A Pro-Nerve Growth Factor (proNGF) and NGF Binding Protein, α₂-Macroglobulin, Differentially Regulates p75 and TrkA Receptors and Is Relevant to Neurodegeneration Ex Vivo and In Vivo

Pablo F. Barcelona,a,b,c H. Uri Saragovi,a,b,c

Lady Davis Institute-Jewish General Hospital, a Department of Pharmacology and Therapeutics, b Department of Oncology and the Cancer Center, c McGill University, Montreal, Quebec, Canada

Nerve growth factor (NGF) is generated from a precursor, proNGF, that is proteolytically processed. NGF preferentially binds a trophic tyrosine kinase receptor, TrkA, while proNGF binds a neurotrophin receptor (NTR), p75NTR, that can have neurotoxic activity. Previously, we along with others showed that the soluble protein α₂-macroglobulin (α₂M) is neurotoxic. Toxicity is due in part to α₂M binding to NGF and inhibiting trophic activity, presumably by preventing NGF binding to TrkA. However, the mechanisms remained unclear. Here, we show ex vivo and in vivo three mechanisms for α₂M neurotoxicity. First, unexpectedly the α₂M-NGF complexes do bind TrkA receptors but do not induce TrkA dimerization or activation, resulting in deficient trophic support. Second, α₂M makes stable complexes with proNGF, conveying resistance to proteolysis that results in more proNGF and less NGF. Third, α₂M-proNGF complexes bind p75NTR and are more potent agonists than free proNGF, inducing tumor necrosis factor alpha (TNF-α) production. Hence, α₂M regulates proNGF/p75NTR positively and mature NGF/TrkA negatively, causing neuronal death ex vivo. These three mechanisms are operative in vivo, and α₂M causes neurodegeneration in a p75NTR- and proNGF-dependent manner. α₂M could be exploited as a therapeutic target, or as a modifier of neurotrophin signals.

Soluble hormones and growth factors (GF) are first produced as precursors that are processed by proteolytic cleavage to mature forms (1). Precursors and mature growth factors often mediate distinct signals. One example is the family of growth factors called neurotrophins that includes nerve growth factor (NGF). A precursor proNGF binds with high affinity to a neurotrophin receptor (NTR), p75NTR (2, 3), an interaction generally considered to be neurotoxic. proNGF induces tumor necrosis factor alpha (TNF-α) production by p75NTR-expressing activated glia (4–6) and directly causes death in p75NTR-expressing neurons (7–9).

proNGF is proteolytically cleaved to produce mature NGF (2). Mature NGF binds with high affinity to a receptor tyrosine kinase called TrkA, an interaction that is neuroprotective. Mature NGF also binds to p75NTR, and TrkA and p75NTR cross-regulate ligand affinity and signal transduction positively or negatively (10–12).

The balance of neurotrophic versus neurotoxic signals depends on many factors, including receptor density and stoichiometry, the cell type and environment, and the proteolytic processing that influences the ratio of proNGF to mature NGF ligands. Thus, we hypothesized that additional mechanisms might exist to tightly regulate these heterogeneous and sometimes opposing signals.

Proteins called growth factor binding proteins (BPs) regulate the proteolytic processing, half-life or stability, extracellular matrix interactions, and receptor binding specificity of growth factors. For example, insulin-like growth factors (IGFs) are bound by several IGF BPs, each regulating a distinct feature of IGF functions (13). A protein present in serum and in tissues, α₂-macroglobulin (α₂M), has been reported as an NGF binding protein: α₂M can bind to mature NGF (dissociation constant [Kd] of 172 nM) making stable α₂M-NGF complexes (14). α₂M inhibits NGF bioactivity (14–16), an effect interpreted as neutralization by α₂M sequestration of NGF (15, 16). However, this interpretation relies on the presumed absence of TrkA binding by α₂M-NGF complexes or on actions mediated by the low-density lipoprotein receptor-related protein 1 (LRP1) (17), the cognate receptor for α₂M, but these have not been studied.

Interestingly, in neurodegenerative diseases, neuronal death in vivo is preceded by increases in local α₂M protein in the tissue (4, 14, 18–22). Moreover, increasing α₂M in a normal tissue causes neuronal death (4, 14, 23). Inhibition of NGF by α₂M could account for some of these in vivo data, but addition of exogenous NGF does not revert α₂M neurotoxicity (23, 24). Hence, there may be additional mechanisms not yet explored.

The issues which we aim to examine in the present paper are (i) the mechanism for the functional inhibition of mature NGF by α₂M, (ii) potential effects of α₂M on the precursor proNGF, (iii) whether α₂M affects NGF or proNGF receptor binding, and (iv) the mechanisms by which increased α₂M in tissue in vivo causes neurodegeneration. Here, we show that α₂M is a genuine proNGF and NGF binding cofactor, forming stable complexes. The complexes bind neurotrophin receptors with kinetics and selectivity similar to those of free proNGF or free NGF. α₂M-NGF binds.
TrkA and p75NTR, while αM-proNGF binds p75NTR only. Formation of αM-proNGF complexes protects proNGF from proteolysis and potentiates proNGF-mediated activation of p75NTR, leading to p75NTR-mediated increases in TNF-α and neurotoxicity. Formation of αM-NGF complexes does not alter TrkA binding but reduces the ability of NGF to induce TrkA dimerization, with a consequent lack of receptor activation and trophic support. These mechanisms were demonstrated in cell cultures and were also confirmed in vivo.

We propose that local inhibition of αM could be a therapeutic target to reduce proNGF neurotoxicity and to increase NGF neuroprotection. In addition, αM or αM variants may be useful tools to modulate neurotrophic signals.

MATERIALS AND METHODS

Ligands. We used recombinant mouse NGF (Prince Laboratories), recombinant wild-type human NGF (a gift from Genentech), recombinant wild-type mouse proNGF made in Escherichia coli (Alomone), recombinant wild-type human proNGF made in E. coli (ProSpec), bovine serum albumin factor Y (BSA) (Wisent, Inc.), recombinant human p75NTR-Fc chimera (R&D Systems), and lipopolysaccharide (LPS) (Sigma Chemicals).

αM protein. Native αM is a plasma protease inhibitor with broad specificity. Cleavage of native αM by proteases causes a conformational change to the active αM form. Activated αM does not possess protease inhibitor activity (15). Recombinant rat αM and human αM (Sigma Chemical) were transformed to the activated forms by incubating αM with 200 mM methylamine-HCl for 6 h at pH 8.2, as described previously (15).

Antibodies. Rat anti-mouse β-NGF monoclonal antibody (MAb) NGF30 (IgG2a) recognizes mouse NGF and mouse proNGF but not human NGF or human proNGF (25). Thus, in enzyme-linked immunosorbent assays (ELISAs) detecting the presence of mouse (pro)NGF, the human counterparts can be used as competitors. Rabbit polyclonal anti-αM R-19 recognizes mouse, rat, and human αM (Santa Cruz). Rabbit polyclonal anti-LRP1 H-80 (Santa Cruz) recognizes mouse, rat, and human LRP1. Mouse anti-rat p75 MAB MC192 (IgG1) and mouse anti-human TrkA MAB 5C3 (IgG1) (26) were prepared and purified in house with protein G-Sepharose (Pharmacia). We purchased mouse antiphosphotyrosine (anti-p-Tyr) antibody 4G10 (Upstate), rabbit polyclonal anti-NGF H-20 (Santa Cruz), which recognizes human NGF and human proNGF, rabbit polyclonal anti-TrkA (Santa Cruz Biotechnology) recognizes total TrkA, rabbit polyclonal anti-phospho-TrkA (Tyr490) (Cell Signaling), rabbit monoclonal anti-phospho-p44/42 mitogen-activated protein kinase (MAPK; extracellular signal-regulated kinase 1/2 [ERK1/2]) (Thr202/Tyr204) (Cell Signaling), rabbit polyclonal anti-phospho-Akt (Ser473) (Cell Signaling), and rabbit polyclonal anti-TNF-α (Millipore). For mouse samples the anti-TNF-α antibody recognizes bands of 26 kDa, 28 kDa, and 38 kDa (Cell Signaling data sheet 3707) reported to be TNF-α isoforms (27–29).

Cell lines. B104 (p75NTR−/−) and its stable transfectants 4-3.6 (p75NTR−/−) and C10 (p75NTR−/−) are rat neutroblastoma lines. 4-3.6 cells were transfected with human trkA CDNA and express ∼50,000 TrkA receptors/cell. The C10 cell line is a subclone of 4-3.6 cells and expresses ∼50,000 TrkA receptors/cell, and no p75NTR can be detected by reverse transcription-PCR (RT-PCR) or Western blotting (11). rMC-1 is a rat Muller cell line, characterized as p75NTR−/− (30).

Labeling of proteins and antibodies. Proteins were conjugated with an Alexa Fluor 488 protein labeling kit or with Alexa Fluor 594 monoclonal antibody labeling kit (Molecular Probes) as per the manufacturer’s instructions. Horseradish peroxidase (HRP) coupling was performed with an EZ-Link activated peroxidase kit (Pierce) as specified by the manufacturer’s instructions. The resulting fluorescently labeled (FL) protein is indicated with a superscript (e.g., αMFL).

ELISAs. (i) αM immobilized on wells to detect neurotrophin binding. Assays were performed by modifications of previously described ELISAs (25). The readouts interrogate αM-neurotrophin interactions and αM-neurotrophin-p75 interactions. To each well of a 96-well microtiter plate (Becton Dickinson) 50 ng of rat αM, human αM, or control BSA was immobilized. After samples were blocked with binding buffer (BB) (phosphate-buffered saline [PBS], 0.5% BSA) for 60 min at room temperature, the wells were washed five times with BB supplemented with 0.05% Tween 20.

For testing neurotrophin binding, 10 ng/well of soluble mouse NGF or soluble mouse proNGF was added for 60 min at room temperature. In controls, 10 ng of soluble human NGF or 10 ng of soluble human proNGF (not recognized by the anti-mouse neurotrophin antibody used in the ELISA) was added as a cold competitor. Competition was done either by adding competitor concomitantly or by preincubation, with similar results.

Two different detection methods were used. In one method, the presence of mouse NGF or mouse proNGF bound to αM was detected by ELISA using labeled antineurotrophin MAB NGF30 (25). In a second method, the presence of mouse NGF or mouse proNGF bound to αM was detected by ELISA using a labeled p75-Fc chimera. The difference between these detection methods is that the first interrogates αM-neurotrophin interactions, and the second interrogates αM-neurotrophin-p75 interactions. Peroxidase activity was determined colorimetrically using 2,2-azinobis(3-ethylbenzothiazoline sulfonic acid) and an Ultra TMB (3,3′,5,5′-tetramethylbenzidine) ELISA (Thermo Scientific). The optical density was measured at 414 nm in a microplate reader (Bio-Rad). Assays were repeated in at least three independent experiments, with each experiment performed in quintuplicate.

(ii) Neurotrophin immobilized on wells to detect αM binding. Assays were performed by modifications of previously described ELISAs (25). The readout interrogates neurotrophin-αM interactions. Ten nanograms per well of mouse NGF, mouse proNGF, or control BSA was immobilized as described above. After the plates were washed, soluble rat αM or human αM was added (50 ng/well), and the presence of bound αM was detected by ELISA using labeled anti-αM MAB. This detection method interrogates neurotrophin-αM interactions. Assays were repeated in at least three independent experiments, and each experiment was performed in quintuplicate.

FACS analysis. The TRP1 receptor and the neurotrophin receptors were phenotyped in B104 (p75NTR−/−) (expresses low levels of LRP1), 4-3.6 (p75NTR−/−) (expresses low levels of LRP1), and C10 (p75NTR−/−) cells. Cells in 100 μl of FACS binding buffer (PBS, 0.5% BSA, and 0.1% NaN3) were immunostained as described previously (26). Saturating anti-TrkA 5C3, anti-p75 MC192, anti-LRP1, or control nonbinding IgGs were added to cells for 1 h at 4°C, excess primary antibody was washed off, and cells were immunostained with the corresponding fluorocescinated secondary antibody, fluorescein isothiocyanate (FITC)-tagged goat anti-rabbit IgG or goat anti-mouse IgG.

Fluorescently labeled αM (αMFL) was used to determine binding to LRP1, which was low and equal for all cells. Also, αMFL in a complex with proNGF (αMFL-proNGF) and with NGF (αMFL-NGF) was bound to the surface of the different cells for 1 h at 4°C to interrogate the relevance of each neurotrophin receptor for binding. Live cells were acquired on a FACSscan, and data from bell-shaped histograms were analyzed using the LYSIS II program.

proNGF digestion assays. Digestion assays were performed using 1 unit of furin (New England BioLabs), as described previously (31, 32). Furin (1 U) was added to 100 ng of proNGF with or without αM or a BSA control. All proteins were diluted in 100 μl of buffer (100 mM HEPES, 0.5% Triton X-100, 1 mM CaCl2, 1 mM 2-mercaptoethanol, pH 7.5, at 25°C). After 0 to 90 min, products were fractionated by SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride (PVDF) membranes (Xymotech Biosystems), and immunoblotted with anti-hu-
man NGF H-20 (Santa Cruz). Blots were visualized using an enhanced chemiluminescence (ECL) system (NEN).

Ligand-dependent TrkA signals. (i) Cell survival assays. Assays of ligand-dependent survival of cells cultured in serum-free medium (SFM) were performed as described previously with readouts by MTT [3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide]. Cell cultures in SFM die by apoptosis. Death can be prevented by mature NGF acting through TrkA, optimally at 2 nM NGF (11, 25). Cells plated in SFM were either untreated or supplemented with NGF (2 nM final) premixed with or without α,M (120 nM final). Wells containing all culture conditions but no cells were used as blanks. The metabolic state of the cells was quantified by MTT (Sigma) after 36 h.

(ii) TrkA activation. The effect of activated α,M on neurotrophin-promoted TrkA phosphorylation in C10 cells was assessed using immunoprecipitation and Western blotting techniques (11). Briefly, 10^6 C10 cells in SFM were exposed to NGF or proNGF (2 nM final) premixed with or without α,M (120 nM final) or controls for 10 and 20 min at 37°C. After cells were washed with Hanks balanced salt solution (HBBS), they were detergent solubilized (1% NP-40 with protease inhibitor cocktail, 0.02 M EDTA, 50 mM sodium orthovanadate, 0.02 M Tris-HCl, 137 mM NaCl, pH 7.5). The protein concentrations in each sample were determined, and the lysates were immunoprecipitated with rabbit anti-TrkA (Santa Cruz) antibody and protein A-Sepharose beads. Samples were fractionated by SDS-PAGE under reducing conditions, transferred to PVDF membranes (Xyromtech Biosystems), and immunoblotted with primary antibodies as indicated in the figure legends. Blots were visualized using an enhanced chemiluminescence (ECL) system (NEN).

(iii) TrkA dimerization. Receptor cross-linking assays were carried out as reported elsewhere (33, 34). C10 cells (10^6 cells/group) were exposed to the ligand (untreated control, 2 nM NGF, 2 nM NGF premixed with 120 nM α,M, or 120 nM α,M alone) for 30 min at 4°C. Following a washing step, cells were chemically cross-linked with 1 mM disuccinimidyl suberate (DSS; Pierce) for 7 min. Unreacted DSS was quenched with a 5 M excess of ammonium acetate, and the cells were washed twice with HBBS at 4°C. Then each cell pellet was detergent solubilized (1% NP-40 containing protease inhibitors). Protein concentration for each of the cleared lysates was determined using a detergent-compatible kit (Bio-Rad). An equal protein amount (25 μg/lane) of each sample was resolved by SDS-PAGE, and after transfer the membranes were analyzed by Western blotting with the highly specific mouse anti-human TrkA MAb SC3. Equal loading was further verified by Coomassie blue staining of the gels.

Cell death assays. B104 cells (5000 cells/well) were cultured in 96-well plates (Falcon) under normal culture conditions (5% serum containing ~120 nM α,M). In cells cultured in 5% serum, death can be induced by proNGF acting through p75NTR (4). Cells were either untreated or supplemented with proNGF (2 nM final) or with or without NGF (2 nM final), and neurotrophins were premixed with or without exogenous α,M (final concentration, 120 nM). Wells containing all culture conditions but no cells were used as blanks. The metabolic state of the cells was quantified by MTT (Sigma) 36 h after plating; this corresponds also to a growth/survival profile.

TNF-α detection in response to p75NTR activity. (i) Soluble TNF-α protein. rMC cells were treated as indicated in the legend to Fig. 4, and supernatants were collected and used for measuring secreted soluble TNF-α by ELISA (TNF-α detection ELISA development kit; Peprotech). Assays were repeated at least three independent experiments, each in triplicate.

(ii) TNF-α mRNA. The same rMC cells from which supernatants were collected were frozen, and RNA was isolated by using an RNAeasy mini- or microkit (Qiagen). The quality of the RNA was analyzed with an Agilent 2100 Bioanalyzer (Agilent). RNA was transcribed to cDNA with random primers (Promega) and SuperScript II reverse transcriptase (Invitrogen) on a thermocycler (Biometra). CDNA was subjected to quantitative RT-PCR (qRT-PCR) using PerfeCta SYBR green PCR FastMix, Low ROX (6-carboxy-X-rhodamine) (Quanta Biosciences). The primers used to generate specific fragments were as follows: rat TNF-α forward, 5′-CTA TGTTGCTCCTACCCCA-3′; rat TNF-α reverse, 5′-TGGAAAGACTCC TCCAGGTA-3′. All primers were used at 100 nM. The reaction was performed in triplicate with an Mx3000P quantitative PCR system (Stratagene). Finally, amplification reaction data were analyzed using the complementary Mx3000P analysis software. Target gene expression was normalized to the average expression of the housekeeping gene rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer, 5′-CACACCCTTGTAGTCTAC-3′; reverse primer, 5′-AGGCCCAAACA TCCCTG-3′).

Intraocular injections of drugs. Adult male wild-type C57BL/6j mice (~24-g body weight, 8 to 10 weeks old) from Jackson Laboratories (Bar Harbor, ME) were used. All animal protocols were approved by the McGill University IACUC. Solutions of activated α,M (Sigma), or without and with a pharmacological antagonist of p75NTR (small-molecule inhibitor THX-B) or an antagonist of proNGF (neutralizing anti-proNGF MAb) or vehicle control PBS were injected. The intraocular injections were performed in 2 μl volumes containing a total of 2 μg of α,M with or without 2 μg of THX-B or 2 μg of anti-proNGF MAb as described previously (4, 14, 25). In the vitreous chamber the molarity of α,M is elevated from endogenous level of ~1 to 10 nM α,M to ~80 nM α,M after injection, a concentration that mimics pathological levels seen in human and rodent retinal diseases. Seven days later, the eyes were collected and retinas were dissected as described for flat mounting and terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) and for analyses of TNF-α levels (4, 14, 23) (n = 5 mice per group). The contralateral eye of each mouse served as an internal vehicle control.

TUNEL assays and image analyses of flat-mounted retinas. Retinas were prepared from treated or control eyes and stained with the DeadEnd Fluorometric TUNEL system (Promega). Four groups were studied: eyes injected once with α,M alone, eyes injected with α,M plus THX-B, eyes injected with α,M plus anti-proNGF, or control contralateral normal eyes from each mouse. Whole retinas were dissected and fixed in 4% formaldehyde (4% PFA) overnight, washed three times in PBS (pH 7.4), and treated for 15 min at room temperature (RT) with a solution of PBS–2% Triton X-100 and then with a solution containing 20 μg/ml proteinase K; samples were postfixed (30 min at RT with 4% PFA) and then washed for 15 min. Whole retinas were incubated in equilibration buffer for 20 min and then in the TdT reaction mixture solution for 120 min at 37°C in humidified chambers. The reaction was stopped with 2X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min, and the retinas were washed and mounted with Vectashield with 4′,6′-diamidino-2-phenylindole (DAPI) to visualize nuclei.

For each flat-mounted whole retina, three digital images per quadrant (superior, temporal, inferior, and nasal) were taken using confocal fluorescence microscopy at a ×20 magnification, for a total of 12 images per retina taken in a blinded fashion (n = 5 retinas per group). Images were taken between 0.5- and 0.7-mm, 1.0- and 1.2-mm, and 2.0- and 2.2-mm distances from the optic disc (areas 1, 2, and 3, respectively) and analyzed per area to account for known variations in retinal ganglion cell (RGC) density in each area. Total TUNEL-positive (TUNEL+) cells and total nuclei of RGCs were counted in each picture. Each ×20 magnification field exposes 0.0285 mm², and in each independent experiment images spanning 10.97 to 21.93 mm² per group were analyzed.

Biochemical analyses of retinal samples. Experimental animals for TNF-α quantification in vivo were injected intravitreally once in the right eye with α,M alone (n = 9). The left eye was injected with α,M plus the THX-B antagonist of p75NTR (n = 3), with α,M plus anti-proNGF (n = 3), or with the vehicle control (n = 3). Retinas were dissected at day 3 postinjection and were detergent solubilized (1% NP-40 containing protease inhibitors, with sonication at 4°C). Protein concentration for each of the cleared lysates was determined using a detergent-compatible BioRad kit. An equal protein amount (25 μg/lane) of each sample was resolved by SDS-PAGE, and after transfer the membranes were analyzed by Western blotting with antibodies to TNF-α as a surrogate marker of p75NTR-activ
The interactions of proNGF or NGF with the ectodomain in the absence of other membrane proteins. p75<sup>NTR</sup> is a receptor for proNGF and NGF. To evaluate whether p75<sup>NTR</sup> is engaged by α<sub>2</sub>M-proNGF or by α<sub>2</sub>M-NGF complexes, we developed a variation of the ELISA: α<sub>2</sub>M protein was immobilized, followed by addition of soluble proNGF or soluble NGF to bind to immobilized α<sub>2</sub>M. After samples were washed, a recombinant p75-Fc was added, followed by HRP-conjugated secondary anti-Fc antibody. Peroxidase activity was determined colorimetrically. Negative controls are wells coated with BSA but containing all other reagents and wells where NGF or proNGF was omitted. ns, not significant; ***, P < 0.001.

**FIG 1** Specific binding of α<sub>2</sub>M with NGF or with proNGF. All assays were repeated at least three independent times, and each assay was performed in triplicate. (A) Soluble NGF and soluble proNGF bind to immobilized α<sub>2</sub>M. Rat or human α<sub>2</sub>M or control bovine serum albumin was immobilized on ELISA plates, and wells were incubated with labeled NGF or proNGF. After the plates were washed, specific binding was quantified. (B) Soluble α<sub>2</sub>M binds to immobilized NGF or proNGF. NGF or proNGF or control bovine serum albumin was immobilized on ELISA plates, and wells were incubated with labeled rat or human α<sub>2</sub>M. Addition of unlabeled α<sub>2</sub>M demonstrated specificity (data not shown). (C) NGF and proNGF binding to immobilized α<sub>2</sub>M is competed by unlabeled NGF. Rat or human α<sub>2</sub>M or control bovine serum albumin was immobilized on ELISA plates, and wells were incubated with labeled NGF or proNGF. Cold competition by addition of unlabeled NGF at doses equimolar to the labeled ligands demonstrated specificity. (D) Specific binding of α<sub>2</sub>M-proNGF complex to p75<sup>NTR</sup> ectodomain in vivo. Rat or human α<sub>2</sub>M or control bovine serum albumin was immobilized on ELISA plates, followed by incubation with NGF or proNGF. After the wells were washed, recombinant soluble p75-Fc was added, followed by HRP-conjugated secondary anti-Fc antibody. Peroxidase activity was determined colorimetrically. Negative controls are wells coated with BSA but containing all other reagents and wells where NGF or proNGF was omitted. ns, not significant; ***, P < 0.001.

**RESULTS**

**ProNGF and NGF make stable complexes with α<sub>2</sub>-macroglobulin.** The interactions of proNGF or NGF with α<sub>2</sub>M were evaluated by ELISA. Human α<sub>2</sub>M or rat α<sub>2</sub>M was immobilized, and binding of labeled recombinant mouse proNGF or labeled recombinant mouse NGF was used as the readout.

Each ligand (proNGF and NGF) bound to the plate-immobilized human α<sub>2</sub>M or rat α<sub>2</sub>M at a level ~15-fold higher than that of the BSA control (Fig. 1A). Similar data were obtained whether native α<sub>2</sub>M or activated α<sub>2</sub>M was used (data not shown). For that reason, all assays thereafter were carried out using activated α<sub>2</sub>M. In a reciprocal ELISA, proNGF or NGF was immobilized on plates, and binding of labeled α<sub>2</sub>M was used as the readout. Human α<sub>2</sub>M and rat α<sub>2</sub>M bound to the immobilized recombinant mouse proNGF or to immobilized recombinant mouse NGF to levels ~10-fold higher than the level of binding to the BSA control (Fig. 1B). In cold competition studies, an equimolar amount of unlabeled NGF can block ~50% of the labeled proNGF or labeled NGF binding to plate-immobilized α<sub>2</sub>M (Fig. 1C).

Hence, the motifs that proNGF or NGF use to associate with α<sub>2</sub>M may be similar. From competition assays where NGF stoichiometrically blocks proNGF-α<sub>2</sub>M binding, we estimate that the affinities of proNGF and NGF for α<sub>2</sub>M are relatively similar.

BIAcore measurements showed that mature NGF binds to α<sub>2</sub>M with relatively high affinity and saturability (K<sub>d</sub> of 172 nM) (14). Based on cold competition ELISAs, we estimate a K<sub>d</sub> of ~260 nM for proNGF binding to α<sub>2</sub>M. Unfortunately, confirmation of the K<sub>d</sub> for proNGF binding to α<sub>2</sub>M by direct BIAcore measurements was not feasible due to limiting amounts/concentrations of proNGF. However, the data show that proNGF interactions with α<sub>2</sub>M are stable, similar to NGF interactions with α<sub>2</sub>M, and are not species specific.

**Complexes of α<sub>2</sub>M-proNGF or α<sub>2</sub>M-NGF bind to p75<sup>NTR</sup> ectodomain in the absence of other membrane proteins.** p75<sup>NTR</sup> is a receptor for proNGF and NGF. To evaluate whether p75<sup>NTR</sup> is engaged by α<sub>2</sub>M-proNGF or by α<sub>2</sub>M-NGF complexes, we developed a variation of the ELISA: α<sub>2</sub>M protein was immobilized, followed by addition of soluble proNGF or soluble NGF to bind to immobilized α<sub>2</sub>M. After samples were washed, a recombinant p75

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**Complexes of α<sub>2</sub>M-proNGF or α<sub>2</sub>M-NGF bind to p75<sup>NTR</sup> ectodomain in the absence of other membrane proteins.** p75<sup>NTR</sup> is a receptor for proNGF and NGF. To evaluate whether p75<sup>NTR</sup> is engaged by α<sub>2</sub>M-proNGF or by α<sub>2</sub>M-NGF complexes, we developed a variation of the ELISA: α<sub>2</sub>M protein was immobilized, followed by addition of soluble proNGF or soluble NGF to bind to immobilized α<sub>2</sub>M. After samples were washed, a recombinant p75
“receptor body” (the ectodomain of human p75NTR linked to a human Fc domain) was added to the wells.

This assay detects the presence of p75 receptor body and interrogates whether the p75NTR ectodomain alone is sufficient for the interaction (e.g., in the absence of the p75NTR (transmembrane domain or other membrane proteins such as SorC2), proNGF and NGF bound to immobilized αM, and each complex then enabled the binding of the p75 receptor body (Fig. 1D). In controls, omission of neuropotrivins in the assay precluded any signals, indicating that the p75 receptor body does not bind to immobilized αM directly. In further controls, there was no signal when BSA was immobilized instead of αM.

These data demonstrate that the p75NTR ectodomain binds to complexes of αM-proNGF or αM-NGF in the absence of other membrane proteins, that αM does not interfere with neurotrophin-p75NTR binding, that αM does not bind p75NTR, and that p75NTR binding is driven by the neurotrophin present within the αM complex.

Binding of αM-proNGF and αM-NGF complexes to cell surface neurotrophin receptors is similar to that of free proNGF and free NGF. We then asked whether complexes of αM-proNGF or αM-NGF bind to cell surface neurotrophin receptors. We used detection of fluorescently labeled αM (αM Fc) by quantitative FACScan assays to evaluate binding to receptors on the cell surface. In these experiments we tested binding to three cloned variants of the B104 cell line: B104 cells expressing only p75NTR, C10 cells expressing only TrkA receptors, and 4-3.6 cells expressing p75NTR and TrkA receptors. These cell lines were previously characterized (10).

αM Fc-proNGF complexes bind to B104 cells expressing p75NTR (Fig. 2A) but not to C10 cells expressing TrkA (Fig. 2B). αM Fc-proNGF also binds to 4-3.6 cells expressing p75NTR and TrkA (Fig. 2C). Thus, the presence of TrkA does not interfere with αM Fc-proNGF binding to p75NTR. Given that proNGF binds p75NTR preferentially, the receptor binding specificity seems to be directed by the proNGF within the complex.

αM Fc-NGF complexes bind to all cell types: B104 cells expressing p75NTR only (Fig. 2A), C10 cells expressing TrkA only (Fig. 2B), and 4-3.6 cells expressing p75NTR and TrkA (Fig. 2C). Given that mature NGF binds p75NTR and TrkA, these data confirm that the receptor binding specificity is directed by the NGF within the complex.

The low-density lipoprotein receptor-related protein 1 (LRP1) is the cognate receptor for αM (17). Phenotyping using anti-LRP1 antibodies as well as αM Fc binding showed low and equal LRP1 expression levels in all of these cells (Fig. 3A). This is important to exclude significant baseline interference by αM Fc potentially binding to LRP1. Hence, the assay measures specific neurotrophin-dependent binding of the complexes to the neurotrophin receptors. In the absence of neurotrophin, αM Fc binding to LRP1 was quantified to ~2-fold above background levels in all cells, regardless of neurotrophin receptor expression. The binding in the presence of neurotrophin was significantly higher. The binding in each assay was standardized to the background level, and fold changes were quantified (n = 3 independent experiments).

A reverse FACScan experiment was done detecting binding of biotinylated NGF (NGF biotin). In these assays we quantified direct NGF binding to neurotrophin receptors on the cell surface and the potential effect of αM on NGF binding. Different concentrations of NGF biotin (2 nM, 0.2 nM, and 0.02 nM) with or without a 60-fold excess of αM (120 nM, 12 nM, or 1.2 nM) were tested (Fig. 3B).

The stoichiometry used to preform the complexes had a constant 60-fold molar excess of αM to minimize dissociation within the time frame of the experiment. Complexes of αM-NGF biotin bound to p75NTR (in B104 cells) or to TrkA receptors (in C10 cells) in a manner indistinguishable from that of free NGF biotin when αM was omitted. Hence, αM has no apparent impact on NGF binding kinetics. The binding in each assay was standardized to the background level, and fold changes were quantified (n = 3 independent experiments) (Fig. 3B).

Unfortunately, this experiment could not be carried out using proNGF biotin due to limiting amounts/concentrations of proNGF that preceded biotinylation. Together, these data demonstrate that αM-proNGF and αM-NGF complexes bind to cell surface neurotrophin receptors in a manner that is instructed by the neurotrophin within the complex, that the presence of αM in the complex does not interfere with neurotrophin receptor binding, and that the presence of p75NTR and TrkA on the same cell surface does not alter the outcome.

Complexes of αM-proNGF protect proNGF from proteolytic cleavage. Next, we asked whether αM would protect proNGF from proteolytic degradation by furin, which gives rise to mature NGF. There are no furin cleavage sites in αM that could act as substrate competitors, and as a control for protein, we used BSA because it is also a globular protein lacking furin cleavage sites.

Furin (1 U) was mixed with 100 ng of proNGF with or without BSA or of proNGF plus αM for 0 to 90 min. Samples were fractionated by Western blotting, and as the readout we quantified (pro)NGF with an anti-NGF antibody that recognizes all forms of the protein (Fig. 4A). Furin cleavage of proNGF (32 kDa), causing a time-dependent increase in the mature NGF subunits (26 kDa and 13 kDa) as digestion products. The remaining proNGF that was not processed after furin treatment was very heterogeneous (28 to 33 kDa), as reported by other researchers (31, 32). In contrast, proNGF plus αM (e.g., within an αM-proNGF complex) was fully protected from furin cleavage, and no products were detected even after a 90-min incubation. Additionally, proNGF remained homogeneous (32 kDa) (Fig. 4A).

These data indicate that αM can prevent proteolytic processing of proNGF. This result is not due to activated αM acting as a protease inhibitor since the methylamine-activated form of αM is not a protease inhibitor (16), and αM did not protect a different furin substrate (data not shown).

Complexes of αM-proNGF are biologically more potent than proNGF at activating p75NTR. Given that the binding of proNGF to p75NTR is not affected by αM and that proNGF is more stable when it is in a complex with αM, we evaluated whether formation of the complex would enhance proNGF bioactivity in two assays: p75-dependent induction of TNF-α production by glia (4–6) and p75-dependent induction of cell death (7–9).

The p75NTR, expressing glial cell line rMC is induced to produce TNF-α mRNA and protein when cultured with proNGF for 6 h, as quantified by a commercially available ELISA (Fig. 4B) and RT-PCR (Fig. 4C). Due to the short 6-h duration of this assay, it can be performed under serum-free conditions without stressing the cells. Serum-free conditions are needed to avoid the αM that
Regulation of proNGF by the Binding Protein α₂M

is present in serum (total of 2 g/liter or 2.5 μM; hence, in cultures with 5% serum, α₂M would be present at ~125 nM).

Significant TNF-α increases were induced by proNGF compared to levels in the untreated control (P < 0.001). TNF-α increases were induced by 500 pM proNGF but were not induced by 10 pM proNGF (Fig. 4B and C). This allows testing of whether α₂M potentiates proNGF activity. A combination of 10 pM proNGF plus 150 pM α₂M caused a significant increase in TNF-α (P < 0.001). The effect was synergistic (Fig. 4D). It is noteworthy that extremely high concentrations of α₂M (7,500 pM α₂M) induced TNF-α, but even this high concentration was synergistic with proNGF (Fig. 4D). Indeed, α₂M-proNGF complex (7,500 pM α₂M plus 500 pM proNGF) significantly potentiated production of TNF-α to levels comparable to those induced by the control LPS (1 μg/ml) (Fig. 4B and C).

In another p75NTR bioassay, we tested the role of α₂M in proNGF-induced cell death (Fig. 4E). The neuroblastoma B104 cell line (TrkA− p75NTR+) dies by apoptosis when exposed to proNGF (4). Compared to survival of untreated cells, 2 nM proNGF causes a loss of ~20% (P < 0.001). These cell death assays were carried out in the presence of 5% serum (normal culture conditions). Thus, from the serum there was ~120 nM α₂M present in the culture, and addition of free neurotrophin to the cultures could result in formation of α₂M-neurotrophin complexes. Nonetheless, a preformed complex of α₂M-proNGF was more potent, causing a loss of ~40%. There was a significant difference
between the ~20% loss caused by proNGF and the ~40% loss caused by 2M-proNGF (P < 0.001). Thus, simply doubling the 2M concentration in culture significantly potentiates proNGF activity at p75NTR. In controls, B104 cells are not affected by 120 nM exogenous 2M alone, by control 2 nM mature NGF alone, or by a preformed complex of 2M-NGF (Fig. 4E).

The proNGF-induced death is mediated by p75NTR because B104 cells lack TrkA. However, some reports contend that proNGF also activates TrkA (35,36). Hence, we tested whether proNGF and/or 2M can induce TrkA tyrosine phosphorylation (p-TrkA) in C10 cells (TrkA p75NTR). Free proNGF, free 2M, or 2M-proNGF complexes did not activate p-TrkA significantly, while the positive control free NGF did (Fig. 4F). In further controls, we verified that 2M-NGF does not activate p-TrkA significantly (Fig. 4F), as reported previously (14,16). Given that 2M-NGF complexes do bind to TrkA (Fig. 2 and 3), we explored in more detail the possible mechanisms that reduce NGF/TrkA bioactivity.

Complexes of 2M-proNGF bind TrkA but do not afford TrkA dimerization or activation. Free NGF was compared to 2M-NGF using three assays quantifying TrkA activation in C10 cells (TrkA p75NTR): (i) ligand-dependent and time-dependent activation of p-TrkA (Fig. 5A), (ii) ligand-stabilized TrkA dimerization (Fig. 5B), and (iii) ligand-induced cell survival (Fig. 5C).

Free NGF very efficiently induced p-TrkA in a dose-dependent and time-dependent manner, but 2M-NGF complexes did not induce p-TrkA efficiently even after 20 min of exposure (Fig. 5A). Hence, in spite of binding TrkA, NGF does not activate TrkA signal transduction.

Free NGF induces/stabilizes TrkA-TrkA homodimers that can be covalently bonded by chemical cross-linking (33). The nonreduced TrkA monomer has a mass of ~120 kDa, and the nonreduced homodimer has a mass of ~270 kDa. However, binding by 2M-NGF complexes does not induce significant levels of cross-linked TrkA-TrkA homodimers or other high-molecular-mass complexes (Fig. 5B).

Absence of chemically cross-linked TrkA-TrkA homodimers can be interpreted as 2M interfering with the exposed amines required for cross-linking or the formation of any combination of different complexes that include 2M (mass of 180 kDa or 720 kDa) plus TrkA (120 kDa or 280 kDa) and/or NGF (25 kDa). Nonetheless, in the samples treated with 2M-NGF, the TrkA within high-molecular-mass cross-linked aggregates is similar to that of control samples not treated with ligands.

Biological assays of NGF-induced cell survival provided additional evidence of no TrkA signal transduction. C10 cells cultured under serum-free conditions die by apoptosis. Death can be inhibited by addition of mature NGF in a dose-dependent manner. The 2M-NGF complexes supported significantly less survival than NGF or provided no support at all (Fig. 5C). Controls testing 2M alone (range of 6 nM to 240 nM) did not accelerate C10 cell death under serum-free conditions (data not shown), and similar

![FIG 3](http://mcb.asm.org/ on June 30, 2017 by guest)
FIG 4 α₂M-proNGF complexes protect proNGF from proteolytic cleavage and potentiate p75NTR bioactivity in glia and neurons. (A) proNGF in the presence or absence of α₂M or a BSA control was exposed to furin (100 ng of proNGF/unit of furin) at 24°C for the times indicated. The products of furin cleavage were quantified by Western blotting. The presence of α₂M inhibited proteolytic cleavage of proNGF. (B) α₂M-proNGF complexes potentiate the production and secretion of TNF-α in supernatants of a rat Müller glial cell line (rMC). Conditioned medium was collected after 6 h of treatment, and protein was quantified by a TNF-α ELISA kit. ***, * P < 0.001 versus the untreated control; ns, not significant (C) The levels of TNF-α transcript were quantified by quantitative real-time PCR from the same cells after 6 h of the indicated treatment. Dose-dependent effects were measured, and for simplicity only one of the synergistic combinations is shown. LPS treatment is shown as a positive control. ***, P < 0.001 versus the untreated control. (D) Complexes act in synergy to promote TNF-α production in rMC cells. Combinations of proNGF and α₂M shift the p75-mediated biological response to the left of the hypothetical additive effect. proNGF and α₂M were used as follows (respectively): dose 1, 10 pM and 150 pM; dose 2, 50 pM and 750 pM; dose 3, 500 pM and 7,500 pM. Single treatments with dose 1 are ineffective and nonsignificant from relative to production of the untreated control (concentration 0). The combination treatment with dose 1 is significantly elevated compared to the hypothetical additive effect (open circles) and compared to the effect obtained with each agent alone at the maximal dose 3. ***, P < 0.001 for the effect of each combination treatment (filled triangles) compared to that of the corresponding hypothetical additive effect (open circles) (one-way ANOVA with Bonferroni’s multiple comparison tests); ###, P < 0.001, for the effect of the dose 1 combination treatment compared to that of single treatments at maximal dose 3 (500 pM proNGF or 7,500 pM α₂M) (one-way ANOVA with Bonferroni’s multiple comparison tests). (E) α₂M-proNGF complexes potentiate the apoptosis of B104 neurons expressing p75NTR. proNGF was added at 2 nM and α₂M at was added at 120 nM. MTT data are relative to the level in untreated control cells (100% growth ± SD; n = 5 independent experiments, each in quadruplicate). ***, P < 0.001 versus results in the untreated control. The P values above brackets show differences between the groups indicated by brackets. (F) C10 cells (TrkA+ p75NTR+) were treated with the indicated ligands for 20 min; cells were detergent solubilized, and whole-cell lysates were studied by Western blotting for p-Tyr with antiactin as a loading standard.
FIG 5 α2M-NGF complexes do not activate TrkA signals. C10 cells (TrkA<sup>−/−</sup> p75<sup>NTR−/−</sup>) were studied. (A) The effects of mature NGF and α2M-NGF complexes were tested for biochemical activation of TrkA and p-Tyr after 10 and 20 min of ligand or control treatment. Total TrkA was immunoprecipitated and samples were studied by Western blotting (A), by ligand-dependent promotion of TrkA dimerization, which was stabilized by chemical cross-linking (B), and by ligand-dependent promotion of cell survival during culture under proapoptotic serum-free conditions with or without NGF or α2M at the indicated concentrations (C). *, P < 0.05; ** P < 0.01; *** P < 0.001, versus results for the untreated control. The asterisks show differences between the groups indicated by brackets.

Data were reported using PC12 cells (14). Additional controls testing C10 cells growing in 5% serum showed that α2M alone (range of 6 nM to 240 nM) had no effect whatsoever on growth/survival, indicating no toxicity (data not shown).

Thus, the data from these three assays are consistent and indicate that even though TrkA is bound by α2M-NGF complexes, there is impaired receptor dimerization and activation and lack of trophic support. Although there are precedents for allosteric or conformational mechanisms of inhibition of receptor tyrosine kinases (37), failure of NGF to activate TrkA after successful binding represents a previously unknown mechanism for inhibition.

Together, the data show increased proNGF/p75<sup>NTR</sup> activity and loss of NGF/TrkA activity, a combination that would be neurodegenerative. The potential physiological relevance was thus evaluated in vivo.

**In vivo the neurotoxicity of α2M requires p75<sup>NTR</sup> and proNGF.**

An association of increased α2M and neurodegeneration was reported in animal models and in human diseases including glaucoma (4, 14, 23), diabetic retinopathy (21, 22), and cognitive impairment (8, 38). For example, in a normal eye the aqueous humor has ~1 to 10 nM α2M, which is in part produced by glial/Muller cells in the retina (23). In neurodegenerative diseases there is an increase in α2M mRNA in retinal glia/Muller cells, and α2M protein in retina and in aqueous humor increases to >40 nM, leading to the hypothesis that α2M may be a mediator of neuronal death.

In a normal retina intravitreal injection of α2M (final concentration of ~100 nM α2M to mimic pathological levels) causes the death of retinal neurons called ganglion cells (RGCs). In diseased retina, such as during glaucoma, neutralization of elevated α2M reduces RGC death (23). However, the in vivo mechanism that brings about RGC death is unknown. We hypothesized that the mechanism of action may involve α2M stabilizing and potentiating proNGF, enhancing p75<sup>NTR</sup> activity, and/or causing loss of NGF trophic activity at TrkA.

We first interrogated whether α2M stabilizes proNGF in vivo. Normal eyes received by intravitreal injection ~80 nM α2M (final concentration), and the contralateral eye was a vehicle-treated control. After 24 h the retinas were collected, and the levels of proNGF and NGF were quantified (Fig. 6A). In all experimental mice (n = 4) α2M caused a >2-fold increase in proNGF (P < 0.01) and a >10-fold decrease in mature NGF (P < 0.001) levels.

Then, using TNF-α production as the readout or p75<sup>NTR</sup> activity (4, 5), we evaluated whether proNGF that is stabilized by α2M enhances p75<sup>NTR</sup> activity in vivo. Normal eyes received by intravitreal injection ~80 nM α2M (final concentration), and the contralateral eye was a vehicle-treated control. After 3 days the retinas were collected, and the levels of TNF-α were quantified (4) (Fig. 6B). In all experimental mice (n = 5) α2M caused an ~3-fold increase in TNF-α (P < 0.001). In α2M-treated retinas there was a significant increase in the 26-kDa form of TNF-α, and higher bands of 30 kDa and 38 kDa (27) also increased compared to the bands in vehicle-treated retinas. These higher-molecular-mass bands of mouse TNF-α are likely glycosylated or membrane-bound TNF-α isoforms (28, 29). Combinant treatment with a p75<sup>NTR</sup> agonist (THX-B) or a proNGF agonist (antibody NGF30) significantly prevented the α2M-induced increases in all forms of TNF-α (quantification is shown in Fig. 6B). The data indicate that α2M-mediated increases in TNF-α are p75<sup>NTR</sup> dependent and proNGF dependent. Note that these experiments rely on the activity of endogenous proNGF that is present in the retina (39) and is stabilized by exogenous α2M.

Increased TNF-α in the retina can cause RGC neuronal death (4, 5). We quantified TUNEL<sup>+</sup> RGCs after intravitreal injection of α2M with or without concomitant delivery of a p75<sup>NTR</sup> antagonist or proNGF antagonist (n = 5 mice per group). This experiment evaluated whether RGC death caused by α2M is p75<sup>NTR</sup> dependent and proNGF dependent. Retinas were processed 7 days after intravitreal injection of α2M or vehicle (4, 23, 40) (Fig. 6C).

RGC density is highest near the optic nerve head and thinner toward the periphery of the retina. Thus, standard quantification accounts for the relative position with respect to the optic nerve head (Fig. 6C). In area 1 (with the highest RGC density) α2M induced TUNEL<sup>+</sup> RGCs at ~250 per mm<sup>2</sup>. Coadministration of a p75<sup>NTR</sup> antagonist or a proNGF antagonist significantly reduced RGC death (Fig. 6C). Similar data were quantified for all three areas of the retina, except that the absolute number of TUNEL<sup>+</sup> RGCs per square millimeter differed depending on the area quantified. Standardization of the α2M-induced TUNEL<sup>+</sup> RGCs to 100% illustrates that α2M-promoted RGC death is reduced to ~35 to 50% by treatment with antagonists, with the p75<sup>NTR</sup> antagonist being more efficient.

Together, these data indicate that exogenous administration of soluble α2M to mimic pathological levels causes RGC death...
through a mechanism involving proNGF protein stabilization (day 1 postinjection), increased p75NTR activity leading to upregulation of TNF-α (day 3 postinjection), and subsequent RGC death (day 7 postinjection).

α2M reduces NGF/TrkA signals in vivo. Last, we evaluated whether administration of soluble α2M affects TrkA function in vivo as this has been reported ex vivo. Quantification p-TrkA and downstream adaptors (p-ERK1/2 and p-AKT) at day 3 after intravitreal injection (n = 5) showed significantly reduced activation in α2M-treated retinas compared to that in vehicle-treated contralateral eyes (Fig. 7).

Note that in these experiments α2M inhibits endogenous NGF present in the retina (39). Inhibition consists of reduced maturation of NGF from proNGF, as well as a reduced ability of NGF to activate TrkA. RGC death ensues because NGF/TrkA function is required for function and maintenance of these neurons (41, 42). Disease states replicate the conditions of this experiment because α2M is endogenously upregulated in vivo and causes RGC death (23).

In sum, the mechanisms of action shown for α2M ex vivo in cells in culture (increased proNGF/p75NTR activity, increased TNF-α, loss of NGF/TrkA activity, and neuronal death) are also operational in vivo.

FIG 6 Exogenous α2M induces neuronal death in vivo by potentiation of proNGF-p75NTR activity and reduction of NGF-TrkA activity. (A) α2M was injected intravitreally to mimic the levels found in retina in pathological states, and the contralateral eye was injected with vehicle PBS. The levels of proNGF in the retina were quantified after 24 h by Western blotting, with values normalized to actin and standardized to the PBS control (n = 4 mice). **, P < 0.01, versus the control. (B) In the indicated groups a p75NTR antagonist (THX-B) or a proNGF antagonist (NGF30) was injected concomitantly with α2M. Retinas were dissected 3 days later, and TNF-α was quantified by Western blotting, with values normalized to β-actin and standardized to the vehicle control. The P values above brackets show differences between the indicated groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (C) Graphic representation of the experiment showing injections and areas quantified in each retina. After 7 days, TUNEL+ cells were quantified in flat-mounted retinas. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant versus the untreated control. The asterisks above brackets show differences between the indicated groups.
DISCUSSION

This work extends previous work on the biological features of α₃M binding to mature NGF and is the first demonstration that α₃M can form stable functional complexes with proNGF, providing information on the biological role of α₃M.

α₃M in complex with neurotrophins does not interfere with neurotrophin receptor binding. In fact, α₃M-proNGF and α₃M-NGF complexes bind to cell surface neurotrophin receptors with selectivity imparted by the neurotrophin present within the complex. Thus, functional regulation of neurotrophins by α₃M is not due to regulation of neurotrophin receptor binding.

Mechanisms for regulation of neurotrophins by α₃M were demonstrated ex vivo. The α₃M-proNGF complex prevents proteolytic processing of proNGF, making this p75NTR agonist more potent. Simply doubling the α₃M concentration significantly potentiates proNGF activity. Note that these levels (~120 nM α₃M in 5% serum and ~240 nM α₃M in the experimental group) approximate the α₃M concentrations reported in healthy and diseased tissues, respectively. Enhanced proNGF stability in turn increases proNGF/p75NTR activity and TNF-α production by glia.

Enhanced proNGF stability also results in lower levels of mature NGF. Moreover, the remaining NGF is in an α₃M-NGF complex that is inefficient at TrkA activation due to reduced TrkA homo- or hetero dimerization with other proteins or to the absence of putative conformational changes in preformed homodimers. The result of combining these mechanisms is activation of proinflammatory signals in glia and neuronal death ex vivo.

Importantly, these mechanisms were also demonstrated in vivo. Delivery of α₃M to a normal retina caused increased levels of proNGF and reduced levels of mature NGF, an increase in TNF-α, and the p75NTR-dependent and proNGF-dependent death of RGCs. These data highlight that in a normal retina there is sufficient proNGF and that increasing the tissue concentration of α₃M to levels found during disease is sufficient to produce a pathological outcome. These data also validate that α₃M is implicated in disease etiology rather than being a simple marker of neurodegenerative disease. The data are summarized in Tables 1 and 2.

Relevance of α₃M to neurotrophin receptors. In addition to previous literature showing that α₃M regulates NGF and the present report that α₃M regulates proNGF and NGF, there is evidence of an intriguing but not fully explored potential cross talk of neurotrophin receptors with the LRP1 receptor for α₃M (17).

Native α₃M is a plasma protease inhibitor with broad specificity. Cleavage of native α₃M by proteinases creates activated α₃M. Activated α₃M does not possess protease inhibitor activity but acquires binding to the cell surface receptor LRP1 (17). LRP1 is a receptor for multiple ligands, including α₃M, myelin-associated glycoprotein (MAG), apolipoprotein E, activated complement, and amyloid β (Aβ). LRP1 is involved in clearance of some ligands such as α₃M, Aβ, and complement through endocytosis and can regulate inflammatory mediators (43–45).

It is intriguing that p75NTR can also bind to or be functionally associated to LRP1 ligands such as MAG and apolipoprotein E. For example, MAG can help LRP1 to recruit p75NTR, causing subsequent RhoA activation. Curiously, p75NTR was not recruited by α₃M (45).

### TABLE 1 Summary of ex vivo results

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<th>Target</th>
<th>Test</th>
<th>Ligand*</th>
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<th>α₃M-proNGF complex</th>
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<tr>
<td></td>
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<td>Death in SFM</td>
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* α₃M alone had no effect in any assay at the physiological tissue concentrations tested.

FIG 7 Exogenous α₃M induces neuronal death in vivo by potentiation of proNGF-p75NTR activity and reduction of NGF-TrkA activity. Biochemical activation of p-TrkA, p-ERK1/2, and p-AKT in retinas treated with vehicle for 3 days was quantified from Western blots of samples prepared from whole-retina detergent lysates (n = 5 individual retinas per group).

### TABLE 2 Summary of in vivo test results

<table>
<thead>
<tr>
<th>Test</th>
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<td>Signal</td>
<td>p-TrkA ↓, p-ERK1/2 ↓, p-AKT ↓</td>
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<td>Ligand</td>
<td>proNGF ↑, NGF ↓</td>
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<tr>
<td>Bioactivity</td>
<td>TNF-α and RGC death, which is p75NTR dependent and proNGF dependent</td>
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</table>

* α₃M was injected in normal retina at levels found during disease. ↑, increase; ↓, decrease.
suggested selectivity. Unfortunately, the fact that LRP1 has over 40 different ligands makes studies of receptor cross talk challenging.

Relevance of αM to neurodegenerative disease. In the serum of healthy mammals the αM concentration is ~2.500 nM, and in acute inflammatory diseases the levels increase severalfold. Given that serum αM is at micromolar concentrations and that it has nanomolar affinity for NGF and proNGF, it is likely that all the serum proNGF and NGF proteins are in the αM-bound state. This may represent a strategy to stabilize circulating proNGF and protect it from serum proteases while it remains in circulation and to prevent circulating NGF from activating vascular endothelium (46, 47) or lymphoid cells (48, 49) that reportedly express TrkA.

In healthy tissues (e.g., retina) αM is present at <20 nM; hence, most of the proNGF and NGF is likely in the free state. However, in animal models of neurodegeneration and in human disease (4, 8, 14, 21–23, 38), αM increases locally to >100 nM. Hence, it is likely that a significant portion of the proNGF and NGF in diseased or injured tissue is in the αM-bound state. This would result in potentiation of proNGF and neutralization of mature NGF in diseased tissue, thereby promoting the death of injured cells.

At this time our data do not resolve which of the mechanisms (enhanced proNGF protein and activity or reduced mature NGF protein and activity) are more important in neurodegeneration in vivo. We speculate that the relative relevance may be disease specific and perhaps time dependent such that proNGF may be more relevant at early stages of disease while loss of NGF is more relevant at later stages of disease.

It is noteworthy that in some diseases—e.g., diabetic retinopathy, cognitive impairment, and vascular diseases—the local increase in αM is due to αM extravasation from the vasculature, whereas in glaucoma there is also reported local production by resident glia (14). Regulation of tissue αM in healthy versus disease states may be a strategy to maintain proNGF protein at low levels in healthy tissue (due to rapid degradation) and proNGF protein at high levels in compromised tissue. At the same time, low αM in healthy tissue may be a strategy to maintain TrkA activity high in normal states, while local increases during disease or injury would reduce TrkA activity in compromised tissue, perhaps to allow other repair signals.

p75NTR and αM disease mechanisms in vivo. In retina there was a reported p75NTR-dependent TNF-α production (4–6) through unclear mechanisms. Here, we show that αM participates in regulation of TNF-α production through p75NTR. Overall αM can affect p75NTR directly via proNGF and perhaps indirectly via and LRP1, MAG, and apolipoprotein E. Thus, these proteins may cooperate in a vicious cycle resulting in neurotoxicity.

In addition, αM reduces homeostatic neurotrophic maintenance by NGF/TrkA. Thus, elevated αM would pose a serious challenge for neuronal survival during disease. Targeting αM (23), proNGF, p75NTR (4, 14), or TNF-α (50) might be a viable therapeutic strategy to reduce toxicity and/or to enhance neuroprotection. In addition, αM or αM variants may be useful experimental tools to modulate the signals of proNGF or NGF and perhaps other neurotrophins.

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