic receptors (nAChRs). Because nicotine activates \(\alpha4\beta2\) and other nAChR subtypes, whereas 10 nM MLA inhibits the \(\alpha7\) subtype, we propose the involvement of a neuronal circuitry-dependent mechanism for nicotinic neuroprotection. The effect of nicotine downstream from the receptors was investigated using inhibitors of cell signaling. The results suggest that the effect of nicotine is mediated by tyrosine receptor kinases, 1,2-phosphatidylinositol-3 kinase, and the mitogen-activated extracellular signal-regulated kinases. Although nicotine neuroprotection is \(Ca^{2+}\)-dependent, neither L-type \(Ca^{2+}\) channels nor calmodulin-dependent protein kinase II is involved in the effect of nicotine. In summary, these results suggest that in acute slices nicotinic protection is initiated either by direct activation of \(\alpha4\beta2\) or indirectly by inhibition of \(\alpha7\) followed by signal transduction involving tyrosine kinases, phospholipid-dependent kinases, and mitogen-activated kinases.

The measurement of the area of population spikes (PSs) to assess the degree of damage has the advantage that the size of the PS is directly proportional to the number of functionally active pyramidal neurons (Andersen et al., 1971). This acute preparation is well suited to study the early functional neuronal damage before the onset of cell death. Many neuroprotective drugs and treatments were evaluated with acute hippocampal slices. Although nicotine and other nAChR agonists were shown to be neuroprotective in a variety of experimental systems, they were never studied in acute slices. Nicotinic neuroprotective activity was reported to be dependent on active \(\alpha7\) nAChRs (Shihomaha et al., 1998) and \(Ca^{2+}\) (Dajas-Bailador et al., 1998; 1999).

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ABBREVIATIONS: PS, population spike; NMDA, \(N\)-methyl-D-aspartate; nAChR, nicotinic acetylcholine receptor; [\(Ca^{2+}\)], intracellular calcium concentration; VGCC, voltage-gated \(Ca^{2+}\) channel; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; PI-3 kinase, phosphatidylinositol 3-kinase; \(\alpha\)-Bgt, \(\alpha\)-bungarotoxin; DHJBE, dihydro-beta-erythroidine; MLA, methyllycaconitine; ACF5, artificial cerebrospinal fluid; DMSO, dimethyl sulfoxide; CaM, calmodulin; CaM-dependent protein kinase; SU6656, 2-oxo-(3,4,5,6,7-tetrahydro-1H-indol-2-yl)methylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide; LY 294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PD98059, 2-amino-3-methoxyflavonone, KN-62, 1-[\(N\),\(O\)-bis(5-isouquinolinesulfanyl)-\(N\)-methyl-L-tyrosyl]-4-phenylpiperazine; K252a, (8S,11S)-(–)-9-hydroxy-9-methoxy carbonyl-8-methyl-2,3,910-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cyclo-octadecadien-1-one.
et al., 2000; Kihara et al., 2001). There are indications that the α4β2 nAChRs also participate in neuroprotection (Kihara et al., 1998). When coapplied with NMDA, nicotine is neuroprotective without decreasing the NMDA-mediated [Ca\(^{2+}\)]\(_i\) rise (Minana et al., 1998; Dajas-Bailador et al., 2000; Kihara et al., 2001). This paradox is generated because depending on the pathway of Ca\(^{2+}\) entry, different downstream effectors are activated (Bading et al., 1993; Ghosh and Greenberg, 1995). Ca\(^{2+}\) is one of the main players in the regulation of excitotoxicity and neuronal survival. There are several routes of Ca\(^{2+}\) entry into neurons, of which the NMDA-receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid-receptor, and the VGCC cannot be ignored, but most relevant for our purpose here are the neuronal nAChRs α7 and α4β2. Nicotine-induced [Ca\(^{2+}\)]\(_i\) increase mediated by α4β2 or perhaps other heteromeric nAChRs is inhibited by tetrodotoxin, but the [Ca\(^{2+}\)]\(_i\) increase mediated by α7 is not. The latter is inhibited by α7 antagonists showing that this receptor contributes directly to Ca\(^{2+}\) entry, whereas α4β2 contributes mainly indirectly through VGCC (Mulle et al., 1992; Tsuneki et al., 2000).

Ca\(^{2+}\) is a second messenger that regulates many processes in the brain. Among others, Ca\(^{2+}\) influx modulates cell signaling by activation of mitogen-activated protein kinases, leading to adaptive changes that include activation of transcription factors (Dolmetsch et al., 2001). One of the branches of mitogen-activated protein kinases, the Raf-MEK-ERK cascade is usually involved in neuroprotection. Neuronal survival is also promoted, in a Ca\(^{2+}\)-dependent manner, by phosphatidylinositol 3-kinase (PI 3-kinase) (Kihara et al., 2001; Perkinton et al., 2002).

This is the first study showing that neuronal function is protected against early electrophysiological NMDA-induced excitotoxic damage by nicotinic effectors in acute hippocampal slices. The pharmacology of the nicotinic neuroprotection is partially elucidated as well as the cell signaling processes downstream from the receptor.

### Materials and Methods

**Materials.** All common chemicals were from Sigma-Aldrich (St. Louis, MO). Raf-1 kinase inhibitor I, PD98059, wortmannin, SU6656, LY294002, K252a, and α-bungarotoxin (αBtx) were from Calbiochem (La Jolla, CA). CN-62 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). (-)-Nicotine di-d-tartrate (nicotine), dihydro-β-erythroidine (DHβE), and methyllycaconitine (MLA) were from Sigma/RBI (Natick, MA). NMDA, choline, and genistein were from Sigma-Aldrich.

**Slice Preparation and Electrophysiological Recordings.** Hippocampal slices from female Sprague-Dawley rats (120–200 g) from our colony were prepared and maintained using standard methods. All procedures have been reviewed and approved by the Institutional Animal Care and Use Committee. For dissection and incubation, a standard artificial cerebrospinal fluid (ACSF) was used: 125 mM NaCl, 3.3 mM KCl, 1.25 mM NaH\(_2\)PO\(_4\), 2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 25 mM NaHCO\(_3\), and 10 mM glucose. Dissections were done at ice temperature; transverse slices were cut 400 μm in thickness with a manual slicer and transferred to a chamber. The lower part of the chamber was filled with H\(_2\)O kept at 37.4 ± 1°C and continuously bubbled with 95% O\(_2\), 5% CO\(_2\). The slices were kept at the gas-liquid interface at 34 ± 1°C on an acrylic plate covered with a nylon mesh (Hanes) above the H\(_2\)O. Before entering the chamber the ACSF was continuously bubbled with 95% O\(_2\), 5% CO\(_2\). The exterior of the chamber was kept at 30 ± 1°C. Strict control of the temperatures at the three levels (outside, nylon mesh, and water bath) was indispensable to avoid variability of results because the toxicity of NMDA is temperature-dependent (Ferchmin et al., 2000). A concentric bipolar electrode placed in stratum radiatum was used to stimulate the slices with a constant current for 0.2 ms. The PSs were recorded in stratum pyramidale with a glass micropipette filled with 2 M NaCl and impedance ranging from 1 to 5 MΩ. The testing of slices started 1 h after dissection. Each slice was stimulated with a stimulus strength twice that required to elicit a threshold PS. This initial response was recorded and compared with the response elicited by the same stimulus and recorded from the same position 1 h after the corresponding experimental treatment. The percentage of the initial response remaining at the end of the experiment was used as a measure of recovery. The incubation chamber contained three lanes with independent perfusion lines exposed to the same gaseous phase. Immediately after dissection, 10 to 30 slices were distributed among the three lanes; when slices from more than one animal were used, they were equally distributed among the lanes.

**Solutions.** Dimethyl sulfoxide (DMSO) was routinely used to dissolve hydrophobic inhibitors; therefore, DMSO was added in the same final concentration to the ACSF for all experimental groups in a given experiment. At the concentrations used [≤0.1% (v/v)], DMSO had no effect on the recovery of PSs.

**Procedure for Testing Neurotoxicity.** The procedure was based on Schurr et al. (1995) as modified by us (Ferchmin et al., 2000). Unless otherwise stated, the excitotoxic stimulus used was the application of 0.5 mM NMDA for 10 min in the presence of 95% O\(_2\), 5% CO\(_2\), and 10 mM glucose. Application of 0.5 mM NMDA for 3 min is known to cause widespread delayed death in neuronal cultures (Hartley and Choi, 1989). The relationship between NMDA concentration and loss of PS was standardized to recover an average of 20% of the PS area (Ferchmin et al., 2000). The general experimental design was as follows: 1 h after slice preparation, the initial PS was recorded. After that, the slices were superfused for 15 min with a putative inhibitor of nicotine action, followed by nicotine in the presence of the inhibitor for 1 h, immediately after with 0.5 mM NMDA for 10 min, and then for 1 h with ACSF. Then the final PS was determined. Controls without inhibitor or without nicotine were perfused with ACSF for the same amount of time. Variations from this general protocol are indicated under Results.

**Data Analysis.** The areas of the PS (millivolts per millisecond) were acquired and analyzed with the Labman program (gift from Dr. T. J. Teyler WWAMI Medical Education Program, University of Idaho, Moscow, ID). The data were statistically analyzed using SigmaStat version 2.03 (SPSS Science, Chicago, IL). Analysis of variance was used whenever the data were distributed normally; otherwise, Kruskal-Wallis one-way analysis of variance on ranks was used followed in each case by the appropriate post hoc test. When two groups were compared, the t test was used.

### Results

This study describes the neuroprotective effect of nicotine and other nicotinic drugs on the early functional effects of NMDA. The compounds used to inhibit the neuroprotective effect of nicotine were tested to determine their effect on the population responses with or without NMDA treatment. Figure 1A shows that the incubation for 1 h with inhibitors of regulatory kinases and of two nicotinic antagonists was null. MLA and its combination with DHβE were also without effect. αBtx was tested in experiments of different design. Slices were stimulated every minute and recording of PSs was done continuously. The first hour, the slices were kept in normal ACSF, followed by 1 h in 10 nM αBtx, and the last hour in 1 μM αBtx. The results of five independent experiments were averaged. Average areas of the PS after ACSF,
axon potentials (Andersen et al., 1971). Slices incubated in ACSF without further treatment conserved most of the area of the PS at the end of the experiment. After application of NMDA, approximately 20% of the PS was recovered. However, when 1 μM nicotine was applied for 1 h before NMDA, the recovery was statistically indistinguishable from ACSF-incubated slices (Figs. 3A and 4A). Ca²⁺ is one of the main factors involved in neuronal nicotinic signaling and in excitotoxicity. Therefore, it was of interest to describe its effect. As expected, NMDA was mostly ineffective when applied in Ca²⁺-free ACSF (Fig. 4B). Nicotine applied immediately after NMDA treatment was as effective as nicotine before NMDA (Fig. 4C). The protective effect of nicotine was partially dependent on the presence of Ca²⁺ in the solution (Fig. 4D).

The dose dependence of nicotine neuroprotection showed the optimal concentration near micromolar. Nicotine was neuroprotective from 0.25 to 1 μM, and there was no increase in the neuroprotection from 1 to 10 μM (Fig. 5). The neuroprotective effect of nicotine was inhibited by coinubcation with 1 μM DHβE before NMDA application (Fig. 6A). DHβE per se did not alter the toxicity of NMDA (Figs. 2C and 6B). Because DHβE inhibited the effect of nicotine, we concluded that the α4β2 heteromeric subtype is involved.

Most studies in neuronal cultures have shown that nicotinic neuroprotection is mediated by the α7 subtype of nAChRs. α7 antagonists were used to test whether inhibition of α7 receptors affected nicotine neuroprotection. nBtx (1 μM) applied for 1 h did not inhibit the neuroprotective effect of 1 μM nicotine (Fig. 7A). In addition, this toxin had a modest neuroprotective effect in the absence of nicotine. Choline, a low-affinity α7 agonist, was significantly neuroprotective (Fig. 7B). Although the neuroprotection by choline seems to be smaller than that of nicotine, the difference between the slices pretreated with nicotine and choline was not significant. DHβE only partially decreased the protection exerted by choline (Fig. 7C).

To further pursue the role of the α7 receptor, MLA, a specific α7 antagonist was tested. MLA was neuroprotective at 10 nM and 1 μM when preapplied for 1 h before NMDA (Fig. 8B). The magnitude of neuroprotection exerted by MLA was similar to that of nicotine (Fig. 8A). When MLA was coapplied with nicotine, it did not block the neuroprotective effect of nicotine (Fig. 8C). As in the case of nicotine, the neuroprotection by MLA was inhibited by 1 μM DHβE (Fig. 8D).

The finding that the neuroprotective effect of nicotine was decreased after exposure to Ca²⁺-free ACSF (Fig. 4D) and effective after NMDA application (Fig. 4C) suggested that it triggers a neuroprotective cell-signaling program and that nicotine does not directly block the NMDA receptor or its effect. To briefly evaluate the pharmacology of the cell signaling steps involved, inhibitors of protein kinases were used to inhibit nicotine neuroprotection. The inhibitors were used at a concentration near 10-fold the IC₅₀ of the intended target where they are in general regarded as reasonably specific. Figure 9 shows the effect of seven inhibitors of cell signaling: Preincubation with 10 or 100 nM K252a, a nonselective Trk inhibitor (Howe et al., 2002; Roux et al., 2002), for 1 h, significantly reduced nicotine-mediated neuroprotection (Fig. 9, A and B). The broad-range inhibitor of tyrosine protein kinase, genistein, at 50 μM, also inhibited the neuropro-
Protection of the capability to produce PSs by 1 μM nicotine and the toxicity of NMDA depend on the presence of Ca²⁺. Nicotine (1 μM) was applied in ACSF for 1 h, and 0.5 mM NMDA was applied for 10 min. The sequence of solutions applied is shown under each bar. B and D, NMDA or nicotine was applied in Ca²⁺-free ACSF in the following manner: Ca²⁺-free ACSF was applied without the drugs for about 20 min until the PS ceased to be evoked with a test stimulus. Then, according to the experiment, either nicotine or NMDA was applied in Ca²⁺-free ACSF. After the Ca²⁺-free treatment, Ca²⁺ was restored until the PS recovered its initial size. Therefore, in the second and third bar in B, NMDA was applied when there was no PSs. In the third bar, in D, nicotine was applied when there was no PSs, but NMDA was applied after restoring Ca²⁺ and the PSs. Bars represent the mean percentage of recovery of PSs ± S.E.M. after each treatment. For every experimental group, the statistical significance is indicated as follows: ***, p < 0.001; *, p < 0.05 and for secondary comparison, †, p < 0.05. The number of slices per experimental group was n = 14 (A), n = 21 (B), n = 14 (C), and n = 28 (D).
involved (Fig. 9D). LY294002 (10 nM) and 10 nM wortmannin, inhibitors of PI3-kinase that mediates Akt/PKB activation, robustly inhibited the effect of nicotine (Fig. 9E). The effective inhibition of nicotinic neuroprotection by Raf-1 kinase inhibitor I (Lackey et al., 2000) (Fig. 9F) and PD98059 (Hetman et al., 1999) (Fig. 9G) indicate the involvement of the Raf-MEK-ERK cascade. On the other hand, nifedipine (10 μM), a blocker of L-type VGCC, and the inhibitor of CaM kinase KN-62 (9 μM) (Tokumitsu et al., 1990) did not decrease the effect of nicotine (Fig. 10).

Discussion

The acute hippocampal slices have a particular advantage as a model of early electrophysiological excitotoxic damage; however, its viability decreases after 8 to 10 h. For this reason, few studies have addressed delayed cell death in acute slices (Wallis and Panizzon, 1995; Wang et al., 1999). The comparison of the early effect of experimental ischemia on the electric activity in acute slices with delayed neuronal...
death in cultured slices was consistent with the concept that both represent the same event in a different time scale (Small et al., 1997).

Nicotine applied before or after NMDA protected the PSs against NMDA (Fig. 4, A and D). The effect of nicotine was tested from 0.1 to 10 μM (Fig. 5). This range is similar to the nicotine concentration, from 1 to 100 μM, found to be neuroprotective in studies with neuronal cultures (Akaike et al., 1994; Marin et al., 1994; Kihara et al., 1998, 2001; Dajas-Bailador et al., 2000). Minana et al. (1998) showed neuroprotection by 10 nM nicotine against glutamate and 10 μM NMDA. In our experiments, both the toxic effect of NMDA and the neuroprotective effect of nicotine were at least in part Ca\(^{2+}\)-dependent (Fig. 4, B and D). The excitotoxic effect of NMDA and the neuroprotective effect of nicotine that persisted in Ca\(^{2+}\)-free ACSF were probably sustained by Ca\(^{2+}\) lingering in the slice. It takes about 1 h to reach equilibrium between the concentration of Ca\(^{2+}\) in the ACSF and the inside of the slice (Dingledine and Somjen, 1981). However, Ca\(^{2+}\)-independent effects cannot be discarded at this time.

The neuroprotective effect of nicotine was not observed in the presence of 1 μM DHβE (Fig. 6A). The \(K_t\) of DHβE for α4β2 was estimated to be from 0.014 to 1.9 μM, for α7 from 25 to 58 μM, and for α3β4 near 220 μM (Sharples and Wonnacott, 2001). This demonstrates that active α4β2 nAChRs are involved.

Unexpectedly, 1 μM αBtx applied during 1 h not only did not inhibit the neuroprotective effect of nicotine but also was neuroprotective per se (Fig. 7A). To further study the role of α7 receptors choline was tested. Choline at 10 mM is an α7 agonist with a possible physiological role (Alkondon et al., 1999). Choline applied before NMDA provided neuroprotection comparable with nicotine (Fig. 7B). In the presence of 1 μM DHβE, the neuroprotection exerted by choline significantly decreased (\(p < 0.02\)) but was still significant (\(p < 0.001\)) (Fig. 7C). Besides acting as an agonist, choline also causes desensitization of α7 nAChRs (Alkondon et al., 1999). Therefore, choline and αBtx could have a similar final effect on the function of α7 receptors by desensitization and inhibition, respectively. Because choline is a low-affinity α7 ligand, it is used at 10 mM. However, at this concentration it is likely to have effects unrelated to nAChRs. Choline blocks the pore of the NMDA receptor (Villarroel et al., 1995) and has muscarinic neuroprotective activity (Minana et al., 1996). Therefore, choline is not specific enough for mechanistic studies.

MLA is an α7 nicotinic antagonist that diffuses more easily than αBtx because of its smaller molecular weight. MLA from 10 nM to 1 μM was also neuroprotective per se. This result
was reproduced in five independent experiments with 21 slices per condition in each (Fig. 8, A–D). The neuroprotective effect of MLA was inhibited by 1 μM DHβE, showing that MLA neuroprotection requires α4β2 activity (Fig. 8D). The Kᵢ of MLA for the α7 receptor is in the nanomolar range, whereas the Kᵢ values for α4β2 and α3β2 are in the micromolar range (Sharples and Wonnacott, 2001). Therefore, MLA neuroprotection is mediated by inhibition of α7 nAChRs. This contradicts the work with neuronal cultures (Minana et al., 1998; Shimohama et al., 1998; Dajas-Bailador et al., 2000; Kihara et al., 2001) where nicotinic neuroprotection is mediated by α7 activation. There are several reasons that could account for the difference. The hippocampal slice has a nearly intact functional neuronal circuitry that is absent in dissociated neuronal cultures. It is possible that the specific neuronal circuitry and distribution of nAChRs in the CA1 area are responsible for the interaction between α7 and α4β2 nAChRs. However, Prendergast et al. (2001) reported inhibition of nicotine-mediated neuroprotection by MLA in organotypic hippocampal slices. Although, organotypic slices conserve the basic neuronal circuits, they might have suffered changes in receptor distribution during maturation in vitro. In addition, the difference between our findings could be attributed to the variables measured (propidium iodide uptake versus electrophysiological activity) and the time at which the effect of NMDA is measured. In acute slices, neuroprotection of the electrophysiological activity is measured within 2 h after NMDA application, whereas in culture it is determined 24 h later. Both systems are probably measuring different stages of the same process.

In conclusion, for this part of the work, the results indicate that activation of α4β2 is neuroprotective regardless of the state of α7 activation, whereas the inhibition of α7 is neuroprotective provided that α4β2 is not inhibited.

We propose a model that explains these findings (Fig. 11 and legend). This model invokes a highly localized action of nAChRs, thus explaining why a major effect of nicotinic agents alone on the PS area was not seen (Fig. 1). The proposed model might be restricted to electrophysiological parameters of neuroprotection in the area CA1 of acute slices within a limited time window. It remains to be determined whether it will be applicable to delayed neuronal death.

The fact that the neuroprotective effect of nicotine is Ca²⁺-dependent and effective after NMDA application suggests that nicotine triggers a neuroprotective program involving neurotrophins and protein kinases. Ca²⁺ entry increases the phosphorylation of transcription factors followed by neurotrophin synthesis (Shieh and Ghosh, 1999). There is evidence that neuroprotection mediated by nAChRs involves neurotrophins (Belluardo et al., 2000). The involvement of protein tyrosine kinases in nicotinic neuroprotection was supported by the inhibitory effect of 50 μM genistein on the neuroprotection (Fig. 9C). To test for the participation of neurotrophin receptors, K252a was used. K252a is best known as a Trk inhibitor; however, it also inhibits other protein kinases. K252a inhibits CaM kinase with an IC₅₀ value of 1.8 nM (Howe et al., 2002), mixed lineage kinases with IC₅₀ values of approximately 5 nM (Roux et al., 2002), cyclic nucleotide-dependent kinases and protein kinase C with Kᵢ values ranging from 18 to 25 nM (Kase et al., 1987), and Trk kinases with IC₅₀ values of 3 nM (Tapley et al., 1992). The inhibition of mixed lineage kinases and CaM kinase is not relevant here.

Mixed lineage kinases are not neuroprotective (Roux et al., 2002), and CaM kinase did not mediate nicotinic neuroprotection in our system because KN-62 did not decrease the effect of nicotine (Fig. 10). A careful analysis of the inhibition of Trk and kinases of IC₅₀ 17 nM or higher reveals that 10 nM K252a inhibits 77% of the activity of Trk but only 37% of the other relevant kinases known to be inhibited by K252a. At 100 nM, K252a inhibits 97% of Trk but also inhibits 86% of protein kinase C and cyclic nucleotide-dependent kinases. Figure 9, A and B, shows that decreasing the concentration of K252a from 100 to 10 nM did not decrease the inhibition of the protective effect of nicotine, suggesting that K252a was inhibiting nicotinic neuroprotection by binding with high affinity to a relevant kinase likely to be Trk.

Fyn, a subtype of Src, was reported to be involved in nicotinic neuroprotection in cultured neurons mediated by α7 nAChRs (Kihara et al., 2001). The involvement of Src kinase in our system was discarded because SU6656, a selective inhibitor of the Src family (Blake et al., 2000), did not block neuroprotection. This part of the model explains why nicotine neuroprotection is decreased by 1 μM DHβE and why external Ca²⁺ is needed. B, difference between cultures and slices points to the role of the hippocampal circuitry. It is generally accepted that “the majority of the nicotinic cholinergic activity in the hippocampus would be mediated by presynaptic/preterminal nAChRs located on GABAergic neurons”. Both α7 and α4β2 subtypes participate in this function (Alkondon and Albuquerque, 2001). Thus, inhibition of α7 receptors would decrease the liberation of GABA. C, GABA decreases acetylcholine (ACh) release in the hippocampus (Moor et al., 1998). Thus, if there are α7 receptors on the GABAergic terminals that modulate the release of ACh, then α7 inhibition should increase the liberation of ACh. This increased level of endogenous ACh would activate the α4β2 receptors, which lead to neuroprotection.
the effect of nicotine at a concentration 10 times the IC50 value for most Src kinases (Fig. 5D).

The survival-promoting activity of the Trk neurotrophin receptors involves, among others, the docking of adapter proteins that activate Ras. Ras activates the PI-3 kinase and the Raf-1, MEK, ERK kinase cascade (Patapoutian and Reichardt, 2001). Indeed, both cell signaling pathways were involved here. LY294002 and wortmannin inhibited the effect of nicotine, implicating PI 3-kinase in neuroprotection (Fig. 9E). The effectiveness of Raf-1 kinase inhibitor I (Fig. 9F) and PD98059 (Fig. 9G) to block the neuroprotection indicated that the ERK cascade was involved. The simultaneous involvement of PI-3 kinase and ERK cascades in neuroprotection has been observed previously (Hetman et al., 1999). Calcium signaling mediated by L-type channels and CAM kinase was reported to lead to survival in cultured neurons (Dolmetsch et al., 2001). However, neither nifedipine nor the CAM kinase inhibitor KN-62 blocked the effect of nicotine in our preparation (Fig. 10).

In conclusion, we propose a neuroprotective cell signaling pathway triggered by the activation of nAChRs. Ca2+ entry, mediated directly or indirectly by α4β2 nAChRs, enhances the signaling through Trk receptors, leading to Ras activation. Ras leads to activation of PI-3 kinase and the Raf-1-MEK-ERK kinase known to promote neuroprotection.

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