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Le rôle des cellules gliales de Müller dans la mort des cellules ganglionnaires de la rétine par des mécanismes cellulaires non-autonomes.

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RÉSUM É

Les cellules gliales sont essentielles au fonctionnement du système nerveux. Dans la rétine, les cellules gliales de Müller assurent à la fois l'homéostasie du tissu et la protection des neurones, notamment celle des cellules ganglionnaires de la rétine (CGRs).

L'hypothèse principale de la thèse est que les cellules de Müller joueraient un rôle primordial dans la survie neuronale tant au plan de la signalisation des neurotrophines/proneurotrophines par suite d'une blessure que lors des mécanismes d'excitotoxicité.

Contrairement au *brain-derived neurotrophic factor* (BDNF), le *nerve growth factor* (NGF) n'est pas en mesure d'induire la survie des CGRs après une section du nerf optique. Le premier objectif de la thèse a donc été de localiser les récepteurs $p75^{NTR}$ et TrkA du NGF dans la rétine adulte et d'établir leur fonction respective en utilisant des ligands peptidomimétiques agonistes ou antagonistes spécifiques pour chacun des récepteurs. Nos résultats ont démontré que TrkA est surexprimé par les CGRs après l'axotomie, tandis que $p75^{NTR}$ est spécifiquement exprimé par les cellules de Müller. Alors que NGF n'est pas en mesure d'induire la survie des CGRs, l'activation spécifique de TrkA par des ligands peptidomimétique est nettement neuroprotectrice. De façon surprenante, le blocage sélectif de $p75^{NTR}$ ou l'absence de celui-ci protège les CGRs de la mort induite par l'axotomie. De plus, la combinaison de NGF avec l'antagoniste de $p75^{NTR}$ agit de façon synergique sur la survie des CGRS. Ces résultats révèlent un nouveau mécanisme par lequel le récepteur $p75^{NTR}$ exprimé par les cellules gliales de Müller peut grandement influencer la survie neuronale.

Ensuite, nous avons voulu déterminer l'effet des proneurotrophines dans la rétine adulte. Nous avons démontré que l'injection de proNGF induit la mort des CGRs chez le rat et la souris par un mécanisme dépendant de $p75^{NTR}$. L'expression de $p75^{NTR}$ étant exclusive aux cellules de Müller, nous avons testé l'hypothèse que le proNGF active une signalisation cellulaire non-autonome qui aboutit à la mort des CGRs. En suivant cette idée, nous avons montré que le proNGF induit une forte expression du *tumor necrosis factor* α (TNFα) dans les cellules de Müller et que l'inhibition du TNF bloque la mort neuronale induite par le proNGF. L'utilisation de souris knock-out pour la protéine $p75^{NTR}$, son co-récepteur sortiline, ou la protéine adaptatrice NRAGE a permis de montrer que la production de TNF par les

cellules gliales était dépendante de ces protéines. Le proNGF semble activer une signalisation cellulaire non-autonome qui cause la mort des neurones de façon dépendante du TNF *in vivo*.

L'hypothèse centrale de l'excitotoxicité est que la stimulation excessive des récepteurs du glutamate sensibles au *N*-Methyl-D-Aspartate (NMDA) est dommageable pour les neurones et contribue à plusieurs maladies neurodégénératives. Les cellules gliales sont soupçonnées de contribuer à la mort neuronale par excitotoxicité, mais leur rôle précis est encore méconnu. Le dernier objectif de ma thèse était d'établir le rôle des cellules de Müller dans cette mort neuronale. Nos résultats ont démontré que l'injection de NMDA induit une activation du nuclear factor κB (NF-κB) dans les cellules de Müller, mais pas dans les CGRs, et que l'utilisation d'inhibiteurs du NF-κB empêche la mort des CGRs. De plus, nous avons montré que les cellules de Müller en réaction à l'activation du NF-κB produisent la protéine TNFα, laquelle semble être directement impliquée dans la mort des CGRs par excitotoxicité. Cette mort cellulaire se produit principalement par l'augmentation à la surface des neurones des récepteurs AMPA perméables au Ca^{2+}, un phénomène dépendant du TNFα. Ces donnés révèlent un nouveau mécanisme cellululaire non-autonome par lequel les cellules gliales peuvent exacerber la mort neuronale lors de la mise en jeu de mécanismes excitotoxiques.

Mots-Clé : Cellules ganglionnaires de la rétine, cellules de Müller, interaction neurone-glie, survie neuronale, nerve growth factor, pro-NGF, $p75^{NTR}$, excitotoxicité, facteur nécrosant des tumeurs, Nuclear Factor κ B.

SUMMARY

Glial cells are essential for the functioning of the nervous system. In the retina, the Müller glial cells ensure the homeostasis of this tissue as well as the protection of neurons including the retinal ganglion cells (RGCs).

The main hypothesis of this thesis is that Müller cells play a predominant role in neuronal survival both at the levels of neurotrophin/proneurotrophin signaling following injury and excitotoxic mechanisms.

Unlike the brain-derived neurotrophic factor (BDNF), the nerve growth factor (NGF) is unable to induce RGCs survival following optic nerve transection. The first objective of the thesis was therefore to describe the expression of the two receptors of NGF, $p75^{NTR}$ and TrkA, in the adult retina and to address their functional role by using peptidomimetic agonistic or antagonistic ligands specific for each receptor. Our results showed that TrkA is overexpressed by RGCs following axotomy, whereas $p75^{NTR}$ is specifically expressed by Müller cells. While NGF by itself does not promote RGC survival, selective activation of TrkA receptors using peptidomimetic ligands is markedly neuroprotective. Surprisingly, selective blockers of $p75^{NTR}$, or the absence of $p75^{NTR}$, protect RGCs from axotomy-induced death. Moreover, combination of NGF or TrkA agonists with $p75^{NTR}$ antagonists functions synergistically to enhance RGC survival. These results reveal a new mechanism by which $p75^{NTR}$ expression by Müller glia may profoundly influence neuronal survival.

Next, we wanted to address the effect of proneurotrophins in the adult retina. We showed that injection of proNGF induces the death of RGCs in rats and mice by a $p75^{NTR}$-dependent signaling mechanism. Expression of $p75^{NTR}$ in the adult retina being confined to Müller glial cells, we tested the hypothesis that proNGF activates a non-cell autonomous signaling pathway to induce RGC death. Consistent with this notion, we showed that proNGF induced a robust expression of tumor necrosis factor α (TNFα) in Müller cells, and that genetic or biochemical ablation of TNFα blocked proNGF-induced death of retinal neurons. Mice rendered null for $p75^{NTR}$, its co-receptor sortilin, or the adaptor protein NRAGE were defective in proNGF-induced glial TNFα production and did not undergo proNGF-induced retinal ganglion cell death. We concluded that proNGF activates a non-cell autonomous signaling pathway that causes TNFα-dependent death of retinal neurons *in vivo*.

The central hypothesis of excitotoxicity is that excessive stimulation of neuronal *N*-Methyl-D-Aspartate (NMDA)-sensitive glutamate receptors is harmful to neurons and contributes to a variety of neurological disorders. Glial cells have been proposed to participate in excitotoxic neuronal loss, but their precise role is poorly defined. In this *in vivo* study, we showed that NMDA induces a strong NF-κB activation in Müller glia, but not in retinal neurons. Intriguingly, NMDA-induced death of retinal neurons was effectively blocked by inhibitors of NF-κB activity. We demonstrated that TNFα protein produced in Müller glial cells via an NMDA-induced NF-κB dependent pathway plays a crucial role in the excitotoxic loss of retinal neurons. This cell loss occurs mainly through a TNFα-dependent increase in Ca^{2+}-permeable AMPA receptors on susceptible neurons. Thus, our data reveal a novel non-cell-autonomous mechanism by which glial cells can profoundly exacerbate neuronal death following excitotoxic injury.

Mots-Clé : Retinal ganglion cells, Müller cells, neuron-glia interaction, neuron survival, nerve growth factor, pro-NGF, $p75^{NTR}$, excitotoxicity, Tumor necrosis factor, Nuclear Factor κB.

TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

CHAPITRE 1

CHAPITRE 2

CHAPITRE 3

CHAPITRE 4

CHAPITRE 5

LISTE DES ABRÉVIATIONS

ADN	Acide DésoxyriboNucléique
Akt	Protéine Kinase B
AMPA	Acide (S)-alpha-amino-3-hydroxy-5-methyl-4-isoxazolpropionique
AMPAR	Récepteur de l'acide (S)-alpha-amino-3-hydroxy-5-methyl-4-isoxazolpropionique
AP-1	Activating Protein-1
Apaf-1	Apoptosis protease activating factor-1
ARMS	Ankyrin Rich Membrane Spannin
ARN	Acide RiboNucléique
ARNm	Acide RiboNucléique messager
ATP	Adenosine Triphosphate
BDNF	Brain-Derived Neurotrophic Factor
Ca^{2+}	Calcium
CAMKII	Calcium/Calmodulin-dependent Protein Kinase II
CCG	Couche des Cellules Ganglionnaires
CGL	Corps Genouillé Latéral
CGRs	Cellules Ganglionnaires de la Rétine
CNE	Couche Nucléaire Externe
CNI	Couche Nucléaire Interne
CNTF	Ciliary NeuroTrophic Factor
CPE	Couche Plexiforme Externe
CPI	Couche Plexiforme Interne
CRALBP	Cellular Retinaldehyde-Binding Protein
CREB	cAMP Response Element Binding
DD	Death Domain
Erk1/2	Extracellular signal-Regulated Kinase1/2
FG	FluoroGold
FGF2	Fibroblast Growth Factor 2
Gab1	Grb2-Associated Binder
GABA	Gamma-Amino-Butyric Acid
GFAP	Glial Fibrillary Acidic Protein
Grb2	Growth factor receptor-bound protein 2
GS	Glutamine synthétase
GSK-3	Glycogen Synthase Kkinase-3
GYKI	1-[4-aminophenyl]-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine
ICAM	Intracellular Adhesion Molecule
Ig	Immunoglobuline
IGF	Insulin Growth Factor
IRAK	Interleukin-1 Receptor Associated Kinase
K^+	Potasium
kDa	kiloDalton
LIF	Leukemia Inhibitory Factor

LINGO	Leucine-rich and Ig domain containing NoGO receptor interacting protein
MAG	Myelin-Associated Glycoprotein
MAGE	Melanoma Associated Antigen
$MAPK_{1/2}$	Mitogen Activated Protein $Kinase_{1/2}$
MEK	Mapk/Erk Kinase
Mg^{2+}	Magnésium
mGluR	Metabotropic Glutamate Receptor
MK-801	Maléate de Dizocilpine
MCP-1	Monocyte Chemoattractant Protein-1
MIP-1α	Macrophage Inflammatory Protein 1α
Na^{+}	Sodium
NADE	$p75^{NTR}$-Associated Death Executor
NF-κB	Nuclear Factor κ B
NGF	Nerve Growth Factor
NgR	Nogo-66 Receptor
NMDA	N-Méthyl-D-Aspartate
NMDAR	Récepteur du N-Méthyl-D-Aspartate
NO	Nitric Oxide/Oxyde Nitrique
NOS	Nitric Oxide Synthase
NR	NMDA Receptor subunit
NRAGE	Neurotrophin Receptor-Interacting MAGE homolog
NRIF	Neurotrophin Receptor-Interacting Factor
NT	Neurotrophine
NT3	Neurotrophin 3
NT4/5	Neurotrophin 4/5
O_2^{-}	Anion superoxyde
OMgp	Oligodendrocyte Myelin glycoprotein
PDK-1	Serine/threonine kinases 3 Phosphoinositide-Dependent Kinase-1
PI-3K	Phosphatidyl Inositol-3 Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC-γ	PhosphoLipase C-γ
ProNT	ProNeurotrophines
RANKL	Receptor-Activator of NF-κB Ligand
RIP2	Receptor-Interacting Protein-2
RSK	Ribosomal S6 Kinase
TGN	Réseau Trans-Golgien
Trk	Tropomyosin-Receptor-Kinase
Shh	Sonic Hedgehog
SLN	Signal de Localisation Nucléaire
SNC	Système Nerveux Central
SNP	Système Nerveux Périphérique
SOD1	Superoxyde Dismutase 1
SOS	Son Of Sevenless
SR	Sérine Racémase

STAT3	Activateur de Transcription 3
TGFβ	Transforming Growth Factor β
TNFα	Tumor Necrosis Factor α
TNFR	Tumor Necrosis Factor Receptor
TRAF	TNFR Associated Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
Trk	Tropomyosin-related kinase
TWEAK	TNF-like Weak inducer of apoptosis
VCAM	Vascular Cell Adhesion Molecule)
VEGF	Vascular Endothelial Growth Factor

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``Chaque publication scientifique ne sert qu'à poser 10, 20 questions. Chaque découverte scientifique est passionnante parce qu'elle ouvre un univers de questions. Si les questions vous angoissent ne soyez pas scientifique.´´

Boris Cyrulnik

``Quand un ouvrage scientifique se trompe, quelqu'un finit par découvrir l'erreur, et elle est corrigée dans les ouvrages suivants. Il est évident que ce n'est pas ce qui se passe pour les livres saints.´´

Richard Dawkins

CHAPITRE 1

INTRODUCTION GÉNÉRALE

1. INTRODUCTION GÉNÉRALE

1.1. Les cellules gliales, les autres cellules du système nerveux central.

C'est en 1856 que le pathologiste allemand Rudolf Virchow découvrit les cellules gliales. Au début du XXe siècle, on estimait que le cerveau contenait à peu près neuf fois plus de cellules gliales que de neurones. C'est cette abondance des cellules gliales qui a donné naissance au mythe voulant que nous n'utilisions que 10 % de notre cerveau pour penser. Cette idée préconçue serait d'autant plus difficile à soutenir de nos jours que nous savons maintenant que le cerveau humain compte environ le même nombre de cellules gliales et de neurones, même si la proportion de ces deux types cellulaires varie d'une région à l'autre du cerveau (Azevedo et al., 2009). En fait, *glia* est un mot grec qui signifie colle. Les cellules gliales ont reçu leur nom du fait que l'on pensait à tort qu'elles n'étaient qu'un support (ou colle), pour les neurones qui transmettent l'information. Elles devaient isoler et refroidir les synapses qui risquaient la surchauffe à cause des impulsions électriques constantes qu'elles subissaient. C'est Carl Ludwig Schleich (1859-1922), un chirurgien et anesthésiste Allemand, qui souligna le rôle essentiel joué par la neuroglie dans le système nerveux central. Il suggéra que les interactions entre les neurones et la glie étaient essentielles et qu'on devait considérer le cerveau comme un système neurone-glie dont la performance était dépendante des deux types cellulaires.

1.1.1. Les différents types de cellules gliales

Les recherches menées au cours des 20 dernières années montrent le rôle primordial joué par les cellules gliales dans l'activité cérébrale et font d'elles les partenaires essentiels des neurones. Les trois principaux types de cellules gliales : astrocytes, oligodendrocytes et microglie assurent des fonctions de protection, de nutrition et de communication. Les rôles de la microglie seraient comparables à ceux du système immunitaire. Contrairement aux autres cellules gliales qui proviennent, comme les neurones, de l'ectoderme dorsal, les microglies sont d'origine mésodermique et sont dérivées des mêmes précurseurs que les cellules du système immunitaire. Les cellules de Schwann dans le système nerveux périphérique (SNP) et les oligodendrocytes dans le système nerveux central (SNC) entourent les axones des neurones de leur gaine de myéline qui isole et protège les fibres nerveuses et

permet une propagation rapide de l'influx nerveux sur de longues distances. Les astrocytes représentent la troisième grande catégorie de cellules gliales et on leur distingue plusieurs formes et fonctions dans le SNC. Ils sont divisés en deux familles en fonction de leur morphologie et de leur localisation. Les astrocytes protoplasmiques, retrouvés dans la matière grise, sont étroitement associées aux synapses et aux vaisseaux sanguins, alors que les astrocytes fibrillaires font contact avec les nœuds de Ranvier dans la matière blanche. Cependant, il existe des astrocytes spécialisés comme les cellules de Müller de la rétine et la glie de Bergmann du cervelet, qui ressemblent aux astrocytes protoplasmiques dans la mesure où ils font contact avec plusieurs synapses (Barres, 2008). Les astrocytes sont principalement responsables de la communication entre les neurones, les capillaires sanguins et les cellules épendymaires. Ils sont spécialisés dans l'apport aux neurones d'éléments nutritifs et dans l'élimination de leurs déchets. Comme la plupart des cellules gliales, ils sont une source importante de facteurs neurotrophiques, de molécules d'adhésion et de protéines de la matrice extracellulaire, ce qui leur fait jouer un rôle dans la migration et la survie neuronale (Allen and Barres, 2009) (Fig.1).

1.1.2. La communication neurone-glie

Contrairement aux neurones, les cellules gliales sont électriquement inexcitables puisqu'elles ne déclenchent pas de potentiel d'action. Cependant, ce sont des cellules tout aussi actives que les neurones et qui communiquent par des signaux chimiques. Comme les neurones, elles expriment une panoplie de canaux ioniques, de transporteurs membranaires, de neurotransmetteurs et de récepteurs neurotrophiques (Stevens, 2003). La communication entre les neurones et les cellules gliales est bi-directionnelle en ce sens que les cellules gliales sont capables à la fois d'émettre des signaux vers les neurones et dans recevoir de ceux-ci. Un seul astrocyte peut entourer et entrer en contact avec des milliers de synapses formés sur plusieurs neurones. Cette relation étroite entre la glie et les synapses est à l'origine du concept de la synapse tripartite, incluant une composante gliale péri-synaptique qui entoure les composantes pré- et post-synaptique neuronales (Perea et al., 2009). Comme les astrocytes sont munis de plusieurs récepteurs de neurotransmetteurs que possèdent les neurones, le relâchement par les neurones de neurotransmetteurs peut aussi activer des cascades de signalisation dépendantes du calcium dans les astrocytes. Les astrocytes peuvent

à leur tour relâcher des gliotransmetteurs, formant ainsi une boucle d'activation neurone-glie. Les différents types de molécules sécrétées par les astrocytes peuvent à la fois inhiber ou augmenter l'activité neuronale (Allen and Barres, 2009).

1.1.3. La communication entre les cellules gliales

Les cellules gliales communiquent entre elles principalement en produisant des vagues calciques, qui peuvent se propager sur une certaine distance. Cette propagation semble dirigée et sugère l'existence d'une organiation en réseau des astrocytes (Wang and Bordey, 2008). L'activation des cellules gliales peut aussi engendrer une réponse calcique dans les neurones environnants. Les mécanismes à l'origine des vagues calciques incluant la diffusion de seconds messagers à travers des jonctions serrées et la signalisation de composés chimiques tout au long du réseau astrocytaire. Ainsi, la relâche d'ATP par les cellules gliales stimulées active les récepteurs purinergiques des cellules gliales adjacentes, lesquelles propagent l'influx calcique sur une certaine distance (Allen and Barres, 2009).

1.1.4. La comunication entre les cellules gliales et les vaisseaux sanguins

Les cellules gliales servent de ponts ou de médiateurs entre les neurones et le système sanguin. La barrière hémato-encéphalique et la barrière hémato-rétinienne s'interpose dans un réseau complexe constitué de cellules endothéliales et de neurones. Dans le SNC, ses éléments gliaux sont de types astocytaire; dans la rétine, ce sont les cellules de Müller (Kim et al., 2006). Ces barrières permettent une diffusion sélective et bi-drectionnelle entre les vaisseaux sanguins et les cellules gliales. Par exemple, les cellules gliales transportent le glucose et de l'oxygène du système sanguin vers les neurones. Outre l'échange de composés, cette association rapprochée entre les cellules gliales et les vaisseaux sanguins permet aux astrocytes d'exercer un contrôle sur le débit sanguin. En réponse à activité neuronale accrue, les astrocytes peuvent signaler aux vaisseaux d'augmenter le débit sanguin, ce qui entraîne une augmentation de l'apport d'oxygène et de glucose vers la région du cerveau activé (Allen and Barres, 2009).

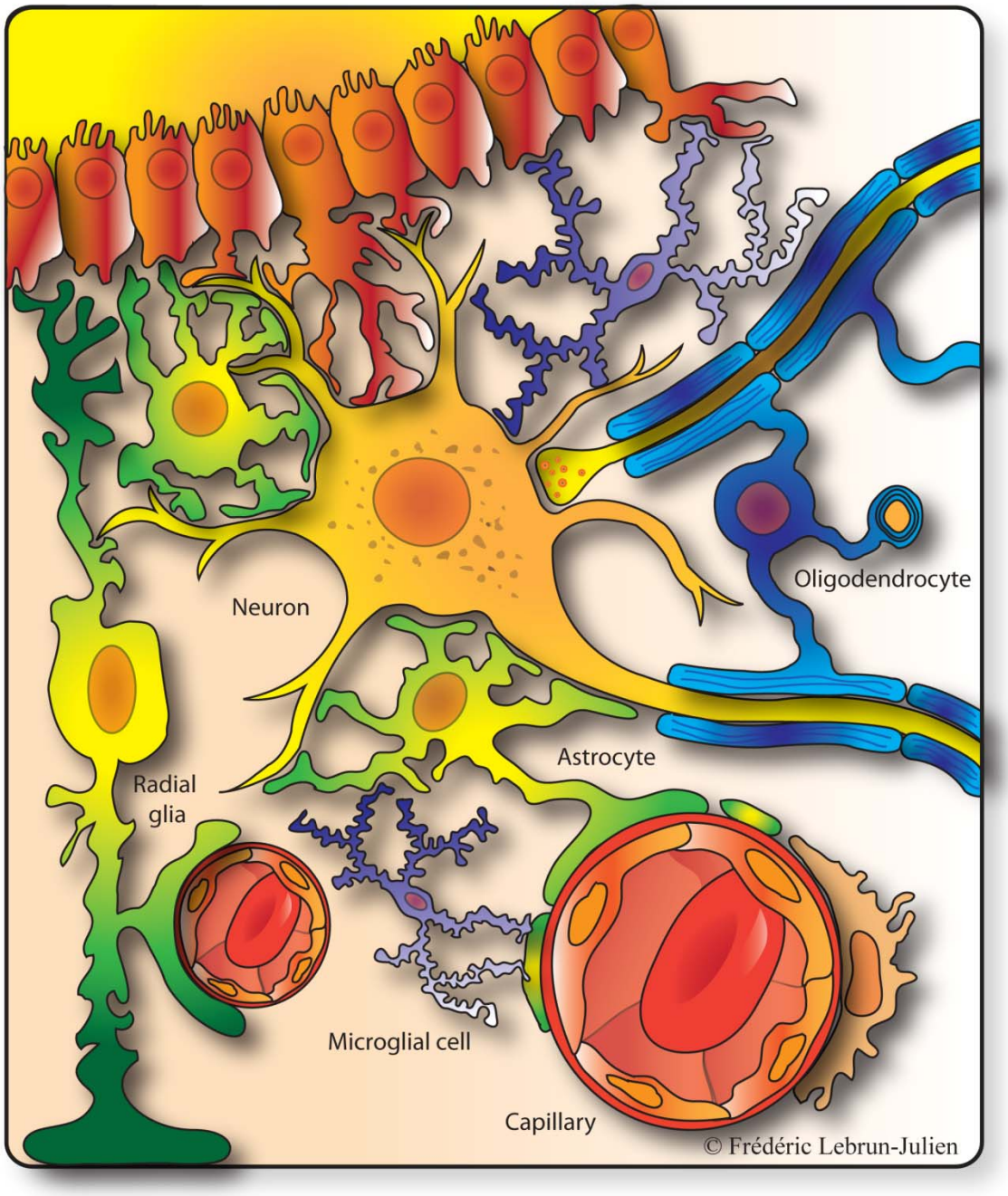

Figure 1. Les cellules gliales. Différents types de cellules gliales interagissent avec les neurones et les vaisseaux sanguins. Les oligodendrocytes entourent les axones de leur gaine de myéline. Les astrocytes entourent les vaisseaux sanguins et les synapses de cours prolongements. Les cellules microgliales gardent le système nerveux central sous surveillance contre les infections et les blessures.

1.1.5. Le rôle des cellules gliales dans les mécanismes neurodégénératifs.

Le fait que les cellules gliales et les neurones communiquent activement, en utilisant une signalisation réciproque, ne laisse nul doute sur le rôle essentiel joué par la glie dans le système nerveux. Par le fait même, un dysfonctionnement de la glie peut être mis en cause dans plusieurs maladies. Autant les cellules gliales peuvent être essentielles à la survie des neurones, autant peuvent elles devenir réactives et relâcher des signaux dommageables aux neurones et aux autres cellules gliales par suite d'une blessure ou d'une mutation. Il y a maintenant plusieurs indices de mécanismes cellulaires non-autonomes, par lesquels des proccessus neurodégénératifs sont exacerbés du fait de la toxicité ou de l'expression de protéines mutantes par des cellules neuronales et non-neuronales de l'environnement de neurones vulnérables. Par exemple, dans la sclérose latérale amyotrophique, diverses données suggèrent l'implication de mécanismes neuroinflammatoires déclenchés par la gliose de cellules astrocytaires et microgliales (Boillée et al., 2006; Maragakis and Rothstein, 2006). Dans la sclérose en plaques, ce sont les oligodendrocytes qui causent une réaction auto-immune entraînant la démyélinisation des axones (Lobsiger and Cleveland, 2007). On pourrait citer plusieurs autres exemples d'interaction neurone-glie dans les processus neurodégénératifs. Les cellules gliales régissant plusieurs activités neuronales, il est primordial de mieux comprendre les mécanismes de collaboration entre ces deux populations cellulaires. La rétine, avec ses couches cellulaires bien définies, offre un système de choix pour étudier le rôle des cellules gliales dans les mécanismes de neurodégénérescence du SNC. Les cellules gliales de Müller, principales cellules gliales de la rétine, sont des astrocytes spécialisés qui apparaissent essentiels à l'homéostasie de la rétine et la survie des neurones rétiniens. Pourtant, les rôles joués par ces cellules dans les différentes rétinopathies ou par suite à une blessure restent encore bien mal définis.

1.2. Le système rétino-colliculaire

La rétine, qui est la partie nerveuse de l'œil, est un modèle idéal et unique pour étudier les divers mécanismes neuronaux et l'interaction neurone-glie. Bien qu'elle soit à la périphérie, elle fait partie du SNC, ce qui permet un accès privilégié aux neurones et la glie du SNC. La rétine est formée de cinq types de neurones et de cellules gliales, dont la population la plus importante est celle des cellules de Müller. Seules les cellules

ganglionnaires de la rétine (CGRs) étendent leurs axones via le nerf optique jusqu'aux cellules cibles du cerveau. La configuration des voies optiques varie considérablement d'une espèce à l'autre. Chez les souris et les rats, la vaste majorité des axones des CGRs se dirigent contra-latéralement vers le collicule supérieur et l'aire prétectale du mésencéphale, et 30 % d'entre eux émettent des collatérales vers le corps genouillé latéral (CGL) et le thalamus (Isenmann et al., 2003). Chez les rongeurs, on parle donc d'un système rétino-colliculaire. Par contre, chez l'humain, la grande majorité des axones se terminant dans le CGL, soit la partie dorsale du thalamus, le relais principal d'une voie qui mène au cortex visuel primaire à l'arrière du cerveau. On parle dons plutôt d'une voie rétino-géniculo-corticale (Usrey, 2002).

1.2.1. Le développement de la rétine

Au cours du développement embryonnaire, la rétine se forme à partir d'une excroissance du diencéphale, appelée vésicule optique, qui s'invagine ensuite pour constituer la cupule optique. La paroi interne de la cupule optique donne naissance à l'épithélium neuronal, la rétine, tandis que sa paroi externe se transforme en épithélium pigmentaire, mince couche cellulaire chargée de mélanine. Nous savons depuis la fin des années 80 que tous ces types cellulaires sont générés par un seul type de cellule progénitrice, multipotente (Turner and Cepko, 1987; Turner et al., 1990). Les progéniteurs sortent du cycle cellulaire et acquièrent un phénotype cellulaire distinct sous l'influence de facteurs génétiques intrinsèques et de facteurs extrinsèques présents dans le milieu extracellulaire (Livesey and Cepko, 2001). De plus, il y a un ordre temporel de genèse des différents phénotypes cellulaires (Young, 1985; Harman and Beazley, 1989). L'ordre d'apparition des différents types cellulaires est fortement conservé à travers les espèces. Durant la première phase de genèse cellulaire, les cellules ganglionnaires, les cellules horizontales, au moins un type de cellules amacrines et les cônes sont les premières à apparaître. Une vague subséquente produit les types de cellules amacrines restants en plus des cellules de Müller. Une troisième vaque produit les bâtonnets et finalement, les cellules bipolaires (Rapaport et al., 2004) (Fig.2A).

1.2.1.1. Les cellules gliales et le développement de la rétine

Le développement de la rétine se fait en plusieurs étapes incluant la différentiation des progéniteurs en cellules gliales et en neurones, la migration cellulaire et la synaptogénèse. On soupçonne que les cellules gliales de Müller influencent grandement chacune de ces étapes. Cependant, comme nous l'avons mentionné, ces cellules quitent le cycle cellulaire relativement tard lors du développement de la rétine. Dans les autres régions du SNC, la glie radiale sert d'échafaudage pour la migration neuronale (Hatten, 1990). Il n'y a pas eu d'observations démontrant un rôle semblable pour les cellules de Müller, bien qu'elles semblent essentielles pour déterminer la structure de la rétine (Bringmann et al., 2006a). Par exemple, grâce à l'utilisation d'explants de rétine, Wang et ses collègues ont démontré que l'expression de *Sonic hedgehog* (Shh) par les CGRs est primordiale pour l'induction du développement et de l'orientation des cellules de Müller, mais que, par la suite, celles-ci sont essentielles pour le développement du reste de la rétine et notamment de la couche des photorécepteurs (Wang et al., 2002c). Dans certaines conditions, les cellules de Müller nouvellement formées ont la capacité d'entrer de nouveau dans le cycle mitotique et de générer de nouveaux neurones et d'autres cellules de Müller (Fischer and Reh, 2001).

Quant aux astrocytes, ils jouent un rôle important dans le contrôle de l'angiogénèse et la différenciation du système vasculaire. Lors du développement, les astrocytes envahissent la rétine par le nerf optique et migrent pour former un réseau de cellules dans la partie interne de la rétine. Cette migration est contrôlée par les neurones qui sécrètent le facteur de croissance dérivé des plaquettes (PDGF). Le déploiement du réseau astrocytaires est suivi de celui du réseau vasculaire, qui émerge également du nerf optique. Le réseau astrocytaire sert d'échafaudage pour les vaisseaux sanguins en croissance et produit également le facteur de croissance endothéliale vasculaire (VEGF), nécessaire à la migration et la croissance des vaisseaux (Fruttiger, 2007).

1.2.2. Organisation générale de la rétine

L'œil est composé de trois couches : la sclérotique, qui est la couche la plus externe; la choroïde, richement vascularisée, qui se situe au milieu, et la rétine, la couche la plus interne, qui est en contact avec l'humeur vitrée. En tant que partie du système nerveux central, la rétine comporte des circuits nerveux complexes. Les différentes cellules sont présentes à

l'intérieur de la rétine dans des couches cellulaires distinctes donnant une architecture cytologique bien définie. Comme le montre la Fig. 2 B, c'est dans la couche nucléaire externe (CNE) que sont localisés les corps cellulaires des photorécepteurs (cônes et bâtonnets), principales cellules sensibles à la stimulation lumineuse. Les segments externes des photorécepteurs sont rigoureusement alignés à la limite de l'épithélium pigmentaire. La couche plexiforme externe (CPE) contient les axones et les dendrites des cellules horizontales et bipolaires, ainsi que les terminaisons synaptiques des photorécepteurs. La couche nucléaire interne (CNI) contient les corps cellulaires des cellules bipolaires, horizontales et amacrines, alors que la couche plexiforme interne (CPI) est constituée des axones et des dendrites des cellules bipolaires, amacrines et ganglionnaires. La couche la plus interne de la rétine est la couche des cellules ganglionnaires (CCG), dont les axones, qui forment le nerf optique, font connexion avec le cerveau (Kolb, 1994). Les CGRs comptent pour seulement 1 % du nombre total de cellules rétiniennes (Young, 1985) et représentent 50-70 % des cellules de la CCG chez les rongeurs, les autres étant principalement des cellules amarines déplacées (Drager and Olsen, 1981; Perry, 1981b; Jeon and Jeon, 1998). Les photons composant la lumière traversent l'épaisseur de la rétine, des cellules ganglionnaires jusqu'aux segments externes des photorécepteurs où ils sont capturés. L'énergie lumineuse est transformée en potentiels électriques dans les photorécepteurs et ceux-ci sont relayés vers les cellules ganglionnaires, qui sont le dernier maillon de la chaîne neuronale rétinienne. Comme nous l'avons déjà dit, les CGRs sont les seuls neurones de la rétine dont les axones se terminent dans le cerveau.

1.2.3. Les cellules gliales de la rétine

La rétine comprend trois types de cellules gliales. En plus des cellules microgliales, on y trouve deux formes de cellules macrogliales, les astrocytes et les cellules de Müller. Les cellules microgliales, dérivées des cellules immunitaires du sang de la rétine, ont un rôle de défense contre les microorganismes, d'initiation des processus d'inflammation et de réparation des tissus. Elles sont situées principalement au sein des fibres nerveuses issues de la couche des cellules ganglionnaires. Les astrocytes sont également présents dans cette région et jouent un rôle important de soutien, partageant ou séparant la portion axonale des autres structures. Outre leur rôle de soutien, les astrocytes jouent un rôle dans l'absorption

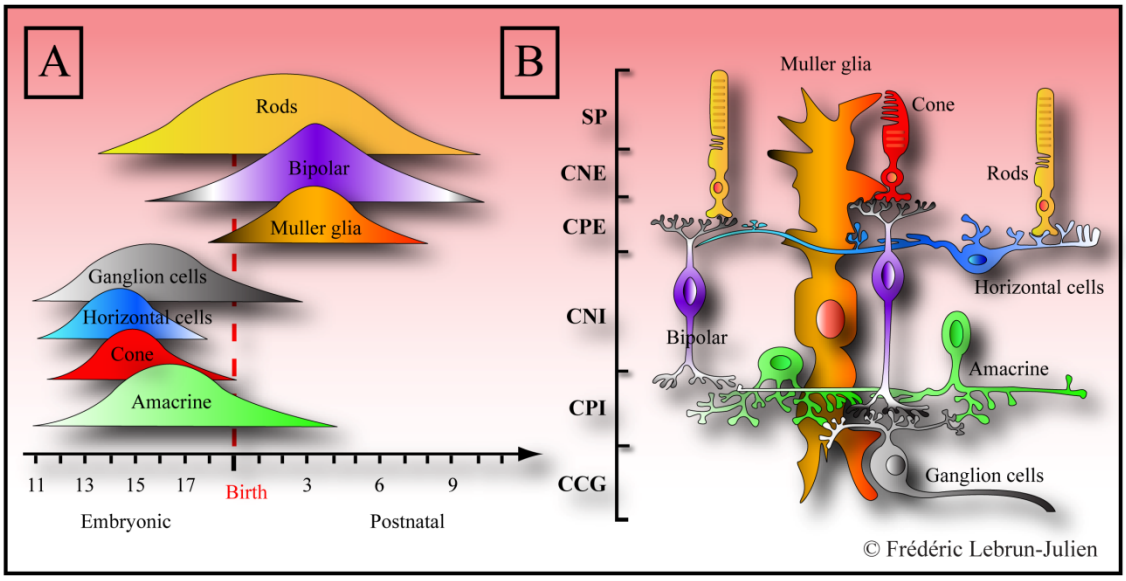

Figure 2. De la chronologie d'apparition des cellules à l'architecture de la rétine adulte. (A) Durant la première phase de genèse cellulaire, les cellules ganglionnaires, les cellules horizontales, un type de cellules amacrines et les cônes sont les premières à apparaître. Une vague subséquente produit les autres types de cellules amarines et les cellules de Müller. Une troisième vague produit les bâtonnets et finalement, les cellules bipolaires. (B) La rétine adulte comprend cinq types de neurones et un type principale de cellules gliales, les cellules de Müller. EP : Épithélium pigmentaire; SP : Segments des photorécepteurs; CNI : Couche nucléaire interne; CPE : Couche plexiforme externe; CNI : Couche nucléaire interne; CPI : Couche plexiforme interne; CCG : Couche des cellules ganglionnaires.

et le tamponnement de certaines substances liées au métabolisme axonal en plus de servir d'intermédiaire entre les neurones et les capillaires sanguins. Finalement, les cellules de Müller représentent le type de cellules gliales principales de la rétine et elles interagissent avec tous les types neuronaux qui s'y trouvent (Bringmann et al., 2006a). Tel que nous l'avons mentionné précédemment, les cellules gliales communiquent entre elles par la propagation de vagues calciques. Dans la rétine, la propagation de ces vagues entre les astrocytes est principalement engendrée par la diffusion de messagers intracellulaires entre les jonctions sérrées des cellules gliales (Zahs and Newman, 1997). Parallèlement, la propagation entre les astrocytes et les cellules de Müller ou entre les cellules de Müller se fait par la relâche d'ATP, qui fonctionne comme un messager extracellulaire. L'ATP peut aussi contribuer à la communication entre les astrocytes (Newman, 2004b).

1.2.3.1. Les cellules de Müller

Les cellules de Müller représentent jusqu'à 90 % des cellules gliales de la rétine chez les vertébrés. Ce sont des cellules astrocytaires radiales spécialisées qui s'étendent dans toutes les couches de la rétine et qui font contact et entourent tous les corps cellulaires et prolongements des neurones rétiniens (Bringmann et al., 2006a). La population des cellules de Müller forme une sorte de palissade dense et régulière parcourant toute la rétine. Elles constituent un lien anatomique entre les neurones rétiniens et les compartiments avec lesquels ils ont besoin d'échanger des molécules, c'est-à-dire les vaisseaux sanguins, l'humeur vitrée et l'espace sous-rétinien. Ce lien n'est pas uniquement anatomique, mais aussi fonctionnel. Une panoplie de canaux ioniques, de récepteurs de ligands, de transporteur de molécules transmembranaires et d'enzymes est spécifiquement exprimé par ces cellules et non par les cellules neuronales (Newman and Reichenbach, 1996). De plus, de récentes études ont démontré que les cellules de Müller agiraient comme des fibres optiques, transmettant la lumière à travers toutes les couches de la rétine jusqu'aux photorécepteurs avec très peu de perte d'énergie (Franze et al., 2007) (Fig.3).

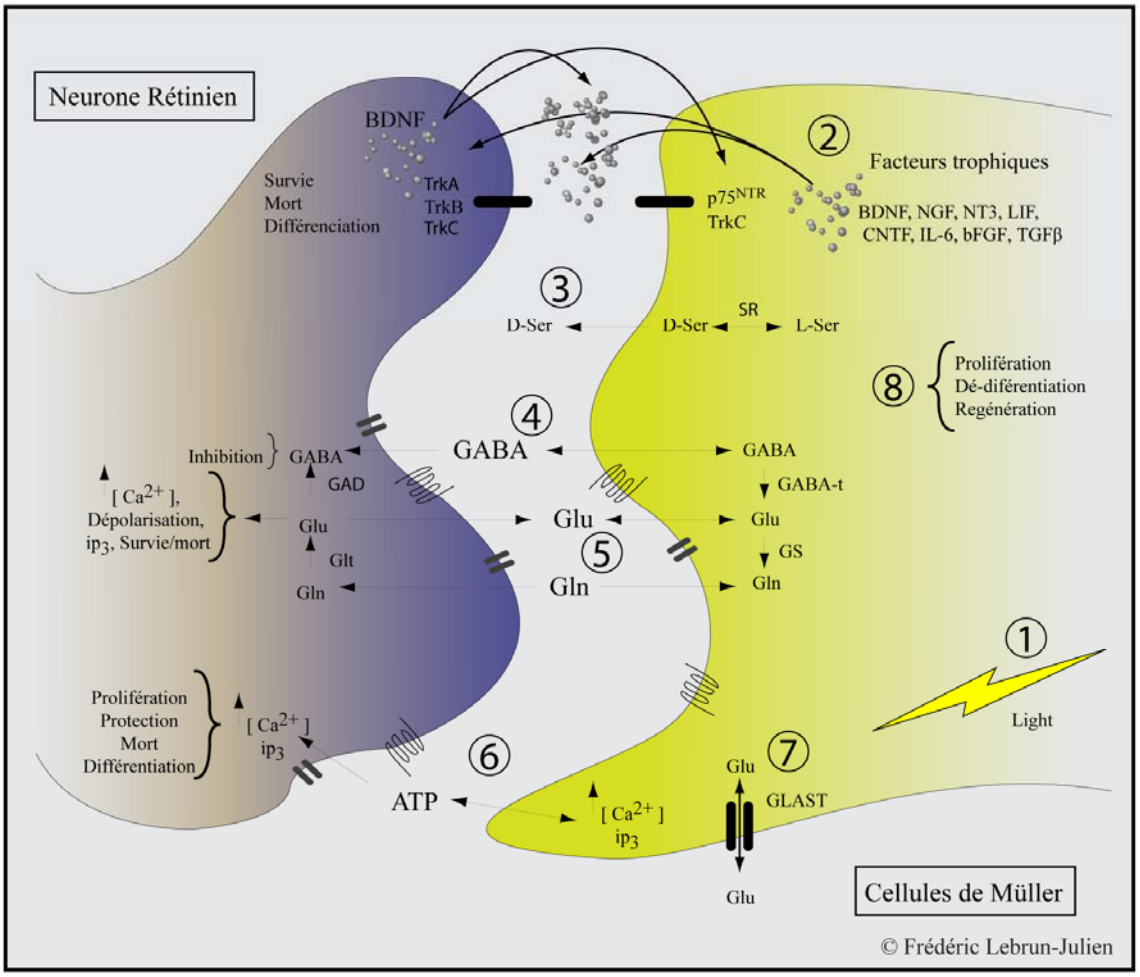
Neurone Rétinien
BDNF
Survie
Mort
Différenciation
TrkA
TrkB
TrkC
p75NTR
TrkC
2
Facteurs trophiques
BDNF, NGF, NT3, LIF,
CNTF, IL-6, bFGF, TGFβ
3
D-Ser
D-Ser
SR
L-Ser
8
Prolifération
Dé-différentiation
Regénération
4
Inhibition
GABA
GAD
GABA
GABA
GABA-t
[Ca2+],
Dépolarisation,
ip3, Survie/mort
Glu
Glt
Glu
Glu
GS
5
Gln
Gln
Gln
1
Light
Prolifération
Protection
Mort
Différentiation
[Ca2+]
ip3
6
ATP
[Ca2+]
ip3
Glu
7
GLAST
Glu
Cellules de Müller
© Frédéric Lebrun-Julien

Figure 3. Les interactions neurone-glie des cellules de Müller. (1) Les cellules de Müller servent de fibre optique transmettant la lumière efficacement vers les photorécepteurs. (2) Un réseau d'échange de facteurs trophiques existe entre les cellules de Müller et les neurones rétiniens régulant plusieurs fonctions comme la survie et la différenciation. (3) La D-sérine, co-agoniste des récepteurs NMDA, est synthétisée dans les cellules de Müller par l'enzyme, *serine racemase* (SR) et peut réguler l'excitation des neurones. (4-5) Les cellules de Müller sont responsables du recyclage de neurotransmetteurs comme le GABA et le glutamate. Elles expriment des transporteurs de ces acides aminés excitateurs, mais probablement aussi certains de leurs récepteurs ionotropiques et métabotropiques. (6) Les purines jouent un rôle important dans la rétine, spécialement l'ATP. Les récepteurs purinergiques ionotropiques et métabotropiques induisent une mobilisation du Ca^{2+} et de seconds messagers dans les neurones et les cellules de Müller. (7) GLAST, le principal transporteur du glutamate est exprimé par les cellules de Müller et enlève l'excès de glutamate de la fente synaptique. (8) Les cellules de Müller sont les dernières à se différencier et peuvent se dédifférencier et proliférer par suite de dommages ou sous l'action de facteurs neurotrophiques.

1.2.3.1.1. Les cellules de Müller et l'homéostasie de la rétine

L'homéostasie de la rétine est indéniablement dépendante des cellules de Müller (Fig.4). En effet, celles-ci sont impliquées dans le métabolisme du glucose, fournissant les neurones rétiniens en nutriments comme le lactate/pyruvate pour le métabolisme oxydatif (Poitry-Yamate et al., 1995; Tsacopoulos and Magistretti, 1996). Par le fait même, elles sont aussi responsables d'éliminer les déchets produits lors des réactions métaboliques (voir section 1.2.3.1.2). Par leur contact avec les vaisseaux sanguins, elles régulent le flux sanguin (Paulson and Newman, 1987) et contribuent à la formation de la barrière hémato-rétinienne (Tout et al., 1993). Elles maintiennent l'homéostasie de l'eau en éliminant le surplus dans le système vasculaire, l'humeur vitrée et l'espace sous-rétinien. La régulation des ions dans la rétine dépend aussi des cellules de Müller, qui contrôlent par le fait même le pH à l'intérieur de la rétine (Newman and Reichenbach, 1996; Bringmann et al., 2009). Les cellules de Müller sont principalement responsables de la régulation des ions potassiques (K^+). Elles tamponnent les ions K^+ dans l'espace intercellulaire, ce qui permet de contrecarrer les changements locaux de concentration du K^+ extracellulaire associés à l'activité neuronale (Newman et al., 1984; Reichenbach et al., 1992). Impliquées dans le recyclage des photopigments, les cellules de Müller expriment la protéine cellulaire de liaison du rétinaldéhyde (CRALBP), qui lie le *all*-transrétinal, le convertit en 11-*cis*-retinol, et le relâche dans l'espace extracellulaire où il est récupéré par les cônes. Les cellules de Müller relâchent aussi des facteurs comme la D-Sérine et le glutamate (voir section 1.2.3.1.3), qui contrôlent l'excitabilité des neurones (Newman and Zahs, 1998; Stevens et al., 2003a). En fait, la D-sérine est un ligand endogène qui peut réguler le site de liaison du récepteur NMDA. Elle est synthétisée dans les cellules gliales par la sérine racémase (SR) et relâchée lors de l'activation de récepteurs NMDA (Stéphane and Jean-Pierre, 2006); cette communication neurone-glie est donc excitatrice.

Il est maintenant établi que les cellules de Müller sont une source essentielle de facteurs neurotrophiques pour les neurones rétiniens (voir section 1.4.4). Un autre aspect de l'effet neuroprotecteur des cellules de Müller vient de leur capacité à défendre la rétine des radicaux libres. Les cellules de Müller synthétisent le glutathion à partir du glutamate, de la cystéine et de la glycine (Pow and Crook, 1995). Le glutathion réduit est ensuite distribué aux neurones et agit comme extracteur des radicaux libres et des composés réactifs

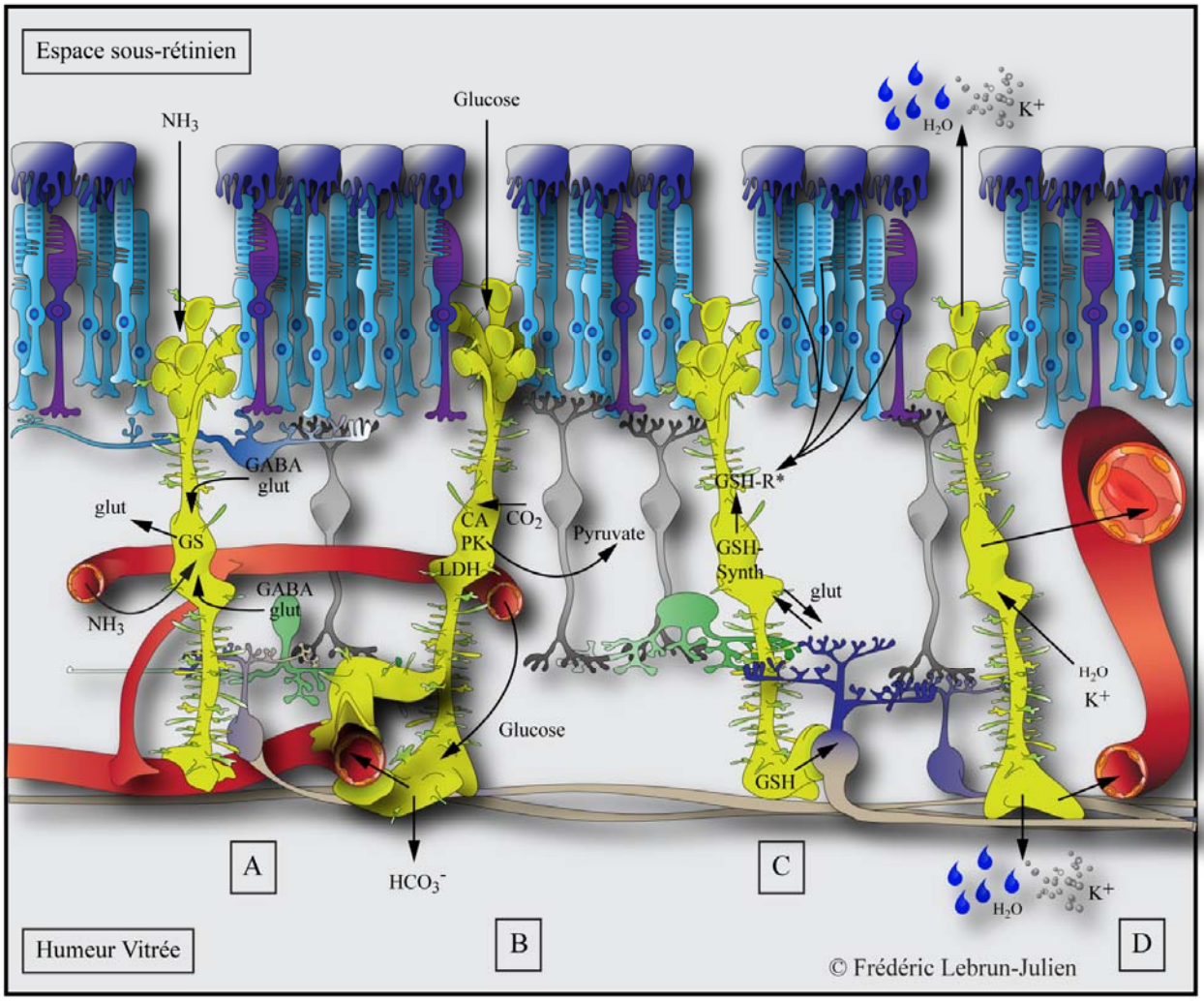
Espace sous-rétinien
NH3
Glucose
H2O
K+
GABA
glut
glut
GS
CA
CO2
PK
LDH
Pyruvate
GSH-R*
GSH-
Synth
glut
GABA
glut
NH3
H2O
K+
Glucose
GSH
A
HCO3-
C
H2O
K+
Humeur Vitrée
B
© Frédéric Lebrun-Julien
D

Figure 4. Les cellules de Müller à l'origine de l'homéostasie de la rétine. (A) Les cellules de Müller sont responsables du recyclage de neurotransmetteurs comme le glutamate (glu) et le GABA. Le GABA récupéré par les cellules de Müller est converti en glutamate qui est à son tour converti en glutamine par la glutamate synthétase (GS) et redistribué aux neurones pour servir de précurseur pour la synthèse de glutamate et de GABA. La GS est aussi responsable de la détoxification de l'ammoniaque à l'intérieur de la rétine. (B) L'interaction entre les cellules de Müller et les neurones rétiniens assure une symbiose métabolique. Par exemple, le métabolisme glycolytique des cellules de Müller produit du lactate qui est converti en pyruvate par les enzymes gliales, *lactate déshydrogénase* (LDH) et *pyruvate kinase* (PK). Le pyruvate est ensuite relâché et récupéré par les neurones pour être utilisé comme substrat pour leur cycle de Krebs. À leur tour, les cellules de Müller récupèrent le CO_2 produit par les neurones et leur enzyme, anhydrase carbonique (CA), transfère le CO_2 au HCO_3^- qui est transporté dans l'humeur vitrée ou les vaisseaux sanguins. Ce mécanisme permet aux cellules de Müller d'éliminer le CO_2 produit par les neurones en l'utilisant pour leur propre métabolisme. Ce type d'interaction est appelé 'symbiose métabolique'. (C) Les cellules de Müller sont essentielles à la défense de la rétine contre les radicaux libres (R^*). Elles synthétisent le tripeptide glutathion (GSH) à partir du glutamate, de la cystéine et de la glycine. Le glutathion réduit est fourni aux neurones et agit comme extracteurs de radicaux libres et de composés réactifs à l'oxygène. (D) Les cellules de Müller jouent un rôle crucial dans l'équilibre des ions potassiques (K^+) à l'intérieur de la rétine en tamponnant et en redistribuant les ions K^+ dans les différents espaces de la rétine, ce qui équilibre les changements locaux de concentration des ions K^+ associés à l'activité neuronale. Elles assurent aussi l'équilibre de l'eau dans la rétine.

à l'oxygène (Schütte and Werner, 1998). De plus, la glutamine synthétase (GS) des cellules de Müller, outre sa fonction essentielle dans le cycle du glutamate (voir section 1.2.3.1.3.), représente la seule enzyme disponible pour la détoxification de l'ammoniaque dans la rétine, ses substrats étant le glutamate et l'ammoniaque.

1.2.3.1.2. La symbiose métabolique des cellules de Müller et des neurones rétiniens

Le métabolisme énergétique spécialisé des cellules de Müller les rend plus résistantes que les neurones. Comme dans la plupart des cellules gliales, il y a glycolyse anaérobique même en présence suffisante d'oxygène, ce qui les rend résistantes à l'anoxie (Poitry-Yamate et al., 1995; Winkler et al., 2000). Quand l'oxygène est disponible, elles sont résistantes à l'absence de glucose, car d'autres substrats comme le lactate, le pyruvate, le glutamate ou la glutamine peuvent être métabolisés pour générer des substrats énergétiques par le cycle de Krebs (Tsacopoulos et al., 1998; Winkler et al., 2000). Ces substrats métaboliques peuvent être relâchés par les cellules gliales et recaptés par les neurones, qui les utilisent pour leur propre cycle de Krebs (Poitry-Yamate et al., 1995). Cette interaction est bénéfique à la fois pour les neurones, qui récupèrent des substrats pour leur métabolisme sans dépense d'énergie, et par les cellules gliales, qui se débarrassent de produits acides issus de leur métabolisme. Inversement, le CO^2 et l'eau générée par les neurones lors du métabolisme aérobie sont récupérés par les cellules de Müller qui les utilisent pour leur propre métabolisme via l'enzyme spécifique aux cellules gliales, l'anhydrase carbonique (Joachim, 2002). Les cellules de Müller inactivent donc un produit dangereux (CO^2) que les neurones ne peuvent eux-mêmes éliminer, tandis que les neurones fournissent un substrat pour le métabolisme des cellules gliales. Ce type d'interaction neurone-glie est appelé symbiose métabolique.

1.2.3.1.3. Le recyclage des neurotransmetteurs

Les cellules de Müller jouent un rôle primordial dans le recyclage des neurotransmetteurs à partir d'un mécanisme d'interaction neurone-glie. L'espace synaptique est rapidement nettoyé par un système de re-captage des neurotransmetteurs exprimé à la surface des cellules de Müller. Des acides aminés tels que le glutamate, l'acide γ-

aminobutirique (GABA) et la glycine (Ana et al., 2002; Vijay et al., 2005) sont ainsi régulés par les cellules de Müller. Le glutamate étant le neurotransmetteur principal dans la rétine (voir section 1.3.3.2), son élimination de la fente synaptique est essentielle au fonctionnement normal de la synapse excitatrice et à la prévention de la neurotoxicité (Barnett and Pow, 2000). L'élimination du glutamate des sites extracellulaires dans la rétine implique au moins cinq transporteurs d'acides aminés excitateurs (EAAT1-5) (Kanai and Hediger, 2004). Les cellules de Müller expriment le transporteur glutamate/aspartate dépendant du sodium, GLAST ou EAAT1 (Otori et al., 1994), lequel est le transporteur prédominant pour l'élimination du glutamate dans la rétine (Harada et al., 1998). Un mauvais fonctionnement de ce transporteur dans les cellules de Müller a pour effet d'augmenter le niveau extracellulaire de glutamate, ce qui contribue aux dysfonctionnements neuronaux et à l'apoptose dans les rétinopathies, via l'activation excessive des récepteurs ionotropiques du glutamate sur les neurones (Kashii et al., 1996; Barnett and Pow, 2000). Après avoir été capté par les cellules de Müller, le glutamate est converti en glutamine par la GS, laquelle est uniquement exprimée par les cellules de Müller (Linser and Moscona, 1979). La glutamine est ensuite redistribuée aux neurones comme précurseur pour la synthèse de glutamate et de GABA, ce qui complète le cycle glutamate-glutamine dans la rétine (Pow and Crook, 1996). Quant aux synapses GABAergiques dans la rétine, elles se retrouvent principalement dans les cellules amacrines et horizontales qui régulent latéralement le flux radial des informations transmises par les synapses glutamatergiques entre les photorécepteurs, les cellules bipolaires et les CGRs (Calaza et al., 2006). L'action GABAergique est terminée par le captage du neurotransmetteur par les transporteurs (GATs) membranaires des terminaisons neuronales présynaptiques et/ou des cellules gliales (Kanner, 2006). Finalement, outre les transporteurs des neurotransmetteurs, les cellules de Müller expriment aussi des récepteurs ionotropiques et métabotropiques GABAergiques, glutaminergiques, purinergiques, noradrénergiques, muscariniques et dopaminergiques (Newman, 2003; de Melo Reis et al., 2008) et autres. Les neurotransmetteurs peuvent donc agir également sur les cellules de Müller. Les cellules gliales ne produisent pas de potentiel d'action, cependant l'action d'un neurotransmetteur peut produire des vagues d'augmentation calcique intracellulaire qui peuvent se propager d'une cellule de Müller à une autre par libération d'ATP (Newman, 2003).

1.2.3.1.4. Le rôle des cellules de Müller dans la neuroprotection

Aux premiers stades du développement de la rétine, les cellules de Müller sont responsables de former et de maintenir l'architecture de la rétine, ensuite elles sont responsables de réguler l'homéostasie dans la rétine, de favoriser la survie des neurones et de participer à la transmission de l'information (Newman and Reichenbach, 1996). De plus, elles sécrètent une panoplie de facteurs neurotrophiques essentiels aux neurones rétiniens (voir section 1.4.4). La destruction spécifique des cellules de Müller engendre une dysplasie rétinienne, l'apoptose des photorécepteurs et la dégénérescence subséquente de la rétine (Dubois-Dauphin et al., 1999). Il est évident que les cellules de Müller sont un élément clé de la survie des neurones rétiniens. Par exemple, Harada et ses collègues ont démontré dans un modèle de dégénérescence des photorécepteurs par sur-exposition à la lumière que les cellules de Müller peuvent sécréter le *basic Fibroblast Growth Factor* (bFGF), lequel contribue à préserver la structure et la fonction des photorécepteurs (Harada et al., 2000b). De plus, cette production de bFGF peut-être modulée à la hausse par le blocage des récepteurs $p75^{NTR}$ sur les cellules de Müller, un constat qui va de pair avec les conclusions de cette thèse, sur lequel nous reviendrons plus en détail. En 2000, Fisher et ses collègues ont démontré qu'une perforation ou une blessure du cristallin induisait une forte survie des CGRs axotomisées (Fischer et al., 2000). Nous savons désormais que la production de CNTF serait en partie responsable de cette survie des CGRs. En effet, la production de CNTF par les cellules de Müller et les astrocytes s'accompagne d'une activation de l'activateur de transcription 3 (STAT3) dans les RGCs, une voie de signalisation découlant de l'activation des récepteurs du CNTF (Muller et al., 2007). Finalement, les cellules de Müller pourraient aussi constituer une cible idéale pour la thérapie génique. Ainsi, le groupe du Dr. Albert J. Aguayo a déjà utilisé un vecteur adénovirus contenant le gène du BDNF pour obtenir une infection et une expression sélective du BDNF par les cellules de Müller, laquelle a temporairement augmenté la survie de CGRs axotomixées (Di Polo et al., 1998b).

1.2.3.1.5. La gliose des cellules de Müller

Contrairement aux neurones davantage susceptibles aux différentes conditions de stress, les cellules de Müller sont plus résistantes à certaines conditions pathologiques comme l'ischémie (Silver et al., 1997). En survivant mieux à la plupart des blessures de la rétine,

elles représentent un acteur disponible et influant lors d'évènements pathologiques. Par exemple, en conditions pathologiques, les cellules de Müller peuvent produire des cytokines pro-inflammatoires (Caspi and Roberge, 1989; Roberge et al., 1991) ou phagocyter des fragments de cellules ou des corps étrangers (Mano and Puro, 1990; Stolzenburg et al., 1992; Francke et al., 2001). En fait, les cellules de Müller deviennent réactives en réponse à presque toute forme d'altération de la rétine. Cette réaction s'appelle la gliose des cellules de Müller. La gliose des cellules de Müller peut être une réponse spécifique ou non-spécifique. Par exemple, la sur-expression de la protéine GFAP est une des réponses considérée comme non-spécifique et utilisée comme marqueur de blessure de la rétine (Bignami and Dahl, 1979a; Eisenfeld et al., 1984). Inversement, l'expression de la GS dans les cellules de Müller peut être spécifiquement modulée à la baisse ou à la hausse selon la rétinopathie en cause (Reichenbach et al., 1995). Par suite de section du nerf optique, il y a translocation de la GS des cellules de Müller vers les prolongements entourant les CGRs, où la lésion des CGRs peut entraîner un relâchement excessif de glutamate (Hao and Arthur, 2002).

La gliose des cellules de Müller peut être considérée comme à la fois bénéfique et néfaste pour les neurones (Bringmann and Reichenbach, 2001). De façon précoce, la gliose peut contrecarrer les effets d'une blessure en sécrétant des facteurs trophiques et des anti-oxydants qui protègent les tissus (Schütte and Werner, 1998; Honjo et al., 2000). Néanmoins, d'autres facteurs relâchés par les cellules de Müller activées, tel que le VEGF, peuvent au contraire exacerber la lésion en induisant une néovascularisation et un dysfonctionnement de la vascularisation existante (Bringmann et al., 2006a). Lors de l'ischémie, la gliose des cellules de Müller s'accompagnent d'une augmentation de l'expression de l'oxyde nitrique synthétase et de la formation d'oxyde nitrique (NO) (David et al., 1994; El-Asrar et al., 2001). Le NO peut-être bénéfique en contrecarrant l'ischémie par dilatation des vaisseaux sanguins, et protéger les neurones de la toxicité du glutamate en causant la fermeture des récepteurs à canaux ioniques NMDA (Kashii et al., 1996); mais il peut éventuellement devenir cytotoxique pour les neurones via la formation de radicaux libres (Goureau et al., 1999).

Finalement, l'activation des cellules de Müller par suite d'une lésion peut entraîner une dé-différenciation de ces cellules. Il s'en suit un changement de la conductance de la membrane des cellules de Müller affectant l'interaction neurone-glie (Bringmann et al., 2000). Dans les cas plus prononcés de gliose, la dé-différenciation des cellules contribue à la

mort neuronale en conséquence d'un dysfonctionnement du recyclage des neurotransmetteurs et un dérèglement de l'homéostasie des ions et de l'eau par suite de sous-régulation des canaux potassiques. Bref, la dé-différenciation cause un dysfonctionnement général de tous les mécanismes vitaux sous la gouverne des cellules de Müller. Cependant, cette dé-différenciation peut être aussi causée par l'action de facteurs neurotrophiques comme le FGF-2, qui provoque également une prolifération (Fischer and Reh, 2001; Andy and Thomas, 2003). Cette capacité des cellules de Müller laisse présager qu'elles pourraient également être une source d'éléments contribuant à la régénération neuronale. L'ensemble de ces données montrent clairement qu'il est essentiel de mieux comprendre le phénomène de gliose des cellules de Müller et ses effets protecteurs ou dévastateurs dans les cas de blessure ou de rétinopathie.

1.2.4. Les paradigmes expérimentaux de section du nerf optique

1.2.4.1. Le modèle de l'axotomie

Un élément essentiel du système visuel est le nerf optique, véritable câble responsable de la transmission de l'information visuelle de la rétine au cerveau. Une maladie comme le glaucome, où il y a augmentation de la pression intraoculaire, peut endommager le nerf optique et compromettre la vision. Le glaucome est la première cause de cécité irréversible dans le monde et touche principalement les cellules ganglionnaires. Le mécanisme responsable de la mort des CGRs dans le glaucome est mal connu, mais plusieurs données suggèrent que la mort neuronale dans cette maladie survient par apoptose (Li et al., 1999; Quigley, 1999a). L'une des hypothèses de cette mort neuronale est que l'élévation de la pression intraoculaire cause un blocage du transport axonal rétrograde des neurotrophines par les CGRs. Afin de mieux comprendre les mécanismes de mort des CGRs, plusieurs modèles ont vu le jour. Depuis déjà quelques années, suite aux travaux d'Albert J. Aguayo et ses collaborateurs, la blessure du nerf optique est utilisée comme modèle de référence pour étudier les mécanismes dégénératifs de la mort neuronale dans le système nerveux central (Aguayo et al., 1987). La section des axones des cellules ganglionnaires de l'œil entraîne une mort progressive par apoptose des CGRs (Garcia-Valenzuela et al., 1995a). L'œil donne un accès privilégié à ces neurones du SNC et l'injection de composés dans l'humeur vitrée

permet de tester leurs effets sur la survie des CGRs. Le système visuel s'impose donc comme un terrain fertile pour étudier la survie neuronale *in vivo*.

L'axotomie est bien caractérisée aussi bien au point de vue des mécanismes et des facteurs impliqués dans la régulation de la dégénérescence des CGRs que dans la chronologie de leur mort. Malgré la grande variété des pathologies et des types de lésion, les mécanismes de mort amenant à l'apoptose des CGRs devraient être communs à plusieurs de ces situations (Nickells, 1996; Cellerino et al., 2000; Levin and Gordon, 2002; Watanabe and Fukuda, 2002). Les traitements neuroprotecteurs établis pour un type de blessure des CGRs comme l'axotomie pourraient s'avérer utiles dans d'autres circonstances comme l'ischémie rétinienne ou le glaucome. De plus, de nombreuses données expérimentales suggèrent que les principes de neuroprotection provenant de l'étude de la mort des CGRs après section du nerf optique puissent aussi s'appliquer à d'autres maladies du SNC comme les maladies neurodégénératives chroniques et les ischémies cérébrales aiguës (Isenmann et al., 2003). Ceci fait donc de l'axotomie un modèle de choix pour mieux comprendre les pathologies neuronales du SNC.

1.2.4.2. La section du nerf optique et le patron de mort des cellules ganglionnaires

Chez les mammifères, une blessure des neurones du SNC entraîne une mort par apoptose ou par nécrose et souvent, les deux phénomènes se superposent. L'interruption d'un axone reliant le corps cellulaire d'un neurone à sa cible empêche le transport rétrograde de facteurs neurotrophiques requis pour la survie (Oppenheim, 1991). Le patron de mort des CGRs suite à l'axotomie est très bien défini. En fait, il a été démontré qu'une rétine de rat adulte Sprague Dawley contient approximativement 100 000-105 000 RGCs, distribués sur une surface d'un peu plus de 50 mm^2, ce qui donne une densité autour de 2000-2100CGRs/mm2 (Mansour-Robaey et al., 1994a). Chez le rat, la section du nerf optique à environ 2 mm de la rétine entraîne une mort progressive des CGRs. Les cellules commencent à disparaître après 5 jours (Villegas-Perez et al., 1988; Mansour-Robaey et al., 1994a). Ensuite la mort est très rapide. Environ 50% des CGRs sont mortes après une semaine et elles ne sont plus que 5-10% de survivantes après deux semaines. La mort par suite d'axotomie est principalement apoptotique (Berkelaar et al., 1994a; Garcia-Valenzuela et al., 1994). L'atrophie des CGRs

en absence de réponse inflammatoire et la fragmentation des brins d'acide désoxyribonucléique (ADN) ont été démontrés sur des rétines étalées et des coupes de rétine avec la technique de TUNEL (Gavrieli et al., 1992a). Cependant, quelques CGRs pourraient mourir par nécrose à la suite des dommages mécaniques ou inflammatoires causés par l'axotomie (Thanos et al., 1993; Cui and Harvey, 1995; Isenmann et al., 1997b).

Chez les rongeurs, le système rétino-colliculaire facilite le marquage spécifique des CGRs. Les CGRs peuvent être marquées de façon rétrograde par l'application de Fluorogold (FG) à la surface des collicules supérieurs (Fig.5). De plus, le nerf optique des rongeurs est facilement accessible par chirurgie. Il peut être sectionné par des techniques microchirurgicales, sous contrôle visuel d'un microscope. Il est ensuite facile de venir injecter dans l'humeur vitrée des composés tels que des facteurs neurotrophiques ou des agents excitotoxique comme le NMDA, qui peuvent diffuser dans la rétine (Vidal-Sanz et al., 1988; Villegas-Perez et al., 1993). Le marquage des CGRs permet une analyse quantitative de leur survie sur des rétines complètes et étalées ou des analyses histochimiques sur des coupes transversales de rétine (Fig.6).

La plupart des données sur la mort des CGRs axotomixées proviennent du rat. Les études chez la souris sont très rares, malgré l'intérêt que représente les souris transgéniques et les souris *knockout*. Il y plusieurs raisons qui expliquent le petit nombre d'études chez la souris, dont la plus grande difficulté d'intervenir chirurgicalement sur le nerf optique d'une souris comparativement à celui d'un rat. De plus, il faut tenir compte compte des variations importantes du nombre des CGRs entre les différentes souches de souris (Williams et al., 1996). Le modèle souris offre néanmoins des avantages inapréciables lorsqu'il s'agit de déterminer le rôle particulier de certaines protéines.

1.2.4.3. La réaction des cellules gliales après une lésion du nerf optique

Il y a activation des cellules gliales de la rétine dans pratiquement tous les processus pathologiques incluant l'axotomie, le glaucome, l'ischémie et la micro-lésion du nerf optique. Tel que mentionné précédemment, ce phénomène est appelé la gliose réactionnelle. La réponse des astrocytes est généralement associée à la formation d'une cicatrice, qui fait obstacle à la régénération axonale dans le SNC. La cicatrice gliale produite par

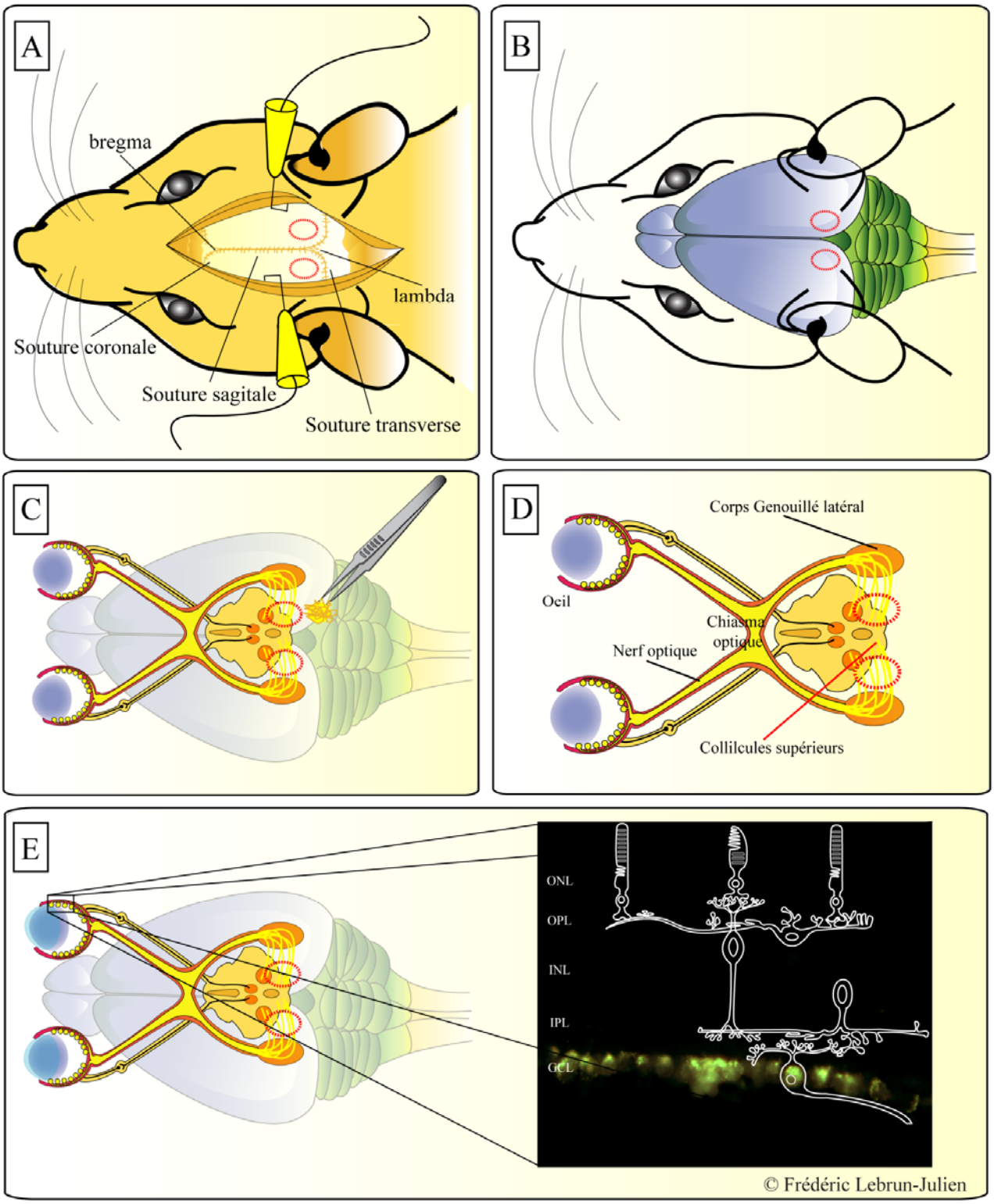
A
bregma
lambda
Souture coronale
Souture sagitale
Souture transverse
B
C
D
Corps Genouillé latéral
Oeil
Chiasma optique
Nerf optique
Collilcules supérieurs
E
ONL
OPL
INL
IPL
GCL
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Figure 5. Le marquage rétrograde des cellules ganglionnaires de la rétine. (A) La peau et les muscles doivent être écartés pour exposer le crâne et les sutures osseuses(sagittale, coronale et transverse). (B) Des trous doivent être percés à 0,5 mm de chaque côté de la suture sagittale, à 0.5 mm devant la suture transverse. La matière cérébrale est aspirée jusqu'aux collicules supérieurs. (C) Le trou est rempli de Gelfoam imbibé de Fluorogold (FG) au dessus des collicules supérieurs. (D) Représentation du système rétino-colliculaire. Chez le rat, environ 98% des CGRs se dirigent vers le collicule supérieur (CS) contralatéral. (E) Coupe transversale d'une rétine dont les CGRs sont marquées au FG. L'application de FG à la surface des collicules supérieurs permet de marquer spécifiquement une grande majorité des CGRs, qui peuvent alors être visualisées en microscopie à fluorescence.

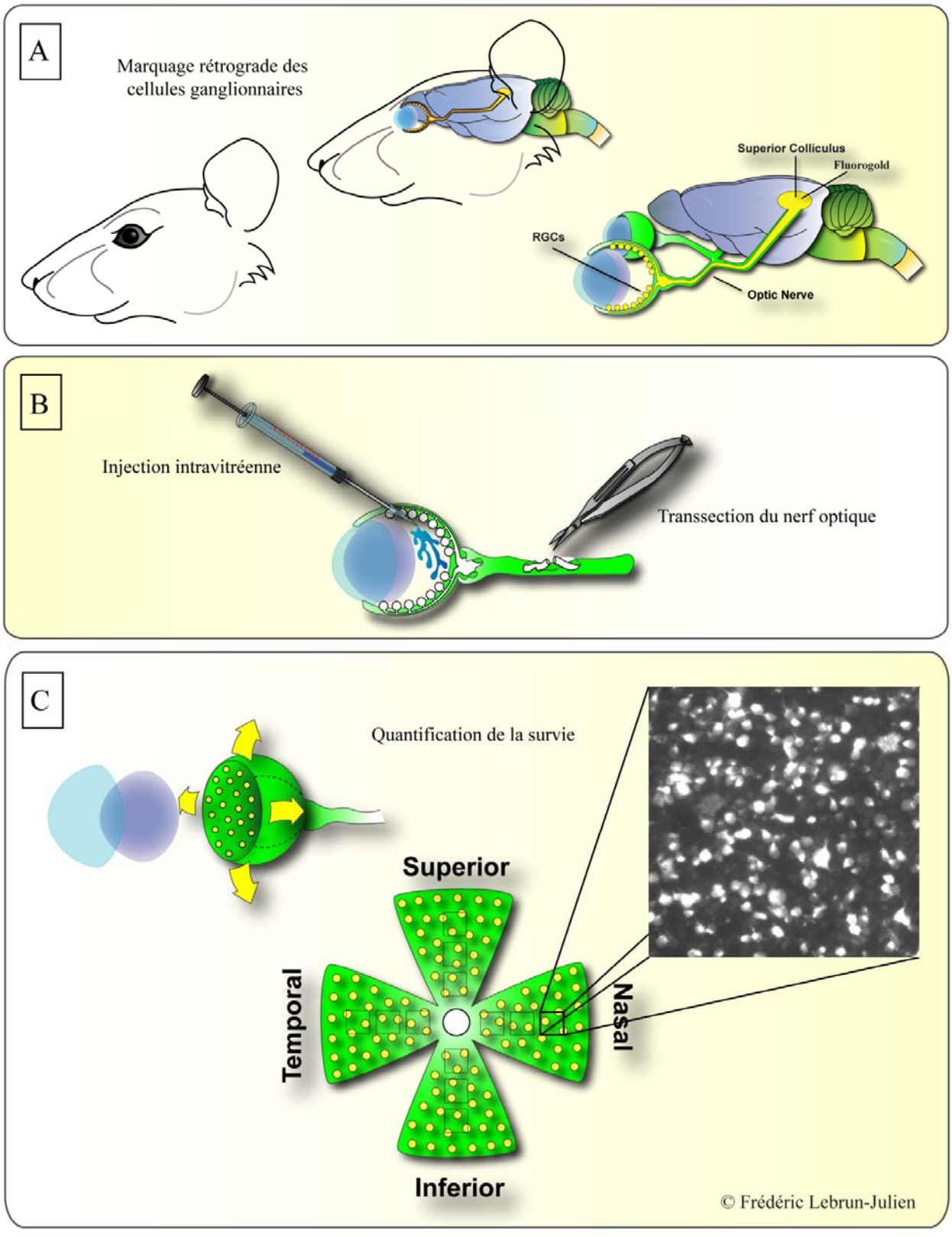
A
Marquage rétrograde des cellules ganglionnaires
Superior Colliculus
Fluorogold
RGCs
Optic Nerve
B
Injection intravitréenne
Transsection du nerf optique
C
Quantification de la survie
Superior
Temporal
Nasal
Inferior
© Frédéric Lebrun-Julien

Figure 6. Le protocole expérimental d'étude de la survie après axotomie.

(A) Les cellules ganglionnaires sont préalablement marquées de façon rétrograde avec du Fluorogold (FG) une semaine avant toute autre procédure. (B) Le nerf optique est précautionneusement exposé hors de sa gaine tout en évitant de toucher à l'artère ophtalmique. Il est ensuite sectionné à environ 1 mm de la rétine avec des micro-ciseaux et délicatement replacé dans sa position initiale. Ensuite, la sclérotique est délicatement percée avec le bout d'une aiguille afin d'injecter les composés d'intérêt dans l'humeur vitrée avec une seringue angulée à 45°. Cet angle d'injection évite toute blessure de l'iris ou du cristallin. (C) Entre 1 et 2 semaines après l'axotomie, les animaux sont euthanasiés et les cellules ganglionnaires marquées au FG ayant survécu sont visualisées en microscopie à fluorescence sur des rétines étalées. Les CGRs sont quantifiées sur 3 grilles placées à 1, 2 et 3 mm de la papille optique du rat à la surface de chacun des quatre cadrans (supérieur, temporal, inférieur, nasal) de la rétine pour un total de 12 grilles. La surface de rétine échantillonnée par les 12 grilles donne le nombre de CGRs pour 1 mm^2. Chez la souris, la quantification se fait comme chez le rat, mais les grilles sont placées à 0,25; 0,625; et 1mm de la papille optique.

les astrocytes est considérée comme une cause importante de l'absence de régénération axonale dans le SNC par rapport au SNP (Dezawa and Adachi-Usami, 2000). Les macrophages, normalement rares dans la rétine saine (Garcia-Valenzuela and Sharma, 1999; Leon et al., 2000b), stimulent la croissance axonale et la survie neuronale après micro-lésion du nerf optique en sécrétant entre autre l'oncomoduline (Leon et al., 2000b; Cui et al., 2009). Après l'axotomie, les cellules microgliales prolifèrent et leur état d'activation devient reconnaissable par leur forme amiboïde. L'activation de la microglie est proportionnelle au taux de mort des CGRs lésées et contribue à la phagocytose de leurs débris (Thanos et al., 1993; Fischer et al., 2000). Les cellules de Müller participant également à ces phénomènes pathologiques (voir section 1.2.3.1.5). Leur rôle exact dans les processus dégénératifs est encore obscur, mais depuis plusieurs années une multitude d'études ont fais état de l'expression *de novo* de la protéine GFAP par suite d'axotomie (Huxlin et al., 1995), lors du glaucome (Tanihara et al., 1997; Kim et al., 1998; Kanamori et al., 2005), après décollement de la rétine (Erickson et al., 1987; Okada et al., 1990), ou lors de dommages lumineux (Grosche et al., 1995) ou de lésions mécaniques (Bignami and Dahl, 1979b). Habituellement associée aux astrocytes, la protéine GFAP est un filament intermédiaire qui est peu exprimé dans la rétine intacte par les cellules de Müller. Bien que sa fonction ne soit pas bien comprise dans la gliose réactive, la GFAP pourrait jouer un rôle dans le remaniement des prolongements des cellules de Müller. Il est aussi connu que, sous l'action d'un stimulus de stress, les cellules de Müller peuvent relâcher des facteurs neurotrophiques comme le CNTF (Cao et al., 1997), et probablement plusieurs autres.

1.3. Les mécanismes de mort cellulaire dans le systeme nerveux central

1.3.1. L'apoptose

La mort cellulaire programmée ou apoptose vient du mot grec qui signifie "chute de pétales ou de feuilles" (Kerr et al., 1972). Ce terme fait référence à un processus naturel de mort cellulaire tout comme la perte de feuilles pour un arbre. L'apoptose est une forme physiologique de mort cellulaire hautement régulée qui est nécessaire tant au cours du développement que lors de processus pathologiques. C'est un mécanisme d'autodestruction des cellules lorsque celles-ci ne sont plus utiles, qu'elles sont endommagées ou dysfonctionnelles. Les premières manifestations morphologiques se caractérisent par une

compaction de la chromatine nucléaire, une convolution des membranes nucléaires et cytoplasmique, une condensation du cytoplasme. Le noyau se fragmente ensuite, chaque fragment étant entouré d'une double enveloppe. Des corps apoptotiques sont ensuite relâchés pour être phagocytés par les cellules voisines, la microglie ou les macrophages, sans aucune réaction inflammatoire (Elmore, 2007).

1.3.1.1. Les caspases

C'est chez le nématode *Caenorhabditis elegans* (C.elegans) que l'existence de la mort cellulaire programmée a d'abord été mise en évidence. Chez C.elegans, on retrouve 4 protéines-clés: Ced-3, une cystéine protéase à substrat aspartate (caspase), synthétisée sous forme de précurseur inactif par la cellule; Ced-4, un activateur qui se lie à Ced-3 pour produire la caspase active; Ced-9, qui interagit avec Ced-4 pour empêcher d'activer Ced-3; Egl-1, qui se lie à Ced-9 pour libérer Ced-4, qui peut alors se lier à Ced-3 pour l'activer. Des homologues de ces protéines ont été identifiés chez les mammifères : la caspase 1 est l'homologue de Ced-3, Apaf celui de Ced-4, Ced-9 a pour homologues les protéines anti-apoptotiques de la famille Bcl-2 et Egl-1 a pour homologues les protéines pro-apoptotiques de la famille Bcl-2 à domaine BH3. Les membres de la famille de Bcl-2 peuvent donc stimuler ou inhiber l'apoptose. Par exemple, l'expression de Bcl-2 et de Bcl-xL inhibe l'apoptose (Boise and Thompson, 1997), alors que celle de bax et bak la stimule (Oltvai et al., 1993; Chittenden et al., 1995; Farrow et al., 1995; Kiefer et al., 1995).

Les caspases sont une famille de protéases qui exécutent le démantèlement et la démolition des cellules apoptotiques. Cette famille comprend au moins 14 protéines différentes. Elles sont synthétisées sous forme de larges précurseurs inactifs (procaspases) et deviennent actives lorsqu'elles sont clivées, habituellement par d'autres caspases. Par conséquent, ces protéases ont été classifiées en deux grandes catégories : les caspases initiatrices (p. ex., caspases 1,2,4,5,8,9 et 10) qui activent d'autres caspases et les caspases effectrices (p. ex. 3,6,7 et 14), qui clivent des substrats spécifiques impliqués dans l'élimination de la cellule (Indrajit et al., 2008).

Il existe 2 voies principales d'induction de l'apoptose dans la cellule: la voie mitochondriale, induite par la relâche de protéines mitochondriales et la voie des récepteurs à domaine de mort, située dans la membrane plasmique. Lorsque l'apoptose est provoquée par des signaux provenant de l'extérieur, on parle d'une voie extrinsèque ou de mort sur

commande; lorsqu'elle résulte de l'absence de signaux externes qui l'ihnibent, on parle de voie intrinsèque ou de mort par défaut.

1.3.1.2. Voie des récepteurs de mort ou voie extrinsèque

La fixation d'un ligand de mort tétramérique comme le ligand FasL à son récepteur de mort (Fas) provoque la trimérisation des domaines de mort cytoplasmiques (DD) du récepteur de mort. Il y a alors recrutement de protéines adaptatrices, comme la protéine FADD, qui s'attachent via leurs domaines DD aux domaines DD des récepteurs de mort. Ces protéines recrutent à leur tour des caspases initiatrices comme la procaspase-8, via des interactions entre les domaines effecteurs de mort des protéines adaptatrices et ceux des procaspases initiatrices. Ceci induit l'auto-activation des caspases initiatrices et la formation des caspases initiatrices hétérotétramériques correspondantes (caspase-8). Les caspases initiatrices activent alors, par protéolyse, les caspases effectrices (procaspase-3) pour produire les caspases effectrices hétérotétramériques (caspase-3), qui vont catalyser des clivages protéolytiques aboutissant à l'apoptose. Les ligands de mort comme FasL appartiennent à la famille du facteur nécrosant des tumeurs, dont le membre fondateur, TNFα, a été initialement caractérisé comme une cytokine tuant les cellules tumorales en induisant l'apoptose et non pas la nécrose (Chang and Yang, 2000; Guicciardi and Gores, 2009).

1.3.1.3. Voie dépendante des mitochondries ou voie intrinsèque

Différents stimuli ou l'absence de stimuli provoquent la libération du cytochrome C par la mitochondrie, à partir de l'espace intramembranaire. Ce processus est induit par l'activation des membres pro-apoptotiques de la famille Bcl-2, telle que Bid, une fois que Bid a subi un clivage par la caspase-8 pour produire tBid ou Bad phosporylée. Ce processus est inhibé par les membres anti-apoptotiques de la famille Bcl-2, comme Bcl-2 et Bcl-x_L. Le cytochrome C libéré se fixe à Apaf-1 et forme l'apoptosome. Celui-ci se fixe à la procaspase-9, qu'il active de telle sorte qu'elle clive les procaspase effectrices comme la procaspase-3. Ces procaspases catalyseront les clivages protéolytiques entraînant l'apoptose (Chang and Yang, 2000).

1.3.1.4. L'apoptose et la neurodégénérescence des cellules ganglionnaires de la rétine.

Alors qu'un certain niveau d'apoptose est associé à l'homéostasie normale des tissus, une apoptose excessive et incontrôlée est caractéristique de la neurodégénérescence. De nombreuses données expérimentales montrent que les CGRs meurent par apoptose (Hughes and La Velle, 1975; Rager and Rager, 1978; García-Porrero et al., 1979) et que la mort cellulaire développementale pourrait être régulée de la même façon que la mort des CGRs lors d'une blessure du nerf optique. La mort apoptotique après section du nerf optique a été démontrée de plusieurs façons. Par exemple, le cytochrome-c libéré par les mitochondries peut être alors visualisé dans le cytoplasme des CGRs par immunohistochimie (Isenmann et al., 1997a; He et al., 2004). Avant même la diminution du nombre de cellules, la procaspase 3 inactive est clivée en caspase 3 active dans les CGRs (Klocker et al., 1999a; Klocker et al., 2000). Une étude a démontré que l'inhibition de la caspase-3 par des inhibiteurs protéiques permet de réduire la mort des CGRs axotomixées *in vivo* (Kermer et al., 1998a; Chaudhary et al., 1999a). De plus, la caspase-8 est exprimée par les CGRs et semble pouvoir être activée, suite au dysfonctionnement des mitochondries, indépendamment de l'activation des récepteurs de mort (Weishaupt and Bahr, 2001). Finalement, la survie des CGRs peut être stimulée en inhibant les molécules pro-apoptotiques ou en activant les molécules anti-apoptotiques. Cependant, l'inhibition ciblée des caspases ne réduit que modestement l'apoptose après lésion du nerf optique (Kermer et al., 1998a; Klocker et al., 1999a; Cheung et al., 2004). Plus spécifiquement, l'injection intraoculaire d'ARN antisens inhibant la synthèse de Bax réduit la mort des CGRs (Isenmann et al., 1999). L'utilisation d'ARN d'interférence dirigé contre Apaf-1 atténue la perte de CGRs après lésion du nerf optique (Lingor et al., 2005). Par ailleurs, si Bcl-2 est surexprimée dans les CGRs de souris transgéniques (bcl-$2^{+/+}$), l'apoptose causée par la blessure du nerf optique est fortement réduite.

L'apoptose est aussi le mécanisme principal de mort des CGRs dans plusieurs rétinopathies comme le glaucome (Garcia-Valenzuela et al., 1995a; Quigley, 1995; Quigley et al., 1995b; Hosking, 1998b; Quigley, 1999a). Les caspases initiatrices-8 et -9 sont activées dans le modèle expérimental du glaucome, alors que l'inhibition de la caspase-3 effectrices protège les CGRs (Hanninen et al., 2002a; McKinnon et al., 2002d; McKinnon et al., 2002a).

Comprendre les mécanismes déployés lors de l'apoptose est essentiel pour l'établissement des bases d'une stratégie de neuroprotection.

1.3.2. La nécrose

On distingue très nettement l'apoptose de la nécrose bien que les deux phénomènes puissent se chevaucher. La nécrose est une mort cellulaire dite accidentelle qui survient lors d'un dommage tissulaire et implique des groupes de cellules. Lors de la nécrose, la cellule devient enflée, puis la membrane cellulaire éclate déversant le contenu cellulaire dans le tissu environnant et provoquant l'inflammation. Les mitochondries et le noyau restent intacts tout au long de ce processus. Une panoplie d'enzymes est alors relâchée et peut venir affecter les cellules environnantes. Lors d'une lésion, il y a souvent un continuum entre apoptose et nécrose (Isenmann et al., 1997a; Nicotera et al., 1997).

1.3.3. L'excitotoxicité

1.3.3.1. Le concept

Le phénomène d'excitotoxicité a été rapporté pour la première fois dans la rétine. En 1957, D.R. Lucas et J.P. Newhouse se sont aperçus que du glutamate monosodique, donné en nourriture aux souriceaux, détruisait les neurones de la rétine (Lucas and Newhouse, 1957). C'est une décennie plus tard que John Olney démontra que le phénomène n'était pas limité à la rétine, mais concernait tout le cerveau. Il détermina également que les neurones post-synaptiques étaient les cibles de cette mort cellulaire. Les dendrites des neurones cibles étaient gonflées tandis que les terminaisons pré- synaptiques étaient épargnées (Olney, 1969). Il démontra que ces effets étaient annulés par l'ajout d'antagonistes des récepteurs du glutamate et avança donc l'hypothèse que le glutamate détruisait les neurones par un mécanisme semblable à celui mis en jeu aux synapses glutamatergiques excitatrices, d'où le nom d'excitotoxicité.

L'excitotoxicité désigne en fait le processus d'altération pathologique des neurones par une sur-activation des récepteurs du glutamate. Dans des conditions physiologiques normales, la concentration du glutamate libéré dans la fente synaptique atteint des niveaux élevés (~1 mM), mais cette valeur n'est maintenue que pendant quelques millisecondes. Si des concentrations anormalement hautes de glutamate s'accumulent dans l'espace

intercellulaire, l'activation excessive des récepteurs du glutamate provoque une entrée massive dans la cellule de calcium. Le Ca^{2+} active à son tour une plusieurs enzymes, dont des phospholipases C, des endonucléases et des protéases comme la calpaïne. Ces enzymes dégradent alors les structures cellulaires comme le cytosquelette, la membrane cellulaire et l'ADN. Diverses données suggèrent que l'excitotoxicité soit impliquée dans la plupart des maladies neurodégénératives du SNC, incluant les lésions aiguës telles que l'ischémie et les accidents vasculaires cérébraux, aussi bien que des maladies chroniques comme le Parkinson et la maladie l'Alzheimer (Obrenovitch et al., 2000; Sapolsky, 2001; Rao and Balachandran, 2002).

1.3.3.2. L'excitotoxicité dans la rétine.

Tout comme les neuropathies du SNC, les rétinopathies comme l'ischémie rétinienne, la rétinite pigmentaire, la rétinopathie diabétique et possiblement le glaucome sont associées à des dommages excitotoxiques. Le glutamate est le principal neurotransmetteur excitateur dans le système visuel. Il est, en particulier, le médiateur libéré par les photorécepteurs, et assure ainsi la transmission synaptique entre cellules bipolaires, les cellules amacrines et les cellules ganglionnaires. L'homéostasie du glutamate est largement prise en charge par les cellules de Müller, qui possèdent des transporteurs du glutamate pour récupérer le glutamate et l'enzyme glutamine synthétase qui convertit le glutamate en glutamine (Higgs and Lukasiewicz, 1999; Matsui et al., 1999). Dans la rétine, l'excès de glutamate pourrait être à l'origine des phénomènes neurodégénératifs provoquées par le glaucome ou l'occlusion de l'artère rétinienne (Hare et al., 2001a; Hare et al., 2004a; Casson, 2006a; Seki et al., 2008). En fait, la dégénérescence des CGRs dans le glaucome présente plusieurs aspects de l'excitotoxicité et de l'apoptose (Garcia-Valenzuela et al., 1995a; Martin et al., 2002). Entre autres, les CGRs sont particulièrement sensibles au glutamate (Olney, 1982; Sisk and Kuwabara, 1985; Siliprandi et al., 1992b). Chez le rat, une absorption orale prolongée de glutamate augmente le niveau de glutamate intravitréal et induit la mort des CGRs, une gliose et l'atrophie de la rétine (Ohguro et al., 2002). On sait aussi que le niveau de glutamate augmente dans la rétine par suite d'une blessure du nerf optique (Yoles and Schwartz, 1998). D'un autre côté, le blocage pharmacologique de la signalisation induite par les récepteurs métabotropiques du glutamate (mGluR) n'a pas permis d'atténuer la mort des

CGRs après axotomie ou injection de NMDA dans l'humeur vitrée (Kermer et al., 2001), ce qui démontre que le mécanisme d'excitotoxicité dépend principalement des récepteurs ionotropiques NMDA. L'entrée massive de Ca^{2+} engendrée par le NMDA active une panoplie d'enzyme dans les CGRs comme la Ca^{2+}/*calmodulin-dependent protein kianse II* (CAMKII) qui à son tour active la caspase 3 et cause le fractionnement de l'ADN (Laabich et al., 2000, 2001). Il a aussi activation de la synthase du monoxyde d'azote (NOS), qui produit du NO, neurotoxique lorsqu'il réagit avec l'anion superoxyde (O_2^-). De plus, il semble avoir activation de la p38, une protéine kinase activée dans des conditions de stress, et du NF-κB (Manabe and Lipton, 2003). Finalement, l'activation plus générale d'endonucléases, de lipases et de la calpaïne vient participer au processus apoptotique et nécrotique (Bonne et al., 1998a; Bonne et al., 1998b) (Fig.7A).

Même si les cellules de Müller sont étroitement impliquées dans le cycle du glutamate, pourtant peu d'études ont tenté de préciser le rôle de ces cellules gliales lors d'événements excitotoxiques dans la rétine. Notre groupe a cependant effectué quelques travaux sur ce sujet. Dans un modèle expérimental d'excitotoxicité au NMDA chez le rat, il a été démontré que les cellules de Müller augmentent l'expression de l'arginase I, une enzyme responsable de la synthèse des polyamines. Les polyamines lient un site spécifique sur les récepteurs NMDA et régulent leur activation. Cette étude a aussi révélé que l'inhibition de la synthèse des polyamines par les cellules de Müller est neuroprotectrice pour les CGRs (Pernet et al., 2007b). Outre la sécrétion de facteurs pouvant réguler l'activité des récepteurs ionotropiques, les cellules de Müller sont aussi responsables de réguler la concentration de neurotransmetteurs dans le milieu extracellulaire. De plus, comme nous l'avons déjà mentionné, elles expriment des récepteurs des neurotransmetteurs. Toutes ces caractéristiques portent à croire qu'elles doivent jouer un rôle important dans les mécanismes excitotoxiques afffectant la rétine.

1.3.3.3. Les récepteurs du glutamate

Il y a deux deux types de récepteurs du glutamate: les ionotropiques et les métabotropiques. Les récepteurs ionotropiques comportent deux sous-types : NMDA et non-NMDA (i.e. AMPA et Kainate). Les CGRs expriment ces deux sous-types, tout comme des récepteurs métabotropiques du glutamate (Siliprandi et al., 1992a; Rorig and Grantyn, 1993;

Matsui et al., 1998). La présence des sous-unités (NR1, NR2A-C et NR3) de récepteurs NMDA a été démontrée dans les CGRs de différents modèles d'animaux (Vandenbranden et al., 2000; Kim et al., 2007; Nakanishi et al., 2009). Les cellules de Müller expriment aussi différents types de récepteur glutamatergiques, ionotropiques et métabotropiques, sans que l'on sache lesquels précisément. Certaines études font état de la présence de récepteurs NMDA (Puro et al., 1996b; Lopez et al., 1997; Lamas et al., 2005a) et AMPA (López-Colomé et al., 1993; Lopez et al., 1994), alors que d'autres rapportent la présence de récepteur AMPA, mais non des NMDA dans les cellules de Müller isolées (Sarthy, 2001). On a également rapporté la présence de NMDA sur les oligodendrocytes et les astrocytes (Lipton, 2006). L'expression de tous ces récepteurs semble cependant présenter de grandes variations d'une espèce à une autre.

1.3.3.3.1. Le récepteur NMDA

Les récepteurs NMDA tirent leur nom de l'agoniste du glutamate, le *N*-methyl-D-aspartate. Le récepteur NMDA est un récepteur couplé à un canal ionique, les ions Na^+, K^+ et Ca^{2+} peuvent y circuler, mais c'est le calcium qu'il laisse entrer de façon privilégiée dans la cellule (MacDermott et al., 1986; Cline and Tsien, 1991). Il a été démontré que l'augmentation intracellulaire de calcium dans les neurones par suite de l'activation des récepteurs NMDA peut réguler l'activité neuronale, mais aussi causer l'excitotoxicité, si présente en excès. Au potentiel de repos, ce canal calcique est cependant bloqué par des ions magnésium (Mg^{2+}) qui empêchent l'entrée de calcium dans le neurone, même si le glutamate si fixe. Pour que les ions magnésium se retirent du canal, le potentiel membranaire de la dendrite doit être dépolarisé. Contrairement aux récepteurs AMPA (voir ci-dessous), les récepteurs NMDA sont caractérisés par une cinétique de déclenchement relativement lente; leur constante de désensibilisation est aussi plus lente (Ozawa et al., 1998). Ceci suggère que ces récepteurs sont principalement impliqués dans une neurotransmission lente et durable. La glycine est requise pour l'activation des récepteurs NMDA pour lesquels elle agit comme co-agoniste (Boje and Skolnick, 1992). La D-sérine, libéré par les cellules gliales, dont les cellules de Müller, peut aussi agir au site de la glycine et agir comme régulateur des récepteurs NMDA (Stevens et al., 2003b).

Il existe au moins cinq sous-unités du récepteur NMDA : NR1 et NR2A à NR2D (Ozawa et al., 1998). Cependant, plus récemment de nouvelles sous-unités ont été clonées, NR3A et NR3B (Cull-Candy et al., 2001). Ces sous-unités s'assemblent en différentes combinaisons pour former les hétérotétramères qui constituent le canal ionique. Dans le SNC mature, les récepteurs NMDA sont principalement formés des sous-unités NR1 et NR2A À NR2C (Dingledine et al., 1999). Les sous-unités peuvent aussi présenter différentes variantes d'épissage. Par exemple, chez le rat, NR1 peut avoir jusqu'à huit formes différentes (Durand et al., 1992; Sugihara et al., 1992). La sous-unité NR1 sert en fait de pilier fondamental aux récepteurs NMDA; sans elle le récepteur ne peut pas fonctionner. Les sous-unités NR2A-D serviraient d'unités régulatrices (Luo et al., 1997; Chazot, 2004). La sous-unité NR1 est généralement responsable de la liaison de la glycine/ D-sérine, alors que la sous-unité NR2 possède le site de liaison du glutamate (Stevens et al., 2003a) (Fig.7B). De plus, les récepteurs NMDA gliaux et neuronaux diffèrent fonctionnellement et structurellement. Par exemple, les récepteurs NMDA de la glie sont très faiblement sensibles au blocage par le Mg^{2+}, ce qui indiquerait une prédominance de l'expression de la sous-unité NR3 qui empêche le blocage par le Mg^{2+} (Verkhratsky and Kirchhoff, 2007).

1.3.3.3.2. Le récepteur AMPA

Le récepteur AMPA est un hétérotétramère constitué d'une combinaison des sous-unités GluR1 à GluR4 (Hollmann and Heinemann, 1994; Pellegrini-Giampietro et al., 1997). Il tire son nom de l'analogue du glutamate, l'acide α-amino-3-hydroxy-5-methylisoxazole-4-propionic (AMPA) (Fig.7C). La plupart des récepteurs AMPA sont fortement perméables au Na^+ et au K^+, ce qui leur permet d'effectuer une neurotransmission glutamatergique rapide. Lorsque le glutamate se fixe au récepteur AMPA, l'entrée de sodium dans le neurone post-synaptique entraine une dépolarisation locale de la dendrite et, si cette dépolarisation atteint le seuil de déclenchement du potentiel d'action, l'influx nerveux se propage au neurone suivant. Contrairement au récepteur NMDA, une activation prolongée est nécessaire (~60 min) pour entraîner la mort neuronale (Koh et al., 1990). La majorité des neurones expriment des récepteurs AMPA contenant la sous-unité GluR2, ce qui les rend imperméables au Ca^{2+}. Les cellules contenant des récepteurs AMPA sans la GluR2 sont plus sensibles à l'excitotoxicité. Par exemple, les neurones contenant la NADPH-

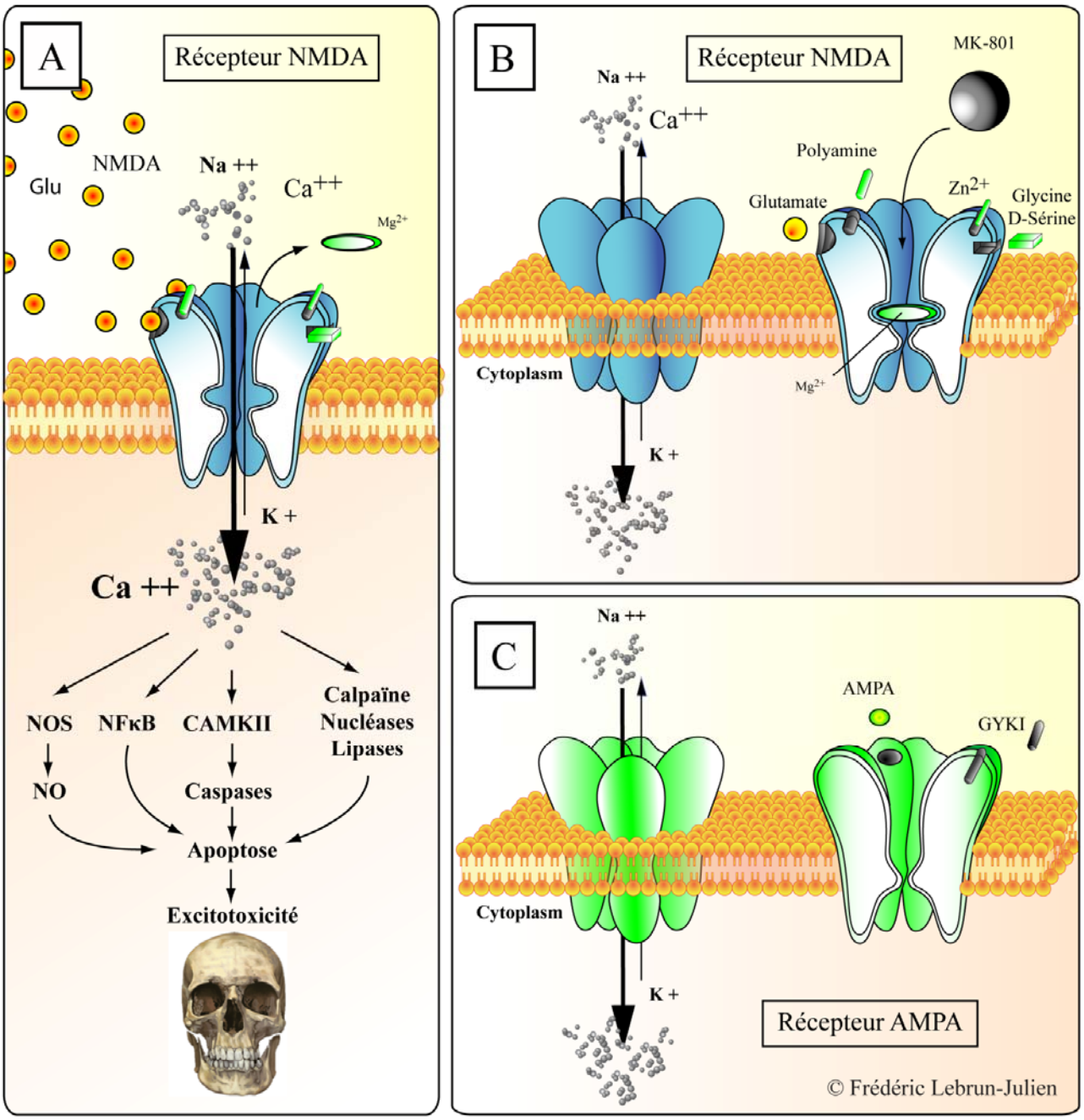
A
Récepteur NMDA
Glu
NMDA
Na ++
Ca++
Mg2+
K +
Ca ++
NOS
NFκB
CAMKII
Calpaïne
Nucléases
Lipases
NO
Caspases
Apoptose
Excitotoxicité
B
Récepteur NMDA
MK-801
Na ++
Ca++
Polyamine
Glutamate
Zn2+
Glycine
D-Sérine
Cytoplasm
Mg2+
K +
C
Na ++
AMPA
GYKI
Cytoplasm
K +
Récepteur AMPA
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Figure 7. Les mécanismes d'excitotoxicité dans les cellules ganglionnaires de la rétine. (A) L'hypothèse principale dérière la théorie de l'excitotoxicité est que la sur-activation des récepteurs NMDA entraîne une entrée massive de calcium. L'influx massif de calcium dans la cellule provoque l'activation d'une panoplie d'enzymes et la production de molécules oxydantes qui stimulent l'apoptose. (B) Le récepteur NMDA est le récepteur principal impliqué dans l'excitotoxicité. Le récepteur NMDA est un récepteur du glutamate ionotropique qui tire son nom de son agoniste sélectif, le *N*-methyl *D*-aspartate (NMDA). Il permet l'entré de sodium et la sortie de potassium, mais c'est le calcium qu'il laisse entrer de façon privilégiée dans la cellule. Au potentiel de repos, ce canal calcique est cependant bloqué par des ions magnésium (Mg^{2+}) qui, même si le glutamate s'y fixe, empêche l'entrée de calcium dans le neurone. Pour que ces ions se retirent du canal, le potentiel membranaire du dendrite doit être dépolarisé. Plusieurs molécules peuvent moduler l'activité du récepteur NMDA, dont les les polyamines et la glycine/D-sérine. Il est possible d'inhiber son activité en utilisant le MK-801, qui vient bloquer le pore ionique. (C) Le récepteur AMPA est également un récepteur du glutamate couplé à un canal ionique et qui laisse entrer le sodium et sortir le potassium. Par contre, ce récepteur est imperméable au calcium. Il est spécifiquement activé par le α-amino-3-hydroxy-5-méthylisoazol-4-propionate (AMPA) et son activation ne nécessite pas la présence d'un co-agoniste. Son activité peut être bloquée en utilisant un antagoniste non-compétitif, comme le GYKI.

diaphorase du cortex et du striatum expriment des récepteurs AMPA perméables au Ca^{2+}, et peuvent entrer en dégénérescence par suite d'une brève exposition (~10 min) au AMPA (Weiss et al., 1994). La présence de la sous-unité GluR2 dans les récepteurs AMPA minimise la participation de ces récepteurs aux phénomènes d'excitotoxicité. L'injection intraoculaire d'AMPA chez le rat montre que les neurones rétiniens sont plutôt résistants à ce composé (Andrés et al., 2003). Il est possible inhiber les récepteurs AMPA avec des antagonistes non-compétitifs, comme le GYKI (1-[4-aminophenyl]-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine).

1.3.3.3.3. Le récepteur Kainate

Les récepteurs Kaïnate sont des récepteurs ionotropes qui réagissent aussi bien au glutamate, qui est leur ligand physiologique, qu'au kaïnate,qui est un médicament isolé de l'algue rouge *Digenea simplex*. Le canal ionique qu'ils forment est perméable aux ions sodium et potassium. Comparativement aux récepteurs AMPA et NMDA, les récepteurs Kainate sont plus fréquemment présynaptiques. Leur structure est similaire à celle des récepteurs AMPA, avec les sous-unités GluR5, 6 et 7 qui peuvent former des homotétramères, et les sous-unités KA1 et 2 qui forment des hétérotétramères avec l'une des trois autres sous-unités (Kessels and Malinow, 2009). Les récepteurs Kainate ont une distribution limitée dans le cerveau, et leur fonction est mal définie, bien qu'ils aient été impliqués dans l'épileptogénèse, la transduction sensorielle, et la plasticité synaptique. On les retrouve également en position post-synaptique. L'injection intravitréenne de Kaïnate chez le rat détruit spécifiquement les cellules amacrines gabaergiques et sérotoninergiques (Osborne et al., 1995).

1.3.3.3.4. Les récepteur métabotropiques

Les récepteurs métabotropiques du glutamate sont des protéines transmembranaires avec sept segments hydrophobes, couplés aux protéines G hétérotrimériques (RCPG). Les mGluRs sont classés en trois groupes, selon leur homologie de séquence, leur pharmacologie et la voie de signalisation à laquelle ils sont couplés. Les récepteurs mGluR1 et mGluR5 font partie du premier groupe. Ils sont couplés à la phospholipase C, via des protéines G de type Gq. Le groupe 2 inclut les récepteurs mGluR2 et mGluR3, et le groupe 3 les récepteurs

mGluR4, mGluR6, mGluR7 et mGluR8. Tous les mGluRs du groupe 2 et 3 sont couplés négativement à l'adénylate cyclase via une protéine G de type Gi/Go. La caractéristique structurale des mGluR est la présence du site de fixation du ligand dans un large domaine N terminal extracellulaire, rattaché par un domaine riche en cystéines au domaine transmembranaire formé de sept hélices. Les mGluRs possèdent également un domaine C terminal intracellulaire plus ou moins long selon les variants d'épissage (De Blasi et al., 2001). Comme de nombreux récepteurs couplés aux protéines G, les mGluRs forment des homodimères (Romano et al., 1996). Fonctionnellement, les mGluRs régulent et participent à la transmission synaptique par le biais de divers effecteurs comme la protéine kinase C (PKC), les récepteurs à l'inositol tri-phosphate, ou les canaux ioniques membranaires. Les mGluRs des groupes II and III modulent la libération de glutamate en agissant comme auto-récepteurs pré-synaptiques de manière homo ou hétéro-synaptique, alors que les mGluRs du groupe I sont principalement post-synaptiques (Coutinho and Knopfel, 2002).

Les mGluR sont connus pour réguler l'activité d'autres récepteurs comme les récpteurs NMDA (Skeberdis et al., 2001; Lea et al., 2002). Ils peuvent donc influencer la vulnérabilité des neurones à l'excitotoxicité. Par exemple, les mGluR du type I peuvent amplifier les dommages aux neurones causés par le NMDA (Bruno et al., 1995), tandis que les mGluR de type II et III tendent à protéger les neurones de l'excitotoxicité (Ambrosini et al., 1995; Faden et al., 1997). Comme les autres récepteurs du glutamate, les mGluR peuvent être impliqués dans la plasticité synaptique et la neurotoxicité (Endoh, 2004; Baskys et al., 2005). De plus, le groupe du Dr. Manev a démontré que l'activation des mGluR protège la rétine de la toxicité du NMDA (Siliprandi et al., 1992a). La variété des rôles tenus par les récepteurs métabotropiques du glutamate se réflète dans la diversité de leur localisation autour de la synapse glutamatergique et sur les cellules gliales.

1.3.3.4. Le modèle d'excitotoxicité au NMDA

L'effet toxique du glutamate peut être reproduit expérimentalement en utilisant une forte concentration de l'agoniste du glutamate, le NMDA (Fig.7A). L'injection intraoculaire de NMDA peut reproduire les conditions pathologiques où la concentration excessive de glutamate sur-active ses ionotropiques. L'application de NMDA a été testée dans plusieurs modèles *in vitro* et *in vivo* (Hahn et al., 1988; Vorwerk et al., 1996; Sucher et al., 1997; Lam

et al., 1999). La neurodégénérescence induite expérimentalement par l'excitotoxicité des récepteurs NMDA ou non-NMDA conduit à une mort neuronale en continuum apoptose-nécrose, où le phénotype de mort neuronale est influencé par le niveau de maturation du cerveau et les types de récepteurs stimulés (Martin et al., 1998). Des ces modèles expérimentaux, l'activation des récepteurs NMDA peut-être régulée par l'ajout d'antagonistes comme le maléate de dizocilpine (MK-801) et la mémantine (Hahn et al., 1988; Osborne et al., 1996; Lam et al., 1997). Le MK-801 est un antagoniste non-compétitif du NMDA qui se lie à l'intérieur du canal ionique empêchant tout échange avec l'extérieur (Coan et al., 1987). Cependant, le blocage des récepteurs NMDA par le MK-801 est loin de représenter une solution miracle, car plusieurs groupes ont montré que l'inhibition complète des récepteurs NMDA entraîne la mort des neurones (Ikonomidou et al., 1999; Ikonomidou et al., 2000; Hansen et al., 2004), du fait que l'activité de ces récepteurs est essentielle au fonctionnement cellulaire. De plus, l'utilisation clinique d'antagonistes des récepteurs NMDA peut entraîner plusieurs effets secondaires, comme des hallucinations, une augmentation de la pression sanguine et même un état catatonique et l'anesthésie (Kemp and McKernan, 2002a).

1.4. Les facteurs neurotrophiques

1.4.1. La théorie des facteurs neurotrophiques

Au début des années 1950, Rita Levi-Montalcini en collaboration avec Stanley Cohen faisait la découverte du *Nerve Growth Factor* (NGF) (Levi-Montalcini, 1987), la première protéine d'une famille de facteurs neurotrophiques appelés ultérieurement, les neurotrophines. Les recherches entourant la découverte du NGF visaient à élucider l'effet délétère de l'amputation de tissus cibles sur la survie des neurones moteurs et sensitifs (Levi-Montalcini, 1987). De cette découverte a découlé la théorie des facteurs neurotrophiques proposant que la survie des neurones dépende de neurotrophines produites par leurs organes cibles, où aboutissent les terminaisons axonales (Oppenheim, 1989) (Fig.8). Durant le développement, un surplus de neurones est généré à la fois dans les SNP et le SNC (Oppenheim, 1991). De même, les CGRs sont produites en excès durant l'ontogénie. Dans la rétine de poulet, deux vagues de mort cellulaire programmée ont été décrites entre le jour embryonique E5 et E7 (García-Porrero et al., 1979), chevauchant la période de neurogénèese,

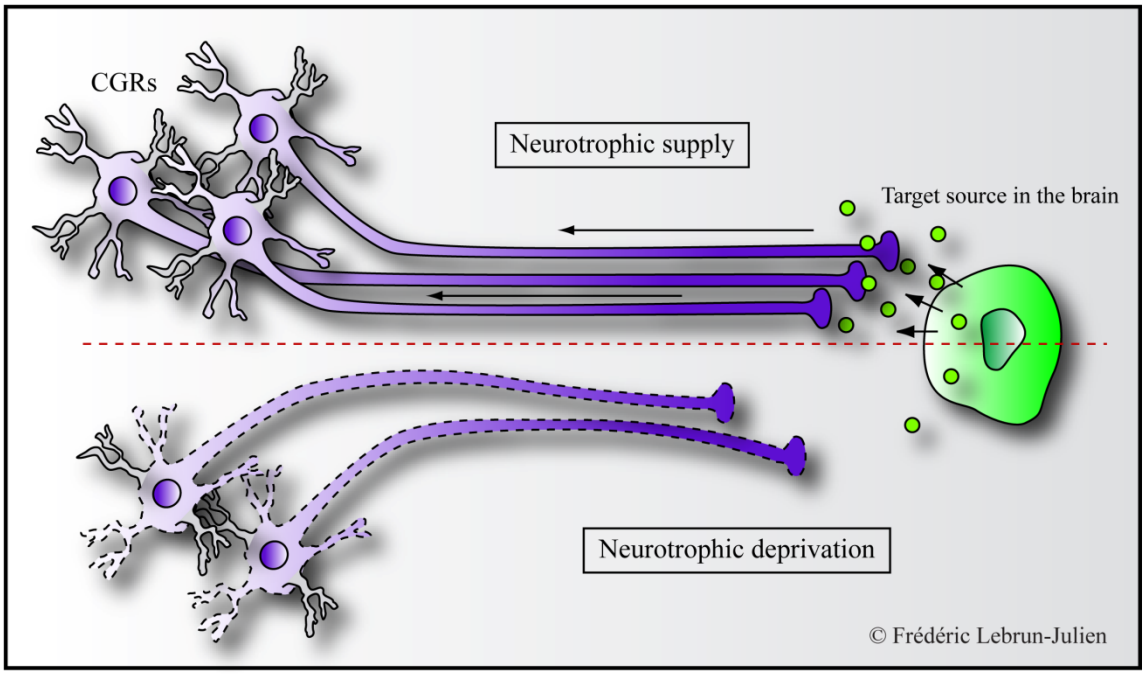

Figure 8. La théorie des facteurs neurotrophiques. La théorie des facteurs neurotrophiques est basée sur des observations montrant que les neurones compétitionnent pour une quantité limitée de facteurs neurotrophiques sécrétés par leurs tissus cibles afin d'assurer leur survie. Les neurones qui ont atteint leur cible les premiers et qui ont absorbé suffisamment de facteurs neurotrophiques survivent tandis que les neurones restants sont éliminés.

de différenciation et de migration des CGRs, et, un peu plus tard, vers E10-E14 (Rager and Rager, 1978; Franklin Hughes and La Velle, 1979), quand les CGRs deviennent dépendantes du support trophique de leurs cibles. Chez le rat, jusqu'à 50% des CGRs nouvellement générées meurent après avoir atteint leurs cibles (Galli-Resta and Ensini, 1996), sans compter toutes celles qui sont mortes avant même de les avoir atteint.

Outre leur rôle majeur au cours du développement, plusieurs autres rôles fondamentaux ont été rapidement attribués aux neurotrophines, aussi bien dans des systèmes neuronaux que non-neuronaux (Huang and Reichardt, 2001a; Sofroniew et al., 2001). De façon générale, les facteurs neurotrophiques sont des facteurs de croissance actifs sur les neurones ou les cellules gliales dont ils règlent la croissance, la prolifération et la différenciation. Ils sont à la base même du contrôle de l'apoptose ou la mort cellulaire programmée.

1.4.2. Les neurotrophines

Le NGF est le membre le mieux caractérisé et représente l'archétype d'une famille de neurotrophines reliées à la fois structurellement et fonctionnellement incluant le *brain derived neurotrophic factor* (BDNF) (Barde et al., 1982a), la *neurotrophin* 3 (NT-3)(Hohn et al., 1990) et la *neurotrophin* 4/5 (NT-4/5) (Berkemeier et al., 1991; Hallbook et al., 1991) chez les mammifères. Deux autres neurotrophines (NT-6 et NT-7) ont été isolées chez le poisson (Gotz et al., 1994; Lai et al., 1998). Au départ identifiée comme une famille de facteurs de croissance qui régule la survie neuronale, leur étude a ensuite démontré leur implication dans plusieurs autres fonctions comme le guidage axonale (Tucker, 2002), la plasticité synaptique (Schinder and Poo, 2000), la différenciation cellulaire et la neuroprotection par suite d'une lésion (Blesch et al., 1998; Di Polo et al., 1998a). De façon similaire à d'autres facteurs de croissance sécrétés, les neurotrophines sont préalablement synthétisées sous forme de précurseurs qui sont ensuite clivés en protéines matures (Voir section 1.4.3). À l'échelon moléculaire, les neurotrophines s'autodimérisent et exercent leurs effets par l'activation de deux types de récepteurs structurellement différents : le récepteur $p75^{NTR}$, un membre de la famille du TNF, et les récepteurs *tropomyosin-receptor-kinase* (Trks) à activité tyrosine kinase. Outre sur le plan structurel, les deux types de récepteurs diffèrent aussi en termes d'affinité de liaison. Alors que $p75^{NTR}$ est capable de lier toutes les neurotrophines

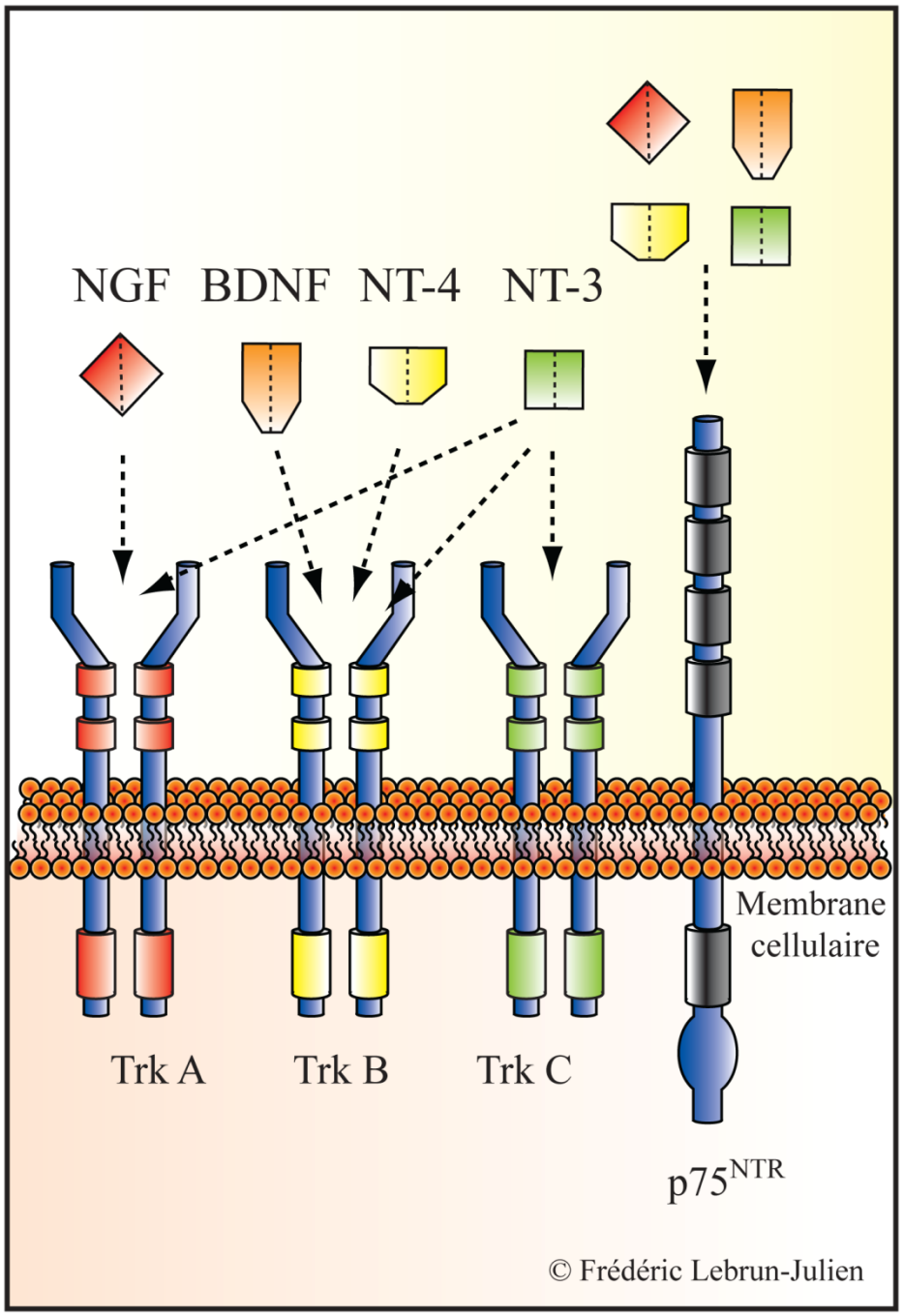

Figure 9. Les neurotrophines et leurs récepteurs. L'effet biologique des neurotrophines est engendré par la liaison aux récepteurs Trks : TrkA est le récepteur du NGF; TrkB est le récepteur du BDNF et de la NT-4; TrkC est le récepteur de la NT-3. Le récepteur p75 ($p75^{NTR}$), de la famille du récepteur du facteur nécrosant des tumeurs, lie le NGF, le BDNF, NT-3 et NT-4 avec la même affinité.

matures avec la même affinité (Rodriguez-Tebar et al., 1990; Frade and Barde, 1998), la famille des récepteurs Trks démontre une sélectivité pour leur ligand (Fig.9). En effet, NGF démontre de l'affinité pour le récepteur TrkA et le BDNF et NT-4 pour TrkB, tandis que la NT-3 lie principalement TrkC, mais se fixe aussi avec une affinité plus faible à TrkA et TrkB (Ip et al., 1993). Les premières études des récepteurs des neurotrophines neuronaux avaient suggéré deux cinétiques d'activités différentes, l'une de l'ordre du picomolaire (Kd~10^{-9} M) et l'autre de l'ordre de 10^{-11} M. Le récepteur $p75^{NTR}$ avait alors été hâtivement identifié comme le récepteur de faible affinité (Chao et al., 1986). Par la suite, il s'est avéré que ni TrkA ni $p75^{NTR}$ ne pouvait former un site de liaison de haute affinité lorsqu'ils sont exprimés séparément, mais que la co-expression des deux récepteurs entrainait la formation d'un site de haute affinité pour le NGF (Hempstead et al., 1991).

1.4.3. Les ProNeurotrophines

Le proNGF est synthétisé par épissage alternatif en deux transcrits de 34 KDa et 27 KDa qui, une fois la séquence signal enlevée, donne deux isoformes de 25 et 32 KDa, lesquelles peuvent être glycosylées pour donner une forme de 40 KDa (Fahnestock et al., 2001; Buttigieg et al., 2007). La maturation protéique est assurée par le clivage enzymatique des pré-séquences dans le réticulum endoplasmique et l'excision des pro-domaines dans le réseau transgolgien et dans les vésicules de sécrétion immatures (Lessmann et al., 2003). Le clivage intracellulaire du proNGF est effectué par des protéases telles que γ-NGF, la kallikrein (Berger and Shooter, 1977; Edwards et al., 1988), la furine ou d'autres proprotéine convertases (Seidah et al., 1996). Cependant, le proNGF peut aussi être sécrété et clivé dans le milieu extracellulaire par la plasmine pour former la forme mature de 13 KDa, le NGF, ou être dégradé par les métalloprotéinases de la matrice extracellulaire (Lee et al., 2001; Bruno and Cuello, 2006).

L'attention toute particulière portée aux neurotrophines dans le milieu scientifique a été quelque peu détournée lorsque des études ont démontré la capacité intrinsèque de leur pro-forme à régir des fonctions intracellulaires bien distinctes. Depuis quelques années, les proneutrophines (proNTs) ne sont plus confinées au rôle de précurseur; il a été établi qu'elles peuvent être sécrétées par les cellules et qu'elles sont biologiquement actives (Lee et al., 2001). De plus, de récentes données semblent montrer que, dans le SNC, les neurotrophines

sont principalement sécrétées sous leur pro-forme (Lee et al., 2001; Margaret et al., 2004; Bruno and Cuello, 2006). En utilisant une forme mutée du proNGF résistant aux enzymes de clivage, le groupe de Barbara Hempstead a démontré que le proNGF liait le récepteur $p75^{NTR}$ avec une grande affinité et qu'il était probablement un acteur important de la mort par apoptose induite par $p75^{NTR}$ dans les neurones sympathiques et les oligodendrocytes (Lee et al., 2001; Nykjaer et al., 2004a). Ces études ont aussi montré que le proNGF ne liait pas le récepteur TrkA et suggéré qu'il était un ligand pro-apoptotique spécifique pour $p75^{NTR}$. Des études subséquentes du groupe de Nykjaer ont révélé que ces formes immatures des neurotrophines liaient en fait avec une forte affinité le complexe protéique formé du récepteur $p75^{NTR}$ et de la sortiline, un membre de la famille VSP10 (voir section 1.4.5.2.3.1). Elles activent des voies de signalisation dépendantes de $p75^{NTR}$, qui dans plusieurs cas, à l'opposé des neurotrophines, vont favoriser l'apoptose (Lee et al., 2001). Ceci a conduit à l'hypothèse voulant que le NGF puisse agir comme ligand pro-apoptotique ou pro-survie selon son état de maturation.

Depuis quelques années déjà, plusieurs équipes de recherche s'efforcent de démontrer, hors de tout doute, l'effet neurotoxique du proNGF. Le rôle joué par les proNTs est encore loin d'être complètement élucidé, mais de récentes études ont grandement fait avancer le sujet. Des niveaux élevés de proNGF ont été mesurés dans plusieurs parties du cerveau adulte (Fahnestock et al., 2001), et des valeurs anormalements hautes retrouvées dans des cerveaux atteints de la maladie d'Alzheimer (Peng et al., 2004; Pedraza et al., 2005) ou par suite de lésion du SNC (Harrington et al., 2004b). Quelques études *in vitro* ont réussi à démontrer un effet neurotoxique des proNTs sur certaines populations neuronales (Raya et al., 2007; Raya et al., 2008), mais c'est dans le cas de neurones embryonnaires et postnataux que cet effet a été démontré de la façon la plus convaincante (Nykjaer et al., 2004a; Pedraza et al., 2005; Volosin et al., 2006b). Une autre étude a démontré que le niveau de proBDNF augmente dans le cerveau de la souris au cours des premières semaines post natales, de concert avec l'expression de $p75^{NTR}$, ce qui signifie que chez les souris juvéniles, proBDNF est plus abondant que le BDNF mature (Yang et al., 2009) et pourrait jouer un rôle dans la régulation des populations neuronales. Par ailleurs, une étude après axotomie des neurones corticospinaux adultes, a montré une production de proNGF, sa liaison au $p75^{NTR}$ et que l'application d'anticorps contre le proNGF induit une survie de ces neurones (Harrington et

al., 2004b). Ces données laissent supposer que les proNTs pourraient jouer un rôle dans la rétine adulte intacte ou par suite d'une lésion comme la section du nerf optique. Cependant, compte tenu que ces études *in vivo* sur la neurotoxicité du proNGF ne font pas consensus, l'établissement d'un modèle *in vivo* pertinent pour déterminer le rôle de ces précurseurs dans le SNC adulte et les maladies neurodégénératives est essentiel.

1.4.4. Les sources de facteur neurotrophique dans la rétine

Les neurotrophines peuvent être sécrétées par les dendrites et agir sur les cellules pré-synaptiques de façon rétrograde (Lohof et al., 1993; Schinder and Poo, 2000) ou migrer le long de l'axone pour influencer les cibles post-synaptiques (Levine et al., 1995; Altar and DiStefano, 1998). Outre les facteurs neurotrophiques issus du transport rétrograde à partir des tissus cibles comme les collicules supérieurs (Ma et al., 1998b; Isenmann et al., 1999), des sources locales de neurotrophines à l'intérieur de la rétine (Herzog and von Bartheld, 1998a) peuvent aussi jouer un rôle prédominant pour la survie des CGRs (Fig.10). Les données en faveur de ce concept proviennent d'études où le tissu cible des CGRs a été éliminé chez des animaux adultes et la mort neuronale n'a été détectée dans la rétine que plusieurs mois plutard (Carpenter et al., 1986a; Pearson and Stoffler, 1992b; Pearson and Thompson, 1993a). Ce résultat suggère que les facteurs neurotrophiques dérivés de la rétine, agissant de façon paracrine et/ou autocrine, supportent la survie des CGRs qui ont été déconnectées de leurs cibles. L'inverse a aussi été proposé, soit que des facteurs trophiques dérivés des tissus cibles puissent agir pour compenser une déficience de support trophique local dans la rétine (Murphy and Clarke, 2006b). On sait aussi que, elles-mêmes synthétisent des neurorotrophines qu'elles peuvent transporter par voie axonale pour influencer leurs cibles post-synaptiques (von Bartheld et al., 1996; Caleo et al., 2000; von Bartheld and Butowt, 2000). Les CGRs peuvent également répondre aux neurotrophines qu'elles produisent de façon autocrine. Par exemple, les cellules ganglionnaires secrètent le BDNF (Ma et al., 1998b; Seki et al., 2003) tout en exprimant fortement son récepteur TrkB (Mey and Thanos, 1993a; Mansour-Robaey et al., 1994a). Outre les différents types neuronaux, les cellules gliales sont une source importante de facteurs trophiques. Certaines études ont démontré que les cellules de Müller exprimaient dans la rétine et en culture l'ARNm du NGF et la protéine correspondante (Chakrabarti et al., 1990). Le BDNF et la NT-3 ont aussi été détectés dans les

cellules de Müller (Taylor et al., 2003; Seki et al., 2005), sans compter les facteurs neurotrophiques appartenant à d'autres familles comme le CNTF (Cao et al., 1997), le *Leukemia Inhibitory Factor* (LIF) (Neophytou et al., 1997), le bFGF (Harada et al., 2000b) ou le *Transforming Growth Factor β* (TGFβ) (Anderson et al., 1995). Par contre, l'expression par les cellules de Müller de facteurs de croissance et l'action de ceux-ci sur ces cellules reste encore à démontrer.

1.4.5. Les récepteurs des neurotrophines

1.4.5.1. Les récepteurs Trk et leur signalisation

La découverte du premier récepteur Trk, en l'occurrence TrkA, est venue plusieurs années après celle du récepteur $p75^{NTR}$ (Levi-Montalcini, 1987). Chez les mammifères, les trois membres de la famille de récepteurs Trk représentent la seconde classe de récepteur des neurotrophines (Chao, 2003; Huang and Reichardt, 2003c). Les récepteurs Trks sont spécifiquement activés par la forme mature des neurotrophines et non par leur forme précurseur (Lee et al., 2001). Tous les récepteurs Trks sont des protéines transmembranaires de type 1 et ils font partie de la grande famille des récepteurs à activité tyrosine kinase (Martin-Zanca et al., 1989). Le domaine extracellulaire des récepteurs Trks contient deux régions riches en cystéine (domaine 1 et 3) entourant un domaine riche en leucine (domaine 2), suivi de deux domaines apparentés aux immunoglobulines (Ig) dans la région juxtamembranaire (domaine 4 et 5) (Windisch et al., 1995). Des études de délétion et de liaison sur TrkA, TrkB et TrkC ont indiqué que le domaine 5 était responsable de la liaison des neurotrophines (Pérez and Caminos, 1995; Urfer et al., 1995; Urfer et al., 1998; Ultsch et al., 1999). Chaque récepteur traverse la membrane une fois et se termine par une région cytoplasmique avec un domaine de tyrosine kinase entouré de plusieurs tyrosines, lesquelles, une fois phosphorylées, servent de site de liaison pour des protéines adaptatrices et des enzymes. L'étude de la structure de NGF lié à TrkA par cristallisation a révélé un complexe avec une stœchiométrie symétrique 2:2 (He and Garcia, 2004). C'est-à-dire qu'un dimère de NGF peut lier et induire la dimérisation de deux récepteurs TrkA. Cette dimérisation des récepteurs Trks causée par la liaison des neurotrophines induit leur activation par

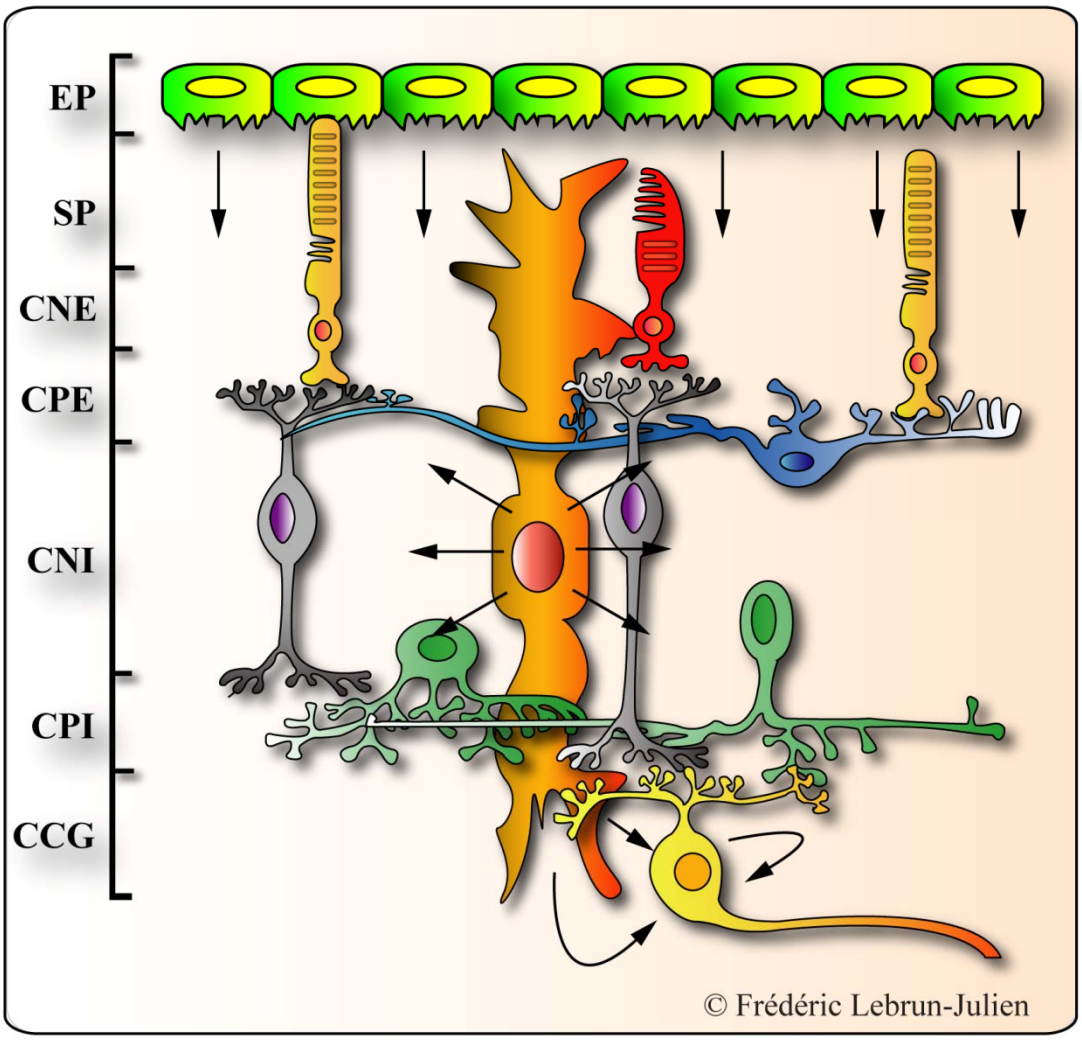

Figure 10. La source des facteurs neurotrophiques dans la rétine. La source locale de facteurs neurotrophiques dans la rétine peut jouer un rôle primordial dans la survie des cellules ganglionnaires rétiniennes matures (CGRs). Des facteurs sécrétés par les cellules de Muller ou l'épithélium pigmentaire rétinien (EPR) peuvent diffuser et agir de façon paracrine afin de promouvoir la survie des CGRs lésées. Des facteurs sécrétés par les neurones, incluant les CGRs, peuvent agir de façon autocrine et/ou paracrine pour stimuler la survie neuronale après une blessure. EP : Épithélium Pigmentaire; SP : Segments des Photorécepteurs; CNI : Couche Nucléaire Interne; CPE : Couche Plexiforme Externe; CNI : Couche Nucléaire Interne; CPI : Couche Plexiforme Interne; CCG : Couche des Cellules Ganglionnaires.

transphophorylation des kinases présentes dans leur domaine cytoplasmique (Huang and Reichardt, 2003a, b). Finalement, les nombreuses fonctions attribuées aux neurotrophines, dont la croissance et la survie cellulaire sont régulées par leurs principales voies de signalisation, soit celles de Ras/MAP Kinase, du phophatidylinositol 3-kinase (PI3-K)/ AKT et de la phospholipase Cγ (PLCγ)/ protéine kinase C (PKC) (Segal and Greenberg, 1996; Kaplan and Miller, 2000a; Roux and Barker, 2002b).

1.4.5.1.1. La voie Ras-MAPK

La voie de signalisation Ras-MAPK régule la survie et la différenciation neuronale par l'activation de la voie *mitogen-activated protein kinases* (MAPKs)/ *extracellular signal-regulated kinase* (ERK) (Grewal et al., 1999; Ballif and Blenis, 2001). La protéine adaptatrice Shc se lie à la tyrosine phosphorylée Y490 sur TrkA, ce qui permet de recruter la protéine Grb2 et *son of sevenless* (SOS) à la membrane. En conséquence, RAS devient activé et il s'en suit une activation de voies de signalisation en aval, qui incluent c-Raf/B-Raf/ERK1/ERK2 et p38MAPK (Xing et al., 1996). Des études ont démontré que l'activation, Erk-dépendante, de p90 ribosomal S6 kinase (RSK) et la phosphorylation subséquente de CREB par RSK et p38MAPK est une voie importante de survie pour les neurones (Xing et al., 1998; Bonni et al., 1999a). D'autres MAPK comme ERK5 peuvent aussi être activées en aval des Trks lors de l'activation de MEK5 par RAS/Raf (Watson et al., 2001).

Le BDNF est la neurotrophine la plus efficace pour protéger les CGRs par suite d'axotomie dans la rétine adulte (Johnson et al., 1986b; Mey and Thanos, 1993b). Les deux voies de signalisation de TrkB sont la voie des MAPK (Johnson and Lapadat, 2002) et la voie PI3Kinase/Akt (Klocker et al., 2000), et les deux sont activées par l'apport exogène de BDNF (Klocker et al., 2000; Nakazawa et al., 2002). Cependant, des études de notre laboratoire ont démontré que la protection des CGRs lors de l'activation de TrkB semble dépend spécifiquement de la voie de la MAPK activant Erk 1/2 et ce, à la fois dans le modèle d'induction du glaucome que celui de section du nerf optique (Cheng et al., 2002a; Pernet et al., 2005b; Zhou et al., 2005a).

1.4.5.1.2. La voie de la PI3 kinase/Akt

La voie PI3-Kinase joue un rôle important dans la survie dépendante des Trks dans plusieurs types neuronaux. L'activation de la PI3-Kinase par les Trks peut se faire de façon dépendante et indépendante de la voie de Ras. La voie indépendante de Ras implique le complexe Shc-Grb2, qui vient lier *Grb-associated binder-1* (Gab-1). Ce complexe induit l'association de la PI3-Kinase qui lui permet d'accéder aux substrats au niveau du feuillet intérieur de la membrane plasmique (Holgado-Madruga et al., 1997; Yamada et al., 1997). Les lipides phophorilés générés par la PI3-Kinase provoquent le recrutement à la membrane de la *serine/threonine kinases 3 phosphoinositide-dependent kinase-1* (PDK1) (Vanhaesebroeck and Alessi, 2000) et son substrat, Akt (protéine kinase B) (Paul and James, 1991). Akt activé par PDK1 phosphoryle en retour des substrats qui régulent la survie neuronale, incluant le membre de la famille Bcl-2, Bad (Datta et al., 1997a; Peso et al., 1997), la caspase 9 (Cardone et al., 1998), la kinase IκB (Kane et al., 1999), la *glycogen synthase kinase-3* (GSK-3) (Cross et al., 1995) et les membres de la famille Forkhead (FKHR) (Brunet et al., 1999). L'activation de PI3-K dépendante de Ras a aussi été observée dans les neurones. Ras peut directement interagir avec PI3-K, et l'inhibition de Ras peut supprimer l'activité de la PI3-K induite par NGF (Klesse and Parada, 1998; Mazzoni et al., 1999). Dans la rétine, une étude démontre qu'il y a activation endogène de la PI3-K suite à une blessure du nerf optique et que l'inhibition de cette activité endogène exacerbe la mort des CGRs (Nakazawa et al., 2003). De plus, l'activation de la PI3-K a été rapportée dans les CGRs en réponse à plusieurs facteurs neurotrophiques, incluant le facteur de croissance de l'insuline (IGF) et le TNFα, et un blocage pharmacologique inhibe son action neuroprotectrice (Kermer et al., 2000b; Diem et al., 2001a).

1.4.5.1.3. La voie de la Phospholipase C

La tyrosine phosphorylée Y^{785} de l'extrémité carboxy-terminale du récepteur sert d'emplacement d'amarrage pour la PLCγ (Obermeier et al., 1994). La phosphorylation de la PLCγ par les Trks génère une enzyme active capable d'hydrolyser le phosphatidyl inositol pour produire l'inositol triphosphate (IP3) et le diacylglycerol (DAG) (Vetter et al., 1991). L'IP3 augmente le niveau cytoplasmique de Ca^{2+}, alors que le DAG active la protéine kinase

C δ (PKCδ), laquelle est impliquée dans la croissance neuritique et l'activation de la cascade ERK (Corbit et al., 1999).

1.4.5.2. Le récepteur $p75^{NTR}$

Le récepteur $p75^{NTR}$, premier récepteur des neurotrophines découvert, a d'abord été identifié comme un récepteur du NGF, puis démontré comme pouvant lier toutes les neurotrophines (Bothwell, 1995). Le récepteur $p75^{NTR}$ est un récepteur protéique transmembranaire de type 1. C'est un membre de la famille des récepteurs du TNF, avec un domaine extracellulaire qui inclut quatre motifs riches en cystéine (CRD1-CRD4), un domaine transmembranaire unique et un domaine cytoplasmique qui comprend un domaine de mort (Liepinsh et al., 1997; He and Garcia, 2004). Tout comme les récepteurs du TNF, $p75^{NTR}$ peut moduler l'apoptose cellulaire (Ashkenazi, 2002). Le $p75^{NTR}$ est considéré comme un membre particulier de cette famille, en raison de sa capacité à dimériser au lieu de former un trimère, d'agir comme co-récepteur des Trks et de lier les neurotrophines qui ne sont pas les ligands typiques de cette famille. Par contre, tout comme les récepteurs de cette famille, $p75^{NTR}$ ne possède pas d'activité catalytique intrinsèque; il doit donc interagir en association avec différentes protéines adaptatrices intracellulaires pour induire sa signalisation (Roux and Barker, 2002b). Alternativement, le gène du $p75^{NTR}$ peut encoder une isoforme variante de $p75^{NTR}$ incapable de lier les neurotrophines (Dechant and Barde, 1997). Cette variante de $p75^{NTR}$ est également une protéine transmembranaire de type 1 avec un domaine intracellulaire et transmembranaire intact (Roux and Barker, 2002b). Il a été démontré que la surexpression d'une protéine recombinante de $p75^{NTR}$, qui de façon similaire ne possède pas de domaine extracellulaire, peut moduler une signalisation apoptotique *in vivo* (Majdan et al., 1997b).

Dans le système nerveux central, $p75^{NTR}$ a été principalement associé au développement, mais il semble aussi jouer un rôle à la suite d'une blessure et dans certaines maladies (Ebadi et al., 1997). Initialement, $p75^{NTR}$ a été défini comme un réservoir de surface pour les neurotrophines sur les cellules gliales, présentant les neurotrophines aux axones en croissance (Taniuchi et al., 1988). Une autre hypothèse le confinait à un rôle de nettoyeur de l'excédant de neurotrophines rencontré par les neurones dans leurs tissus cibles (Bothwell, 1995). Plus tard, $p75^{NTR}$ s'est illustré comme un partenaire des récepteurs Trks en

augmentant la capacité de réponse dans le cas du NGF et de TrkA et en augmentant la spécificité de liaison dans le cas du BDNF et de la NT-3 (Clary and Reichardt, 1994b; Mischel et al., 2001). De plus, l'incapacité d'identifier des molécules de signalisation interagissant avec son domaine cytoplasmique a laissé croire que la principale fonction de $p75^{NTR}$ était de moduler l'affinité et la spécificité de l'interaction des neurotrophines et des récepteurs Trks. Des études utilisant des souris transgéniques dont le gène *$p75^{NTR}$* avait été invalidé ont montré une baisse de la réponse au NGF des neurones sympathiques (Lee et al., 1994). Cependant, il a ensuite été démontré que $p75^{NTR}$ pouvait indépendamment des récepteurs Trks induire à la fois des signaux pro-apoptotique et pro-survie en liant différentes protéines adaptatrices par son domaine cytoplasmique (Roux and Barker, 2002b) .

1.4.5.2.1. La signalisation de $p75^{NTR}$

Les principales voies de signalisation activées par $p75^{NTR}$ comprennent l'activation du facteur de transcription NF-κB, la phosphorylation de la kinase du facteur de transcription c-jun (JNK), ainssi que l'augmentation des céramides, ce qui entraîne la transcription de gènes et/ou l'induction de la mort cellulaire programmée (Meakin et al., 1992). La différence structurale du domaine intracellulaire pour $p75^{NTR}$ et le récepteur du TNF (TNFR) suggère un mécanisme potentiellement différent entre $p75^{NTR}$ et les autres récepteurs de mort. Confirmant cette hypothèse, il a été observé que parmi les protéines interagissant avec $p75^{NTR}$ identifiées jusqu'à présent, incluant NRIF (Casademunt et al., 1999), NADE (Mukai et al., 2000), et les membres de la famille de protéine MAGE (Salehi et al., 2000; Jordan et al., 2001), TRAF-2 (Ye et al., 1999), seul la protéine NADE interagit avec le domaine de mort du récepteur. Une autre protéine, RIP-2, interagit aussi avec le domaine de mort de $p75^{NTR}$, mais cet engagement résulte en une activité anti-apoptotique (Khursigara et al., 2001). L'identification de protéines activées par $p75^{NTR}$ avec une activité anti-apoptotique, telle que RIP-2 (Khursigara et al., 2001), IRAK (Mamidipudi et al., 2002) et TRAF-6 (Khursigara et al., 1999), suggère que l'équilibre entre les cascades de signalisation pro-apoptotique et pro-survie détermine ultimement la conséquence cellulaire de l'activation de $p75^{NTR}$ (Fig.11A).

Quelques études indépendantes ont montré que plusieurs membres de la famille de protéines MAGE lient la région cytosolique de $p75^{NTR}$ et qu'une de ces protéines, nommée

NRAGE (aussi connue sous Maged1 ou Dlxin), est un activateur potentiel de la Jun Kinase (JNK) et une inductrice de l'apoptose (Barker and Salehi, 2002; Bhakar et al., 2003). La protéine NRAGE (Neurotrophin Receptor-Interacting MAGE homolog) est largement exprimée durant le développement et des études *in vitro* ont démontré qu'elle est impliquée dans des fonctions cellulaires aussi variées que la régulation du cycle cellulaire et l'adhésion cellulaire et la régulation de la transcription (Chomez et al., 2001; Kuwajima et al., 2004; Xue et al., 2005). Il faut également suggérer que NRAGE est impliquée dans l'apoptose neuronale qui est régulée par $p75^{NTR}$. En effet, les souris avec une délétion du gène codant NRAGE démontrent une diminution de l'apoptose développementale des neurones sympathiques des ganglions cervicaux supérieurs, identique à celle observée chez les souris *knockout* pour $p75^{NTR}$ (Bertrand et al., 2008b). De plus, la sur-expression de NRAGE perturbe le complexe formé de TrkA et $p75^{NTR}$, ce qui indique que l'interaction entre $p75^{NTR}$ et TrKA ou NRAGE est mutuellement exclusive (Salehi et al., 2000). Jusqu'à présent, les premières données suggèrent que le mécanisme apoptotique engendré par NRAGE passerait par l'activation de JNK et la voie des caspases pour ensuite amener la mort cellulaire apoptotique par activation des caspases dépendantes des mitochondries (Salehi et al., 2002).

Plusieurs études montrent que, dans certaines circonstances, $p75^{NTR}$ peut faciliter la survie cellulaire au lieu de la mort cellulaire indépendamment des récepteurs Trks. Par exemple, NGF protège les cultures de neurones corticaux qui expriment $p75^{NTR}$, mais pas TrkA, contre la cytotoxicité induite par le glutamate (Shimohama et al., 1993; Kume et al., 2000). Récemment, il a été observé que la liaison de neurotrophine à $p75^{NTR}$ induit la survie *in vitro* et *in vivo*. Effectivement, l'activation du NF-κB par $p75^{NTR}$ induit la survie de plusieurs types cellulaires (Maggirwar et al., 1998; Yoon et al., 1998; Hamanoue et al., 1999b; Foehr et al., 2000; Gentry et al., 2000; Hughes et al., 2001). De plus, la voie de signalisation de la PI3-K/Akt, qui joue un rôle important dans la survie cellulaire induite par les récepteurs Trks (Kaplan and Miller, 2000a; Brunet et al., 2001), semble aussi participer aux mécanismes de survie générés par $p75^{NTR}$ indépendamment des récepteurs Trks (Roux et al., 2001).

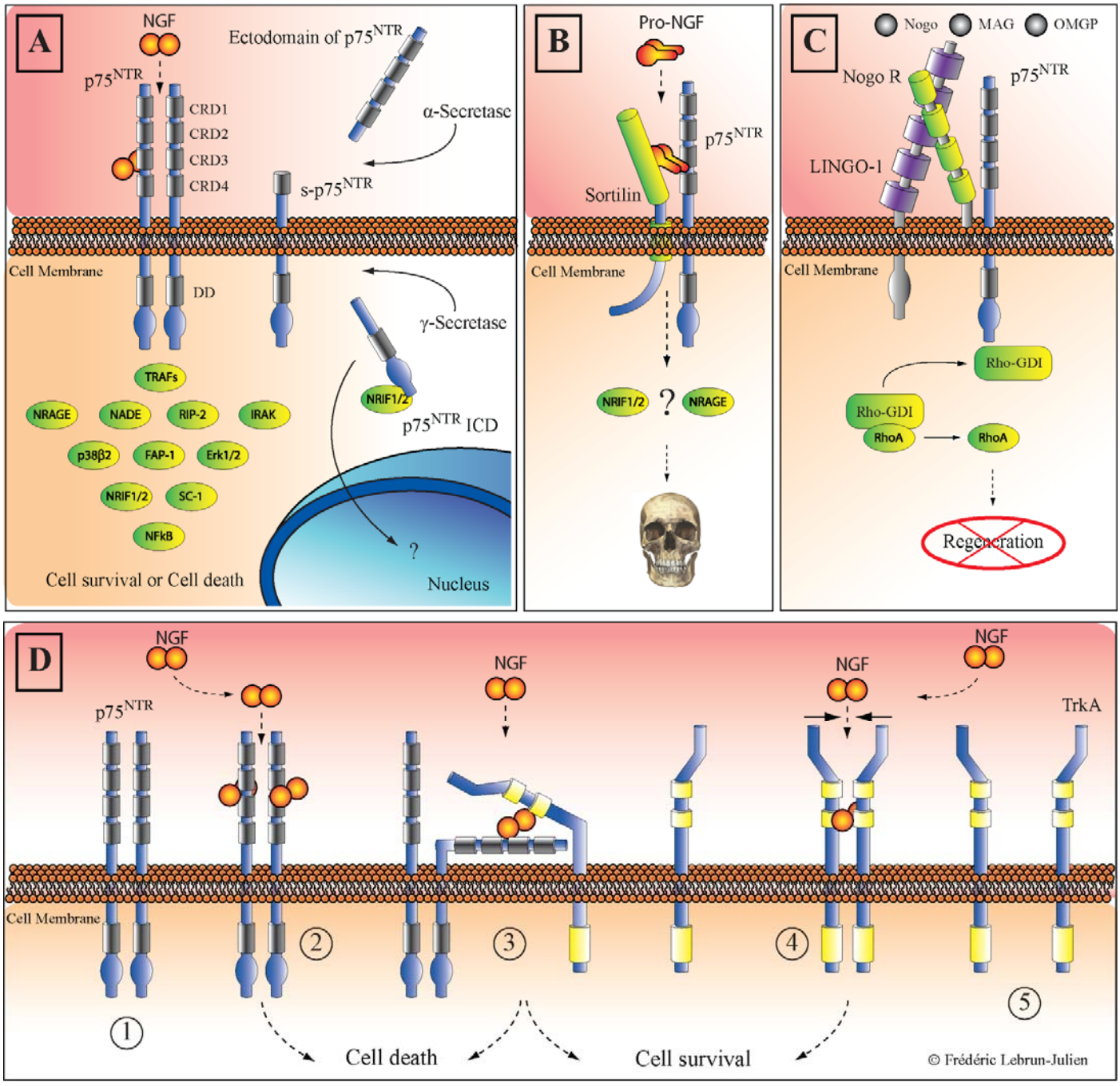
A
NGF
p75NTR
Ectodomain of p75NTR
α-Secretase
CRD1
CRD2
CRD3
CRD4
s-p75NTR
Cell Membrane
DD
γ-Secretase
TRAFs
NRAGE
NADE
RIP-2
IRAK
p38β2
FAP-1
Erk1/2
NRIF1/2
SC-1
NFkB
NRIF1/2
p75NTR ICD
?
Nucleus
Cell survival or Cell death
B
Pro-NGF
p75NTR
Sortilin
Cell Membrane
NRIF1/2
?
NRAGE
C
Nogo
MAG
OMGP
Nogo R
p75NTR
LINGO-1
Cell Membrane
Rho-GDI
Rho-GDI
RhoA
RhoA
Regeneration
D
NGF
p75NTR
NGF
NGF
NGF
TrkA
Cell Membrane
1
2
3
4
5
Cell death
Cell survival
© Frédéric Lebrun-Julien

Figure 11. La signalisation du récepteur p75^{NTR}. (A) Le récepteur p75^{NTR} est un récepteur transmembranaire de type I avec une partie extracellulaire contenant quatre domaines riches en cystéine (CRDs) et une partie intracellulaire contenant un domaine de mort (DD). Le p75^{NTR} n'a pas d'activité enzymatique, mais plusieurs protéines adaptatrices sont responsables de sa signalisation intracellulaire. Le gène *p75^{NTR}* peut donner une version complète du récepteur (p75^{NTR}) tout comme une version alternative tronquée (s-p75^{NTR}). Le p75^{NTR} peut aussi être clivé par l'enzyme α–sécrétase pour donner un fragment extracellulaire (ectodomaine de p75^{NTR}) et par l'enzyme γ–sécrétase pour donner un fragment intracellulaire (p75^{NTR} ICD). (B) Les proNTs lient le complexe protéique formé du récepteur p75^{NTR} et de la sortiline pour induire une signalisation pro-apoptotique. (C) Avec la protéine transmembranaire Lingo, le récepteur p75^{NTR} fait partie du complexe hétérotrimérique du récepteur Nogo qui génère des effets inhibiteurs sur la croissance axonale de trois glycoprotéines associées à la myéline; *Nogo-A*, MAG et OMgp. L'activation de RhoA par suite de l'activation de ce complexe protéique inhibe la polymérisation du cytosquelette dans le cône de croissance, ce qui bloque la régénérescence. (D) En absence de neurotrophines, p75^{NTR} se présente sous forme de dimère, mais TrkA ne forme pas d'homo- ou d'hétérocomplexes (1,5). Le NGF lie p75^{NTR} avec un taux d'association-dissociation élevé. Un dimère de NGF peut lier chacun des récepteurs du dimère, car la liaison du NGF ne défait pas le dimère de p75^{NTR}. Cependant, les deux récepteurs p75^{NTR} ne peuvent lier simultanément un seul dimère de NGF (2). Le NGF lié à p75^{NTR} possède une interface de liaison à TrkA. Le p75^{NTR} et TrkA lient le NGF dans des orientations différentes ce qui permettrait aux deux récepteurs de lier NGF en 'sandwich'. Dans cette orientation, le NGF est présenté à TrkA sous une conformation qui favorise une association rapide avec le récepteur Trk. Le dimère de NGF peut lier une deuxième chaine de TrkA, ce qui permet au récepteur kinase de former un homodimère actif (4).

1.4.5.2.2. La liaison de NGF au recepteur p75^{NTR}

Des études récentes de modélisation 3D de la structure du domaine extracellulaire de p75^{NTR} lié au NGF ont démontré que chacun des domaines CRD participait à cette liaison du NGF (He and Garcia, 2004). La liaison du NGF à p75^{NTR} se fait le long de l'interface entre les deux monomères du NGF et la liaison résulte en un changement de conformation du NGF qui altère l'interface du monomère du côté opposé, ce qui élimine la possibilité qu'un dimère de NGF puisse lier deux monomères p75^{NTR} (Wehrman et al., 2007). Ce constat suggère que la liaison de NGF à p75^{NTR} peut impliquer la dissociation du multimère p75^{NTR} et s'avère compatible avec la possibilité que les récepteurs Trks et un monomère p75^{NTR} lient de façon simultanée le même dimère de neurotrophine (He and Garcia, 2004; Barker, 2007).

1.4.5.2.3. Les partenaires de p75^{NTR}

1.4.5.2.3.1. La sortiline

Les proneurotrophines lient avec une forte affinité le complexe protéique formé de p75^{NTR} et de la sortiline (Nykjaer et al., 2004a; Chen et al., 2005) (Fig.11B). La sortiline est une protéine transmembranaire de type 1 exprimée par une grande variété de tissus, mais plus abondante dans le SNC durant le développement que chez l'adulte. La sortiline et le p75^{NTR} participent directement à la liaison des neurotrophines précurseurs. Il a été démontré que le pro-domaine du NGF lie directement le domaine extracellulaire de la sortiline et forme avec p75^{NTR} un complexe protéique qui lie les proNTs à la surface cellulaire. Elle est un membre de la famille VPS10. Jusqu'à tout récemment, la sortiline était principalement connue pour jouer un rôle important dans le trafic cellulaire du réseau trans-golgien (TGN) aux endosomes et du TGN aux lysosomes. La majorité de la sortiline est localisée dans les compartiments intracellulaires (Nielsen et al., 2001). Puisque les proNTs peuvent lier la sortiline même en absence de p75^{NTR}, et que la sortiline joue un rôle dans le transport vésiculaire, il est possible qu'en plus de son rôle de co-récepteur, la sortiline puisse aussi être responsable du transport intracellulaire des proNTs et des NTs nouvellement synthétisées. Alors que p75^{NTR} est majoritairement exprimé à la membrane plasmique et accessible aux ligands proneurotrophiques, la sortiline, comme nous l'avons mentionné, est principalement localisée dans les membranes intracellulaires, ce qui limite la formation d'un complexe avec son co-récepteur à la surface. Cependant, une étude récente suggère qu'un homologue de

$p75^{NTR}$, NRH2, pourrait agir comme un régulateur du trafic de la sortiline des lysomes à la surface cellulaire, ce qui rendrait les cellules exprimant $p75^{NTR}$ susceptibles à la mort cellulaire induite par les proNTs (Kim and Hempstead, 2009). La cascade de signalisation du complexe $p75^{NTR}$-sortiline reste à élucider, mais plusieurs données semblent indiquer que les protéines adaptatrice NRIF et NRAGE jouent un rôle dans la signalisation pro-apoptotique de ce complexe (Linggi et al., 2005b; Bertrand et al., 2008a; Bertrand et al., 2008b)

1.4.5.2.3.2. Le complexe du récepteur Nogo

Le récepteur $p75^{NTR}$ agit aussi comme sous-unité du complexe du récepteur Nogo (NogoR, Lingo, $p75^{NTR}$), lequel médie les effets inhibiteurs sur la croissance axonale des trois glycoprotéines associées à la myéline : Nogo-A, *myelin-associated glycoprotein* (MAG) et *oligodendrocyte-myelin glycoprotein* (OMgp) *(Wang et al., 2002b; Wong et al., 2002; Mi et al., 2004)*. Des études récentes ont montré que l'expression d'un récepteur $p75^{NTR}$ dépourvu de son domaine intracellulaire permet la croissance des neurites sur un substrat d'inhibiteurs associés à la myéline (Wang et al., 2002a). De plus, les neurotrophines matures ne semblent pas enter en compétition avec Nogo dans les essais biochimiques de liaison à $p75^{NTR}$ (Wang et al., 2002a). Alternativement, un homologue du récepteur du TNF appelé TROY peut remplacer $p75^{NTR}$ pour former un complexe avec le récepteur Nogo (Shao et al., 2005b) (Fig.11C).

1.4.5.3. L'interaction structurelle et fonctionnelle entre les récepteurs Trks et $p75^{NTR}$

La littérature qui porte sur l'interaction entre les deux récepteurs est vaste, et l'on commençe à peine à établir les bases d'un modèle cohérent. L'interaction entre les deux récepteurs sous l'action des neurotrophines parait avoir des conséquences contradictoires. Par exemple, la liaison aux récepteurs Trks engendre presque qu'invariablement la survie et la différenciation cellulaire, alors que le recrutement de $p75^{NTR}$ induit l'apoptose dans bien des cas (DeFreitas et al., 2001; Roux and Barker, 2002b). L'observation que les deux classes de récepteurs ont des ligands préférentiels distincts (Pro-neurotrophines pour $p75^{NTR}$ et neurotrophines pour les Trks) fournit une explication partielle à cette dualité (Lee et al., 2001).

Plusieurs données favorisent une interaction entre les deux récepteurs. En effets, des études ont montré que $p75^{NTR}$ augmente la réponse et la spécificité des récepteurs Trks aux neurotrophines (Bibel et al., 1999), ou qu'une perturbation de la liaison de NGF à $p75^{NTR}$ inhibe l'activation de TrkA par NGF (Barker and Shooter, 1994; Clary and Reichardt, 1994b; Verdi et al., 1994; Lachance et al., 1997). Par ailleurs, les souris knockouts pour $p75^{NTR}$ exigent une concentration plus élevée de NGF pour survivre (Lee et al., 1992; von Schack et al., 2001). Il a donc été proposé que $p75^{NTR}$ agisse comme co-récepteur qui lie NGF et le concentre localement ou qui le présente au récepteur TrkA dans une conformation favorable.

Une autre hypothèse propose que $p75^{NTR}$ et TrkA forment un site de forte affinité pour lequel le NGF aurait une affinité 10 à 30 fois supérieures a son affinité pour chacun de ces récepteurs séparément (Hempstead et al., 1991). Quand les deux récepteurs sont co-exprimés, le taux d'association de NGF à TrkA augmente de 25 fois (Mahadeo et al., 1994). Par contre, ce site de haute affinité ne constituent que 10%-15 % des sites de liaison du NGF dans les cellules transfectées avec les deux récepteurs (Hempstead et al., 1991). Cependant, les études visant à déterminer le domaine protéique responsable de la formation du site de haute affinité sur $p75^{NTR}$ ou TrkA ont abouti à des résultats conflictuels (Hempstead et al., 1990; Battleman et al., 1993; Bilderback et al., 2001; Esposito et al., 2001; Mischel et al., 2001), échouant à fournir une base moléculaire au modèle (Bothwell, 1995; Huang and Reichardt, 2003a). De plus, d'autres études n'ont pas réussi à montrer une influence de $p75^{NTR}$ sur l'affinité des récepteurs Trks (Jing et al., 1992; Bothwell, 1995; Aurikko et al., 2005).

Il y a quelques années, une étude de la structure tridimensionnelle du NGF lié au domaine extracellulaire (DEC) de $p75^{NTR}$ (He and Garcia, 2004) a indiqué que l'interaction d'un seul DEC de $p75^{NTR}$ avec un dimère de NGF amène un changement de conformation du NGF qui empêche une seconde molécule de $p75^{NTR}$ de joindre le complexe. Le changement de conformation induit sur le NGF par la liaison au $p75^{NTR}$ n'altère pas son site de liaison au récepteur TrkA, ce qui suggère la possibilité que NGF lié à $p75^{NTR}$ puisse lier simultanément l'ectodomaine de TrkA (Barker, 2007; Wehrman et al., 2007). D'autres études structurales ont montré que les deux récepteurs avaient des sites différents de liaison sur NGF, mais que ces sites pouvaient se chevaucher, ce qui empêche la possibilité d'une liaison simultanée sur le même côté du NGF pour former un complexe 2:2:2, comme il avait été suggéré précédemment (Wiesmann and de Vos, 2001).

En combinant les solutions structurales des complexes TrkA-NGF et $p75^{NTR}$-NGF, l'équipe de Wehrman (2007) propose un modèle de complexe ternaire, TrkA-NGF- $p75^{NTR}$. Théoriquement, les deux récepteurs pourraient lier NGF dans une stœchiométrie 1:2:1 sans encombrement stérique, ce qui permettrait aux deux différents DEC de lier les côtés opposés du dimère de NGF. La création d'un complexe ternaire requière que les deux DEC se présentent sous une conformation en sandwich autour du NGF, où chaque récepteur est orienté de façon opposé. Même si les domaines extracellulaires de $p75^{NTR}$ and TrkA ne sont pas associés directement (Wehrman et al., 2007), $p75^{NTR}$ et TrkA peuvent être co-immunoprécipités (Bibel et al., 1999), ce qui démontre qu'ils sont certainement à proximité l'un de l'autre. Toutes les données recueillies sur les interactions entre ces deux récepteurs peuvent s'expliquer par un modèle de transfert du ligand selon lequel NGF est rapidement associé avec $p75^{NTR}$ et ensuite présenté au récepteur TrkA dans une conformation favorable à l'association avec ce récepteur (Barker, 2007; Wehrman et al., 2007) (Fig.11D).

1.4.5.3.1. Convergence de la signalisation

La convergence intracellulaire de la signalisation et le partage de molécules adaptatrices par les deux types de récepteurs amènent un autre degré de complexité aux neurotrophines. Par exemple, des études ont montré que $p75^{NTR}$ augmente la phosphorylation des sérines de TrkA (MacPhee and Barker, 1997). De plus, l'activation de PI-3 kinase peut supprimer la production de céramides par $p75^{NTR}$ et peut empêcher l'apoptose induite par $p75^{NTR}$ (Yoon et al., 1998). D'autres études ont utilisé des anticorps et/ou des ligands sélectifs de $p75^{NTR}$ et des Trks pour montrer que les deux récepteurs peuvent activer des cascades de signalisation distinctes, mais convergentes (Ivanisevic et al., 2003). La recherche de protéines interagissant avec $p75^{NTR}$ a mené à la découverte de la protéine ARMS (*ankyrin rich membrane spanning*) qui interagit aussi avec les récepteurs Trks. La convergence de signalisation entre les deux récepteurs n'est pas unidirectionnelle. En effet, plusieurs expériences montrent que l'activation des récepteurs Trks module la signalisation de $p75^{NTR}$. La survie générée par TrkA semble être un signal dominant qui bloque l'apoptose générée par $p75^{NTR}$ (Bamji et al., 1998; Yoon et al., 1998; Salehi et al., 2000). Les mécanismes qui permettent aux Trks de supprimer la mort induite par $p75^{NTR}$ ne sont pas encore bien définis, mais la production de céramide par $p75^{NTR}$ est bloquée par une voie de signalisation de TrkA

qui semble requérir l'activation de la PI-3 kinase (Dobrowsky et al., 1995; Bilderback et al., 2001). Les cavéolines représentent une autre possibilité d'interaction entre les deux récepteurs parce qu'elles interagissent avec les deux (Bilderback et al., 1999). Finalement, le complexe protéique p62-Traf6-IRAK semble aussi fonctionner comme un échafaudage pour l'association de ces deux récepteurs avec Trf6, qui lie $p75^{NTR}$, et p62 avec TrkA (Wooten et al., 2001). Il a été démontré que ce complexe joue un rôle important dans l'internalisation et la signalisation induite par TrkA (Geetha et al., 2005).

1.4.6. Les composés peptidomimétiques

Tel que mentionné dans notre article de synthèse (Lebrun-Julien and Di Polo, 2008), l'utilisation de facteurs neurotrophiques à des fins cliniques ou de recherche fondamentale est quelque peu limitée du à leur courte demi-vie et leurs effets pléiotropiques qui peuvent induire des signaux non-spécifiques et de la toxicité (Barinaga, 1994a; Verrall, 1994b; Jonhagen, 2000a). De plus, il a été démontré que les facteurs neurotrophiques ont une diffusion limitée dans les tissus, qu'ils subissent une dégradation enzymatique rapide et qu'ils ne traversent pas la barrière hémato-encéphalique. Ces restrictions peuvent être surmontées en concevant de petites molécules synthétiques qui imitent l'activité biologique des facteurs neurotrophiques sans avoir leurs effets indésirables. Les ligands peptidomimétiques sont de petites molécules protéolytiquement stables qui imitent les propriétés de liaison et d'activation des ligands endogènes. Ils interagissent spécifiquement avec un récepteur cible et agissent comme un agoniste ou un antagoniste de haute affinité (Saragovi and Gehring, 2000). Le pharmacophore d'une molécule peptidomimétique fait référence à la partie active requise pour une interaction optimale avec la cible biologique (p. ex., les récepteurs des facteurs neurotrophiques) afin de stimuler ou d'inhiber une réponse biologique. Le domaine spécifique d'un récepteur, qui déclenche la réponse biologique lors de la liaison d'un ligand, est désigné comme le point chaud (hot spot) du récepteur (Saragovi et al., 1992b) (Fig.12). Cependant, les points chauds peuvent aussi être activés par des changements conformationnels ou allostériques sans contact direct (Saragovi et al., 1992b).

Les ligands peptidomimétiques ont plusieurs avantages pharmacologiques par rapport aux ligands endogènes, comme leur faible poids moléculaire, leur absence

d'immunogénicité, leur excellent profil pharmacocinétique et leur activité spécifique pour un récepteur. Des ligands peptidomimétiques des NTs avec une activité spécifique pour un ou des récepteurs des NTs ont été développés. Ils représentent un outil idéal pour établir le rôle spécifique du récepteur $p75^{NTR}$ ou TrkA dans la rétine adulte. Récemment, un nouvel agoniste peptidomimétique du récepteur TrkA des NTs, le D3, a été testé comme thérapie neuroprotectrice des neurones cholinergiques dans un modèle de rat aux facultés cognitives altérées. Cette étude a démontré que le traitement avec ce ligand peptidomimétique de TrkA protégeait de façon durable et significative les neurones cholinergiques, avec en conséquence une amélioration des capacités cognitives (Bruno et al., 2004). Plus récemment, ce même composé a démontré une capacité de neuroprotection des CGRs dans un modèle expérimental de glaucome (Shi et al., 2007b). En résumé, ces petits ligands prétéolytiquement stables avec une activité spécifique représentent un outil formidable pour la recherche fondamentale.

1.4.7. La localisation de TrkA et $p75^{NTR}$ dans la rétine adulte.

Il est paradoxal que dans la rétine adulte, les récepteurs TrkA et $p75^{NTR}$ soient pas exprimés par la même population cellulaire. TrkA est exprimé par les cellules ganglionnaires (Rickman and Brecha, 1995; Cui et al., 2002b), alors que des études en microscopie électronique et par fluorescence ont démontré la présence exclusive de $p75^{NTR}$ dans les cellules de Müller et particulièrement dans les prolongements des extensions ces cellules

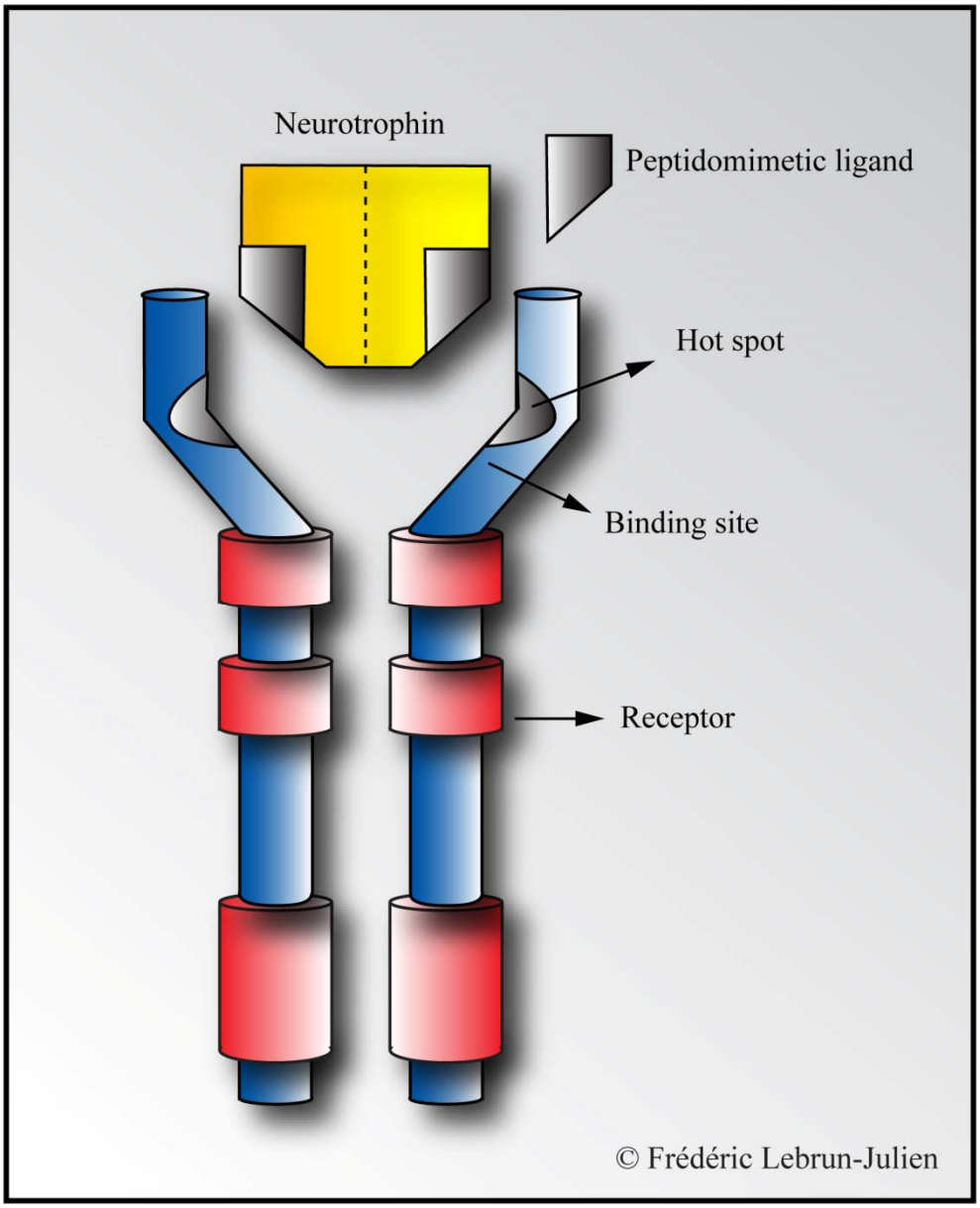

Figure 12. Les composés peptidomimétiques. L'activation des récepteurs par les neurotrophines se produit lors du contact spécifique d'une région du ligand avec la région chaude du récepteur 'Hot Spot' plutôt que la liaison complète de la protéine avec le site de liaison. De petits composés peptidomimétiques peuvent imiter la région de la protéine nécessaire pour activer le 'Hot Spot' du récepteur.

entourant les cellules ganglionnaires (Hu et al., 1998; Hu et al., 1999). Par contre, quelques groupes persistent à dire que $p75^{NTR}$ est exprimé dans une sous-population de cellules ganglionnaires (Ahmed et al., 2006). Malgré cette controverse, tous s'accordent pour dire que le $p75^{NTR}$ est principalement exprimé par les cellules gliales de Müller. L'un des premiers objectifs de cette thèse a permis de démontrer l'absence de $p75^{NTR}$ dans les CGRs, un point sur lequel nous reviendrons dans le chapître 2 et 3. L'injection de neurotrophines ou l'expression endogène de celles-ci doit donc indéniablement causer une réaction des cellules de Müller, un phénomène qui n'a toutefois pas été bien exploré et qui est un des points importants explorés dans cette thèse.

1.4.8. Le rôle des neurotrophines dans la rétine

En accordance avec la théorie des facteurs neurotrophiques, le groupe du Dr. Yves-Alain Barde a démontré en 1989 que la survie des CGRs était dépendante du BDNF durant l'établissement de la projection au collicule supérieur (Rodriguez-Tébar et al., 1989). Le BDNF étant produit en grande quantité par le collicule supérieur et transporté rétrogradement dans les axones jusqu'aux corps cellulaires des CGRs (Herzog and von Bartheld, 1998a; Ma et al., 1998a), le BDNF a rapidement été identifié comme le facteur neurotrophique essentiel à la survie des CGRs. Cependant, d'autres expériences ont démontré que, dans la rétine adulte, la survie des CGRs est moins dépendante du collicule supérieur (Perry and Cowey, 1982), le BDNF étant alors produit de façon plus importante dans la rétine elle-même (Ma et al., 1998a; Seki et al., 2003). Outre le BDNF, le NGF est aussi intimement lié au développement des CGRs. L'importance du NGF dans la rétine a d'abord été mise en évidence lors de la mort cellulaire programmée précoce. En effet, la régulation précoce du nombre de CGRs serait grandement régulée par la liaison du NGF à son récepteur $p75^{NTR}$ (Frade et al., 1996b; Frade and Barde, 1999b). Des expériences utilisant des anticorps contre NGF ou $p75^{NTR}$ ont nettement réduit le nombre de cellules apoptotiques dans la rétine embryonnaire de poulet. De plus, des expériences similaires ont montré une inhibition de la mort cellulaire programmée précoce chez des souris *knockout* pour les gènes du NGF et du $p75^{NTR}$ (Frade et al., 1996b; Frade and Barde, 1999b). Les souris *knockout* pour la protéine adaptatrice NRIF de $p75^{NTR}$ montrent aussi une réduction de la mort développementale dans la rétine, tout comme les embryons de souris *knockout NGF (-/-)* ou *$p75^{NTR}$ (-/-)* (Casademunt

et al., 1999). Cependant, le rôle joué par NGF et son récepteur $p75^{NTR}$ dans la mort cellulaire programmée durant l'embryogenèse et la période post-natale n'est toujours pas clair. Effectivement, même si l'expression de l'ARNm de $p75^{NTR}$ augmente graduellement durant le développement dans la rétine de rongeurs, les CGRs positives au TUNEL chez le rat de 1 jour ne présentent pas de marquage détectable pour $p75^{NTR}$. Par contre, les prolongements des cellules de Müller sont très fortement immunoréactives (Ding et al., 2001).

Dans la rétine adulte, le rôle du NGF reste obscur. On pourrait croire que le BDNF accapare toute l'attention ! Certes, parmi les neurotrophines, le BDNF est le mieux caractérisé et le facteur de survie le plus efficace pour les RGCs lésées. Les CGRs adultes expriment très fortement le récepteur TrkB et la capacité de survie des CGRs axotomixées est nettement améliorée par l'ajout exogène de BDNF (Mey and Thanos, 1993b; Mansour-Robaey et al., 1994a; Peinado-Ramon et al., 1996b; Di Polo et al., 1998b; Klocker et al., 2000; Chen and Weber, 2001a). Le NGF est presque tombé dans l'oubli après la publication de quelques études démontrant son incapacité à promouvoir la survie des cellules ganglionnaires à la fois *in vivo* et *in vitro* (Johnson et al., 1986b; Cohen et al., 1994; Cui and Harvey, 1995; Bosco and Linden, 1999; Shi et al., 2007b). Ces résultats étaient cependant surprenants, puisque l'expression de TrkA a été démontrée dans les CGRs (Rickman and Brecha, 1995; Cui et al., 2002b). Les mécanismes qui pourraient expliquer l'absence de support trophique par le NGF sont inconnus. Parmi les explications possibles, on peut envisager que l'axotomie et/ou l'exposition au NGF puisse entrainer une régulation négative de l'expression du récepteur TrkA par les CGRs. Il se peut également que le NGF ou le proNGF agissant sur le récepteur $p75^{NTR}$ active une voie de signalisation pro-apoptotique qui limiterait la survie neuronale. En fait, ces questions sont à la base de notre démarche scientifique et seront abordées de front dans les chapîtres 2 et 3.

1.5. Dr Jekyll et Mr Hyde, l'étrange cas du NF-κB

1.5.1. Le facteur de transcription NF-κB

Le facteur de transcription NF-κB a été découvert en 1986 dans les lymphocytes *B* grâce à sa capacité de favoriser la transcription de la chaîne légère kappa (κ) des immunoglobulines (Sen and Baltimore, 1986). Sa présence a été retrouvée ensuite dans de nombreux types cellulaires. Nous savons maintenant que le NF-κB est un élément clé de la régulation de

l'expression des gènes de l'immunité, mais aussi de la prolifération, de la différenciation et de la survie cellulaire (Baeuerle and Henkel, 1994; Baldwin, 1996; Bonizzi and Karin, 2004). Il est donc partie plusieurs signalisations intracellulaires. Globalement, il régule parfois positivement mais le plus souvent négativement l'apoptose, selon le type cellulaire en question (Perkins and Gilmore, 2006). La particularité de ce facteur de transcription réside en sa rapidité d'activation. Sa vitesse d'exécution se base sur le fait qu'il est séquestré dans le cytoplasme sous forme inactive en raison d'une interaction avec un inhibiteur appelé IκB. Le signal de stimulation induisant l'activité du NF-κB est transmis par une cascade de protéines kinases aboutissant à la phosphorylation de IκB, qui signale sa destruction par protéolyse. Cet évènement démasque le signal de localisation nucléaire de NF-κB qui est alors véhiculé vers le noyau où il active la transcription de gènes. Cette séquestration dans le cytoplasme permet la disponibilité immédiate du facteur de transcription, sans avoir recours à une synthèse de protéine (Perkins and Gilmore, 2006). Chez les mammifères, la famille de protéines NF-κB/Rel comporte cinq membres : NF-κB1/p50 (et son précurseur p105); NF-κB2/p52 (et son précurseur p100); p65 (RelA), RelB et c-Rel. Le NF-κB est composé d'homo- ou d'hétérodimères formés d'une combinaison de cinq sous-unités, dont le plus commun est le dimère p50/p65 (Baldwin, 1996; Ghosh et al., 1998). Chaque membre de la famille NF-κB/Rel contient, dans sa partie N-terminale, un domaine d'homologie Rel (Rel Homology Domain). Ce domaine contient le signal de localisation nucléaire (SLN) et est responsable de la liaison à l'ADN, de la dimérisation et de l'association avec IκB.

1.5.1.1. Les mécanismes d'activation du NF-κB

Il existe deux principales voies d'activation du NF-κB selon l'inducteur en cause (Imbert et al., 1996; Karin and Ben-Neriah, 2000; Pomerantz and Baltimore, 2002; Dejardin, 2006): la voie classique ou canonique et la voie alternative (Fig. 13).

1.5.1.1.1. Voie classique (canonique) d'activation du NF-κB

La voie canonique induite par le TNFα, l'IL-1β ou le LPS, est initiée par des récepteurs différents, mais converge vers le complexe IKK, composé d'un hétérodimère d'IKKα et IKKβ et d'un homodimère de NEMO. Dans les cellules non induites,

IκBα séquestre NF-κB dans le cytoplasme en masquant son signal de localisation nucléaire (SLN). Dans les minutes qui suivent la stimulation par ces inducteurs, le complexe IKK est activé par phosphorylation et il s'en suit une phosphorylation spécifique d'IκBα par le complexe IKK qui permet sa poly-ubiquitination (Traenckner and Baeuerle, 1995; Rodriguez et al., 1996). IκBα est alors dégradé par le protéasome 26S. Le NF-κB libéré se dirige vers le noyau pour activer la transcription de ses gènes cibles. Parmi ceux-ci, on retrouve le gène codant pour la protéine IκBα, laquelle, nouvellement synthétisée, retourne dans le noyau afin de décrocher NF-κB de l'ADN. En effet, l'affinité du NF-κB pour IκBα est plus forte que pour l'ADN. Ce processus entraîne la terminaison du signal en exportant du noyau le complexe IκBα/NF-κB.

1.5.1.1.2. Voie alternative d'activation du NF-κB

Un mécanisme alternatif d'induction de l'activité du NF-κB a été mis en évidence. Il repose sur la protéolyse inductible de p100, réalisée de manière dépendante d'IKKα et indépendante d'IKKβ et NEMO. Cette voie peut être stimulée par les inducteurs comme LTβ, BAFF, CD40L et TWEAK. Les récepteurs spécifiques de ces ligands sont des membres de la superfamille des récepteurs du TNF. Lorsqu'un de ces ligands se lie à son récepteur, la voie alternative est activée. La protéine NIK (NF-κB-inducing kinase) phosphoryle un homodimère d'IKKα. Cet homodimère ainsi activé phosphoryle p100 dans sa partie C-terminale qui comporte des répétitions ankyrine. Une ubiquitination subséquente entraîne p100 vers le protéasome pour une dégradation partielle. Le p52 obtenu après cette maturation est activé et peut ainsi migrer vers le noyau pour réguler la transcription. En général, p100/p52 est lié à RelB. (Dejardin et al., 2002; Pomerantz and Baltimore, 2002). Cette voie en généralement associée à l'organogénèse lymphatique tout comme au développement et à la survie des lymphocytes B (Senftleben et al., 2001; Falk and Jorge, 2003).

1.5.1.2. Gènes sous le contrôle du NF-κB

Le facteur de transcription NF-κB régule la transcription d'un grand nombre de gènes codant pour des protéines impliquées dans la réponse inflammatoire, la réponse immunitaire, la prolifération cellulaire et l'apoptose, ainsi que l'inhibition du NF-κB. La réponse

inflammatoire induite par NF-κB peut être médiée notamment par des cytokines (TNFα, IL-1β, IL-6, LTβ), des chémokines (IL-8, MIP-1α, MCP-1) et des molécules d'adhésion (ICAM, VCAM). Étant donné l'importance des fonctions biologiques régulées par NF-κB, une activation aberrante de ce facteur de transcription joue un rôle critique dans le développement et la progression des cancers (Pomerantz and Baltimore, 2002).

1.5.1.3. Le rôle du NF-κB dans les neuropathies

Dans le SNC, le NF-κB peut-être activé par plusieurs effecteurs comme le glutamate via son récepteur ionotropique NMDA, ou les neurotrophines comme le NGF via le récepteur $p75^{NTR}$ (Carter et al., 1996; Kaltschmidt et al., 2005). Cependant, le rôle joué par le NF-kB dans les maladies neurodégénératives est controversé. Plusieurs études ont montré que NF-kB est neuroprotecteur par suite d'une blessure (Maggirwar et al., 1998; Hamanoue et al., 1999b; Bhakar et al., 2002a), tandis que d'autres groupes ont montré que l'activation du NF-kB facilitait les dommages aux neurones (Schwaninger et al., 2006b). Un véritable Dr Jekyll et Mr Hyde! En fait, la multitude de gènes transcris sous le contrôle du NF-kB est à l'origine de plusieurs fonctions cellulaires, ce qui explique, que son action puisse être à la fois pro-apoptotique ou pro-survie selon la situation, le type cellulaire ou l'activateur en question. De façon plus générale, il semblerait qu'une perturbation de l'activation physiologique du NF-κB dans les neurones, soit à la baisse soit à la hausse, puisse être néfaste (Kaltschmidt et al., 2005). Par exemple, dans la maladie d'Alzheimer, il y a diminution de l'activation de NF-κB et la régulation à la hausse de sa signalisation est neuroprotectrice (Mattson, 2004). D'un autre côté, le NF-κB hyperactivé est en cause dans plusieurs maladies comme l'ischémie (Schneider et al., 1999a) et le Parkinson (Hunot et al., 1997). Le NF-κB est présent dans les neurones, mais aussi dans les cellules gliales. Dans les cellules gliales, le NF-κB peut-être à l'origine de plusieurs cytokines proinflammatoires cytotoxiques pour les neurones comme,

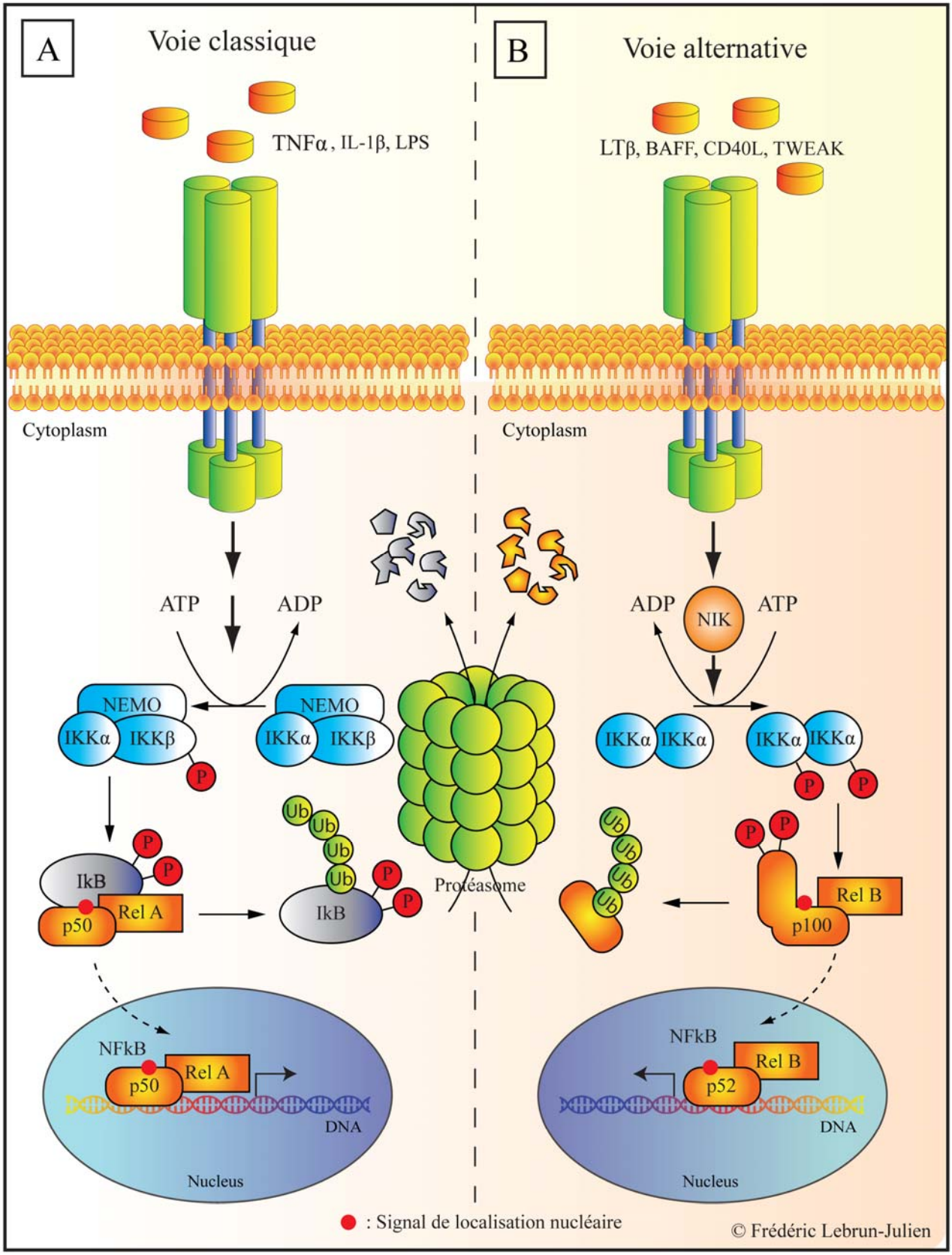
A
Voie classique
TNFα, IL-1β, LPS
Cytoplasm
ATP
ADP
NEMO
IKKα
IKKβ
P
IkB
p50
Rel A
Ub
Protéasome
NFkB
DNA
Nucleus
B
Voie alternative
LTβ, BAFF, CD40L, TWEAK
Cytoplasm
ADP
NIK
ATP
IKKα
Rel B
p100
p52
: Signal de localisation nucléaire
© Frédéric Lebrun-Julien

Figure 13. Les voies d'activation du NF-κB. (A) Via la cascade classique d'activation du NF-κB, une stimulation, active le complexe NEMO/IKK qui phosphoryle ensuite la protéine inhibitrice IκB. Une fois phosphorylée, IκB est ubiquitinylée pour ensuite être dégradée par le protéasome, ce qui libère le signal de localisation nucléaire du complexe NF-κB. Ce complexe est alors transporté dans le noyau et active ses gènes cibles. (B) Par la voie alternative d'activation du NF-κB, sous l'action de stimuli particuliers, il y a activation de la protéine NIK qui phosphoryle IKKα. Cette dernière phosphoryle le précurseur p100, lequel est ensuite ubiquitinylé et partiellement dégradé par le protéasome pour libérer le complexe p52/RelB qui est alors transporté au noyau pour activer ses gènes cibles.

l'interleukine-1 bêta, le TNFα et le ligand des récepteurs de mort, Fas (Mattson, 2005). De plus, le NF-κB peut induire l'activité de la NO synthase des cellules gliales, qui augmente la production de NO et de radicaux libres neurotoxiques (Mattson, 2005). L'activité du NF-κB et le rôle joué par celui-ci dans la rétine lésée a été scruté à la loupe dans le quatrième chapître de cette thèse.

1.6. Le TNFα, une cytokine pro-apoptotique

1.6.1. La signalisation de TNFα

Le TNFα ou facteur nécrosant des tumeurs alpha, est une cytokine pro-inflammatoire qui joue le rôle de médiateur dans l'inflammation, l'immunité, la prolifération, la différenciation et l'apoptose. Le TNFα exerce ses effets à travers la liaison à deux récepteurs distincts, TNFR1 (TNF Receptor type 1) et TNFR2, qui appartiennent à la superfamille des récepteurs au TNF (TNFR) (Baud and Karin, 2001; Chen and Goeddel, 2002). Cette superfamille regroupe un grand nombre du récepteurs, notamment ceux de FasL (*Fas Ligand*), RANKL (*Receptor-Activator of NF-κB Ligand*), TRAIL (*TNF-Related Apoptosis-Inducing Ligand*), LTβ, BAFF, CD40L, TWEAK (*TNF-like Weak inducer of apoptosis*) et $p75^{NTR}$ (Baud and Karin, 2001; Saitoh et al., 2003). Comme $p75^{NTR}$, aucun de ces récepteurs ne possède une activité enzymatique intrinsèque. La transmission du signal est engendrée par le recrutement de molécules intracellulaires adaptatrices. Ces molécules activent des protéines effectrices qui, à leur tour, activent des caspases et des facteurs de transcription comme NF-κB et AP-1 (*Activating Protein-1*) (Baud and Karin, 2001). TNFR1 est exprimé constitutivement par presque tous les types cellulaires alors que TNFR2 est généralement inductible et préférentiellement exprimé par les cellules endothéliales et hématopoïétiques (Idriss and Naismith, 2000).

La liaison de TNFα au récepteur peut induire deux complexes protéiques entraînant des activités distinctes. Le complexe I, lié à la membrane plasmique, comprend TNFR1, TRADD, RIP et TRAF2. La protéine TRAF2 permet le recrutement des IKKs au TNFR1, et RIP est responsable de l'activation du complexe. Ces événements conduisent à l'activation du NF-κB et à l'expression de ses gènes cibles. Ce complexe I participe également à l'activation de la cascade des MAPK. Le complexe II, cytoplasmique, contient TRADD, RIP,

FADD et la caspase-8. Il est essentiel à l'apoptose induite par le TNFα via la cascade des caspases (Shen and Pervaiz, 2006). Le TNFR1 est donc capable d'entraîner deux réponses opposées : la survie et l'apoptose. L'ubiquitination de RIP permet de réguler le changement entre ces deux voies (O'Donnell et al., 2007). La signalisation du TNFα et celle du NF-κB sont étroitement liées, le TNFα est l'un des nombreux activateurs du NF-κB tandis que le gène du TNFα est l'une des nombreuses cibles de la transcription induite par le NF-κB. En fait, l'expression du TNFα est sujet à une autorégulation par le NF-κB activé (Collart et al., 1990).

1.6.2. Le TNFα, un médiateur de mort sécrété par la glie dans la rétine

Le TNFα est sur-exprimé dans de nombreuses maladies du SNC, incluant la sclérose en plaques, la maladie de Parkinson et la maladie d'Alzheimer (Shohami et al., 1999). Dans l'œil, une production de TNFα a été démontrée dans la microglie et les astrocytes du le nerf optique de patients atteints de glaucome (Yan et al., 2000a; Yuan and Neufeld, 2000; Yuan and Neufeld, 2001). Plusieurs études du groupe du Dr. Tezel ont indiqué que le TNFα sécrété par les cellules gliales stressées des rétines glaucomateuses peut induire la mort des CGRs par activation de son récepteur de mort, qui entraîne un dysfonctionnement des mitochondries et des dommages oxydatifs (Tezel and Wax, 2000a, 2004; Tezel et al., 2004a). Le TNFα est toxique pour les CGRs immunopurifiées et les CGRs en culture mixte, quand les cellules gliales sont soumises à un stress (Tezel and Wax, 2000a; Fuchs et al., 2005). Paradoxalement, d'autres études semblent montrer que le TNFα induit une activité neuroprotectrice dans un modèle de CGRs dissociées *in vitro,* voir même par suite d'axotomie *in vivo* (Diem et al., 2001a; Meyer et al., 2001). L'action du TNFα sur les CGRs adultes reste donc mal définie, bien que toutes ces études démontrent au moins une chose : qu'il puisse jouer un rôle dans les rétinopathies et les blessures de la rétine. En fait, le TNFα s'avère un acteur de la signalisation de mort par la glie, capable d'affecter la survie des CGRs dans les deux modèles différents de blessure étudiés dans cette thèse (voir chapître 3 et 4).

1.7. Objectifs de la thèse

Les cellules de Müller sont responsables de l'homéostasie de la rétine et réagissent à toute situation de stress par une réaction appelée gliose. Même s'il est facilement envisageable de penser qu'elles jouent un rôle primordial dans les différents mécanismes de mort entourant les CGRs, peu d'études ont tenté d'établir l'implication de ces cellules gliales dans les processus neurodégénératifs. La première partie de ma thèse s'intéresse au rôle joué par les cellules de Müller dans la signalisation des neurotrophines et notamment du NGF. Comme les cellules de Müller semblaient exprimer le récepteur $p75^{NTR}$ du NGF, et les CGRs, le récepteur TkA, nous avons tenté d'établir le rôle spécifique de chacun de ces récepteurs dans la survie neuronale après section du nerf optique. À cette fin, nous avons utilisé des composés peptidomimétiques agonistes ou antagonistes avec une activité spécifique différente pour chacun des récepteurs du NGF.

Après avoir étudié le rôle des neurotrophines, nous avons tourné notre attention vers leur précurseur, les proNeurotrophines. Les proneurotrophins sont principalement connues comme étant des ligands pro-apoptotiques qui ont comme récepteurs le $p75^{NTR}$, un récepteur, qui comme mentionné précédemment, est largement exprimé par les cellules de Müller de la rétine adulte. Le deuxième objectif de ma thèse a donc été de vérifier l'effet du proNGF dans la rétine adulte et d'établir le rôle joué par son récepteur $p75^{NTR}$ sur les cellules gliales de Müller. Dans ce but, nous avons fait des injections de proNGF dans l'humeur vitrée de rat et de souris préalablement marquées au fluorogold pour ainsi déterminer l'effet du proNGF sur la survie neuronale. Nous avons aussi utilisé des souris *knockout* dont les gènes codant pour le récepteur $p75^{NTR}$, le récepteur sortiline, NRAGE ou TNFα afin de caractériser l'implication de chacun de ces gènes dans la signalisation du proNGF dans la rétine adulte.

Finalement, le dernier objectif de ma thèse a été d'étudier le rôle joué par les cellules de Müller dans les mécanismes d'excitotoxicité. Nous avons établi un modèle basé sur l'injection intravitréenne de NMDA chez la souris. En utilisant en combinaison la technique de marquage rétrograde des CGRs et la technique de TUNEL, qui marque les cellules apoptotiques, nous avons établi la chronologie de mort des cellules de la rétine dans un processus excitotoxique. Nous avons ensuite déterminé quels acteurs moléculaires étaient impliqués dans la mort des CGRs par excitotoxicité.

CHAPITRE 2

PREMIER ARTICLE: "INHIBITION OF P75^{NTR} IN GLIA POTENTIATES TRKA-MEDIATED SURVIVAL OF INJURED RETINAL GANGLION CELLS".

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L'introduction de cette thèse a souligné l'importance des interactions neurones-glie, en ce qui a trait, tout particulièrement, au rôle essentiel des cellules de Müller de la rétine. Cette première étude est fondée sur l'hypothèse d'une participation des cellules de Müller à la signalisation des neurotrophines par suite d'une lésion du nerf optique. L'objectif principal a été de vérifier la localisation des récepteurs TrkA et p75^{NTR} du NGF et de déterminer leurs rôles spécifiques en fonction de leur localisation sur les CGRs ou les cellules gliales de Müller.

INHIBITION OF P75^{NTR} IN GLIA POTENTIATES TRKA-MEDIATED SURVIVAL OF INJURED RETINAL GANGLION CELLS

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2.1. ABSTRACT

Little is known about the molecular mechanisms that limit the ability of retinal neurons to respond to neurotrophic factor stimulation following axonal injury. In the adult retina, nerve growth factor (NGF) binds to TrkA (expressed by neurons) and $p75^{NTR}$ (expressed by Müller glia), but fails to promote the survival of axotomized retinal ganglion cells (RGCs). We addressed the functional role of TrkA and $p75^{NTR}$ in this lack of survival by using peptidomimetic agonistic or antagonistic ligands specific for each receptor. While administration of exogenous NGF failed to rescue axotomized RGCs, administration of selective TrkA agonists led to robust neuroprotection. Surprisingly, we found a remarkable survival of axotomized RGCs following pharmacological inhibition of $p75^{NTR}$ or in $p75^{NTR}$ knockout mice. Combination of NGF or TrkA agonists with $p75^{NTR}$ antagonists further potentiated RGC neuroprotection *in vivo*, an effect that was greater than each treatment alone. NGF can therefore be neuroprotective when acting on neuronal TrkA receptors but engagement of $p75^{NTR}$ on glial cells antagonizes this effect. Our data reveal a novel mechanism by which $p75^{NTR}$ expressed on retinal glia can profoundly influence neuronal survival.

Keywords: Retinal ganglion cells; Nerve growth factor; TrkA; $p75^{NTR}$; Peptidomimetic ligands; Neuronal survival; Knockout.

2.2. INTRODUCTION

The neurotrophins exert a potent survival effect on adult central neurons undergoing degeneration induced by a broad variety of insults. The neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). Their biological effects are mediated by two classes of cell surface receptors: i) the tropomyosin related kinase (Trk) family of receptor tyrosine kinases comprising TrkA, the receptor for NGF; TrkB, the receptor for BDNF and NT-4/5; and TrkC, the receptor for NT-3; and ii) the p75 receptor (p75NTR) which binds all neurotrophins with similar affinity (Huang and Reichardt, 2003; Teng and Hempstead, 2004).

It is now well established that upon binding to Trk receptors, mature neurotrophins stimulate multiple signaling pathways including the extracellular signal-regulated kinase 1/2 (Erk1/2) and the phosphatidylinositol 3/Akt kinase pathways, which promote neuronal survival (Cui, 2006; Reichardt, 2006). In contrast, the role of $p75^{NTR}$ has been considerably more enigmatic partly because $p75^{NTR}$ lacks kinase activity, hence does not signal via conventional pathways, and can interact with several co-receptors (Barker, 2004; Roux and Barker, 2002). While $p75^{NTR}$ can enhance binding of Trk receptors to their preferred ligands, it can also mediate neuronal apoptosis in response to neurotrophins and pro-neurotrophins (Frade et al., 1996; Hempstead, 2006; Nykjaer et al., 2004).

The mammalian retinocollicular system is an excellent model to characterize the biological activity of neurotrophins *in vivo*. Among neurotrophins, BDNF is the best characterized and the most potent survival factor for injured retinal ganglion cells (RGCs) (Chen and Weber, 2001; Di Polo et al., 1998; Klöcker et al., 2000; Mansour-Robaey et al., 1994; Mey and Thanos, 1993; Peinado-Ramon et al., 1996). In contrast, NGF has received less attention because several studies showed that this neurotrophin fails to promote RGC survival both *in vitro* and *in vivo* (Bosco and Linden, 1999; Cohen et al., 1994; Cui and Harvey, 1995; Johnson et al., 1986; Shi, 2007). This is surprising as RGCs have been shown to express TrkA (Cui et al., 2002; Rickman and Brecha, 1995), thus the mechanisms underlying lack of NGF-mediated RGC trophic support are poorly understood. Possible explanations include that: i) axotomy and/or exposure to NGF lead to downregulation of TrkA receptors by RGCs, and ii) NGF or pro-NGF acting on $p75^{NTR}$ may activate proapoptotic pathways that limit neuronal survival.

To test these hypotheses, we asked the following three questions. First, are there axotomy-induced or NGF-induced changes in TrkA levels in adult RGCs? Because TrkA would mediate the survival response of RGCs to NGF, we examined if axotomy or exposure to NGF lead to detectable changes in TrkA protein levels. Second, given that NGF-mediated activation of $p75^{NTR}$ can induce neuronal apoptosis; do adult RGCs express $p75^{NTR}$ protein *in vivo*? Third, what is the functional consequence of $p75^{NTR}$ and/or TrkA activation on the survival of adult RGCs undergoing degeneration? To address this question, we used small molecule peptidomimetic ligands (agonists or antagonists), specific for TrkA or $p75^{NTR}$, to establish the role of each receptor. The peptidomimetic ligands used here have been well characterized both *in vitro* and *in vivo* (Bruno et al., 2004; Debeir et al., 1999a; LeSauteur et al., 1995; Li et al., 2005; Maliartchouk et al., 2000; Saragovi and Zaccaro, 2002; Shi et al., 2007; Zaccaro et al., 2005) and have several advantages including their ability to selectively agonize or antagonize neurotrophin receptors, lack of immunogenicity, capacity to diffuse in the target tissue, water solubility and excellent stability *in vivo*.

In this study, we demonstrate that RGCs upregulate TrkA soon after axotomy and that TrkA levels remain high in the presence of exogenous NGF. $p75^{NTR}$ protein is expressed by Müller glial cells, but not RGCs, demonstrating a distinctive topological and cellular separation of these receptors in the adult rat and mouse retinas. Our functional data indicate that while NGF by itself does not promote RGC survival, selective activation of TrkA receptors using peptidomimetic agonistic ligands is markedly neuroprotective. Surprisingly, selective blockers of $p75^{NTR}$ or absence of $p75^{NTR}$ protect RGCs from axotomy-induced death. Moreover, combination of NGF or TrkA agonists with $p75^{NTR}$ antagonists functions synergistically to enhance RGC survival. Collectively, these results indicate that NGF can be neuroprotective when acting only on TrkA receptors but not on $p75^{NTR}$, and reveal that p75NTR activation in Müller glia may antagonize neuronal survival.

2.3. EXPERIMENTAL PROCEDURES

2.3.1. Peptidomimetic Ligands

The peptidomimetic compounds used in this study were synthesized and validated for their binding specificity to neurotrophin receptors as previously described (LeSauteur et al.,

1995; Pattarawarapan et al., 2002; Zaccaro et al., 2005). The following seven, small peptides were used (Table 1): i) D3, a selective agonist of TrkA that does not bind to $p75^{NTR}$ (Maliartchouk et al., 2000; Bruno et al., 2004), ii) 3Ac, an agonist of both TrkA and TrkC (Zaccaro et al., 2005), iii) 3Aa, a selective agonist of TrkC only (Zaccaro et al., 2005), iv) C92–96, an antagonist of TrkA that selectively inhibits NGF binding to TrkA (LeSauteur et al., 1995; Debeir et al., 1999a), v) C30-35, an antagonistic ligand of $p75^{NTR}$ that selectively inhibits NGF binding to $p75^{NTR}$ (LeSauteur et al., 1995; Longo et al., 1997; Saragovi and Zaccaro, 2002; Botchkarev et al., 2003), vi) C28-35, an antagonist that inhibits NGF binding to both TrkA and $p75^{NTR}$ (LeSauteur et al., 1995; Xie and Longo, 2000; Saragovi and Zaccaro, 2002), and vii) 3Bj, a peptide of similar structure and physicochemical properties but with no activity (Zaccaro et al., 2005). Previous *in vitro* and *in vivo* studies using these peptidomimetic compounds showed lack of toxic effects (Debeir et al., 1999a; Bruno et al., 2004; Li et al., 2005; Shi et al., 2007a).

2.3.2. Experimental Animals and Surgical Procedures

Animal procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care for the use of experimental animals (www.ccac.ca). All surgeries were carried out in adult, female Sprague-Dawley rats (180-200 g) or in $p75^{NTR}$ knockout mice and wild-type littermate controls (kind gift of P. A. Barker, Montreal Neurological Institute, McGill University, QC) under general anesthesia (2% Isoflurane, 0.8 litre/min).

i) Retrograde Labeling and Optic Nerve Axotomy: RGCs were selectively labeled by application of the retrograde tracer FluoroGold (2%, Fluorochrome, Englewood, CO) to the superior colliculus as described by us (Cheng et al., 2002b; Sapieha et al., 2003b; Pernet et al., 2005b; Pernet and Di Polo, 2006b). Briefly, the superior colliculi on both brain hemispheres were exposed and a small piece of gelfoam (Pharmacia and Upjohn, Inc, Mississauga, ON) soaked in FluoroGold was applied to their surface. Seven days is the earliest time for detection of a maximum number of RGCs after application of FluoroGold to the rat superior colliculus (Berkelaar et al., 1994b; Mansour-Robaey et al., 1994b; Peinado-Ramon et al., 1996b; Cheng et al., 2002b). Thus, to ensure that all RGCs were fully labeled prior to axonal injury, axotomy was performed at 7 days after FluoroGold application. The left optic nerve was exposed and carefully transected at 0.5-1 mm from the optic nerve head,

avoiding injury to the ophthalmic artery. Fundus examination was routinely performed immediately after axotomy and 3-5 days later to check the integrity of the retinal circulation after surgery. Animals showing signs of compromised blood supply were excluded from the study.

ii) Intravitreal Injections: NGF (1 μg/μl, human recombinant NGF, PeproTech Inc., Rocky Hill, NJ), peptidomimetic ligands (1 μg/μl) or the polyclonal anti-$p75^{NTR}$ antibody REX (10 μg/μl, gift of P. A. Barker) were injected into the vitreous chamber of the left eye using a Hamilton syringe fitted with a 32-gauge glass microneedle (total volume: 5 μl). Phosphate buffer saline (PBS) was used as vehicle control. The sclera was exposed and the tip of the needle was inserted at a 45° angle through the sclera and retina into the vitreous space using a posterior approach. This route of administration avoided injury to the iris or lens, which can promote RGC survival (Mansour-Robaey et al., 1994b; Leon et al., 2000b). The injection was performed within ~30 sec, after which the needle was gently removed. For combined treatments, animals received two consecutive injections of each compound with a delay of 20 min between each other. Surgical glue (Indermill, Tyco Health Care, Mansfield, MA) was used to seal the injection site. Intraocular injections were performed immediately after optic nerve axotomy. The right eye was never operated on and served as internal contralateral control for each animal.

2.3.3. Quantification of Neuronal Survival and Statistical Analysis

Rats were euthanized at 1 or 2 weeks post-axotomy by transcardial perfusion with 4% paraformaldehyde and both the left (optic nerve lesion) and right (intact control) retinas were dissected and fixed for an additional 30 min. Retinas were then flat-mounted vitreal side up on a glass slide for examination of the ganglion cell layer. FluoroGold-labeled neurons were counted within 3 square areas at distances of 1, 2 and 3 mm from the rat optic disc in each of the 4 retinal quadrants for a total of 12 retinal areas as described by us and others (Villegas-Perez et al., 1993; Mansour-Robaey et al., 1994b; Cheng et al., 2002b; Sapieha et al., 2003b; Pernet and Di Polo, 2006b). In mice retinas, quantification was performed as in rats but the counted areas were at 0.25 mm, 0.625 mm and 1 mm from the optic disc for a total of 12 retinal areas as described (Sapieha et al., 2005a; Murphy et al., 2007). Macrophages and microglia that may have incorporated FluoroGold after phagocytosis of dying RGCs were

distinguished by their morphology and excluded from our quantitative analyses (Thanos, 1991a; Thanos, 1991b). Fluorescent staining was examined with a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Canada, Kirkland, QC), and pictures were captured with a CCD video camera (Retiga, Qimaging, Burnaby, BC) and analyzed with Northern Eclipse software (Empix Imaging, Mississauga, ON). Data analysis and statistics were performed using the GraphPad Instat software (GraphPad Software Inc., San Diego, CA) by a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post-test or by a Student's t-test.

2.3.4. Retinal Immunohistochemistry

Rats (N = 4) or $p75^{NTR}$ knockout mice (N = 4) were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Retina radial cryosections (16 μm) were prepared as previously described by us (Sapieha et al., 2003b). The following primary antibodies were added to the retinal sections in blocking solution and incubated overnight at 4°C: polyclonal anti-TrkA (1 μg/ml; gift of Dr. L. F. Reichardt, University of California, San Francisco, CA), monoclonal anti-cellular retinaldehyde binding protein (CRALBP, 1:1,000, gift from J. C. Saari, University of Washington, Seattle, WA), or polyclonal anti-$p75^{NTR}$ (REX, 2 ng/μl, gift of P.A. Barker, McGill University, QC). Sections were then incubated with the following secondary antibodies: sheep anti-mouse IgG (1 μg/ml, FITC conjugate, Sigma, Oakville, ON) or anti-rabbit IgG (1 μg/ml, Cy3, Jackson ImmunoResearch Laboratories, Inc.). Sections were washed and mounted in anti-fade reagent (SlowFade, Molecular Probes, Eugene, OR). Fluorescent labeling was observed using a microscope Zeiss AxioSkop 2 Plus (Carl ZeissCanada, Kirkland, QC) or confocal microscope Leica TCS-SP1 (Leica Microsystems, Heidelberg, Germany).

2.3.5. Western Blot Analysis

Whole fresh retinas (N = 3 per condition) were rapidly dissected and homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer (20 mM Tris pH 8.0, 135 mM NaCl, 1% NP-40, 0.1% SDS, and 10% glycerol supplemented with protease inhibitors). Retinal lysates were incubated on ice for 30 min and then centrifuged at 14,000 rpm for 5 min; the supernatants containing solubilised proteins were collected. Protein

concentrations were determined with a detergent-compatible protein assay (Bio-Rad Life Science, Mississauga, Ontario, Canada). Retinal extracts (75–100 μg) were resolved on SDS gel or Precast SDS polyacrylamide 4-20% gels (Bio-Rad Life Science) and transferred to nitrocellulose membranes (Bio-Rad Life Science). Blots were incubated in 10 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Tween-20 (TBST) and 5% dry non-fat milk for 1 hr at room temperature to block non-specific signals. Blots were then incubated overnight at 4°C with anti-TrkA (1 μg/ml), polyclonal phospho-Erk1/2 that specifically recognizes Erk1/2 phosphorylated on Thr202/185 and Tyr204/187 residues (0.2 μg/ml, BioSource, International, Camarillo, CA), polyclonal pan Erk1/2 (2.3 μg/ml, BioSource International), or monoclonal β-actin (0.5 μg/ml, Sigma) antibodies. Membranes were washed in TBST and incubated in peroxidase-linked secondary antibodies (0.5 μg/ml, GE Healthcare, Baie d'Urfé, QC) for 1 hr at room temperature. Blots were developed using chemiluminescence reagents (ECL or Plus-ECL, Perkin Elmer Life and Analytical Sciences, Woodbridge, ON) and exposed to autoradiographic film (X-OMAT; Eastman Kodak, Rochester, NY). Densitometric analysis was performed using Scion Image software (Scion Corporation, Frederick, MD) on scanned autoradiographic films obtained from a series of 3 independent western blots each carried out using retinal samples from distinct experimental groups. The densitometric values obtained for TrkA were normalized with respect to their ß-actin loading controls in the same blot to obtain the final ratios. The densitometric values obtained for phosphorylated (active) Erk1/2 were normalized with respect to their loading controls (non-phosphorylated Erk1/2) in the same blot to obtain the final phosphorylated/total protein ratios.

2.4. RESULTS

2.4.1. Optic nerve axotomy increases TrkA protein levels in RGCs

The reported failure of NGF to protect RGCs is intriguing because these neurons have been shown to express TrkA receptors in developing and adult RGCs (Rickman and Brecha, 1995; Cui et al., 2002b). To address the possibility that lack of TrkA in adult RGCs may limit their ability to respond to this neurotrophin, we examined the pattern of TrkA protein expression in mature retinas, both intact and axotomized. Retinal immunohistochemistry demonstrated low levels of TrkA in the ganglion cell layer in the intact retina (Fig. 1A). Following

axotomy, there was robust upregulation of TrkA immunostaining in the ganglion cell layer (Fig. 1B), with most RGCs expressing high levels of this receptor (Fig. 1C-E). Confocal microscopy analysis confirmed that all TrkA-positive cells were RGCs, visualized with the retrograde tracer FluoroGold (Fig. 1F-H).

Ligand-induced downregulation of neurotrophin receptors has been reported both *in vitro* and *in vivo* (Knusel et al., 1994; Knusel et al., 1997). Western blot analysis was carried out to test whether intravitreal injection of NGF altered TrkA protein expression by RGCs and to validate our immunocytochemical findings. Analysis of retinal homogenate samples from intact eyes injected with NGF or PBS (control) showed low levels of TrkA, similar to intact non-injected retinas. Axotomy alone substantially increased retinal TrkA protein expression, and this level remained high whether or not NGF was administered at the time of axotomy (Fig. 1I). Densitometric analysis confirmed that optic nerve transection resulted in a ~3.3-fold increase of TrkA with respect to control retinas (ANOVA, $P < 0.001$), while injection of NGF at the time of axotomy did not change significantly the levels of TrkA with respect to axotomy alone (ANOVA, $P > 0.05$) (Fig. 1J).

To test whether TrkA expressed by RGCs was activated *in vivo*, we examined the levels of phosphorylated (active) retinal Erk1/2 following intraocular injection of NGF or D3, a selective TrkA agonist. The Erk1/2 pathway is a canonical signaling pathway downstream of Trk receptors and has been previously used as a reliable readout of retinal Trk activity *in vivo* (Cheng et al., 2002b). Eyes treated with NGF or D3 showed a marked increase in phospho-Erk1/2 compared to control PBS-treated or intact retinas (Figs. 1K, L) (ANOVA, $P < 0.001$). Collectively, these results are in agreement with previous studies showing that retinal Trk proteins increase following optic nerve injury and ocular hypertension (Cui et al., 2002b; Rudzinski et al., 2004), and extend these observations to show that injection of NGF at the time of axotomy does not alter TrkA protein expression by RGCs but leads, as D3, to receptor activation. These data indicate that TrkA is present in injured RGCs and that signaling by this receptor may contribute to neuronal survival after optic nerve injury.

2.4.2. Selective TrkA peptidomimetic agonists promote survival of axotomized RGCs

To test whether TrkA activation can promote RGC neuroprotection, we analyzed the ability of RGCs to survive in the presence of exogenous NGF or the TrkA agonist D3. In control uninjured retinas, the mean density of FluoroGold-labeled RGCs was 2,347 ± 54 cells/mm^2 (mean ± S.E.M., n=5) (Fig. 2A, Fig. 3). These values did not change during survival times of up to 5 weeks following tracer application. The densities of RGCs at 2, 3, 4 and 5 weeks after retrograde labeling were: 2,217 ± 97 cells/mm^2 (n=5), 2,398 ± 42 cells/mm^2 (n=5), 2,292 ± 33 cells/mm^2 (n=5), and 2,327 ± 51 (n=5), respectively, and were not statistically different from each other (ANOVA, $P > 0.05$). These data indicate that FluoroGold by itself does not induce cell death and does not leak from rat RGCs *in vivo* for at least 5 weeks after retrograde application. Based on this, treated retinas were examined at 1 and 2 weeks following axonal injury to determine the density of surviving RGCs, visualized with FluoroGold (Figs. 2 and 3, Table 1).

Eyes that received a single intraocular injection of D3 showed robust RGC neuroprotection (Fig. 2B) compared to control eyes injected with the inactive peptide 3Bj (Fig. 2C) or non-injected eyes (Fig. 2D), while intraocular injection of NGF (5 μg) did not promote RGC survival (Fig. 2E). This effect was consistent with a previous study showing D3-induced RGC neuroprotection in an ocular hypertension model (Shi et al., 2007a). Combined treatment with D3 (TrkA agonist) and C92-96 (selective TrkA antagonist) blocked D3-induced RGC survival (Figs. 2F, 3). We have previously demonstrated that C92-96 is an antagonist of NGF (LeSauteur et al., 1995; Debeir et al., 1999b; Maliartchouk et al., 2000) and that D3 acts synergistically with NGF *in vivo* (Maliartchouk et al., 2000; Bruno et al., 2004). It is therefore likely that C92-96 antagonizes the effect of D3 on TrkA activation by antagonizing its cooperation with NGF, which is expressed endogenously in the adult retina.

We then quantified RGC densities on retinal flat mounts to confirm our histological observations (Fig. 3). Previous studies, including ours, have demonstrated that virtually all RGCs survive for 5 days after axotomy and then die abruptly: the population of RGCs is reduced to approximately 50% by day 7 and to ~10% on day 14 post-lesion (Berkelaar et al., 1994b; Mansour-Robaey et al., 1994b; Peinado-Ramon et al., 1996b; Clarke et al.,

1998; Klöcker et al., 1998; Bien et al., 1999; Cheng et al., 2002b; Pernet and Di Polo, 2006b). In D3-treated eyes, 80% of RGCs survived at 1 week after axotomy, a number significantly greater than that produced by NGF (65% survival) or the inactive peptide 3Bj (58% survival) at 1 week post-lesion (ANOVA, $P < 0.001$). The effect of D3 was still marked at 2 weeks after axotomy, with 22% of all RGCs remaining alive compared to only 11% or 9% survival afforded by NGF or the inactive peptide 3Bj, respectively (ANOVA, $P < 0.01$). Interestingly, a peptidomimetic agonist for both TrkA and TrkC (3Ac, Fig. 3) also promoted robust RGC neuroprotection at 1 and 2 weeks after axotomy, and was not significantly different to that produced by D3 (ANOVA, $P > 0.05$). Intraocular injection of a selective TrkC peptidomimetic agonist (3Aa), however, failed to promote RGC neuroprotection, leading us to conclude that the survival effect promoted by 3Ac was primarily due to TrkA activation. These data indicate that selective peptidomimetic agonists of TrkA, but not NGF or TrkC agonists, promote RGC neuroprotection following optic nerve injury.

To determine how endogenous neurotrophins, particularly NGF and pro-NGF, could modulate receptor activity and the effect of peptidomimetic ligands, we examined their levels in intact versus axotomized retinas. The level of endogenous NGF was low and did not change between intact and injured retinas (Fig. 4A), while pro-NGF was markedly upregulated after injury (Fig. 4B). Based on previous observations that $p75^{NTR}$ can mediate neuronal apoptosis in response to neurotrophins and pro-neurotrophins (Hempstead, 2006b), we then examined the expression and role of $p75^{NTR}$ in adult RGC survival.

2.4.3. $p75^{NTR}$ is expressed by Müller cells in the adult rodent retina

$p75^{NTR}$ mediates neuronal apoptosis in the early phases of retinal development (Frade et al., 1996a; Harada et al., 2006), thus we asked whether this receptor might play a role in the survival of adult retinal neurons. First, we examined the cellular distribution of $p75^{NTR}$ in the mature retina. $p75^{NTR}$ immunostaining was detected in radial processes that spanned the entire retina, typical of Müller glia (Fig. 5A), and was particularly strong in putative Müller cell end-feet in the ganglion cell layer (GCL). To confirm that Müller cells express $p75^{NTR}$, we carried out co-localization studies using an antibody against cellular retinaldehyde-binding protein (CRALBP), a Müller cell-specific marker (Fig 5B). The superimposition of

$p75^{NTR}$ and CRALBP immunoreactivity revealed that Müller cell bodies and processes contain $p75^{NTR}$ (Fig. 5C). $p75^{NTR}$ staining in the outer plexiform and outer nuclear layers was consistently associated with Müller cell processes in all retinas examined (Fig. 5D-F). In contrast, FluoroGold-positive cells in the ganglion cell layer completely lacked $p75^{NTR}$ immunoreactivity as revealed by epifluorescent (Fig. 5G-I) and confocal (Fig. 5J-L) microscopy. These data indicate that $p75^{NTR}$ is either not expressed by RGCs or is below the levels detected by immunohistochemistry.

To confirm and extend these observations, we examined $p75^{NTR}$ protein expression in wild-type and $p75^{NTR}$ knockout mice (Fig. 5M, N). Similar to rat retinas, $p75^{NTR}$ immunoreactivity was detected in Müller cells of wild-type mouse retinas, but this staining was completely absent in $p75^{NTR}$ null retinas validating the specificity of the REX antibody. To examine whether $p75^{NTR}$ protein levels changed after axotomy, we performed western blot analysis of retinal homogenates with REX (Fig. 5O). Our results showed no detectable changes in the total levels of $p75^{NTR}$ protein between intact and axotomized retinas (48 hrs or 1 week). This finding is consistent with a previous electron microscopy study showing that $p75^{NTR}$ protein levels or distribution did not change after axotomy (Hu et al., 1999).

2.4.4. Inhibition of $p75^{NTR}$ signaling enhances RGC survival following optic nerve injury

To establish the role of $p75^{NTR}$ receptor signaling on RGC survival, we used the peptidomimetic compound C30-35, an antagonistic ligand of $p75^{NTR}$ that selectively inhibits NGF binding to $p75^{NTR}$ (LeSauteur et al., 1995; Longo et al., 1997; Saragovi and Zaccaro, 2002; Botchkarev et al., 2003). Intravitreal injection of C30-35 promoted significant RGC protection from axotomy-induced death (Fig. 6A, Fig. 7, Table 1) compared to control retinas injected with an inactive peptide (Fig. 6B). Indeed, quantitative analysis of RGC densities demonstrated that a single injection of C30-35 promoted the survival of 75% of RGCs at 1 week post-axotomy, and 23% RGC survival at 2 weeks after lesion (ANOVA, $P < 0.001$). To confirm these findings, we used an alternative method to inhibit $p75^{NTR}$ activity by injecting the selective $p75^{NTR}$ function blocking antibody REX. Similar to our findings with C30-35, the $p75^{NTR}$ antibody promoted the survival of 81% and 18% of RGCs at 1 and 2 weeks after axotomy, respectively, validating the specificity of C30-35 *in vivo* (Fig. 7). Another

peptidomimetic ligand (C28-35) that inhibits NGF binding to both TrkA and $p75^{NTR}$ (LeSauteur et al., 1995; Xie and Longo, 2000; Saragovi and Zaccaro, 2002) promoted RGC survival at 1 week after axotomy (~80%); but this effect was much reduced at 2 weeks post-injury (9%) and was not significantly different from retinas exposed to the inactive control peptide (9%) or axotomy alone (11%) (ANOVA, $P > 0.05$) (Fig. 7).

To establish whether $p75^{NTR}$ blockade would enhance neuroprotection by TrkA agonists, we tested the combinatorial effect of C30-35 with NGF or D3 on RGC survival. This combination afforded a substantial potentiation of RGC neuroprotection after axotomy (Figs. 6C, D). Administration of both NGF and C30-35 promoted striking survival of RGCs: 100% of all neurons were alive at 1 week after axotomy, an effect that was still robust at 2 weeks post-injury (34% survival). Overall, the neuroprotective effect of combined C30-35 and NGF or D3 was significantly higher than each treatment alone (Fig. 7, ANOVA, *** = $P < 0.001$, * = $P < 0.05$). Collectively, these data demonstrate that blockade of NGF binding to $p75^{NTR}$ leads to RGC neuroprotection, and that simultaneous blockade of $p75^{NTR}$ and activation of TrkA markedly potentiates neuronal survival.

To unambiguously demonstrate that activation of $p75^{NTR}$ in glia may be detrimental for neuronal survival, we took a genetic (non-pharmacological) approach by examining the densities of axotomized RGCs in $p75^{NTR}$ knockout mice. Similar to our studies in rat retina, mouse RGCs were first labeled by application of FluoroGold to the superior colliculus, then axotomized (7 days after retrograde labeling), and quantified at 1 week after injury. We, and others, have previously demonstrated that RGC densities are higher in the intact mouse (~ 3,500-4,000 RGCs/mm^2) than in intact rat (~2,300 RGCs/mm^2) retinas (Sapieha et al., 2005a; Murphy et al., 2007). Figure 8 shows that lack of $p75^{NTR}$ leads to remarkable neuroprotection of adult RGCs following axotomy: 83% survived in $p75^{NTR}$ (-/-) retinas at 1 week post-axotomy compared to 51% that survived in $p75^{NTR}$ (+/+) controls (ANOVA, $P < 0.001$) (Fig. 8). Of interest, the total density of RGCs in non-injured (intact) $p75^{NTR}$ (-/-) was not significantly different from that found in wild-type littermate controls (ANOVA, $P > 0.05$). This observation is in agreement with a recent study showing that while $p75^{NTR}$ is involved in the regulation of mouse RGC number in the early phases of retinal development, the number of post-natal RGCs is the same in $p75^{NTR}$ (-/-) and $p75^{NTR}$ (+/+)

retinas (Harada et al., 2006). These results further support our conclusion that inhibition of $p75^{NTR}$ in glia potentiates RGC survival after axonal injury.

2.5. DISCUSSION

The present study explored the functional roles of the neurotrophin receptors TrkA and $p75^{NTR}$ in the regulation of adult RGC survival *in vivo*. Our data support four main conclusions. First, TrkA protein expression is substantially increased in adult RGCs following optic nerve axotomy, and these neurons continue to express high levels of TrkA in the presence of exogenous NGF. Second, selective activation of TrkA receptors using small, peptidomimetic agonistic ligands markedly enhances RGC survival. In contrast, NGF, the natural ligand of TrkA, or TrkC ligands, do not promote RGC protection. The failure of TrkC agonists to protect RGCs may be partly because most of the TrkC expressed in the adult retina is the truncated isoform TrkC(T1) (Rudzinski et al., 2004). Third, selective blockade of $p75^{NTR}$ or absence of $p75^{NTR}$, which is expressed by Müller glia, enhances the survival of RGCs. Finally, combined activation of TrkA with inhibition of $p75^{NTR}$ enhances neuroprotection beyond that obtained by activation of TrkA alone.

Neurotrophins have been extensively investigated for their ability to promote the survival of retinal neurons after injury. However, the molecular mechanisms that limit or impair the neuronal response to neurotrophins *in vivo* are poorly understood. Although axotomized RGCs express high levels of TrkA and can respond to NGF, neither endogenous NGF produced in the retina (Amendola et al., 2003; Rudzinski et al., 2004) nor exogenous NGF administered intraocularly promoted neuronal survival. In contrast, selective TrkA agonists were neuroprotective demonstrating that TrkA signaling plays a key role in the survival of adult RGCs following optic nerve injury. Then, why might exogenous NGF fail to promote RGC survival? Our data indicate that exogenous NGF can be neuroprotective when acting on TrkA receptors on neurons but not when it binds to $p75^{NTR}$ on glia, suggesting that NGF may bind to glial $p75^{NTR}$ and trigger signaling pathways that are detrimental for neuronal survival. Interestingly, our data also show that the level of endogenous NGF protein was low and did not increase in axotomized retinas with respect to non-injured retinas, while endogenous pro-NGF was markedly upregulated. This finding suggests that endogenous pro-NGF may play a role in the modulation of RGC

death via $p75^{NTR}$. Indeed, inhibition of $p75^{NTR}$ unmasked a potent neuroprotective effect of NGF. Furthermore, the sole blockade of $p75^{NTR}$ in the absence of NGF promoted RGC survival and a large number of axotomized RGCs survived in $p75^{NTR}$ null retinas. This suggests that there may be sufficient endogenous neurotrophic factors in the retina to promote RGC survival when $p75^{NTR}$ is blocked. The peptidomimetic compound C28-35, which inhibits NGF binding to both TrkA and $p75^{NTR}$ (Xie and Longo, 2000; Saragovi and Zaccaro, 2002), conferred only marginal RGC survival, detectable at 1 week but not at 2 weeks post-injury. These results suggest that blockade of $p75^{NTR}$ initially promotes survival but that TrkA activation is ultimately required for prolonged neuroprotection. This finding may also explain why C28-35 failed to rescue RGCs in a chronic glaucoma model where the analysis of neuronal survival was performed at ≥21 days after administration of this compound (Shi et al., 2007a)**.**

The functional interaction between TrkA and $p75^{NTR}$ is best understood in cells that co-express both of these receptors (Clary and Reichardt, 1994a; Bibel et al., 1999; Dechant and Barde, 2002; Huang and Reichardt, 2003a; Teng and Hempstead, 2004; Barker, 2007). When TrkA/$p75^{NTR}$ ratios are low or when the neurotrophin levels are limited, $p75^{NTR}$ activation can lead to neuronal death. This occurs in the developing chick and mouse retina, where migratory RGCs are eliminated by $p75^{NTR}$-mediated apoptosis via an autocrine effect of NGF (Frade et al., 1996a; Frade et al., 1999; Gonzalez-Hoyuela et al., 2001; Harada et al., 2006). However, this model does not apply to the effects described here in the adult retina where the receptors are segregated topographically and are expressed in different cell populations: RGCs express TrkA and Müller glia express $p75^{NTR}$. Our finding is in agreement with previous electron microscopy studies in mature retina showing that $p75^{NTR}$ is present in Müller cell end-feet, but not in the RGCs themselves (Hu et al., 1998; Hu et al., 1999). A recent study detected very low levels of $p75^{NTR}$ by RT-PCR (40 amplification cycles) in acutely purified, mouse RGCs *in vitro* (Butowt and von Bartheld, 2005), but there are no reports that unequivocally demonstrate the expression of $p75^{NTR}$ in adult RGCs *in vivo*. The absence or very limited expression of $p75^{NTR}$ in mature RGCs is in line with the general observation that neuronal-specific $p75^{NTR}$ plays a prominent role during developmental cell death, but it is markedly downregulated in many neuronal types once they have reached their final targets (Yan and Johnson, 1988). Notably, $p75^{NTR}$ is also

expressed by other non-neuronal cell populations in the CNS including oligodendrocytes, olfactory ensheathing cells and Schwann cells, but its role in glia remains poorly defined (Cragnolini and Friedman, 2007). In the adult retina, our results indicate that pharmacological blockade of $p75^{NTR}$ or genetic deletion of $p75^{NTR}$ in Müller glia enhances RGC survival after axotomy. Furthermore, selective inhibition of $p75^{NTR}$ is required for optimal TrkA-mediated RGC neuroprotection. Collectively, these findings provide evidence for a novel mechanism by which glial cells profoundly influence neuronal survival.

Müller cells are specialized radial glia with processes that closely wrap around neuronal cell bodies and dendrites. They provide metabolic support and regulate the microenvironment (Bringmann et al., 2006b), hence are ideally suited to control neuronal viability. Of interest, Müller cells become reactive after axotomy but they do not die (Scherer and Schnitzer, 1991; Huxlin et al., 1995) making it unlikely that $p75^{NTR}$ expression by Müller glia promotes cell-autonomous death. Although $p75^{NTR}$ protein does not increase in Müller cells following optic nerve axotomy (Hu et al., 1999), it is possible that $p75^{NTR}$ signaling in reactive Müller cells leads to the production of neurotoxic molecules that limit the ability of RGCs to survive after axonal injury. For example, we have shown that endogenous polyamines, produced by Müller glia, enhance NMDA-mediated RGC death (Pernet et al., 2007a). Moreover, blockade of $p75^{NTR}$ on Müller cells prevents photoreceptor apoptosis during light-induced retinal degeneration by increasing synthesis of the photoreceptor survival factor FGF-2 (Harada et al., 2000a). Therefore, Müller cells may regulate neuronal death directly by releasing neurotoxins or indirectly by reducing their supportive functions.

Understanding the requirements of adult RGCs to survive after axonal injury is paramount for the development of neuroprotective strategies applicable to most optic neuropathies. Here we show that selective TrkA agonists and NGF, a neurotrophic factor previously thought to be inadequate to promote RGC survival, are in fact potent neuroprotective agents when acting only on neuronal TrkA receptors but not glial $p75^{NTR}$. Moreover, our data reveal that combined TrkA activation and glial $p75^{NTR}$ blockade potentiate neuronal survival and provide proof-of-principle for the efficacy of this strategy for retina and optic nerve repair.

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2.7. FIGURE LEGENDS

FIGURE 1. Axonal injury increases TrkA levels in RGCs.

Fluorescent microscopy images show low expression of TrkA in intact RGCs (A), while there is marked upregulation of this receptor at 48 hrs after optic nerve transection (B). High magnification of the TrkA staining in RGCs, visualized with FluoroGold (FG) (C-E). Confocal microscopy images show that TrkA-positive cells are FluoroGold-positive RGCs (F-H). Western blots of retinal extracts confirm that TrkA levels in the intact retina are low, either in the presence or absence of NGF, and that optic nerve transection leads to TrkA upregulation, an effect that remains unchanged with NGF (n=3 rats/group). The lower panel represents the same blot as in the upper panel but probed with an antibody that recognizes β-actin used to confirm equal protein loading (I). Densitometric analysis (n = 3/group) shows that axotomy leads to TrkA upregulation with respect to control retinas (ANOVA, * = $P < 0.001$), and that NGF treatment did not alter TrkA expression with respect to axotomy alone (ANOVA, $P > 0.05$) (J). *In vivo* activation of Erk1/2 kinases was detected in retinal homogenates after injection of D3 or NGF compared to control eyes. Blots were probed with an antibody that selectively recognizes Erk1/2 phosphorylated on Thr202/Tyr204 residues. Lower panel shows the same blot probed with an antibody to visualize total Erk1/2 protein (K). Densitometric analysis (n = 3/group) confirmed that a single injection of both D3 and NGF led to a 2-fold increase in phospho-Erk1 (ANOVA, * = $P < 0.05$, ** = $P < 0.01$) and a 2.1-fold increase in phospho-Erk2 (ANOVA, * = $P < 0.05$) with respect to control retinas (L). ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars: 50 μm (A, B), 12.5 μm (C-E), 8 μm (F-H).

FIGURE 2. TrkA peptidomimetic agonists promote RGC survival.

Fluorescent photomicrographs of flat-mounted retinas showing FluoroGold-labeled RGCs in intact retina (A), or in axotomized retinas that received: D3, a peptidomimetic agonist of TrkA (B), 3Bj, an inactive peptidomimetic ligand (C), no treatment (axotomy alone) (D), nerve growth factor (NGF) (E), or a combination of D3 and C92-96, an antagonist of TrkA (F). Scale bar: 100 μm.

FIGURE 3. Quantification of RGC survival using TrkA agonists and antagonists.

Quantitative analysis of RGC survival at 1 and 2 weeks after axotomy in eyes treated with: axotomy alone (light gray bars), inactive peptide (3Bj, dotted bars), nerve growth factor (NGF, dark gray bars), a TrkA agonist (D3, black bars), a combination of D3 and a specific TrkA antagonist (C92-96) (horizontal lines bar), a TrkA and TrkC agonist (3Ac) (vertical lines bars), and a selective TrkC agonist (3Aa) (hatched bars) (n = 4-6 rats per group, see Table 1). The density of RGCs in intact retinas, without treatment or surgery, is shown as reference (open bar). Data are expressed as the mean RGCs ± S.E.M. (ANOVA ** = $P < 0.001$, * = $P < 0.01$)

FIGURE 4. Levels of endogenous NGF and pro-NGF in intact and injured retinas.

(A) Western blot analysis show that endogenous NGF level is low in retinal extracts, but does not change after optic nerve transection. Densitometric analysis (n = 3/group) shows no difference in NGF levels in non-injured versus axotomized retinas (Student's t-test, $P > 0.05$). (B) In contrast, endogenous retinal pro-NGF is upregulated at 48 hrs after axotomy. Densitometric analysis (n = 3/group) confirmed that axotomy led to a 3-fold increase in pro-NGF levels with respect to intact retinas (Student's t-test, $P < 0.001$). Each lower panel represents the same blot as in the upper panel but probed with an antibody that recognizes β-actin used to confirm equal protein loading.

FIGURE 5. $p75^{NTR}$ is expressed by Müller cells in the adult rodent retina.

Double immunolabeling with antibodies against $p75^{NTR}$ (REX) and cellular retinaldehyde-binding protein (CRALBP), a Müller cell-specific marker, shows strong expression of $p75^{NTR}$ in Müller cell bodies and processes (A-C). $p75^{NTR}$ staining in the OPL and ONL co-localized with CRALBP (D-F). Absence of co-localization of $p75^{NTR}$ staining with FluoroGold demonstrates that adult RGCs are devoid of $p75^{NTR}$ (G-I, white arrowheads). Confocal microscopy images show that $p75^{NTR}$ staining is in Müller cell processes surrounding RGCs, but not in the neurons themselves (J-L). $p75^{NTR}$ immunoreactivity is also detected in Müller cells of wild-type mouse retinas, and this staining is completely absent in $p75^{NTR}$ null retinas validating the specificity of our antibody (M, N). Western blot analysis of retinal extracts show that the level of $p75^{NTR}$ protein does not change at 48 hrs or 1 week

after axotomy. The lower panel represents the same blot as in the upper panel but probed with an antibody that recognizes β-actin used to confirm equal protein loading (O). ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars: 50 μm (A-C), 25 μm (D-I), 100 μm (M, N).

FIGURE 6. Blockade of $p75^{NTR}$ enhances RGC survival following optic nerve injury.

Fluorescent photomicrographs of flat-mounted retinas showing FluoroGold-labeled RGCs in retinas treated with C30-35, a $p75^{NTR}$ antagonist (A), 3Bj, an inactive peptidomimetic ligand (B), a combination of nerve growth factor (NGF) and C30-35 (C), or a combination of D3 (TrkA agonist) and C30-35 (D). Scale bar: 100 μm.

FIGURE 7. Quantification of RGC survival using $p75^{NTR}$ antagonists alone or in combination with TrkA agonists.

Quantitative analysis of RGC survival at 1 and 2 weeks after axotomy in eyes treated with: axotomy alone (light gray bars), inactive peptide (3Bj, dotted bars), the $p75^{NTR}$ antagonist C30-35 (dark gray bars), a $p75^{NTR}$ function-blocking antibody (REX, dark gray bars), the $p75^{NTR}$/TrkA antagonist C28-35 (hatched bars), a combination of nerve growth factor (NGF) and C30-35 (horizontal lines bars), or a combination of the TrkA agonist D3 and C30-35 (vertical lines bars) (n = 3-6 rats per group, see Table 1). The density of RGCs in intact retinas, without treatment or surgery, is shown as reference (open bar). Data are expressed as the mean RGCs ± S.E.M. (ANOVA * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

FIGURE 8. Increased survival of axotomized RGCs in $p75^{NTR}$ null retinas.

Quantitative analysis of RGC survival at 1 week after axotomy in eyes of $p75^{NTR}$ knock-out mice (black bars) and $p75^{NTR}$ (+/+) wild-type littermate controls (open bars). There is marked survival of axotomized RGCs in $p75^{NTR}$ null retinas compared to wild-type littermate controls. The density of RGCs in intact mouse retinas is shown as reference. Data are expressed as the mean RGCs ± S.E.M. (ANOVA * = $P < 0.001$).

2.8. TABLES

Table1. Survival of axotomized RGCs after injection of peptidomimetic ligands *in vivo*

		RGCs/mm^2 ± S.E.M. (% of intact contralateral retinas); *n* Time after axotomy	
Agent [a]	**Target**	**1 week**	**2 weeks**
NGF	TrkA natural ligand	1,525 ± 32 (65%), *n*=4	259 ± 21 (11%), *n*=4
D3	TrkA agonist	1,874 ± 73 (80%), *n*=5	523 ± 39 (22%), *n*=5
3Ac	TrkA and TrkC agonist	2,023 ± 43 (86%), *n*=5	501 ± 21 (21%), *n*=5
3Aa	TrkC agonist	1,289 ± 45 (55%), *n*=4	246 ± 6 (10%), *n*=4
C30-35	p75^{NTR} antagonist	1,768 ± 42 (75%), *n*=4	533 ± 16 (23%), *n*=4
REX	p75^{NTR} function blocking antibody	1,895 ± 38 (81%), *n*=3	423 ± 13 (18%), *n*=3
C28-35	p75^{NTR} and TrkA antagonist	1,930 ± 56 (82%), *n*=4	200 ± 27 (9%), *n*=4
3Bj	Inactive peptide	1,366 ± 73 (58%), *n*=3	217 ± 9 (9%), *n*=5
Axotomy only	-	1,276 ± 70 (54%), *n*=6	248 ± 10 (11%), *n*=4
Combined Treatments		**1 week**	**2 weeks**
D3 + C92-96 (TrkA antagonist)		1,385 ± 20 (59%), *n*=4	344 ± 8 (15%), *n*=4
NGF + C30-35		2,348 ± 58 (100%), *n*=4	792 ± 57 (34%), *n*=5
D3 + C30-35		2,104 ± 106 (90%), *n*=4	650 ± 12 (28%), *n*=4

Contralateral intact retinas: 2,347± 54 RGCs/mm^2 (100%); *n* = 5

[a] Intravitreal injection performed at the time of axotomy.

FIGURE 1

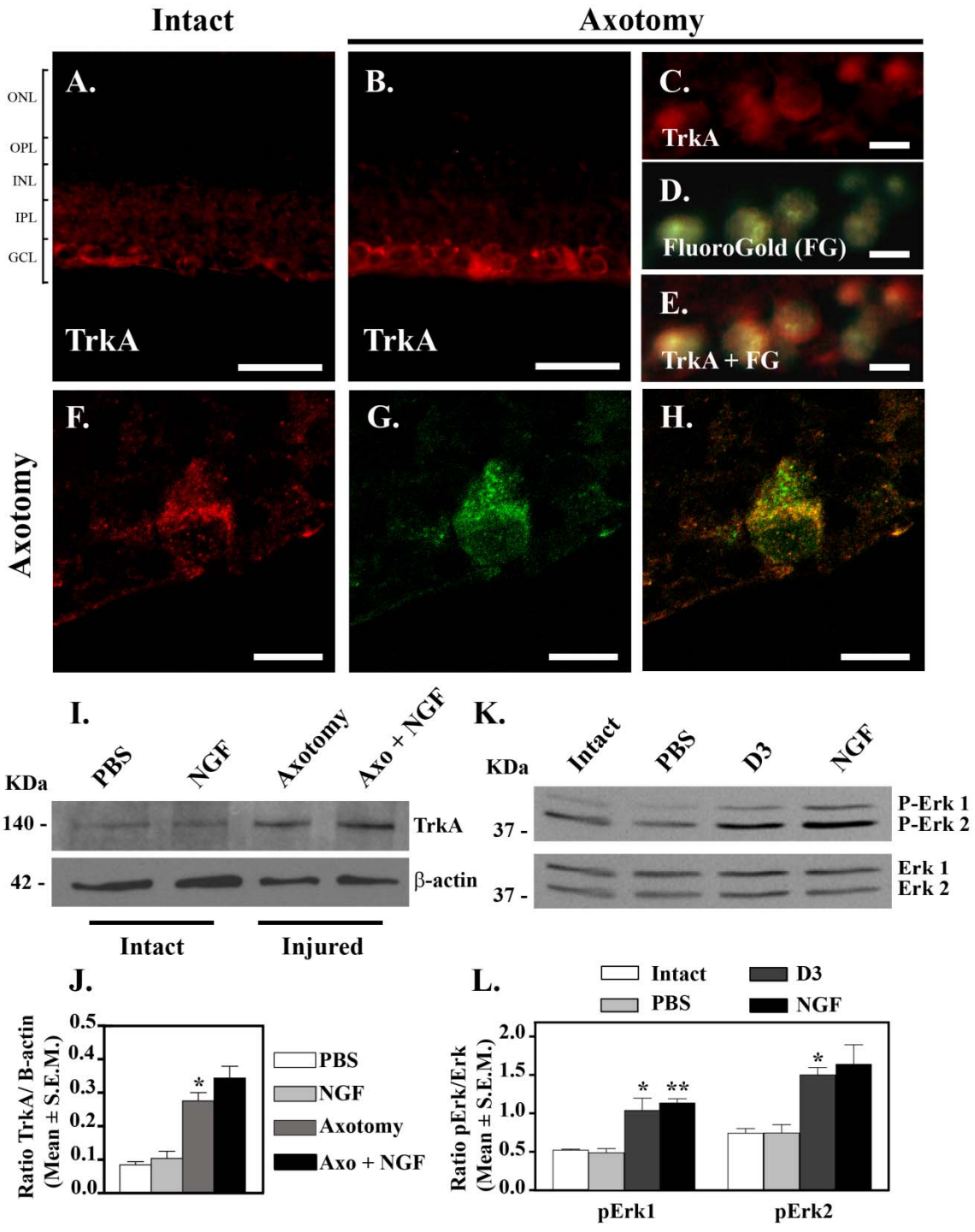

Figure 1

FIGURE 2

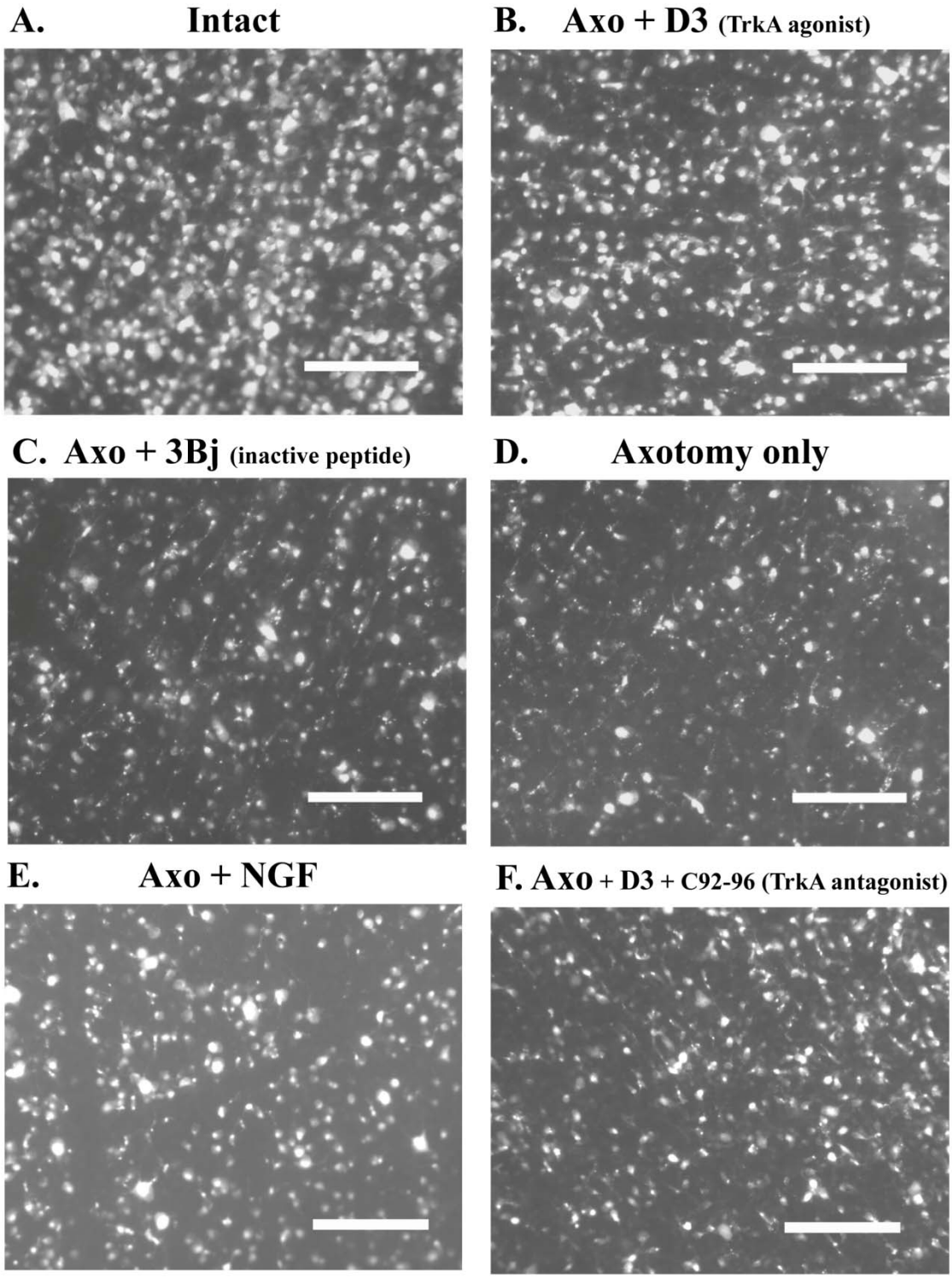

Figure 2

FIGURE 3

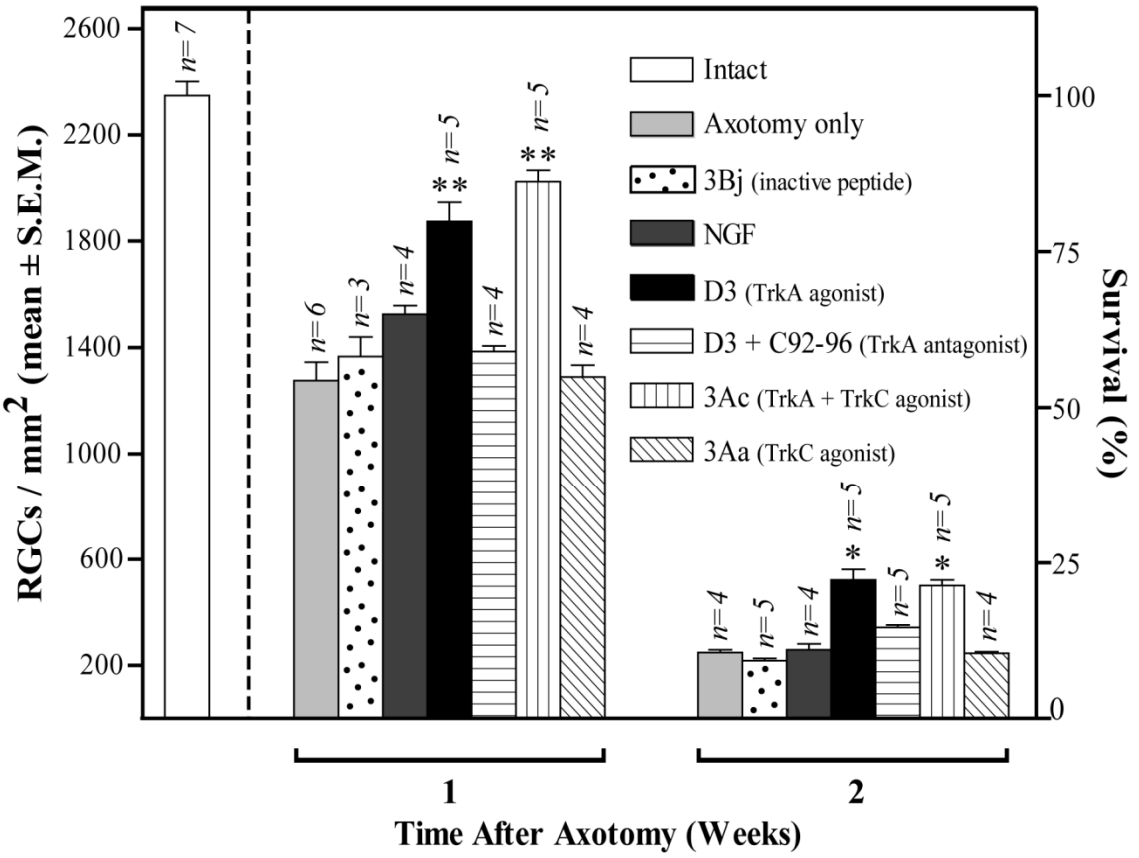

Figure 3

FIGURE 4

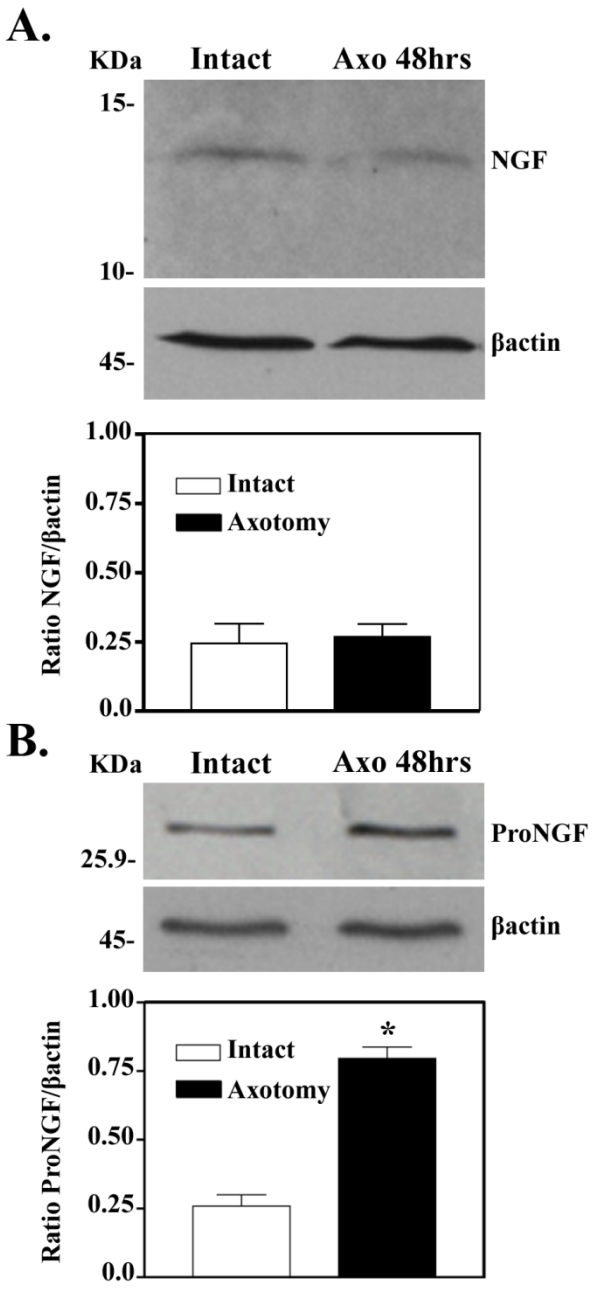

Figure 4

FIGURE 5

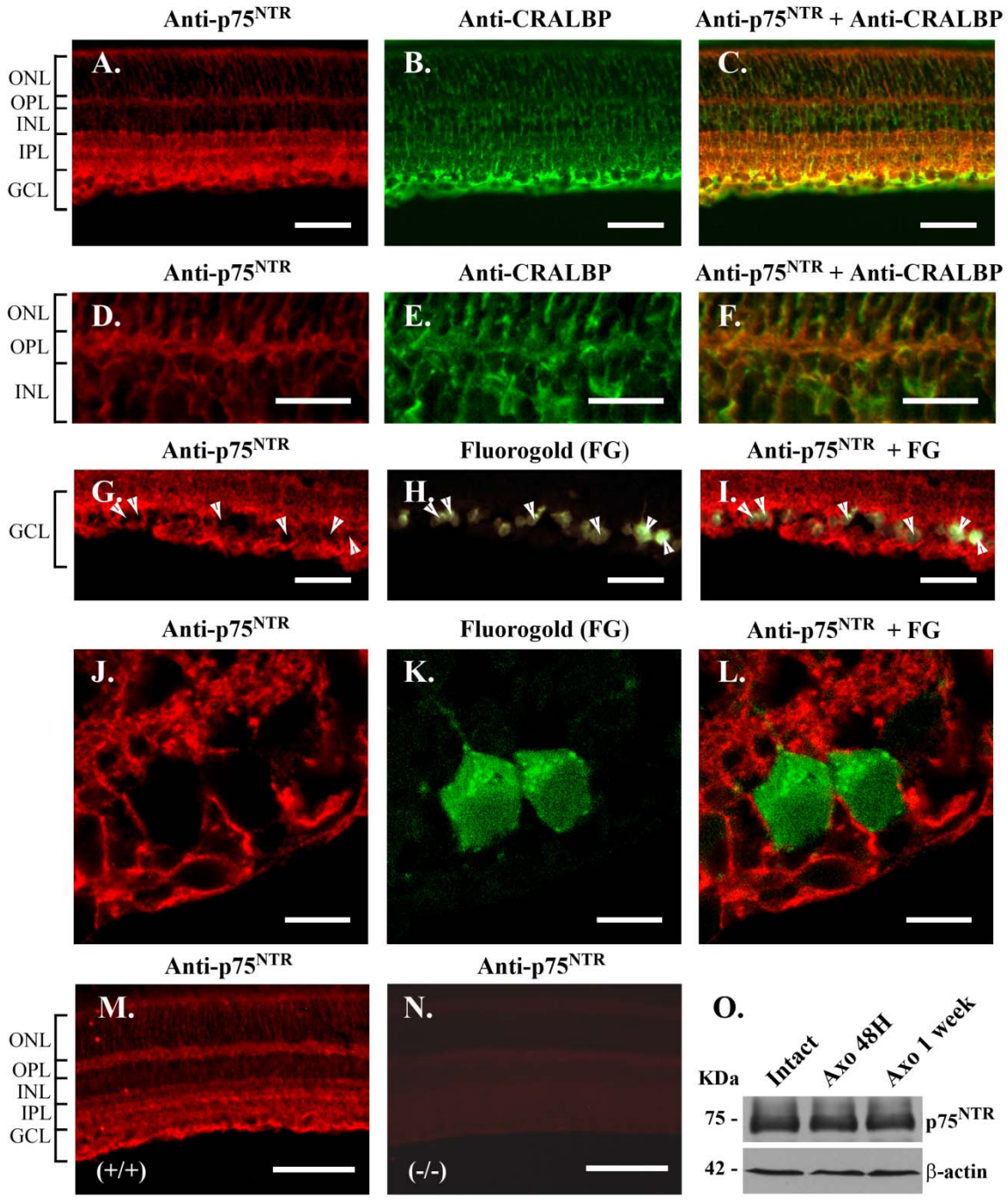

Figure 5

FIGURE 6

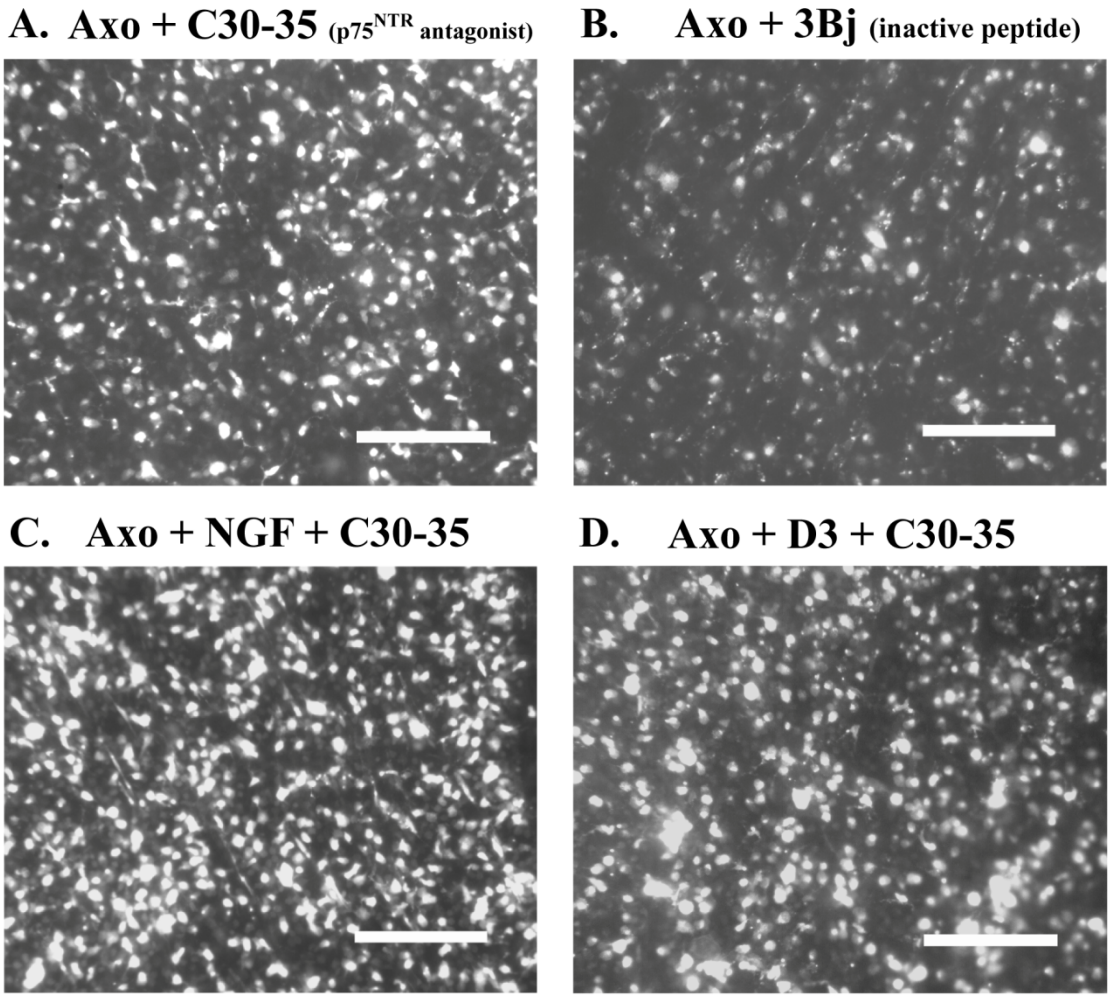

Figure 6

FIGURE 7

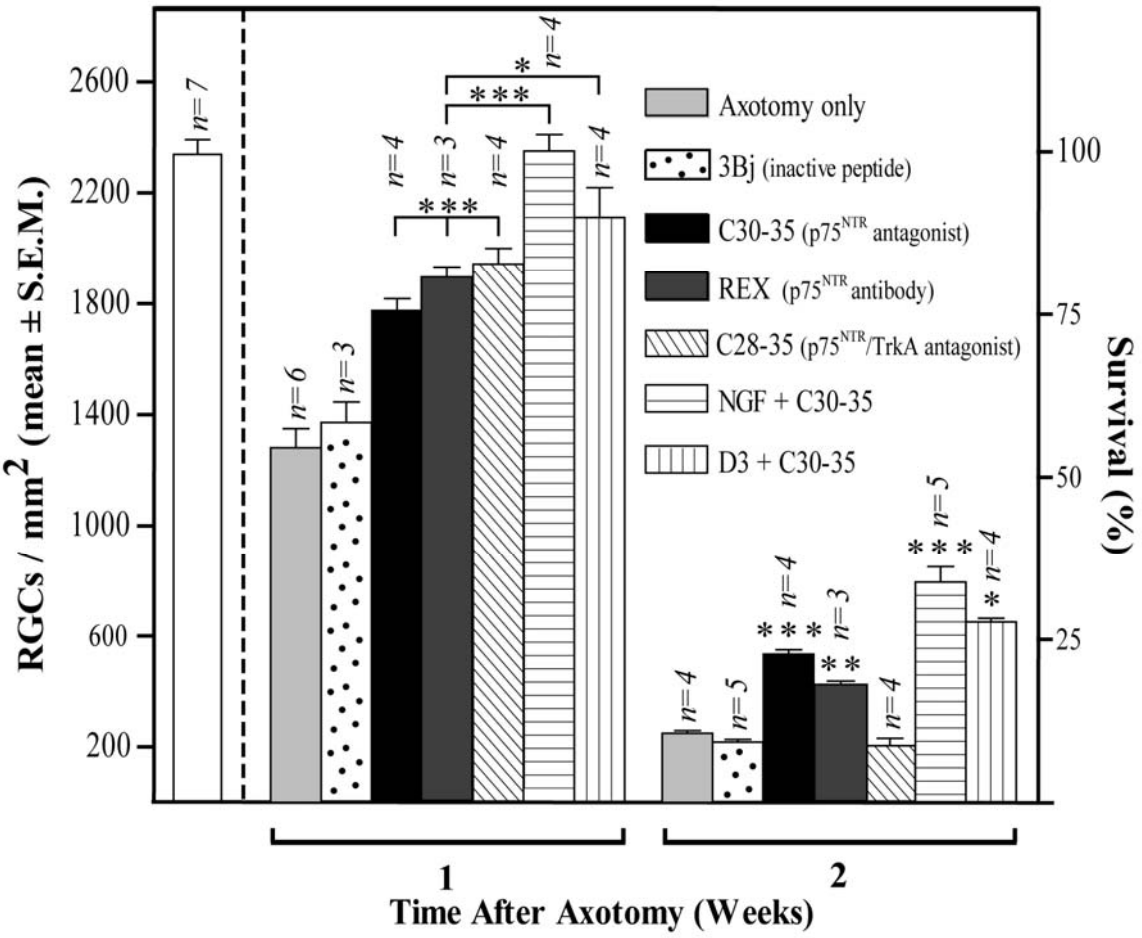

Figure 7

FIGURE 8

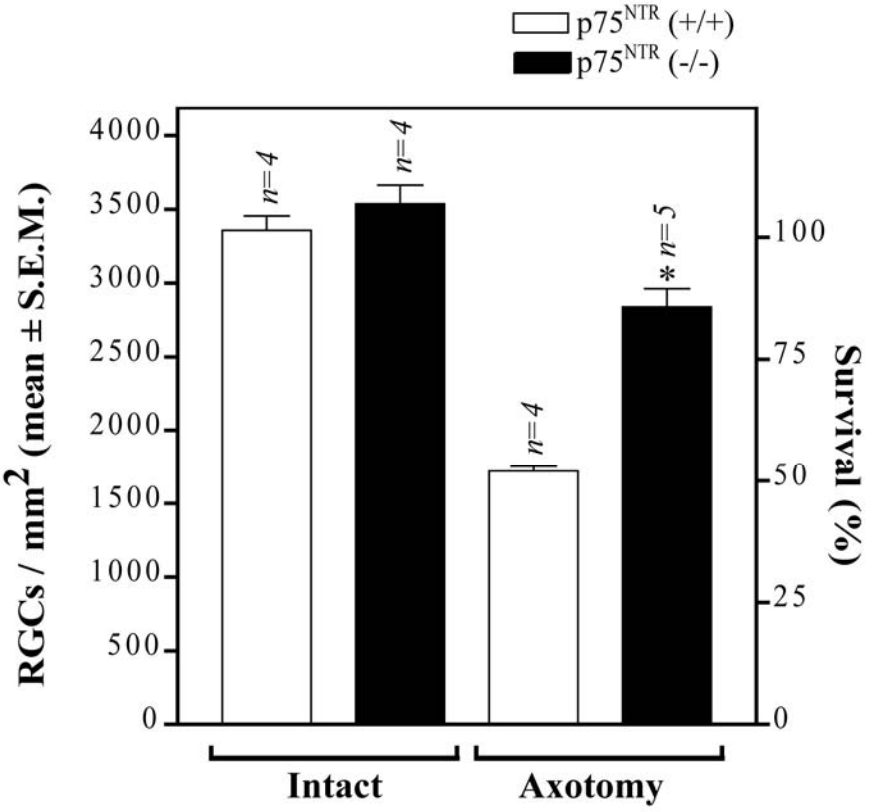

Figure 8

CHAPITRE 3

DEUXIÈME ARTICLE: "PRONGF INDUCES TNFα-DEPENDENT DEATH OF RETINAL GANGLION CELLS THROUGH A P75^{NTR} NON-CELL-AUTONOMOUS SIGNALING PATHWAY".

PNAS (*Proceedings of the National Academy of Sciences), 2010*, sous presse.

La première étude présentée de cette thèse a démontré une production à la hausse de proNGF (et non de NGF) dans la rétine par suite d'axotomie. Ce résultat suggérait que le proNGF, un ligand considéré comme pro-apoptotique, puisse être un acteur important lors de la mort des CGRs. Cette deuxième étude a donc eu pour but de vérifier si le proNGF produit en condition pathologique pouvait être néfaste pour les neurones. Cette étude voulait aussi déterminer quelles protéines étaient impliquées dans la cascade de signalisation du proNGF. Les résultats de cette étude ont suggéré que l'action délétère du proNGF sur les CGRs dépend d'un mécanisme cellulaire non-autonome impliquant les cellules de Müller

PRONGF INDUCES TNFα-DEPENDENT DEATH OF RETINAL GANGLION CELLS THROUGH A P75^{NTR} NON-CELL-AUTONOMOUS SIGNALING PATHWAY

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Running title: ProNGF Induces Non-Cell Autonomous Apoptosis

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3.1. ABSTRACT

Neurotrophin binding to $p75^{NTR}$ activates neuronal apoptosis following adult central nervous system injury, but the underlying cellular mechanisms remain poorly defined. In this study, we show that pro-nerve growth factor (proNGF) induces death of retinal ganglion cells in adult rodents via a $p75^{NTR}$-dependent signaling mechanism. Expression of $p75^{NTR}$ in the adult retina is confined to Müller glial cells, therefore we tested the hypothesis that proNGF activates a non-cell autonomous signaling pathway to induce RGC death. Consistent with this, we show that proNGF induced robust expression of TNFα in Müller cells and that genetic or biochemical ablation of TNFα blocked proNGF-induced death of retinal neurons. Mice rendered null for $p75^{NTR}$, its co-receptor sortilin, or the adaptor protein NRAGE were defective in proNGF-induced glial TNFα production and did not undergo proNGF-induced retinal ganglion cell death. We conclude that proNGF activates a non-cell autonomous signaling pathway that causes TNFα-dependent death of retinal neurons *in vivo*.

3.2. INTRODUCTION

The four mammalian neurotrophins comprise a family of related secreted factors that are required for differentiation, survival, development, and death of specific populations of neurons and non-neuronal cells. Neurotrophins are produced as proforms of ~240 amino acids that are cleaved by furins and proconvertases to yield products of ~120 amino acids. Recent studies have indicated that nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) can be secreted as proforms in the central nervous system (CNS) (Fahnestock et al., 2001; Lee et al., 2001; Bruno and Cuello, 2006), and demonstrated that pro-neurotrophins can function as potent apoptosis-inducing ligands both *in vitro* and *in vivo* (Hempstead, 2006a). However, the precise mechanisms by which pro-neurotrophins lead to neuronal death are poorly defined.

The biological effects of neurotrophins are mediated by binding to TrkA, TrkB, and TrkC receptor tyrosine kinases and to the p75 neurotrophin receptor ($p75^{NTR}$). Trk receptors respond preferentially to mature neurotrophins whereas pro-neurotrophins exert their apoptotic effect via a receptor complex that contains $p75^{NTR}$ and sortilin (Nykjaer et al., 2004b). The precise signaling cascades evoked by occupancy of the $p75^{NTR}$-sortilin complex remain to be elucidated, but several lines of evidence indicate that NRIF and NRAGE adaptor proteins play key roles in death signaling cascades evoked by $p75^{NTR}$ (Linggi et al., 2005b; Bertrand et al., 2008a).

Previous studies have shown that neurotrophins induce cell death via $p75^{NTR}$ during early retinal development (Frade and Barde, 1999a). $p75^{NTR}$ has also been implicated in light-induced photoreceptor death in adult rodents *in vivo* (Harada et al., 2000a) and a proNGF-$p75^{NTR}$ link has been proposed to facilitate apoptosis in a retinal cell line (Srinivasan et al., 2004b). Here, we investigate the role of proNGF in the adult retina and demonstrate that proNGF promotes death of retinal ganglion cells (RGCs) *in vivo*. Importantly, proNGF-induced RGC loss is indirect, and requires the $p75^{NTR}$-dependent production of TNFα by Müller glial cells. We conclude that proNGF-induced neuronal loss in the adult retina occurs through a non-cell-autonomous mechanism that requires TNFα production by central glia.

3.3. RESULTS

3.3.1. ProNGF induces death of retinal ganglion cells in adult rodents

To investigate whether proNGF promotes neuronal death *in vivo*, we first retrogradely labeled RGCs of adult rats by applying fluorogold to the surface of the superior colliculus and then provided a single intraocular injection of proNGF or vehicle. A week later, retinal whole mounts were prepared and RGC densities were quantified. ProNGF caused a profound loss of adult rat RGCs, whereas vehicle injection had no effect on cell death (Fig. 1A). To determine if the effect of proNGF on neuronal survival was specific to the proform of this neurotrophin, we asked whether mature NGF could similarly promote RGC death. Our results demonstrate that neuronal density was not altered by mature NGF treatment (Fig. 1A). The effect of proNGF was not species specific as proNGF also caused a marked loss of RGCs in mice subjected to intraocular proNGF injection (Fig. 1B). We conclude that elevation of proNGF levels within the retina promotes neuronal loss, and used this system as a model for examining the cellular details of proNGF-induced cell death *in vivo*.

3.3.2. $p75^{NTR}$, sortilin and NRAGE are required for proNGF-induced death of retinal ganglion cells

To determine if $p75^{NTR}$ was required for the loss of RGCs that was evoked by exogenous proNGF, we first asked whether co-injection of the $p75^{NTR}$ function blocking antibody REX (Weskamp and Reichardt, 1991) antagonized proNGF-induced neuronal death. Co-administration of proNGF and REX resulted in significant rescue of RGCs in mice, whereas combined proNGF and non-specific Ig did not exert a protective effect (Fig. 2A). As an alternative approach, we assessed the apoptotic effect of proNGF in mice deficient for $p75^{NTR}$, and showed that proNGF-induced loss of RGCs did not occur in $p75^{NTR}$ null retinas. Together, these data suggest that proNGF binding to $p75^{NTR}$ is required to induce RGC death. $p75^{NTR}$ and sortilin have been shown to form a cell surface receptor complex for pro-neurotrophins that is required for activation of downstream apoptotic pathways (Nykjaer et al., 2004b), and we previously demonstrated that NRAGE, a $p75^{NTR}$ adaptor protein, plays an obligatory role in $p75^{NTR}$-dependent death (Bertrand et al., 2008a). We therefore asked whether sortilin or NRAGE were required

for proNGF-induced RGC loss *in vivo*. Our data show that RGCs within sortilin or NRAGE null mice retinas were protected from proNGF-induced death (Figs. 2C, 2D). We conclude that $p75^{NTR}$, sortilin and NRAGE are each required for proNGF-induced death of RGCs in the adult retina.

3.3.3. ProNGF kills retinal ganglion cells through glia-mediated production of TNFα

A simple model to explain these results would have proNGF binding a $p75^{NTR}$-sortilin complex on the surface of RGCs that in turn activates a cell-autonomous, NRAGE-dependent pro-apoptotic pathway. However, it has been reported that Müller glia are the only cells that express $p75^{NTR}$ in the adult retina (Hu et al., 1998; Hu et al., 1999; Harada et al., 2000a; Ding et al., 2001; Lebrun-Julien et al., 2009), implying that proNGF killing of RGCs may involve a more complex mechanism. To confirm and extend the $p75^{NTR}$ expression pattern in the adult mouse retina, RGCs were retrogradely labeled with fluorogold and then stained with antibodies specific for $p75^{NTR}$. In both naïve and proNGF-treated animals, RGCs were uniformly negative for $p75^{NTR}$ whereas neighboring cells and processes typical of Müller glia invariably expressed abundant quantities of this receptor (Fig. 3A). Co-staining with the Müller cell-specific marker CRALBP confirmed that $p75^{NTR}$ is abundantly expressed by Müller glia, but not RGCs (Fig. 3B).

Based on these results, we hypothesized that proNGF promotes RGC death through an indirect pathway by stimulating the production of a pro-apoptotic factor by Müller cells. A candidate pro-apoptotic factor downstream of $p75^{NTR}$ is tumor necrosis factor alpha (TNFα) since exogenous and endogenous TNFα can induce death of retinal neurons (Tezel et al., 2001; Nakazawa et al., 2006; Berger et al., 2008) and because $p75^{NTR}$ activates NF-κB, a transcription complex that is a potent inducer of TNFα production (Shakhov et al., 1990; Hiscott et al., 1993a; Mori and Prager, 1996). To address whether TNFα could play a role in proNGF-induced RGC killing, we first determined if retinal TNFα levels were increased in eyes injected with proNGF. Immunostaining showed that in eyes injected with vehicle or mature NGF, TNFα basal levels were low. In contrast, eyes injected with proNGF showed robust TNFα expression,

both in cell bodies in the inner nuclear layer and within processes that extended radially across the breadth of the retina (Fig. 3C). Double immunocytochemistry using antibodies against CRALBP identified these TNFα-expressing cells as Müller glia (Fig. 3D).

The finding that proNGF stimulates TNFα production by Müller cells prompted us to ask whether RGC death induced by this pro-neurotrophin could be blocked by Etanercept, a recombinant TNFα antagonist in which the extracellular ligand-binding domain of TNFR2 is fused to a Fc fragment (Fantuzzi et al., 2008b). Figure 4A shows that intraocular injection of Etanercept markedly blocked RGC death induced by proNGF. To rule out the possibility that Etanercept may have off-target pharmacological effects and to further substantiate a role for TNFα in proNGF-induced killing, we also examined whether proNGF led to RGC loss in TNFα null mice. Our data show that proNGF administration failed to induce RGC death in TNFα null mice (Fig. 4B), indicating that TNFα plays a crucial role in RGC death induced by proNGF.

These data indicate that proNGF causes RGC death indirectly, by stimulating production of TNFα by Müller glial cells. We therefore tested whether $p75^{NTR}$ and sortilin activated an NRAGE-dependent pathway that resulted in TNFα production. For this purpose, TNFα protein levels were compared in retinas derived from wild-type mice versus mice lacking $p75^{NTR}$, sortilin or NRAGE following proNGF or vehicle injection. In wild-type retinas, robust TNFα protein production was detected within 48 hours of proNGF administration, while PBS injection had no effect on TNFα levels (Fig. 4C). In contrast, proNGF-induced TNFα protein upregulation was entirely lost in $p75^{NTR}$, sortilin and NRAGE null mice (Fig. 4C), correlating with the failure of proNGF to induce RGC death in these null lines (Fig 2B-D). Importantly, intraocular injection of TNFα caused RGC death in both wild-type and $p75^{NTR}$ null strains, indicating that the apoptotic effects of TNFα on RGCs occurred downstream of the action of $p75^{NTR}$ (Supplemental Figure 1). Taken together, these data indicate that sortilin and NRAGE cooperate with $p75^{NTR}$ to transduce proNGF-induced signaling events required for TNFα production by Müller glial cells, and that the TNFα kills RGCs through a $p75^{NTR}$-independent mechanism.

3.4. DISCUSSION

This study reports three major findings. First, proNGF leads to striking neuronal death in the adult rodent retina *in vivo*. Second, proNGF mediates RGC death indirectly via a non-cell autonomous mechanism that involves TNFα production by Müller glia. Third, proNGF-induced death requires $p75^{NTR}$, sortilin and NRAGE, which cooperate to stimulate TNFα production by Müller cells. $p75^{NTR}$ is an important neuronal signaling protein that interacts with numerous ligands and co-receptors to exert a wide range of functions. The role of $p75^{NTR}$ as an apoptotic receptor is well established as it has been shown to facilitate developmental cell death of peripheral sympathetic neurons and early retinal neurons (Majdan et al., 1997a; Frade and Barde, 1999a; Harada et al., 2006; Jansen et al., 2007). $p75^{NTR}$ has also been implicated in cell loss following various forms of CNS injury and promotes apoptosis of cortical and hippocampal neurons, basal forebrain neurons, oligodendrocytes and photoreceptor cells (Frade and Barde, 1999a; Roux et al., 1999; Friedman, 2000; Beattie et al., 2002b; Harrington et al., 2004a; Volosin et al., 2006a). Overall, the functional role of $p75^{NTR}$ in neuronal death is best understood in neurons that endogenously express this receptor. In this study, however, we identify a novel mechanism by which $p75^{NTR}$ in glial cells can profoundly influence neuronal death by activating production of neurotoxic TNFα in the adult retina *in vivo*.

Recent studies have indicated that proNGF, but not NGF, functions as a potent pro-apoptotic ligand that binds to a cell surface complex of $p75^{NTR}$ and sortilin (Nykjaer et al., 2004b). Consistent with this, we showed that fully processed NGF does not share the apoptotic effect of proNGF, and that proNGF is unable to induce retinal cell death in mice rendered null for sortilin. NRAGE is a crucial adaptor protein required for $p75^{NTR}$-dependent apoptosis in cell lines, primary cells and *in vivo* (Bertrand et al., 2008a). Consistent with this, we found that NRAGE is also necessary for proNGF-induced death of RGCs within the adult retina. Previous studies have established that RGCs express abundant $p75^{NTR}$ during retinal development (Harada et al., 2006), and that $p75^{NTR}$ and sortilin collaborate to induce NGF-dependent apoptosis of a subset of these neurons at ~E15 (Frade and Barde, 1999a; Harada et al., 2006; Jansen et al., 2007). Therefore, it is possible that proNGF induces cell death in the adult retina by directly binding to a $p75^{NTR}$-sortilin complex on adult RGCs. This is unlikely, however, because examination

of $p75^{NTR}$ expression in the adult rodent retina using light and electron microscopy has established that retinal $p75^{NTR}$ expression is confined to Müller glial cells (Hu et al., 1998; Hu et al., 1999; Harada et al., 2000a; Ding et al., 2001; Lebrun-Julien et al., 2009). A recent study also reported that sortilin is expressed by Müller glial cells in the adult retina (Xu et al., 2009a). It therefore seemed probable that the association of proNGF with $p75^{NTR}$ and sortilin on Müller glia induces death of RGCs through an indirect, non-cell autonomous pathway.

Pharmacological or genetic blockade of TNFα dramatically reduced proNGF-induced RGC death indicating that Müller cell-derived TNFα plays a crucial role in neuronal loss. There are several mechanisms by which TNFα produced by Müller cells can kill RGCs but two predominate. First, previous work has established that TNFα can directly kill primary cortical neurons via a caspase 8-dependent mechanism in models of excitotoxicity (Velier et al., 1999b; Kaushal and Schlichter, 2008b). It is thus possible that direct caspase activation occurs in RGCs. However, caspase-8 inhibitors that we validated *in vivo* (15) did not block proNGF-induced death of RGCs. Thus, we favor a second possibility, which involves the recent discovery that TNFα promotes the selective insertion of Ca^{2+}-permeable AMPA receptors (AMPARs) into the neuronal cell surface (Ogoshi et al., 2005; Stellwagen et al., 2005). Under physiological circumstances, TNFα-mediated increase in Ca^{2+}-permeable AMPARs plays a role in synaptic scaling (Stellwagen and Malenka, 2006b) but, during injury, TNFα-mediated increase in these channels can facilitate death of primary hippocampal and spinal cord neurons (Ferguson et al., 2008a; Leonoudakis et al., 2008b). Importantly, this mechanism of cell death may play a role in RGC loss after injury. For example, RGCs lacking TNFα receptor 1 (TNFR1) are protected following optic nerve crush (Tezel et al., 2004b), and we recently showed that TNFα-mediated increase in cell surface Ca^{2+}-permeable AMPARs leads to RGC loss *in vivo* (Lebrun-Julien et al., 2009). Significantly, TNFα and TNFR1 are increased in human donor eyes with glaucoma, diabetic retinopathy and age-related macular degeneration (Armstrong et al., 1998; Yan et al., 2000b; Yuan and Neufeld, 2000; Tezel et al., 2001; Nakazawa et al., 2006), and specific TNFα gene polymorphisms have been found in patients with primary open angle glaucoma (Lin et al., 2003b; Funayama et al., 2004a).

Several recent studies have highlighted the important role that non-cell autonomous mechanisms play in neuronal cell death, notably in models of amyotrophic lateral sclerosis, spinocerebellar ataxia and Huntington's disease (Lobsiger and Cleveland, 2007). TNFα is a hallmark of acute and chronic neuroinflammation and has emerged as an important candidate for mediating non-cell autonomous effects in these conditions (McCoy and Tansey, 2008). $p75^{NTR}$ expressed by cholinergic neurons is required for production of an unknown factor that facilitates GABAergic neuron development (Lin et al., 2007), and $p75^{NTR}$ expressed on Müller glial cells mediates light-induced photoreceptor death via a non-cell autonomous pathway (Harada et al. 2000). To determine whether TNFα functions in either of these $p75^{NTR}$-dependent events will be an important priority in futures studies.

In summary, our work demonstrates that proNGF can induce neuronal death in the retina through a non-cell autonomous mechanism that involves the activation of $p75^{NTR}$ and production of TNFα by Müller glial cells. These findings raise the possibility that non-cell autonomous events may be a general feature of $p75^{NTR}$-dependent cell apoptosis *in vivo*.

3.5. MATERIALS AND METHODS

3.5.1. Experimental animals

Procedures were carried out primarily in adult C57BL/6 transgenic or wild-type littermate control mice, with the exception of results shown on Fig. 1A which were carried out using adult Sprague-Dawley rats. All animal procedures were performed in accordance with the policies on the Use of Animals in Neuroscience Research and the Canadian Council on Animal Care guidelines (Olfert et al., 1993). $p75^{NTR}$ (Lee et al., 1992), TNFα (Taniguchi et al., 1997) and NRAGE (Bertrand et al., 2008a) null mice have been previously described. Sortilin null mice were generated by replacing a segment between exons 2 and intron 3 of the sortilin gene with a neomycin resistance cassette using homologous recombination. Chimeric cells were injected into C57BL/6 blastocysts giving rise to chimeric mice, which were then backcrossed to C57BL/6 mice to identify if the germ line transmission of the mutant allele had taken place. These heterozygotes were backcrossed with C57BL/6 mice for a minimum of seven generations before they were used in this study. All genotypes were verified by PCR

using pfuTurbo (Stratagene, Vancouver, BC, Canada). The number of animals used in each experiment (n) is shown in each corresponding graph.

3.5.2. Intraocular injections

A mutant form of proNGF that is resistant to cleavage by proteases (proNGFmut-m, 25 ng/µl, Alomone Labs, Jerusalem, Israel) was injected into the vitreous chamber of the left eye using a 10-µl Hamilton syringe adapted with a 32-gauge glass microneedle (total volume: 2 µl). The average vitreous fluid volume in adult mice is estimated to be ~10 µl (Remtulla and Hallett, 1985; Sharma et al., 2005; Yu and Cringle, 2006); therefore the concentration of proNGF that reached RGCs was 0.1 µM. ProNGF was injected alone or in combination with Etanercept (Enbrel, 25 µg/µl, Wyeth, Montreal, QC), the $p75^{NTR}$ antibody REX (10 µg/µl), or an Fc control (25 µg/µl, Sigma). Control eyes were injected with mature NGF (0.1 µg/µl, human recombinant NGF, PeproTech Inc., Rocky Hill, NJ), tumor necrosis factor-alpha (200 ng/ml, murine recombinant TNFα, R&D Systems, Minneapolis, MN), or vehicle (phosphate-buffered saline: PBS). Intraocular injections were performed under general anesthesia (2% Isoflurane/oxygen mixture, 0.8 liters/min). The needle tip was inserted into the superior hemisphere of the eye, at a 45° angle through the sclera into the vitreous body. This route of administration avoided retinal detachment or injury to eye structures, including the iris and lens, which release factors that induce neuronal survival (Leon et al., 2000a; Fischer et al., 2001a).

3.5.3. Retrograde labeling and quantification of neuronal survival

Retrograde labeling of RGCs was performed using Fluorogold (2%, Fluorochrome, Englewood, CO) in 0.9% NaCl, which was applied to the superior colliculus as described (Sapieha et al., 2005b). Subsequent surgical procedures were performed at 1 week after Fluorogold application (Berkelaar et al., 1994b; Mansour-Robaey et al., 1994b; Peinado-Ramon et al., 1996b; Cheng et al., 2002b). Mice were perfused with 4% paraformaldehyde; eyes were dissected and flat-mounted vitreal side up on glass sides. Fluorogold-positive RGCs were counted in 12 retinal zones: three areas in each eye quadrant (dorsal, ventral, nasal and temporal) located at 0.5 mm, 1.0 mm and 1.5 mm from the optic nerve head were examined (Sapieha et al., 2005b), corresponding

to a total area of 0.5 mm^2. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by non-parametric test (Bonferroni's multiple comparison test) or by Student's *t*-test, as indicated in legends.

3.5.4. Retinal immunohistochemistry

Mice were perfused with 4% paraformaldehyde and retinal sections were prepared as above. Tissue sections were incubated in 3% BSA and 0.3% Triton X-100 (Sigma) to block non-specific binding, then incubated with primary antibodies (see list below) overnight at 4°C, followed by incubation with secondary antibodies at room temperature. Slides were mounted with SlowFade (Molecular Probes, Eugene, OR) and visualized on a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Canada, Kirkland, QC) or confocal microscope (Leica Microsystems, Heidelberg, Germany). Primary antibodies used were: anti-cellular retinaldehyde-binding protein (1:1,000, gift from J. C. Saari, University of Washington, Seattle, WA) anti-$p75^{NTR}$ (REX, 2 ng/µl) (Bhakar et al., 1999), and anti-TNFα (0.4 µg/ml, Chemicon). The secondary antibodies used were sheep anti-mouse IgG (1 µg/ml, FITC conjugate, Sigma, Oakville, ON) or anti-rabbit IgG (1 µg/ml, Cy3, Jackson ImmunoResearch Laboratories, Inc.).

3.5.5. Immunoblot analysis

Retinas were homogenized in lysis buffer (20 mM Tris pH 8.0, 135 mM NaCl, 1% SDS, and 10% glycerol supplemented with protease inhibitors) and centrifuged at 14,000 rpm for 5 min. The supernatants were collected, diluted in Leammli sample buffer (4% SDS, 10% glycerol, 0.004% bromophenol blue, 0.1 M DTT and 0.125 M Tris pH 6.8) and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting following standard protocols. Primary antibodies were against: $p75^{NTR}$ (REX, 1:1,000) (Salehi et al., Neuron 2000), NRAGE (1:1,000) (Salehi et al., Neuron 2000), Sortilin (1:1,000) (BD Biosciences), TNFα (0.2 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA), and β-Actin (1:30,000) (Sigma). Secondary HRP-coupled antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). Protein signals were detected using a chemiluminescence reagent (ECL, Amersham Biosciences) followed by exposure of blots to X-OMAT (Kodak) imaging film.

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3.7. FIGURE LEGENDS

FIGURE 1. Exogenous proNGF leads to marked RGC loss in the adult rodent eye

Quantitative analysis of RGC survival in rat (A) and mouse (B) retinas at 1 week after intraocular injection of proNGF (black), vehicle (PBS, grey), or NGF (horizontal lines). The density of RGCs in intact, non-injected retinas is shown as reference (white). Data are expressed as RGC densities (RGCs/mm^2; mean ± SEM; ANOVA, * = $p<0.001$). The number of animals used in each experiment is shown above the corresponding graph bar.

FIGURE 2. p75^{NTR}, sortilin and NRAGE are required for proNGF-induced death of RGCs

(A) Treatment with REX, a p75^{NTR} function-blocking antibody, promoted RGC survival in the presence of proNGF whereas co-administration of a non-specific, control Ig did not exert a neuroprotective effect (ANOVA * = $p<0.001$). RGCs from mice eyes deficient in p75^{NTR} (B), sortilin (C) or NRAGE (D) were resistant to proNGF-induced death (Student's *t*-test, $p > 0.05$). Values are expressed as RGC densities (RGCs/mm^2; mean ± SEM).

FIGURE 3. p75^{NTR} and p75^{NTR}-induced TNFα are expressed by Müller cells in the adult mouse retina

(A) Confocal microscopy images show absence of p75^{NTR} within Fluorogold-positive RGCs, demonstrating that adult RGCs are devoid of p75^{NTR}. (B) Double immunolabeling with antibodies against p75^{NTR} (REX) and the Müller cell marker cellular retinaldehyde-binding protein (CRALBP) shows strong expression of p75^{NTR} in Müller cell processes surrounding RGCs. GCL: ganglion cell layer. (C) Fluorescent microscopy images show robust TNFα protein induction in retinas exposed to proNGF compared to low, basal levels in eyes injected with vehicle or recombinant, mature NGF. (D) Confocal microscopy images show that TNFα co-localizes with CRALBP-positive Müller cell soma and processes. Scale bars: 10 μm (A, B); 50 μm (C, D).

FIGURE 4. Sortilin and NRAGE cooperate with $p75^{NTR}$ to transduce proNGF-induced signaling events required for TNFα production

(A) Co-administration of proNGF with the TNFα inhibitor Etanercept leads to striking RGC neuroprotection, but RGCs did not survive in Fc-treated control retinas (ANOVA, * = $p < 0.001$). (B) RGCs from TNFα null mice were resistant to proNGF-induced death (Student's *t*-test, $p > 0.05$). (C) Western blot analyses of retinal extracts show that while TNFα protein levels are below the detection limit in wild-type retinas, exposure to proNGF elicits robust TNFα protein upregulation within 48 hrs of proNGF injection. ProNGF-induced TNFα production is completely lost in $p75^{NTR}$ null, sortilin null and NRAGE null animals (n=5 mice/group). Lower panels were probed with antibodies that recognize $p75^{NTR}$, Sortilin, or NRAGE, respectively, and actin was used as control for equal protein loading.

Supplemental Figure 1. Exogenous TNFα kills retinal ganglion cells in wild-type and $p75^{NTR}$ null retinas.

Quantitative analysis of RGC densities after intraocular injection of TNFα shows that RGCs die in the presence of exogenous TNFα in both wild-type and $p75^{NTR}$ retinas (n=5 mice/group). Values are expressed as RGC densities (RGCs/mm^2; mean ± SEM) and the density of RGCs in intact, non-injected wild-type retinas is shown as reference (white).

FIGURE 1

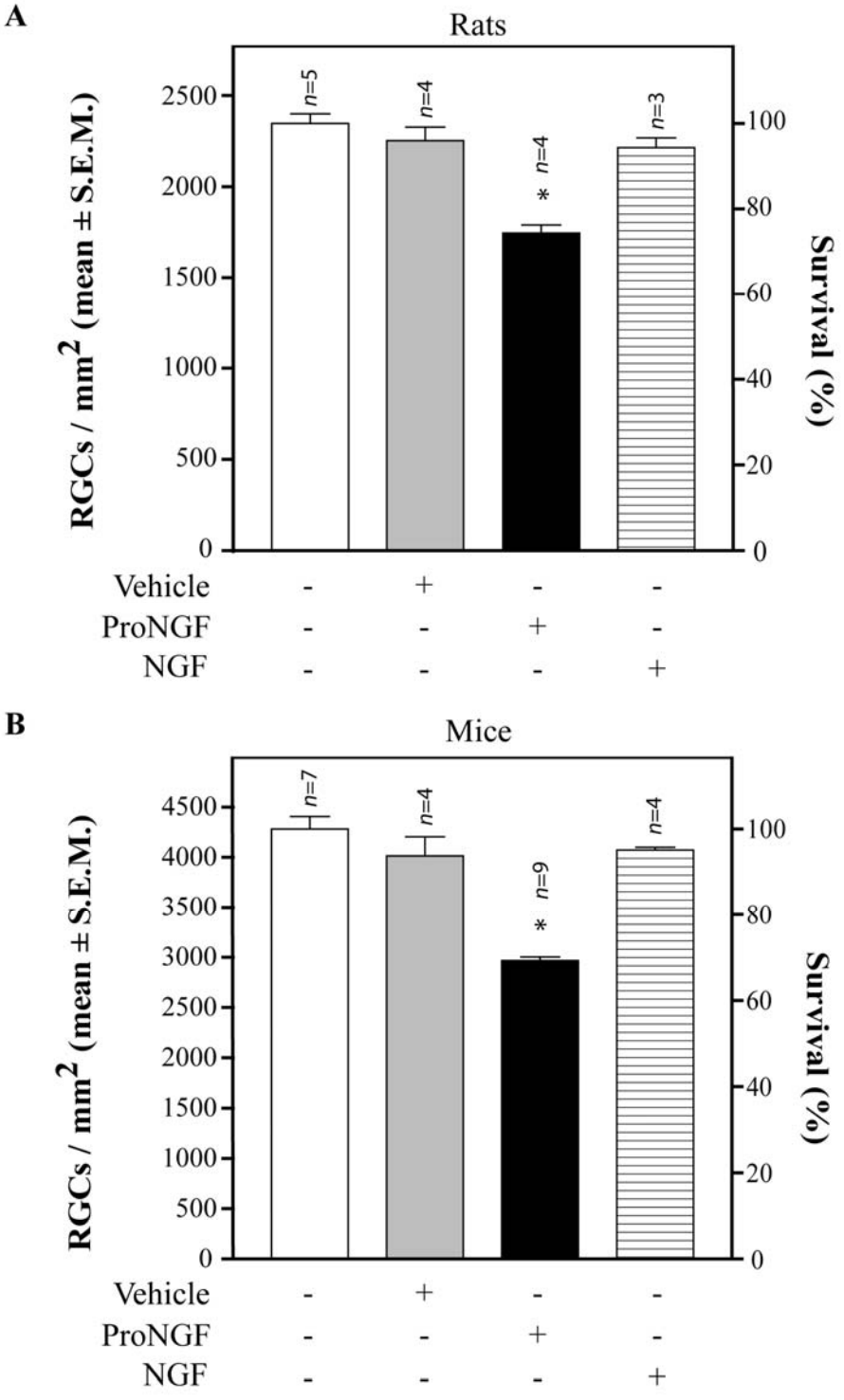


FIGURE 1

FIGURE 2

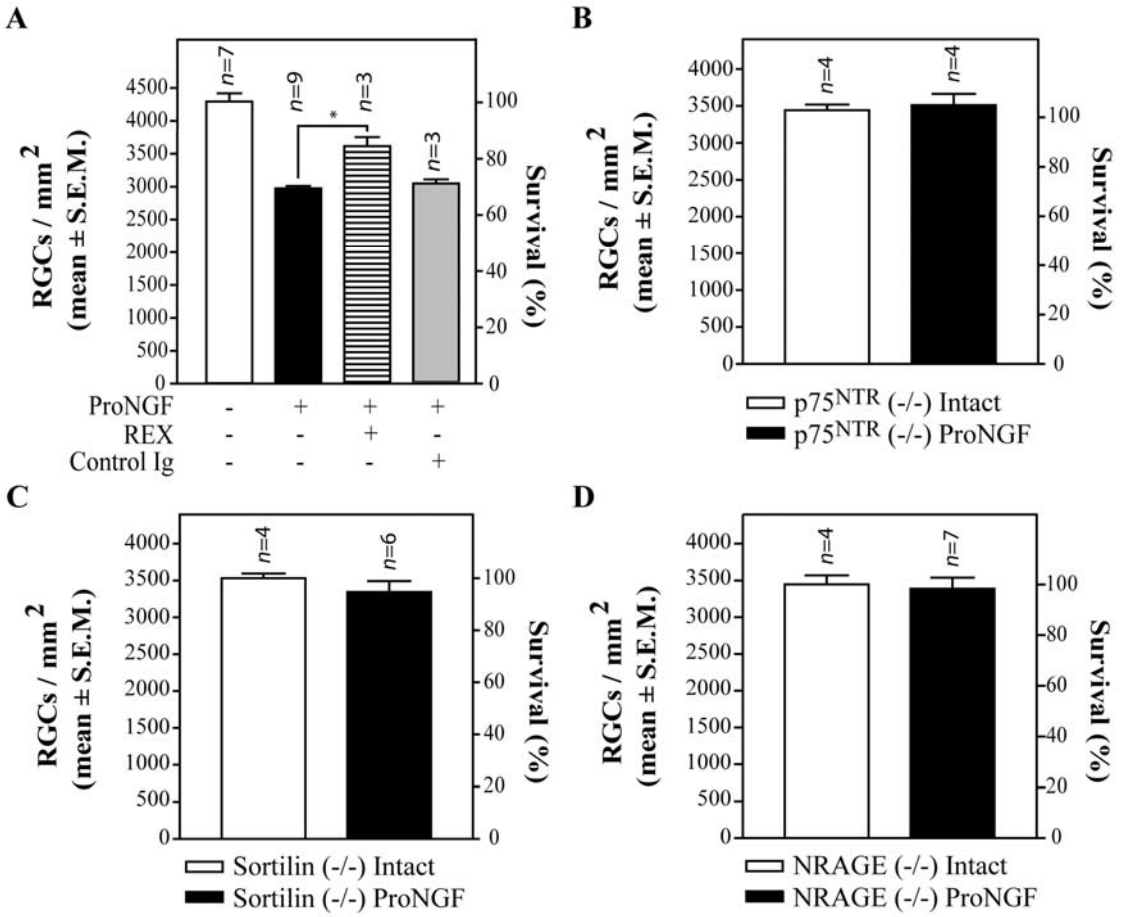


FIGURE 2

FIGURE 3

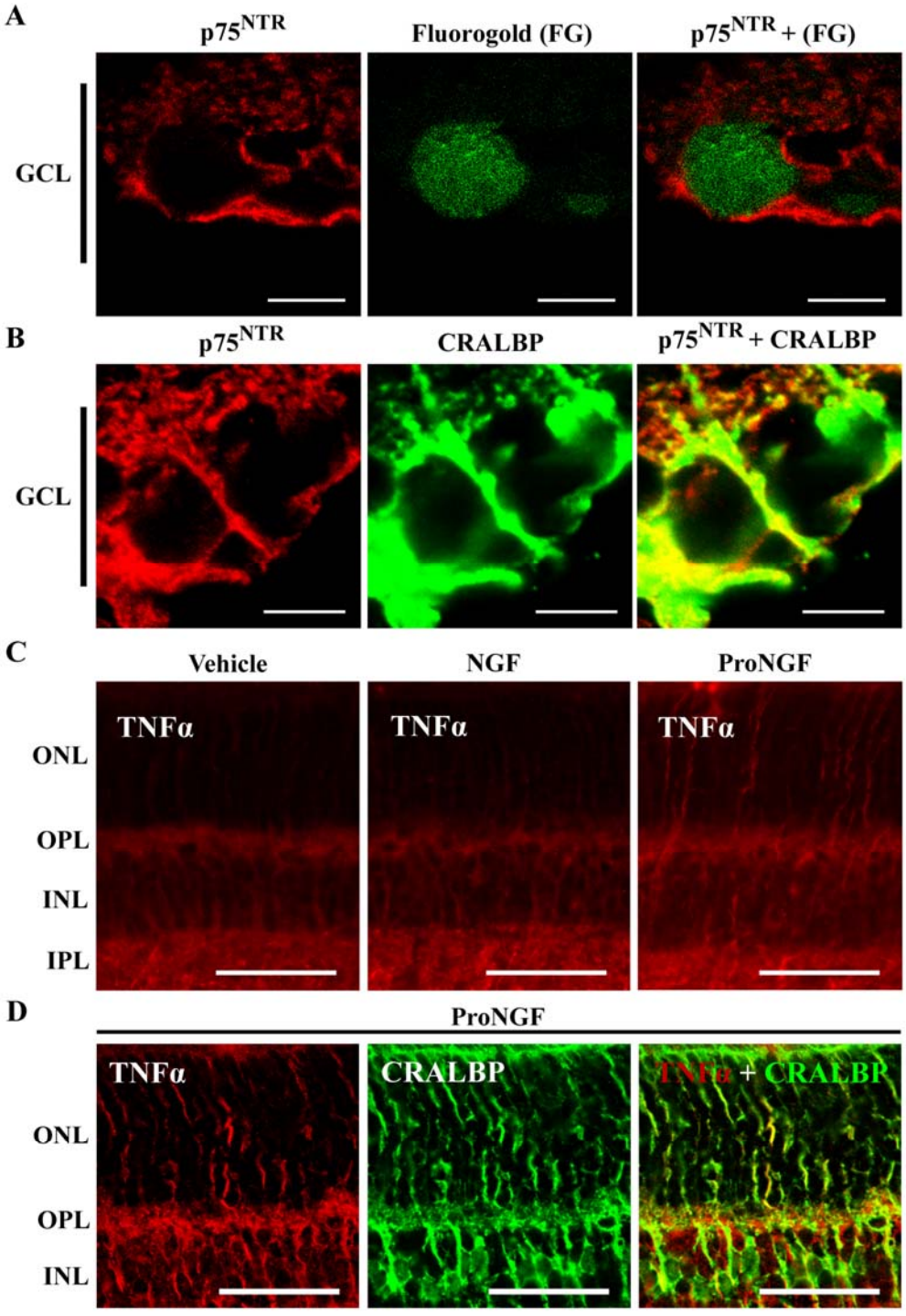


FIGURE 3

FIGURE 4

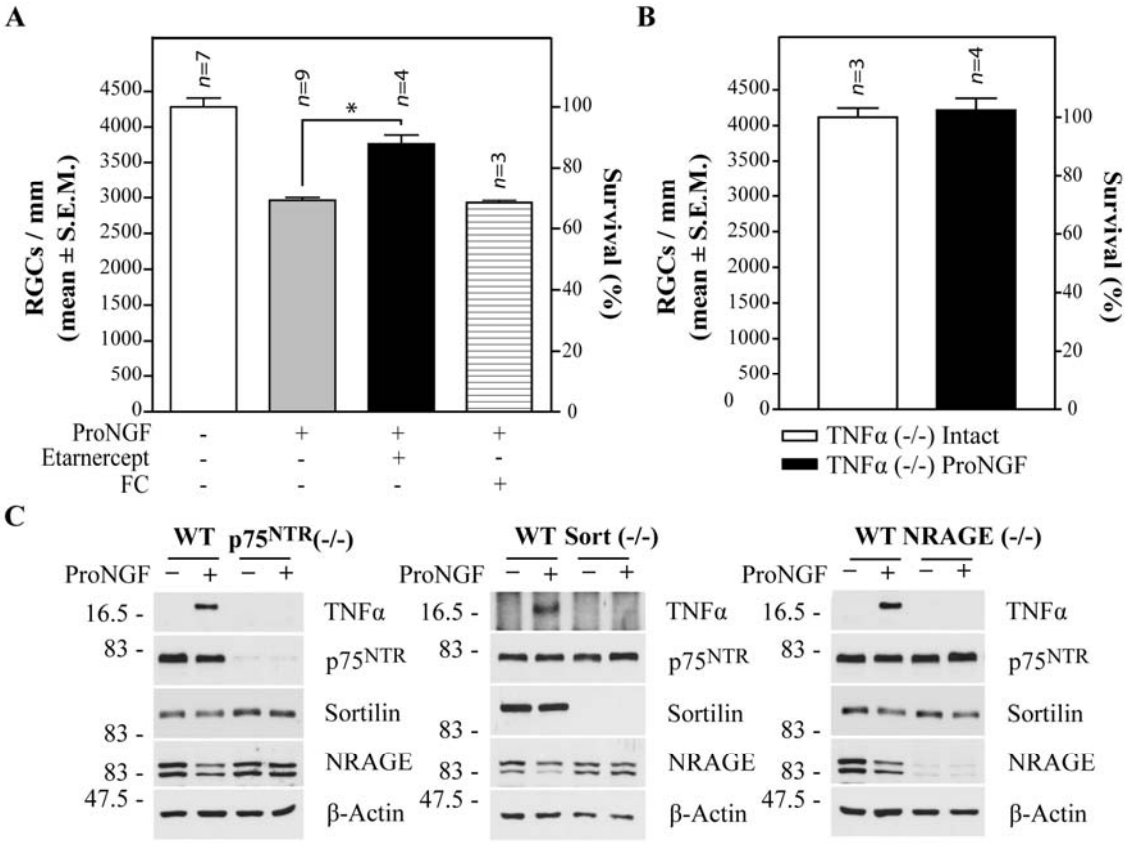
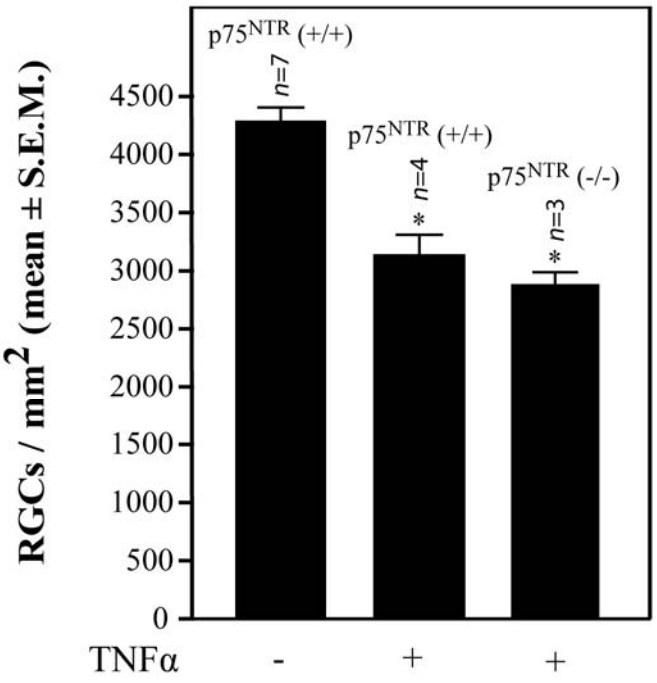


FIGURE 4

SUPPLEMENTAL FIGURE 1



SUPPLEMENTAL FIGURE 1

CHAPITRE 4

TROISIÈME ARTICLE: "EXCITOTOXIC DEATH OF RETINAL NEURONS *IN VIVO* OCCURS VIA A NON-CELL-AUTONOMOUS MECHANISM".

Journal of Neuroscience, 2009 29(17): 5536-45.

Dans le droit fil des deux premières études, nous avons voulu préciser le rôle des cellules de Müller lors d'un dommage excitotoxique des CGRs. La réaction des cellules de Müller a été examinée par suite d'une sur-activation des récepteurs du glutamate pour établir les bases moléculaires de l'interaction entre ces cellules et les CGRs. Les résultats ont montré une activation de NFκβ avec production de TNFα dans les cellules de Müller, laquelle pourrait exacerber l'excitotoxicité via relocalisation de certains récepteurs AMPA à la surface des CGRs.

EXCITOTOXIC DEATH OF RETINAL NEURONS *IN VIVO* OCCURS VIA A NON-CELL-AUTONOMOUS MECHANISM

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Abbreviated title: Retinal glia mediate NMDA-induced neuronal death

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4.1. ABSTRACT

The central hypothesis of excitotoxicity is that excessive stimulation of neuronal *N*-Methyl-D-Aspartate (NMDA)-sensitive glutamate receptors is harmful to neurons and contributes to a variety of neurological disorders. Glial cells have been proposed to participate in excitotoxic neuronal loss, but their precise role is poorly defined. In this *in vivo* study, we show that NMDA induces profound NF-κB activation in Müller glia but not in retinal neurons. Intriguingly, NMDA-induced death of retinal neurons is effectively blocked by inhibitors of NF-κB activity. We demonstrate that TNFα protein produced in Müller glial cells via an NMDA-induced NF-κB dependent pathway plays a crucial role in excitotoxic loss of retinal neurons. This cell loss occurs mainly through a TNFα-dependent increase in Ca^{2+}-permeable AMPA receptors on susceptible neurons. Thus, our data reveal a novel non-cell-autonomous mechanism by which glial cells can profoundly exacerbate neuronal death following excitotoxic injury.

Key words: NMDA/Müller Glia/Nuclear Factor κB/Tumor Necrosis Factor/Retina

4.2. INTRODUCTION

Glutamate is the predominant excitatory neurotransmitter in many regions of the central nervous system (CNS). Elevation of endogenous glutamate and activation of glutamate receptors contribute to a variety of acute and chronic neurological disorders, including hypoxic-ischemic brain injury (stroke), trauma, seizures, and various forms of dementia and neurodegeneration (Kalia et al., 2008). In the retina, excess glutamate has been proposed to underlie common neurodegenerative disorders, including glaucoma and retinal artery occlusion (Hare et al., 2001b; Hare et al., 2004b; Casson, 2006b; Seki and Lipton, 2008). The central hypothesis for excitotoxic injury is that excess glutamate binds to cell surface *N*-Methyl-D-Aspartate (NMDA) receptors on neurons, triggers massive Ca^{2+} influx, and activates pro-apoptotic signaling cascades. However, NMDA antagonists have consistently failed in clinical trials (Lee et al., 1999; Ikonomidou and Turski, 2002; Kemp and McKernan, 2002b) suggesting that other mechanisms contribute to the devastating excitotoxic damage that occurs *in vivo*.

Neighbouring non-neuronal cells may facilitate neuronal damage after excitotoxic injury. For example, activated microglia and astrocytes produce proinflammatory cytokines after excitotoxic injury (Wood, 1995; Barone and Feuerstein, 1999; Jeon et al., 2008; Vogt et al., 2008). Tissue plasminogen activator derived from microglia promotes excitotoxin-induced neuronal death in the hippocampus (Tsirka et al., 1995), while inhibition of microglial activation and proliferation has been shown to reduce excitotoxic injury (Tikka et al., 2001). Collectively, these data support a model in which excitotoxic brain damage activates glial cells which then enhance neuronal death. However, the molecular mechanisms by which glial cells regulate excitotoxic loss of neurons have not yet been defined. A primary aim of this study was to investigate whether non-neuronal cells play a role in excitotoxic injury in the adult retina and, if so, to define the molecular pathways by which they influence neuronal death *in vivo*.

A potential effector of injury in the CNS is the nuclear factor κB (NF-κB). In non-neuronal cells, NF-κB activates transcription of anti-apoptotic genes and production of cytokines and chemokines that mediate inflammation (Hoffmann and Baltimore, 2006). NF-κB also plays important roles in the nervous system, both promoting neuronal

survival and facilitating synaptic function (Mattson and Camandola, 2001; Meffert and Baltimore, 2005). The role of NF-κB in neuropathological settings, however, has been controversial. Several studies have indicated that NF-κB is neuroprotective after injury (Maggirwar et al., 1998; Hamanoue et al., 1999a; Bhakar et al., 2002b), whereas others have shown that NF-κB activation facilitates neuronal damage (Schneider et al., 1999b; Schwaninger et al., 2006a).

In this study, we report that the extensive death of retinal neurons induced by NMDA is effectively blocked with pharmacological inhibitors of NF-κB activity. Using a transgenic NF-κB reporter mouse, we unequivocally identify the cellular site of NMDA-induced NF-κB activation as Müller glial cells. Furthermore, we demonstrate that NF-κB-dependent TNFα production by Müller cells promotes retinal ganglion cell death. Finally, we show that Müller cell-derived TNFα leads to neuronal loss by increasing cell surface expression of Ca^{2+}-permeable AMPA receptors. Our data reveal a novel non-cell-autonomous pathway by which retinal glia can exacerbate neuronal death following excitotoxic injury.

4.3. EXPERIMENTAL PROCEDURES

4.3.1. Experimental animals

Experimental procedures were carried out on C57BL/6 transgenic or wild-type littermate control mice in accordance with the policies on the Use of Animals in Neuroscience Research and the Canadian Council on Animal Care guidelines (Olfert et al., 1993). The NF-κB reporter mice contain an NF-κB responsive minigene composed of three tandem HIV-derived κB binding repeats placed upstream of a minimal SV40 promoter and drive expression of an E. coli ß-galactosidase (ß-gal) gene tagged with a nuclear localization sequence (Bhakar et al., 2002b). TNFα knockout mice were kindly provided by Dr. David Stellwagen (Montreal General Hospital, McGill University). The number of animals used in each experiment (n) is shown on each graph and/or in the figure legend.

4.3.2. Intraocular injections

N-Methyl-D-Aspartate (NMDA, 20 mM, Sigma, Oakville, ON) was injected into the vitreous chamber of the left eye using a 10-µl Hamilton syringe adapted with a 32-gauge glass microneedle (total volume: 2 µl). The vitreous fluid in adult mice is estimated to range from 5-20 µl (Remtulla and Hallett, 1985; Sharma et al., 2005; Yu and Cringle, 2006); therefore the concentration of NMDA that reaches retinal ganglion cells is 2-8 mM. This amount of NMDA allows us to compare our results with previous studies using similar NMDA concentrations (Shimazawa et al., 2005; Ito et al., 2008; Nakazawa et al., 2008).

NMDA was injected alone or in combination with: cell-permeable recombinant SN50 peptide (100 µg/ml, BIOMOL, Research Laboratories, Plymouth, PA), SN50M (100 µg/ml, BIOMOL, Research Laboratories), PS1145 (500 µM, kind gift of Millennium Pharmaceuticals Inc, Cambridge, MA), MK-801 (1 mM, Sigma), Etanercept (Enbril, 25 µg/µl, Wyeth, Montreal, QC), z-IETD-fmk (50 µM, Biovision, Mountain View, CA), GYKI 52466 (500 µM, Tocris, Ellisville, MO) or IEM 13460 (500 µM, Sigma). Eyes injected with vehicle (PBS), control Fc, or non-injected intact eyes served as controls. Intraocular injections were performed under general anesthesia (2% Isoflurane/oxygen mixture, 0.8 liters/min). The needle tip was inserted into the superior hemisphere of the eye, at a 45° angle through the sclera into the vitreous body. This route of administration avoided retinal detachment or injury to eye structures, including the iris and lens, which release factors that induce neuronal survival (Mansour-Robaey S et al., 1994; Leon et al., 2000b; Fischer et al., 2001b). Surgical glue (Indermill, Tyco Health Care, Mansfield, MA) was used to seal the injection site.

4.3.3. β-galactosidase detection

Mice were perfused transcardially with 0.8% glutaraldehyde and the eyes were immediately dissected out. Tissue was incubated overnight at 37°C in staining solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal, Invitrogen, Burlington, ON). Tissue was embedded in optimal cutting temperature (O.C.T.) compound (Tissue-Tek,

Miles Laboratories, Elkhart, IN), retinal cryosections (16 μm) were collected onto gelatin-coated slides and examined under light microscopy.

4.3.4. Retinal Immunohistochemistry

Mice were perfused with 4% paraformaldehyde and retinal sections were prepared as above. Tissue sections were first incubated in 3% BSA and 0.3% Triton X-100 (Sigma) to block non-specific binding, then incubated with primary antibody (see list below) overnight at 4°C, followed by incubation with secondary antibody at room temperature. Slides were mounted with SlowFade (Molecular Probes, Eugene, OR) and visualized on a Zeiss Axioskop 2 Plus microscope. Primary antibodies used were: β-galactosidase (6.6 μg/ml, Chemicon, Temecula, CA), cellular retinaldehyde-binding protein (1:1,000, gift from J. C. Saari, University of Washington, Seattle, WA), protein kinase C (5 μg/ml, BD Biosciences, Mississauga, ON), calretinin (2 μg/ml, Chemicon), glial fibrillary acidic protein (2.5 μg/ml, Chemicon), and macrophage/monocytes (MAC-1, 1:2, gift from S. David, McGill University, Montreal, QC), and TNFα (0.4 μg/ml, Chemicon). The secondary antibodies used were sheep anti-mouse IgG (1 μg/ml, FITC conjugate, Sigma, Oakville, ON) or anti-rabbit IgG (1 μg/ml, Cy3, Jackson ImmunoResearch Laboratories, Inc.).

4.3.5. Immunoblot analysis

Retinas were homogenized in lysis buffer, centrifuged at 10,000 rpm for 5 min and supernatants were collected and analyzed by SDS polyacrylamide gels and immunoblotting. Membranes were incubated with TNFα antibody (0.2 μg/ml, Chemicon) or β-actin (0.5 μg/ml, Sigma), washed in TBST and then incubated with peroxidase-linked secondary antibody (0.5 μg/ml, Amersham Pharmacia). Protein signals were detected using a chemiluminescence reagent (ECL, Amersham Biosciences) followed by exposure of blots to X-OMAT (Kodak) imaging film.

4.3.6. Cell survival assays and quantification

i) TUNEL: The terminal dUTP nick end labeling (TUNEL) assay (Gavrieli et al., 1992b) was performed using the *In Situ* Apoptosis Detection Kit (Chemicon) on retinal

cryosections following the manufacturer's instructions. TUNEL-positive cells in the entire retinal section were counted on 16 sections per eye.

ii) Retrograde labeling: Fluorogold (2%, Fluorochrome, Englewood, CO) in 0.9% NaCl was applied to the superior colliculus as described (Sapieha et al., 2005b). The maximum number of retinal ganglion cells is observed after 7 days of Fluorogold application and it is stable for several weeks thereafter (Berkelaar et al., 1994b; Mansour-Robaey et al., 1994b; Peinado-Ramon et al., 1996b; Cheng et al., 2002b). Therefore, procedures were performed 1 week after Fluorogold application. Mice were perfused with 4% paraformaldehyde; eyes were dissected and flat-mounted vitreal side up on glass sides. Fluorogold-positive retinal ganglion cells were counted in 12 retinal zones: three areas in each eye quadrant located at 0.5 mm, 1.0 mm and 1.5 mm from the optic nerve head were examined (Sapieha et al., 2005b), corresponding to a total area of 0.5 mm^2.

4.3.7. Identification of Ca^{2+}-permeable AMPARs

Ca^{2+}-permeable AMPARs were identified using the Co^{2+} staining technique (Osswald et al., 2007). Retinas were dissected, cut in small pieces and incubated for 30 min in oxygenated assay buffer (5 mM KCl, 2 mM MgCl, 12 mM glucose, 20 mM bicarbonate, 139 mM sucrose, 57.5 mM NaCl and 0.75 mM $CaCl_2$). The tissue was then incubated in 5 mM $CoCl_2$ and 10 mM L-glutamic acid in the presence or absence of AMPA receptor blockers GYKI 52466 (40 µM, Tocris) or IEM 13460 (100 µM, Sigma) for 15 minutes. Co^{2+} was precipitated with 0.24% ammonium sulfide and the retina was then fixed in 0.8% glutaraldehyde and 20 µm sections were prepared. Silver enhancement of the Co^{2+} sulfide precipitate was performed using the Intense kit (Amersham), then retinal sections were rinsed, mounted and photomicrographs were captured with a Zeiss Axioplan 2 imaging microscope.

4.3.8. Statistical Analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by non-parametric test (Tukey's multiple comparison test) or by Student's *t*-test, as indicated in legends.

4.4. RESULTS

4.4.1. NMDA induces NF-κB dependent death of retinal neurons

Retinal neurons, particularly amacrine and retinal ganglion cells, are exquisitely sensitive to excitotoxicity (Siliprandi et al., 1993; Kikuchi et al., 1995; Manabe and Lipton, 2003). To dissect the signaling pathways that contribute to excitotoxic injury in the retina, we first established the time-course of neuronal loss following NMDA intraocular injection. Two complementary techniques were used for quantification of neuronal cell death: TUNEL labeling on retinal cross sections, and Fluorogold retrograde labeling of retinal ganglion cells visualized on retinal whole mounts.

Figure 1A-C shows that TUNEL-positive cells were first detected 3 hours after NMDA exposure, and their number increased markedly by 6 and 24 hours after NMDA injection. TUNEL staining was observed in the inner nuclear layer, where amacrine cells are found; and in the ganglion cell layer, where retinal ganglion cells and displaced amacrine cells are located. Fluorogold retrograde labeling, performed to assess retinal ganglion cell viability, showed that NMDA injection resulted in rapid loss of these neurons: 50% of retinal ganglion cells remained at 6 hours after NMDA exposure, while <10% survived at 24 hours post-NMDA treatment (Figure 1D, E). Microglia and macrophages, which may have incorporated Fluorogold after phagocytosis of dying retinal ganglion cells, were excluded based on morphological criteria. Microglia invariably have smaller cell size, visible process ramifications and lack axons (Thanos, 1991b; Kacza and Seeger, 1997) (Figure 1D insert, arrowheads). Although NMDA was administered in the dorsal (superior) retina, loss of Fluorogold-labeled retinal ganglion cells was similar between dorsal, ventral, nasal and temporal retinal quadrants (ANOVA, $p > 0.05$, Supplementary Figure 1). The apparent higher number of TUNEL+ cells compared to Fluorogold+ cells in the ganglion cell layer (Figures 1A and 1D, 24 hrs), likely reflects that this layer is composed of retinal ganglion cells and displaced amacrine cells in approximately 1:1 ratio (Perry, 1981a). Therefore, TUNEL+ cells in the ganglion cell layer represent both retinal ganglion cells as well as displaced amacrine cells, whereas Fluorogold labels only retinal ganglion cells.

NF-κB transcriptional activity has been reported to promote pro- or anti-apoptotic responses following CNS injury (Maggirwar et al., 1998; Hamanoue et al., 1999a;

Schneider et al., 1999b; Bhakar et al., 2002b; Schwaninger et al., 2006a), but the molecular basis for these divergent effects remains unknown. To determine if NF-κB activity influenced NMDA-induced neuronal death in the retina we asked whether SN50, a cell-permeable peptide that blocks nuclear translocation of NF-κB subunits, altered levels of NMDA-induced cell loss. Intriguingly, co-administration of SN50 with NMDA sharply reduced the number of TUNEL-positive cells, both in the ganglion cell layer and inner nuclear layer (Figure 2A). In contrast, SN50M, a mutant form of SN50 that does not block NF-κB nuclear translocation, had no effect on NMDA-induced TUNEL labeling.

Induction of canonical NF-κB signaling requires activation of IKK1 and IKK2, kinases that phosphorylate IκB family members (Schmid and Birbach, 2008). IKK2 often plays an obligatory role in canonical NF-κB signaling thus we asked whether PS1145, a small-molecule inhibitor of IKK2, altered retinal ganglion cell death induced by NMDA. Figure 2B shows that PS1145 elicited a remarkable inhibition of NMDA-induced retinal ganglion cell death, equivalent to that evoked by SN50 or by the non-competitive NMDA receptor antagonist, MK801. Therefore, NF-κB is strongly activated in the retina following NMDA exposure and inhibitors that block NF-κB transcriptional activity, by different mechanisms, rescue neurons challenged with NMDA. We conclude that NF-κB activation plays a crucial role in NMDA-induced loss of retinal neurons.

4.4.2. NMDA induces NF-κB activity in Müller glia but not in retinal neurons

To determine the cellular localization of NMDA-induced NF-κB activity in the adult retina, we used a well-characterized reporter mouse in which NF-κB responsive elements drive expression of nuclear ß-galactosidase (ß-gal) (Bhakar et al., 2002b). In the PBS-treated, uninjured retina, almost no NF-κB reporter activity was detected (Figure 3A). In contrast, retinas examined at 6, 18 and 24 hours after NMDA injection showed a striking increase in ß-gal activity within the inner nuclear layer (Figure 3B-D). The induction of NF-κB in the retina invoked by NMDA was completely blocked by the NMDA receptor antagonist, MK-801 (Figure 3E), and with the NF-κB inhibitor SN50 (Figure 3F). These results indicate that the ß-gal activity detected in the inner nuclear

layer reflects an NF-κB transcriptional response mediated through activation of NMDA receptors.

The inner nuclear layer of the retina consists of interneurons (amacrine, bipolar, and horizontal cells) and Müller glial cells. To determine which of these cell types exhibit NMDA-induced NF-κB activation, retina derived from NF-κB reporter mice were stained using antibodies for ß-gal and for retinal cell-type markers. After intravitreal injection of NMDA, ß-gal-expressing cells were consistently positive for cellular retinaldehyde-binding protein (CRALBP), a specific Müller cell marker (Figure 4A-C). In contrast, ß-gal staining was never found in cells positive for markers of bipolar and amacrine cells, including protein kinase C (Figure 4D-F) or calretinin (Figure 4G-I) respectively. We conclude that Müller glia is the only cell type within the inner nuclear layer that undergoes NMDA-induced NF-κB activation.

NMDA exposure induced ß-gal expression in a very small number of cells within the ganglion cell layer in the NF-κB reporter mice (see Figure 3B-D). In addition to retinal ganglion cells, this region contains astrocytes, macrophages and displaced amacrine cells. The few ß-gal-labeled cells observed in this region were invariably positive for cell-specific markers of astrocytes (GFAP, Figure 4J-L) or macrophages (MAC-1, Figure 4M-O). ß-gal staining was never observed in retinal ganglion cells retrogradely labeled with Fluorogold (Figure 4P-R) or in calretinin-positive displaced amacrine cells (not shown). These data demonstrate that NMDA induces NF-κB activity in Müller glia but not in retinal neurons. This finding led us to test the hypothesis that a Müller cell-derived factor, regulated by NF-κB, acts in a non-cell-autonomous manner to facilitate neuronal death following NMDA exposure.

4.4.3. TNFα produced by Müller cells promotes NMDA-dependent neuronal loss

TNFα transcription is strongly regulated by NF-κB activity (Shakhov et al., 1990; Hiscott et al., 1993a; Mori and Prager, 1996), and TNFα added exogenously can increase death of retinal neurons (Tezel and Wax, 2000b; Nakazawa et al., 2006; Berger et al., 2008). We therefore tested whether Müller cell-derived TNFα acts as an endogenous factor that facilitates NMDA-dependent neuronal death *in vivo*. Figure 5A shows that

TNFα protein was undetectable in the adult, intact retina but its levels increased dramatically after NMDA exposure. The NMDA-induced increase in TNFα expression was completely blocked by the NF-κB inhibitor SN50, indicating that activation of NF-κB plays a central role in NMDA-dependent expression of this cytokine. Similarly, TNFα was not detected in the uninjured retina by immunocytochemistry (Figure 5B) but was clearly visualized after NMDA injection in presumptive Müller cell bodies and processes in the inner retina (Figure 5C). Moreover, NMDA-induced accumulation of TNFα immunoreactivity was blocked by the NF-κB inhibitor SN50 (Figure 5D). Co-labeling experiments using the Müller cell-specific marker CRALBP confirmed that Müller cells selectively upregulate expression of TNFα in the presence of NMDA (Figure 5E-G). We conclude that Müller glial cells are the main cellular source of TNFα production in the retina following NMDA exposure.

To test if Müller cell-derived TNFα facilitates excitotoxic death of retinal neurons, we examined if the extent of NMDA-induced retinal ganglion cell loss was attenuated in the absence of TNFα. Our data show striking survival of retinal ganglion cells in TNFα knockout mice at 6 hours after NMDA exposure: 76% of these neurons remained alive compared to only 42% in wild-type littermate controls ($p < 0.001$) (Figure 5H). Although the total number of retinal ganglion cells in adult TNFα null mice treated only with vehicle was similar to that in wild-type mice (Figure 5H), it is conceivable that increased neuronal survival following NMDA injection in TNFα null mice reflects a chronic effect of TNFα deprivation. As an alternative approach, we blocked TNFα action in the retina using Etanercept, a soluble Fc fusion protein that contains the extracellular ligand-binding domain of TNFR2 that is used clinically for blockade of TNFα in humans with rheumatoid arthritis or psoriasis (Fantuzzi et al., 2008a; Lyudmila, 2008). Figure 5H shows that Etanercept, but not control Fc, protected retinal ganglion cells from NMDA-induced death. Etanercept protected 75% of these neurons from NMDA-induced death compared to only ~45% in Fc-treated or vehicle-treated controls ($p < 0.001$). We conclude that TNFα derived from Müller glia acts in a non-cell-autonomous manner to promote NMDA-dependent neuronal death in the retina.

4.4.4. Endogenous TNFα exacerbates excitotoxicity by increasing neuronal Ca^{2+}-permeable AMPA receptors (AMPARs)

We then asked what mechanisms are used by endogenous TNFα released from Müller cells to exacerbate NMDA-dependent neuronal loss in the retina. Engagement of the TNFR1 receptor can activate caspase-8 to induce apoptosis (Thorburn, 2004a). We therefore tested whether inhibition of caspase-8 activation reduced NMDA-mediated excitotoxicity. Blockade of caspase 8 activity using z-IETD-fmk, a potent caspase 8 inhibitor previously shown to effectively inhibit caspase 8 *in vivo* at the concentrations used here (Weishaupt et al., 2003), had no effect on NMDA-induced retinal ganglion cell death (Supplementary Figure 2). z-IETD-fmk, by itself, was not toxic and did not promote retinal ganglion cell death. These findings indicate that TNFα-dependent neuronal loss occurs via an alternative pathway.

Exogenous TNFα has been shown to induce selective cell surface expression of Ca^{2+}-permeable AMPARs (Beattie et al., 2002a; Ogoshi et al., 2005; Stellwagen et al., 2005), and their expression has been correlated with enhanced neuronal death in models of ischemia and excitotoxicity (Hermann et al., 2001; Noh et al., 2005; Ferguson et al., 2008b; Leonoudakis et al., 2008a). We tested the hypothesis that TNFα produced by Müller glia increases cell surface Ca^{2+}-permeable AMPARs on retinal neurons, thus rendering them more susceptible to excitotoxicity. Ca^{2+}-permeable AMPARs transport divalent cations such as cobalt (Co^{2+}), therefore we used an *in situ* Co^{2+} staining technique to identify retinal cells that express Ca^{2+}-permeable AMPARs. In the absence of NMDA, Co^{2+} accumulation was restricted to cells in the inner nuclear and ganglion cell layers (Figure 6A) previously identified as horizontal and AII amacrine cells (Osswald et al., 2007) as well as ganglion cells (Zhang et al., 1995). Following NMDA exposure, both amacrine and retinal ganglion cells accumulated Co^{2+} indicating that Ca^{2+} permeability in these cells was markedly enhanced when the retina was challenged with NMDA (Figure 6B). To test whether TNFα was required for NMDA-induced increase of Ca^{2+}-permeable AMPARs on retinal neurons, NMDA was co-administered with the TNFα antagonist Etanercept. Figure 6C shows that Etanercept effectively diminished the NMDA-induced Co^{2+} uptake.

To confirm that increased Co^{2+} accumulation in retinal neurons occurs via Ca^{2+}-permeable AMPARs and not other glutamate receptors, retinas were incubated with GYKI, a non-competitive AMPAR selective antagonist, and IEM, a channel blocker selective for Ca^{2+}-permeable non-NMDA receptors (i.e. AMPA and kainate receptors). Figure 6 (D, E) shows that NMDA-induced Co^{2+} accumulation in these neurons was blocked with IEM and GYKI, indicating that NMDA-induced Co^{2+} accumulation is due to increased flux of divalent cations exclusively through AMPARs. Finally, we tested whether TNFα-mediated increase in neuronal Ca^{2+} permeability influenced neuronal death following NMDA exposure. Figure 6F shows that retinal ganglion cells were markedly protected from NMDA-induced death in the presence of either IEM (63% survival) or GYKI (76% survival) with respect to control retinas (ANOVA, $p < 0.001$). The neuronal survival afforded by AMPAR blockers was similar to that observed in TNFα null mice or following treatment with the TNFα antagonist Etanercept (see Figure 5H). We conclude that Müller cell-derived TNFα plays a crucial role in the NMDA-dependent elaboration of Ca^{2+}-permeable AMPARs and, ultimately, excitotoxic death of retinal neurons.

4.5. DISCUSSION

This study reports four major findings. First, NMDA exposure causes induction of NF-κB activity in Müller glia, but not neurons, in the adult retina. Second, the death of amacrine and retinal ganglion cells induced by NMDA is sharply attenuated when Müller cell NF-κB activation is blocked. Third, TNFα is the target of NF-κB activity in Müller cells and is required for NMDA-induced loss of retinal neurons. Fourth, increased levels of Ca^{2+}-permeable AMPARs evoked by Müller cell-derived TNFα is a crucial mechanism that renders retinal neurons susceptible to excitotoxic injury.

The accessibility and defined cellular architecture of the retina, together with the exquisite sensitivity of retinal neurons to NMDA, make it an ideal system to elucidate the contribution of NF-κB to excitotoxicity. We showed that NF-κB is activated in Müller glia following NMDA exposure. The effect of NMDA on NF-κB activation in Müller glia was blocked by MK-801 confirming that NMDA receptors are involved in this response. NMDA receptors have been described on dissociated Müller cells from human (Puro et

al., 1996a) and chick retinas (Lamas et al., 2005b), and *in situ* hybridization studies have demonstrated expression of NMDA receptors in the rat inner plexiform layer where Müller cells reside (Brandstatter et al., 1994; Hartveit et al., 1994; Fletcher et al., 2000). The functional properties of glial and neuronal NMDA receptors are thought to be different (Lamas et al., 2005b), it is thus possible that NMDA receptors with different oligomeric structures differ in their ability to activate NF-κB. Alternatively, NMDA-dependent NF-κB activation in retinal glia may be indirect and results from a stimulating factor produced by neurons. Candidates for this effect are neuronal release of ATP and activation of glial P2Y receptors (Metea and Newman, 2006) and glutamate, which is released in high amounts by stressed neurons (Fujimoto et al., 2004). Regarding the latter possibility, previous studies have shown that occupancy of metabotropic glutamate receptors on glia can induce NF-κB activation (Caccamo et al., 2005b; Caccamo et al., 2005a; Kaushal and Schlichter, 2008a; Sitcheran et al., 2008).

Blockade of NF-κB activity in Müller cells dramatically reduced amacrine and retinal ganglion cell death, indicating that a Müller cell-derived factor plays a crucial role in NMDA-induced excitotoxicity. We focused on TNFα as a major effector in this pathway because the TNFα gene is tightly regulated by NF-κB, and previous studies have indicated that TNFα can facilitate excitotoxic damage (Chao and Hu, 1994; Hermann et al., 2001; Bernardino et al., 2005; Noh et al., 2005; Ferguson et al., 2008b; Kaushal and Schlichter, 2008a; Leonoudakis et al., 2008a). We showed that TNFα in Müller glia was dramatically increased by NMDA exposure, and our loss of function experiments confirmed that TNFα plays an obligatory role in the loss of retinal neurons induced by NMDA. These results are consistent with a previous study showing that retinal ganglion cells lacking TNFα receptor 1 (TNFR1) are protected following optic nerve crush (Tezel et al., 2004c). Interestingly, TNFα and TNFR1 have been shown to be upregulated in human donor eyes with glaucoma (Yan et al., 2000b; Yuan and Neufeld, 2000; Tezel et al., 2001), diabetic retinopathy and age-related macular degeneration (Armstrong et al., 1998; Oh et al., 1999); and TNFα gene polymorphisms have been found in patients with primary open angle glaucoma (Lin et al., 2003a; Funayama et al., 2004b) supporting a crucial role for TNFα in ocular diseases.

How does NF-κB-mediated production of TNFα by Müller glia kills retinal neurons? Ligand binding to TNFR1 triggers apoptosis by induction of caspase-8 activation which in turn activates caspase-3 and promotes cell death (Boldin et al., 1996; Hsu et al., 1996). Previous studies have shown that TNFα released from microglia can kill primary cortical neurons via a caspase 8-dependent mechanism (Velier et al., 1999a; Kaushal and Schlichter, 2008a), however we found that caspase-8 inhibition had no effect on NMDA-induced apoptosis of retinal neurons *in vivo*. Nevertheless, apoptosis is still a major cell death mechanism in this model based on the widespread TUNEL labeling observed by us and others (Manabe and Lipton, 2003; Nakazawa et al., 2005), and is likely to involve induction of the intrinsic mitochondrial pathway and activation of caspase-3.

Our data support an alternative mechanism that invokes TNFα-mediated increase of Ca^{2+}-permeable AMPARs on neuronal cell surfaces. A series of elegant *in vitro* studies demonstrated that glia-derived TNFα selectively stimulates cell surface insertion of Ca^{2+}-permeable AMPARs, while simultaneously decreasing surface $GABA_A$ receptors, a process required for rapid control of synaptic strength at excitatory synapses (Beattie et al., 2002a; Stellwagen et al., 2005; Stellwagen and Malenka, 2006a). Ca^{2+}-permeable AMPARs have been proposed as mediators of excitotoxic cell death (Kwak and Weiss, 2006b; Liu and Zukin, 2007). Recent studies have shown that exogenous TNFα may contribute to excitotoxicity by increasing cell surface levels of Ca^{2+}-permeable AMPARs in primary hippocampal neurons and spinal cord (Ferguson et al., 2008b; Leonoudakis et al., 2008a), but the cellular source and mechanisms that lead to TNFα production were not addressed in these studies. We provide the first *in vivo* evidence that Müller glia-derived TNFα exacerbates excitotoxic damage by increasing surface levels of Ca^{2+}-permeable AMPARs in retinal neurons.

Our data support a model in which non-cell-autonomous signaling events play a major role in NMDA-triggered excitotoxicity in the retina. We demonstrate that NMDA-dependent activation of NF-κB in Müller glial cells induces production of endogenous glia-derived TNFα, which in turn renders retinal neurons highly sensitive to excitotoxicity by increasing their surface levels of Ca^{2+}-permeable AMPARs. Our loss-

of-function experiments show that this non-cell-autonomous mechanism accounts for >60% of the NMDA-induced neuronal loss in the retina. Given the prevalence of NMDA receptors on retinal neurons (Zhang and Diamond, 2006), direct action of NMDA may play a modest, yet tangible, role in excitotoxicity in this system. However, our findings are a departure from the traditional paradigm of excitotoxic damage in which the death of neurons was entirely attributed to excessive Ca^{2+} influx via neuronal NMDA receptors. Instead, our data indicate that glial regulation of neuronal AMPAR distribution via TNFα is a central requirement for neuronal loss in retinal excitotoxicity.

There is strong evidence that glutamate, the endogenous neurotransmitter involved in excitotoxicity, increases intracellular Ca^{2+} in retinal ganglion cells primarily via NMDA receptors (Hartwick et al., 2008) raising the question: how do AMPARs mediate neuronal death? Interestingly, AMPARs are highly permeable to Zn^{+2} while NMDA receptors are blocked by this cation. Zn^{+2}, known to accumulate in hippocampal neurons following ischemia and prior to the onset of cell death (Koh et al., 1996; Lee et al., 2003), is extremely toxic for neurons (Sensi et al., 1999; Jiang et al., 2001; Kim and Koh, 2002b; Sensi et al., 2003). The elucidation of the precise role of Zn^{+2} in retinal ganglion cell death will be an interesting topic for future studies.

Our results are unexpected because Müller cells have been traditionally attributed a neuroprotective role under physiological conditions (Bringmann et al., 2006b). Indeed, one of the normal functions of Müller cells is the modulation of synaptic transmission and neuronal excitability in the mammalian retina (Newman, 2004a). It is possible that the injury-induced effects reported here represent the end of a physiological continuum; it will be interesting to determine if the low levels of TNFα produced by Müller cells in the uninjured, adult retina alters synaptic transmission through AMPAR-dependent mechanisms.

The failure of NMDA receptor antagonists in clinical trials (Lee et al., 1999; Ikonomidou and Turski, 2002; Kemp and McKernan, 2002; Kalia et al., 2008) presupposes that other molecular mechanisms play a critical role following the initial excitotoxic insult. Our study provides new insight into these events *in vivo* by demonstrating a new role for NF-κB in the injured CNS. Furthermore, we identify TNFα as a key glial-to-neuron signal that exacerbates excitotoxicity through modulation

of Ca^{2+}-permeable AMPARs. The precise delineation of the molecular events that occur in glia versus those in neurons, as shown here, should be useful for the design of novel therapeutic interventions applicable to neurodegenerative diseases and ischemia.

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4.7. FIGURE LEGENDS

Figure 1. Retinal neurons die following intraocular injection of NMDA.

(A) Fluorescent microscopy images of retinal sections from intact or NMDA-treated eyes following TUNEL assay. Quantification of TUNEL-positive cells in: (B) the inner nuclear layer (INL), and (C) ganglion cell layer demonstrated a striking increase in the number of apoptotic cells in the inner retina. (D) Retinal whole-mounts following retrograde labeling with Fluorogold were examined to assess the density of retinal ganglion cells in intact and NMDA-treated retinas. Insert shows an example of the morphological difference between retinal ganglion cells (arrow) and fluorogold-labeled microglia (arrowheads). (E) Quantitative analysis of the number of retinal ganglion cells that survived at 3 hrs, 6 hrs and 24 hrs after NMDA treatment. The density of retinal ganglion cells in intact, uninjured retinas is shown as reference (100% survival, open bar). Values are expressed as mean ± S.E.M. (ANOVA, Tukey's Multiple Comparison

Test, *: p <0.001). ONL: Outer Nuclear Layer, OPL: Outer Plexiform Layer, INL: Inner Nuclear Layer, IPL: Inner Plexiform Layer, GCL: Ganglion Cell Layer. Scale bars: 100 μm (A), 50 μm (D).

Figure 2. NMDA-induced neuronal death is NF-κB dependent.

(A) Quantification of TUNEL-positive cells in the inner nuclear layer (INL) or ganglion cell layer (GCL) at 6 hrs after treatment with NMDA alone, a cocktail of NMDA and SN50, or NMDA and inactive SN50M peptide. In the presence of SN50, but not SN50M, there was a 9-fold and 4-fold reduction in the number of TUNEL-positive cells in the ganglion cell layer and inner nuclear layer, respectively. Values are expressed as mean ± S.E.M. (ANOVA, Tukey's Multiple Comparison Test, *: $p < 0.05$, **: $p < 0.001$).

(B) Treatment with PS1145, a small molecule inhibitor of IKK2 promoted retinal ganglion cell neuroprotection in the presence of NMDA (ANOVA, Tukey's Multiple Comparison Test, *: $p < 0.001$). The neuroprotective effect of PS1145 was comparable to that of MK-801, a non-competitive antagonist of NMDA receptors (ANOVA, Tukey's Multiple Comparison Test, *: $p < 0.001$). Values are expressed as retinal ganglion cell densities (RGCs/mm^2; mean ± S.E.M.), and are compared to intact retinas (100% survival, open bar).

Figure 3. NF-κB is activated in the inner nuclear layer following exposure to NMDA. The cellular localization of NMDA-induced NF-κB activity in the adult retina was examined in NF-κB reporter mice. (A) Almost no NF-κB reporter activity was detected in the PBS-treated, uninjured retina (n=3), while retinas examined at 6 hrs (B),

18 hrs (C) and 24 hrs (D) after NMDA injection showed a striking increase in ß-gal activity in the inner nuclear layer (n=4-6/group). (E) The NF-κB transcriptional activity invoked by NMDA was completely blocked with the NMDA receptor antagonist, MK-801 (n=4), and with the NF-κB inhibitor SN50 (F, n=4). RPE: Retinal Pigment Epithelium, PS: Photoreceptor Segments, ONL: Outer Nuclear Layer, OPL: Outer Plexiform Layer, INL: Inner Nuclear Layer, IPL: Inner Plexiform Layer, GCL: Ganglion Cell Layer. Scale bars: 100 μm.

Figure 4. NMDA induces NF-κB activity in Müller glia, but not in retinal neurons. Double immunohistochemistry using a ß-gal antibody and specific markers for each retinal cell-type was carried out on retinal sections at 24 hrs after NMDA injection. In the inner nuclear layer, cells positive for cellular retinaldehyde-binding protein (CRALBP), a Müller cell-specific marker, were always positive for ß-gal (panels A-C). No co-localization was found between ß-gal and bipolar cell markers, including protein kinase C (PKC) (panels D-F, arrows); or amacrine cell markers, including calretinin (CR) (panels G-I, arrows). The few ß-gal-labeled cells observed in the ganglion cell layer were invariably positive for markers of astrocytes (GFAP) (panels J-L) or macrophages/microglia (MAC-1) (panels M-O). ß-gal staining was never observed in retinal ganglion cells retrogradely labeled with Fluorogold (panels P-R) or in displaced amacrine cells (not shown). All scale bars: 50 μm, except panels G-I: 12.5 μm.

Figure 5. TNFα produced by Müller glia promotes NMDA-dependent neuronal loss. (A) Western blot analysis of retinal homogenates show that TNFα protein levels were

low in the uninjured retina and increased following NMDA intraocular injection. NMDA-induced increase in TNFα protein was blocked with the NF-κB inhibitor SN50. By immunohistochemistry, TNFα was not detected in the uninjured retina (B) but was clearly visualized after NMDA injection (C) in presumptive Müller cell bodies and processes in the inner retina. (D) NMDA-induced TNFα immunoreactivity was blocked by the NF-κB inhibitor SN50. (E-G) Co-labeling experiments using the Müller cell-specific marker CRALBP confirmed that TNFα is upregulated in Müller glia in the presence of NMDA. Scale bars: 100 μm (B-D), 50 μm (E-G). (H) Striking neuroprotection after NMDA exposure was observed in TNFα knockout mice or in the presence of the TNFα blocker Etanercept, while no survival was observed in wild-type littermate or Fc-treated controls (ANOVA, * = $p < 0.001$). Values are expressed as retinal ganglion cell densities (RGCs/mm^2; mean ± S.E.M.), and are compared to intact retinas (100% survival, open bar).

Figure 6. TNFα exacerbates excitotoxicity by increasing neuronal Ca^{2+}-permeable AMPA receptors (AMPARs). (A) In the absence of NMDA, Co^{2+} accumulation was restricted to a few cells in the inner nuclear and ganglion cell layers. (B) Following NMDA exposure, both amacrine and retinal ganglion cells accumulated Co^{2+} indicating that Ca^{2+} permeability in these cells was markedly enhanced. The TNFα antagonist Etanercept (C), as well as the AMPAR blockers IEM-1460 (D) and GYKI (E) effectively reduced NMDA-induced Co^{2+} uptake. Scale bars: 40 μm. (F) Retinal ganglion cells were protected from NMDA-induced death in the presence of either IEM-1460 or GYKI, with respect to control retinas (ANOVA, * = $p < 0.05$, ** = $p < 0.001$). Values are expressed

as retinal ganglion cell densities (RGCs/mm^2; mean ± S.E.M.), and are compared to intact retinas (100% survival, open bar).

Supplementary Figure 1. NMDA-induced retinal ganglion cell loss is similar in all retinal quadrants. Although NMDA was administered in the dorsal (superior) retina, there was no significant difference in the loss of Fluorogold-labeled retinal ganglion cells between dorsal, ventral, nasal and temporal retinal quadrants (ANOVA, $p > 0.05$). These data indicate that NMDA diffuses well and leads to similar neuronal loss throughout the retina.

Supplementary Figure 2. TNFα-mediated neuronal loss is caspase 8-independent. (A) Treatment with z-IETD-fmk, a caspase-8 inhibitor, did not promote retinal ganglion cell survival, an effect that was not significantly different from that observed in vehicle-treated retinas (Student's t-test, *: $p > 0.05$). IETD by itself did not promote neuronal death. Values are expressed as retinal ganglion cell densities (RGCs/mm^2; mean ± S.E.M.) in intact retinas (open bar) or after treatment (black bars). (B) Analysis of whole-mounted retinas confirmed that z-IETD-fmk did not attenuate NMDA-induced retinal ganglion cell survival with respect to vehicle-treated

FIGURE 1

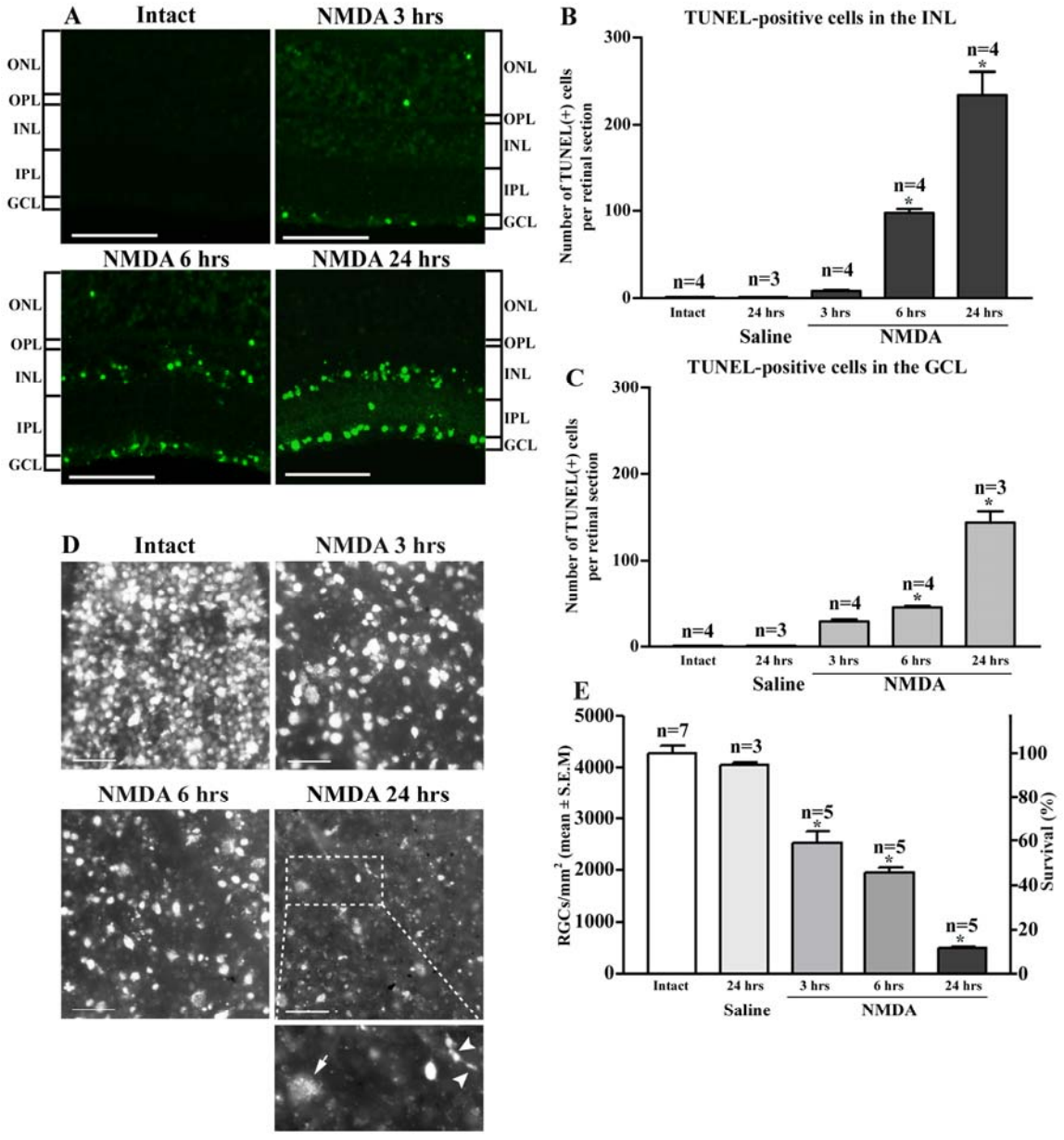


Figure 1

FIGURE 2

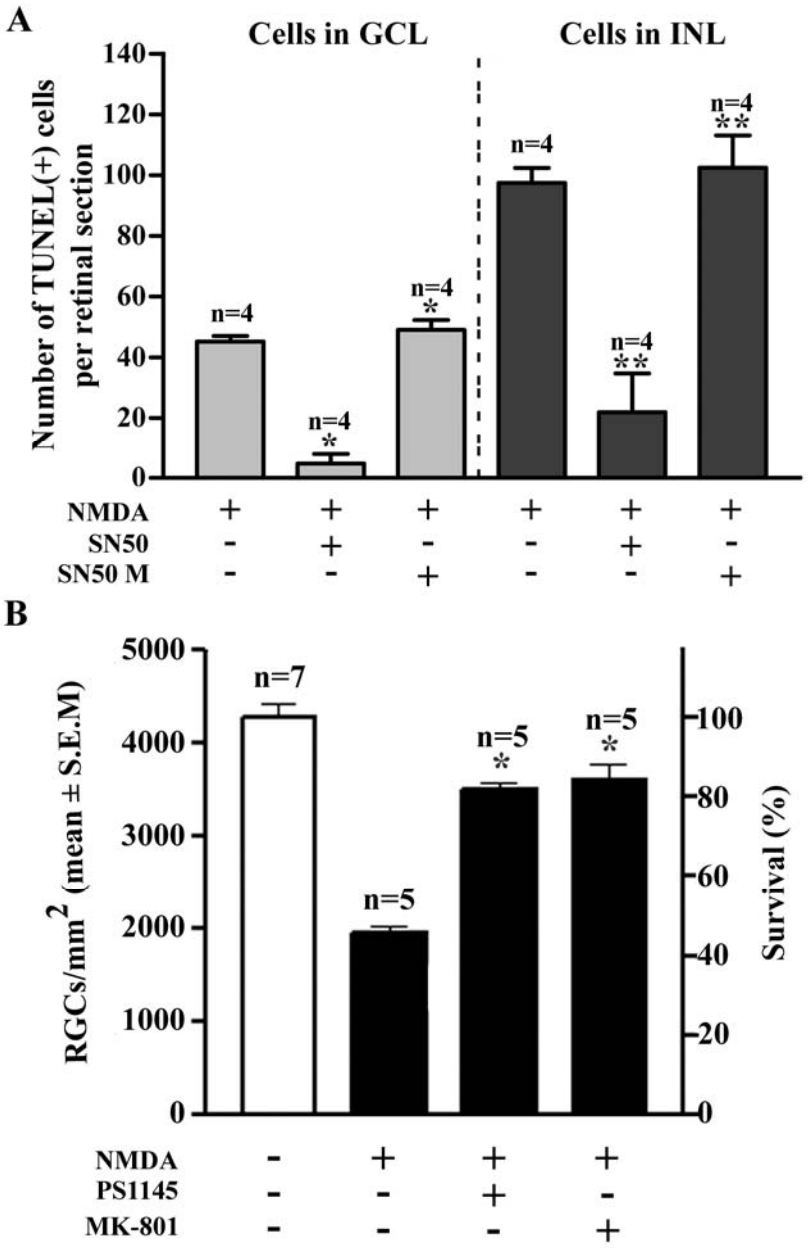


Figure 2

FIGURE 3

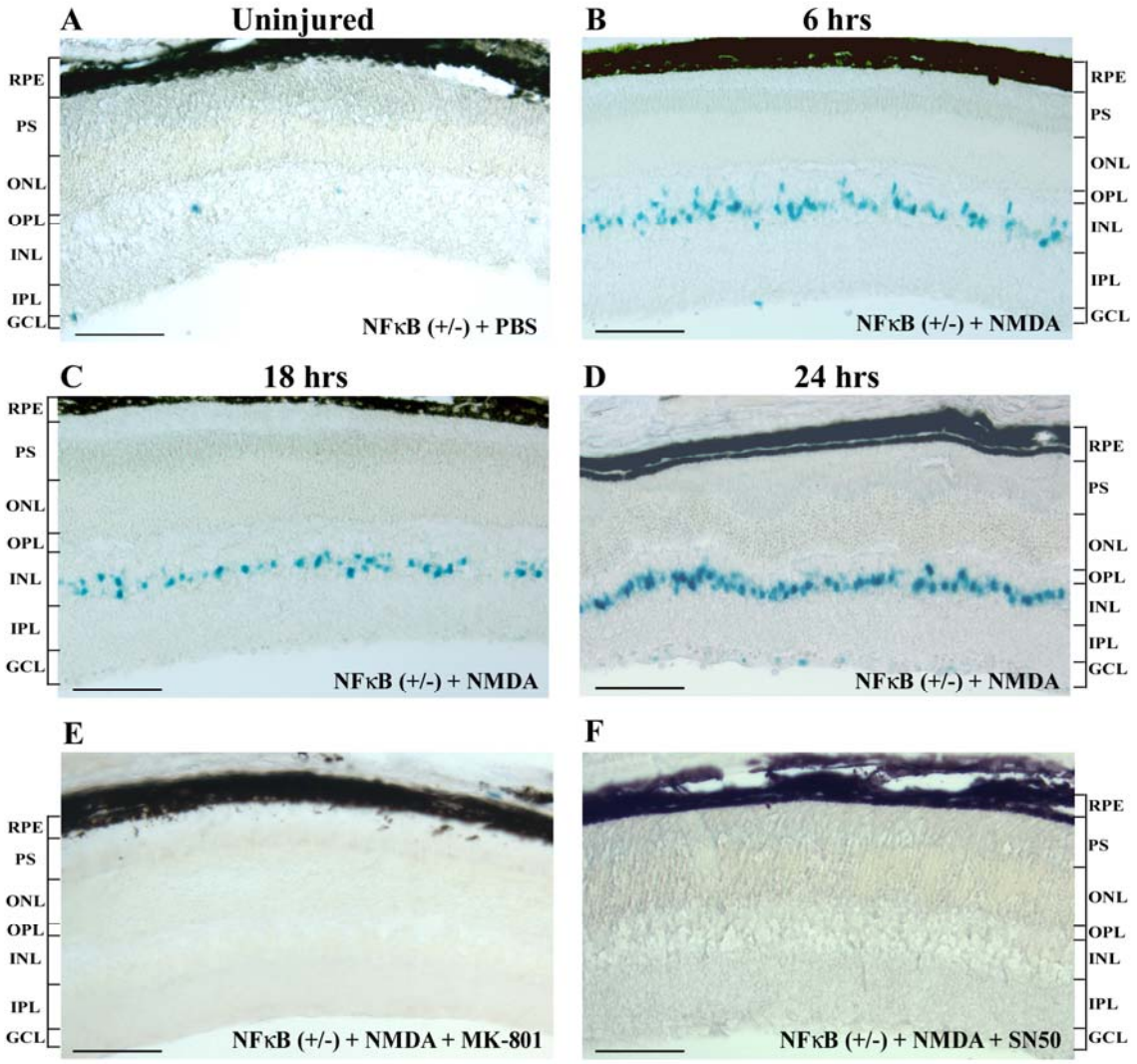


Figure 3

FIGURE 4

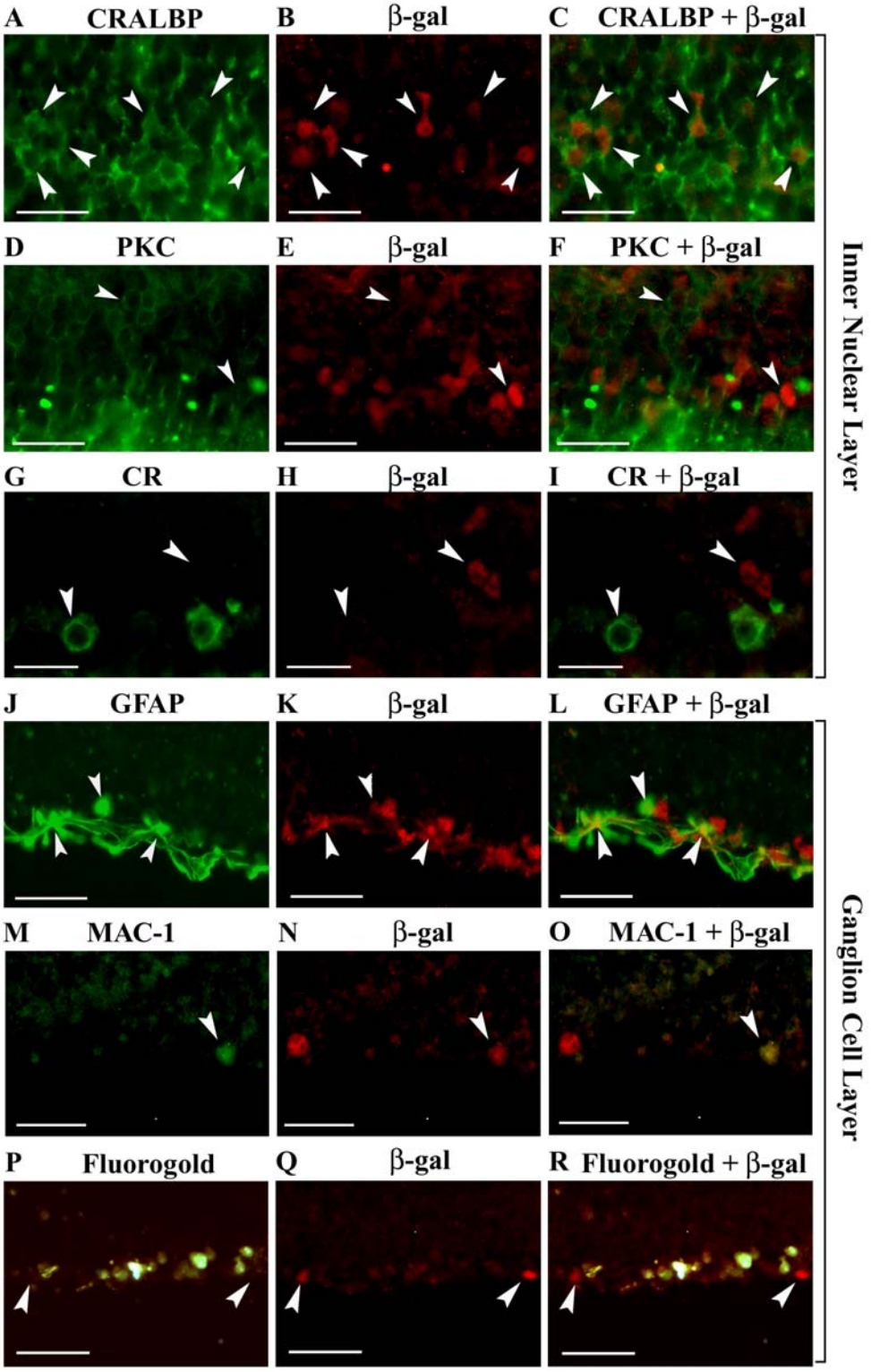


Figure 4

FIGURE 5

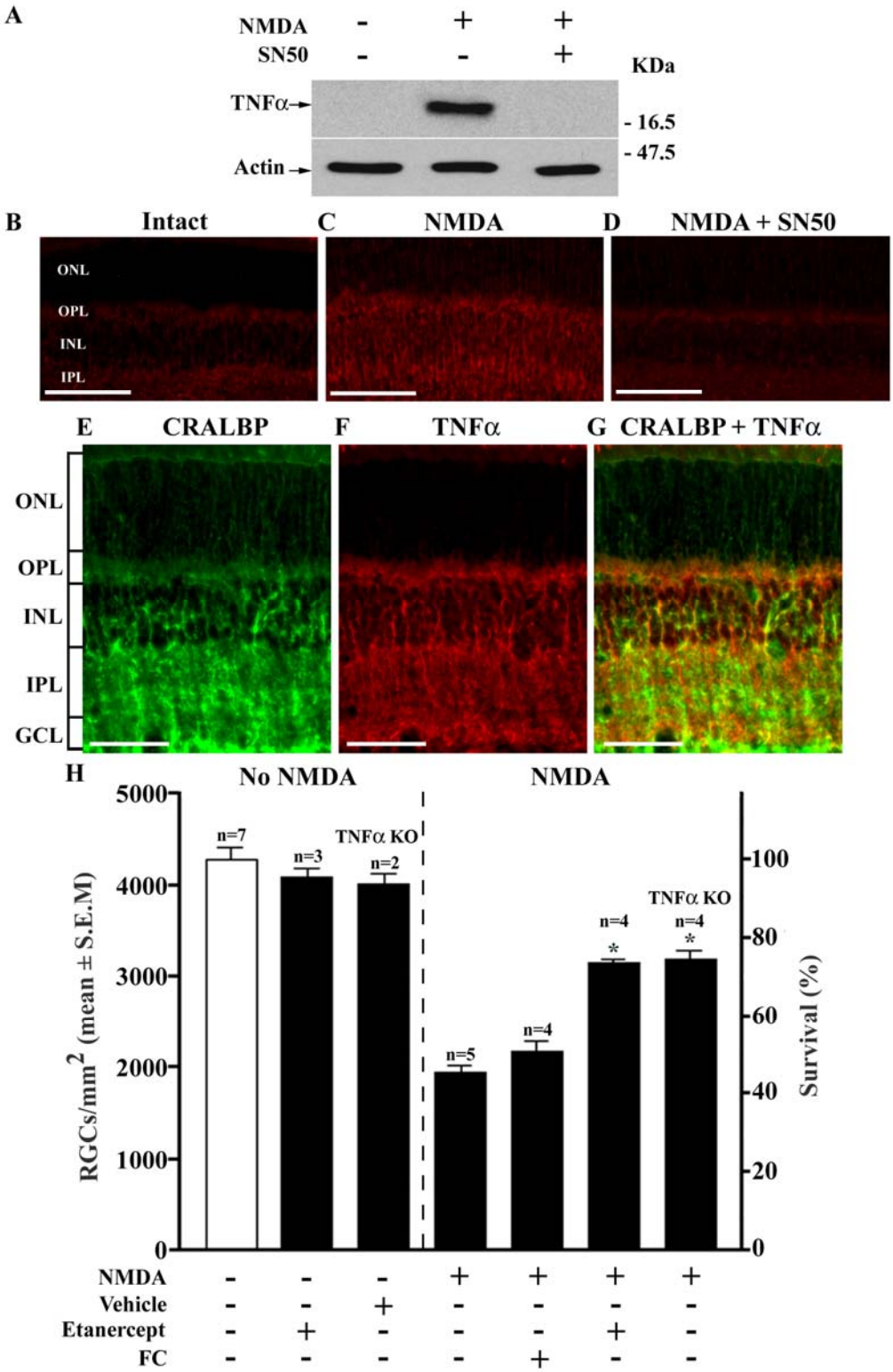


Figure 5

FIGURE 6

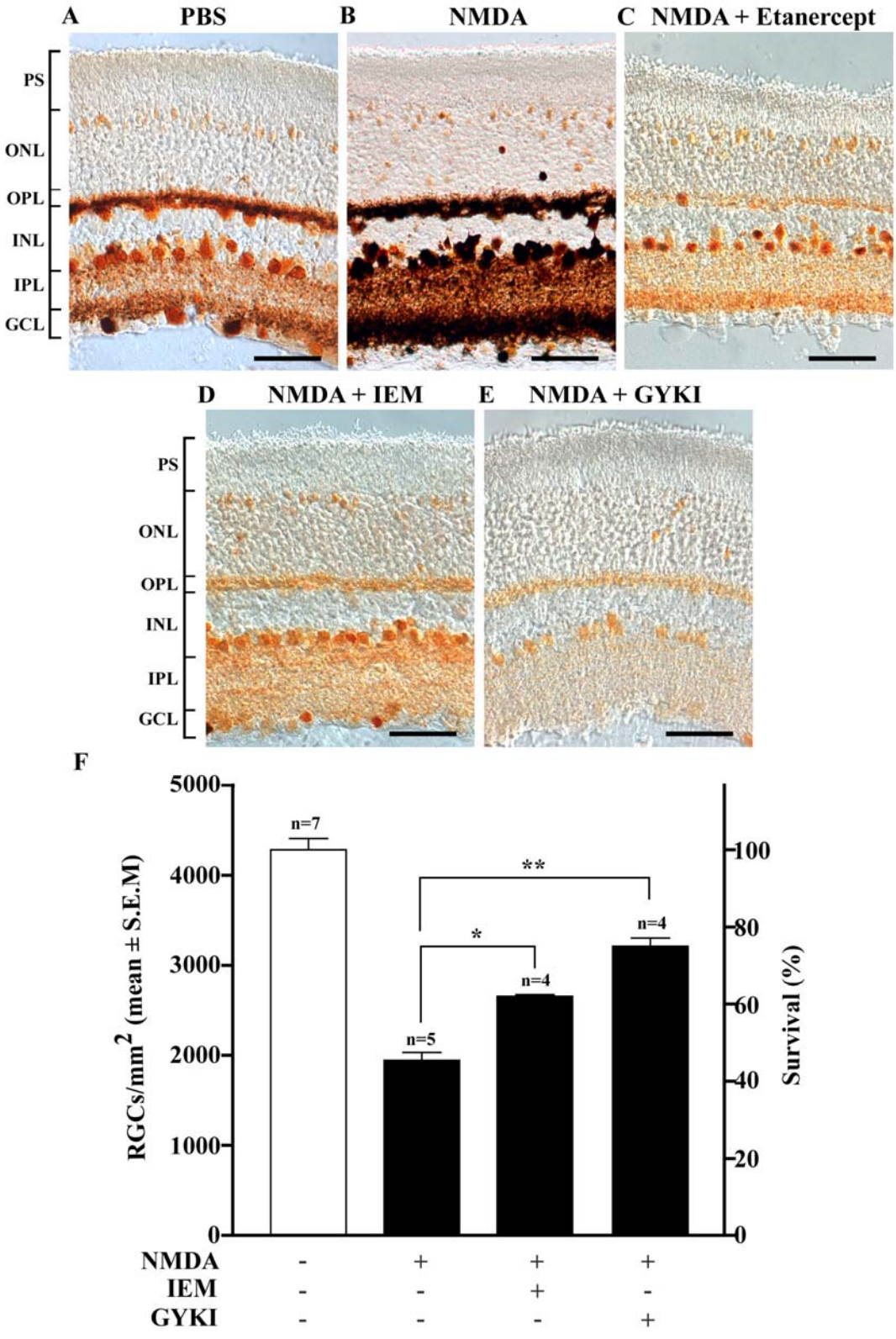
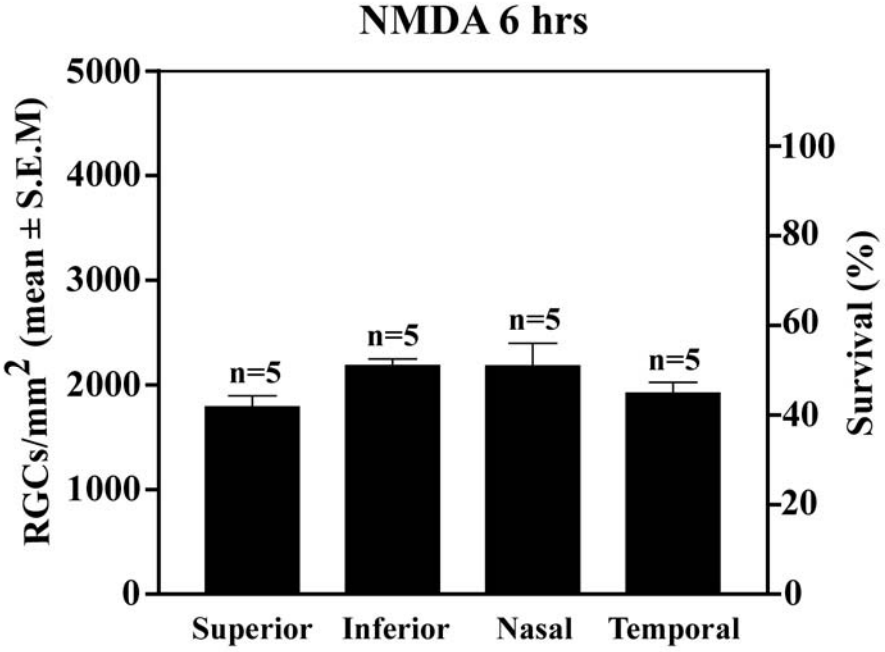


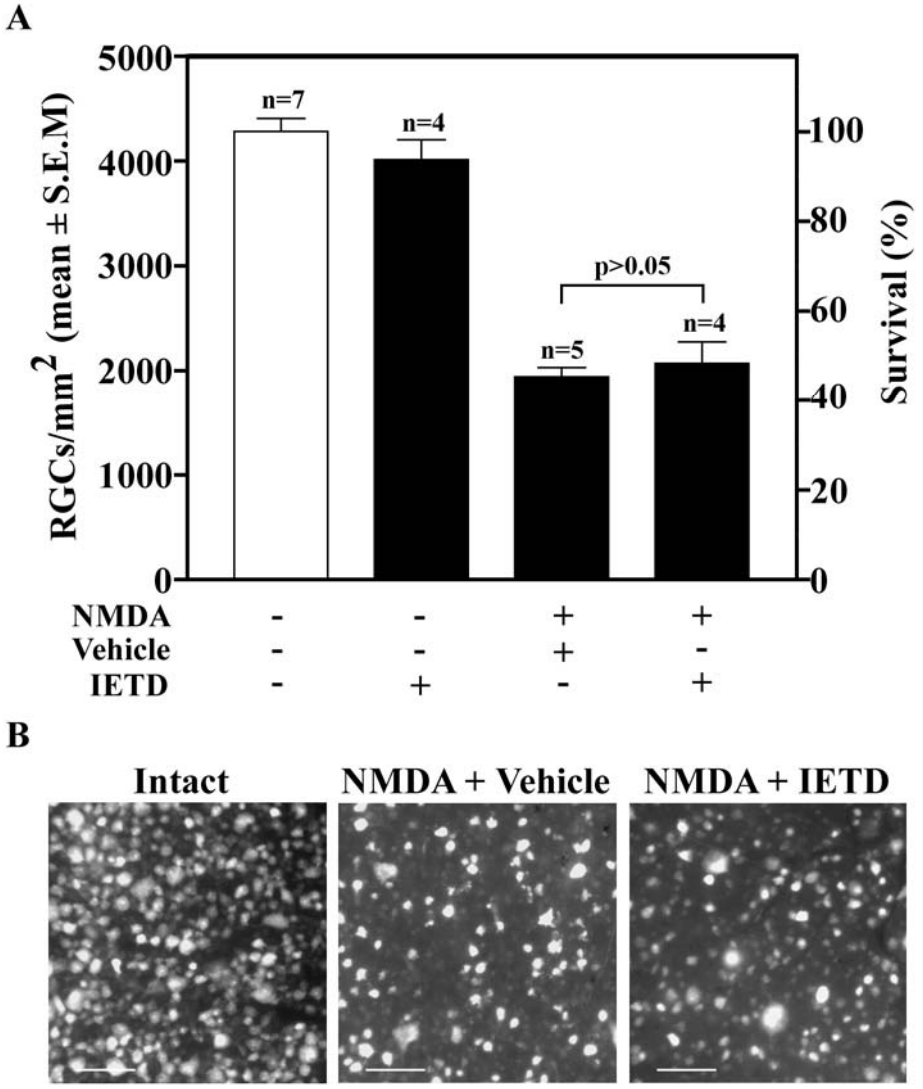
Figure 6

SUPPLEMENTAL FIGURE 1



Supplemental Figure 1

SUPPLEMENTAL FIGURE 2



Supplemental Figure 2

CHAPITRE 5

DISCUSSION GÉNÉRALE

5. DISCUSSION GÉNÉRALE

5.1. Le rôle des cellules gliales de Müller dans la mort des cellules ganglionnaires rétiniennes.

Les cellules gliales sont étroitement associées aux neurones dans le système nerveux. Il a une constante communication bidirectionnelle entre les deux populations. Il n'est donc pas étonnant de constater qu'à la suite d'une lésion affectant les neurones, il y a toujours une réponse des cellules gliales. Le rôle des cellules gliales n'est rien moins que d'assurer l'homéostasie de l'environnement neuronal. Il est légitime et même essentiel de mieux comprendre les mécanismes réactionnels des cellules gliales par suite d'une lésion. C'est à cette fin que les études présentées dans cette thèse ont établi le rôle joué par les cellules de Müller, les principales cellules gliales de la rétine, dans trois modèles de mort affectant les neurones rétiniens. Ces trois modèles ont permis de mettre en évidence un mécanisme cellulaire non-autonome affectant les CGRs et impliquant les cellules de Müller. L'idée d'un mécanisme cellulaire non-autonome est souvent évoquée dans cette thèse. Mais que signifie-t-elle au juste ? Dans notre cas, il s'agit d'un processus ou d'une réaction initié et/ou amplifié par les cellules gliales, et qui a pour finalité d'induire la mort des neurones environnants. C'est une mort des CGRs provoquée en grande partie par des forces qui leurs sont extérieures. L'idée d'un modèle cellulaire non-autonome n'est pas nouvelle. Plusieurs faits démontrent la survenue d'un tel processus dans plusieurs des maladies neurodégénératives comme la sclérose latérale amyotrophique (SLA), l'ataxie spinocérébelleuse, la maladie d'Huntington et le Parkinson (Lobsiger and Cleveland, 2007). Dans l'exemple de la SLA, les neurones moteurs sont touchés, bien que l'étiologie de la plupart des cas reste inconnue; 20% des cas familiaux proviennent d'une mutation de l'enzyme superoxyde dismutase 1 (SOD1), dont l'activité contribue à la destruction de l'oxygène superoxyde (Boillée et al., 2006). En utilisant des souris mutantes où le gène SOD, pouvait être spécifiquement exprimé dans certains types cellulaires, une étude a démontré que la diminution du niveau d'enzyme mutante dans la microglie n'avait qu'un faible effet sur la phase initiale de la maladie, mais atténuait nettement sa progression subséquente (Boillée et al., 2006). En somme, les expériences avec les mutants SOD semblent démontrer que des dommages

intrinsèques aux neurones moteurs sont à l'origine de la maladie, alors que l'expression de SOD mutante dans la microglie environnante est responsable de sa progression rapide. Sans insister sur chacune des maladies neurodégénératives, il semble que la mort neuronale puisse être grandement influencée par la toxicité ou l'expression de protéines mutantes, tant dans les neurones que les cellules gliales qui les entoure : astrocytes, oligodendrocytes et microglies. Ceci étant dit, jusqu'à maintenant, le rôle des cellules de Müller dans la mort des CGRs au cours de différentes rétinopathies n'avait jamais été bien défini.

5.2. L'inhibition de $p75^{NTR}$ dans les cellules de Müller augment la survie induite par TrkA dans les cellules ganglionnaires axotomisées.

5.2.1. Le *nerve growth factor* n'induit pas la survie des cellules ganglionnaires axotomisées.

Comme d'autres l'avaient démontré avant nous, l'apport exogène de NGF ne nous a pas permis d'induire la survie des CGRs après section du nerf optique (Johnson et al., 1986b; Cohen et al., 1994; Cui and Harvey, 1995; Bosco and Linden, 1999; Shi et al., 2007b). Pourtant, le NGF est connu pour induire la survie de neurones dans plusieurs modèles *in vitro* et *in vivo* (Sofroniew et al., 2001). Quelques hypothèses peuvent expliquer l'incapacité du NGF à induire la neuroprotection dans la rétine. À la base de ces hypothèses, certains constats à mentionner. Premièrement, alors que ses deux récepteurs ($p75^{NTR}$/TrkA) sont habituellement co-exprimés dans le même type cellulaire, et que le rapport entre les deux récepteurs peut déterminer leur finalité (pro-survie vs pro-mort), nous avons montré que, dans la rétine adulte, le $p75^{NTR}$ est largement exprimé par les cellules de Müller tandis que TrkA est spécifiquement exprimé par les CGRs. Deuxièmement, bien que le TrkA soit sur-exprimé après section du nerf optique, laissant supposer un possible rôle à la suite d'une blessure, il est probable que le rapport entre $p75^{NTR}$ et TrkA reste largement en faveur de $p75^{NTR}$ dans la rétine adulte, bien que cette possibilité doive être testée autrement que par immunobuvardage et par immunohistochimie (Chapitre 2, Figure 1,5). De plus, il est important de noter que l'injection de NGF ne semble pas affecter la morphologie et la survie des cellules de Müller, la question étant légitime puisque le NGF peut induire la mort d'une autre

population de cellules gliales exprimant le $p75^{NTR}$, les oligodendrocytes (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Gu et al., 1999). Alors que d'autres neurotrophines comme le BDNF n'ont pas d'effet sur les oligodendrocytes, le NGF induit, de façon dépendante à sa liaison à $p75^{NTR}$, une augmentation de l'activité intracellulaire des céramides et de la *c-Jun amino-terminal kinase* (JNK), qui sont connus pour participer à la signalisation conduisant à la mort cellulaire (Casaccia-Bonnefil et al., 1996). Finalement, les cellules de Müller sont réactives après l'axotomie, mais n'entrent pas en apoptose (Scherer and Schnitzer, 1991; Huxlin et al., 1995).

À partir de ces observations, il est raisonnable de supposer que le NGF injecté puisse se lier principalement au $p75^{NTR}$ des cellules de Müller, ce qui masquerait son effet bénéfique sur les cellules ganglionnaires. Ainsi, une autre neurotrophine, le BDNF, est bien connue pour induire un effet neuroprotecteur sur les cellules ganglionnaires axotomixées (Mansour-Robaey et al., 1994a; Cheng et al., 2002a; Pernet and Di Polo, 2006a). Tout comme le NGF, le BDNF est un ligand de $p75^{NTR}$, mais contrairement à TrkA, son récepteur, TrkB, est fortement exprimé par les CGRs dans la rétine intacte (Cheng et al., 2002a). Il est également bien connu que la régulation à la baisse de TrkB dans les jours suivant la blessure est responsable de l'incapacité du BDNF à soutenir la survie des CGRs à long terme (Cheng et al., 2002a). Il est donc évident que la quantité de récepteurs disponibles à la surface cellulaire influence fortement la capacité des neurotrophines à induire la survie et que le niveau d'expression de TrkA ne favorise possiblement pas d'effet de survie par le NGF dans la rétine adulte axotomisée, même si son niveau est augmenté après l'axotomie. Une autre hypothèse à envisager pour expliquer l'incapacité du NGF à induire la survie serait qu'une autre voie de signalisation amorcée par l'activation de $p75^{NTR}$ sur les cellules de Müller vienne contrecarrer de façon indirecte l'effet bénéfique sur les CGRs, une possibilité sur laquelle nous reviendrons dans les prochains paragraphes.

5.2.2. L'activation spécifique de TrkA permet la neuroprotection.

L'utilisation de ligands peptidomimétiques nous a permis de décortiquer le rôle spécifique de chacun des récepteurs du NGF dans la rétine adulte. Alors que le NGF ne peut induire de la survie, l'activation spécifique de TrkA sur les CGRs par son agoniste

spécifique, le D3, est significativement neuroprotectrice. L'action ciblée du D3 a aussi démontré un effet protecteur sur les CGRs dans un modèle de glaucome *in vivo* (Shi et al., 2007b). Lorsque le $p75^{NTR}$ est inhibé sur les cellules de Müller, le NGF devient subitement efficace pour promouvoir la survie des CGRs. L'inhibition de $p75^{NTR}$ semble permettre de focaliser l'action du NGF sur les CGRs, démasquant ainsi son effet neuroprotecteur. Même si nous n'avons pas exploré les mécanismes moléculaires responsables de cette survie, nous avons montré que l'injection de D3, tout comme celle du NGF, entraîne la phosphorylation de Erk 1/2 (Chapitre 2, Figure 1). Il est fort probable que la survie engendrée par ces deux molécules passe par l'activation de la voie de Erk, cette voie ayant déjà été démontrée comme étant principalement impliquée dans la survie des CGRs axotomisées (Cheng et al., 2002a; Pernet et al., 2005b). Nous n'avons pas vérifié si l'injection du D3 et du NGF induit toujours la phosphorylation de Erk 1/2 après l'axotomie. Alors que l'activation de Erk 1/2 dans une rétine intacte semble comparable pour D3 et le NGF, nous pouvons supposer qu'après l'axotomie, un déséquilibre joue en faveur du D3, et que le NGF ne peut maintenir un niveau assez élevé pour permettre la survie. D'un autre côté, puisque l'injection de NGF peut induire l'activation de Erk tout comme celle du D3, il est possible que le NGF active une voie de signalisation via le $p75^{NTR}$ des cellules de Müller, ce qui annulerait son pouvoir neurotrophique sur les CGRs. Ainsi, nous avons montré qu'après l'axotomie, il y a une augmentation du niveau de proNGF, un ligand pro-apoptotique de $p75^{NTR}$, et l'étude qui a suivi a d'ailleurs démontré le lien entre le proNGF et la production par les cellules de Müller de TNFα, néfaste pour les CGRs. Nous reviendrons plus loin sur cette hypothèse. Sans démonter le mécanisme moléculaire en cause, notre étude indique qu'une activation ciblée d'un récepteur des neurotrophines sur les CGRs, ici TrkA, peut induire la survie de ces neurones.

5.2.3. L'inhibition de $p75^{NTR}$ sur des cellules gliales induit la survie neuronale.

De façon assez surprenante, dans notre modèle, l'inhibition de $p75^{NTR}$ ou l'absence de $p75^{NTR}$ sur les cellules de Müller suffit à elle seule pour induire la survie des CGRs, démontrant par le fait même une interaction neurone-glie fonctionnelle. Après la section

du nerf optique, la gliose des cellules de Müller est une réaction qui avait déjà été décrite, ces cellules sur-exprimant le GFAP (Lewis and Fisher, 2003). Par contre, le rôle joué par ces cellules gliales était resté obscur. Nous montrons, pour la première fois, qu'une signalisation initiée dans les cellules de Müller par le récepteur $p75^{NTR}$ peut, de façon indirecte, venir exacerber la mort des CGRs axotomisées, et que l'inhibition de $p75^{NTR}$ permet de prévenir ce phénomène. Ceci suggère que les facteurs de croissance endogènes peuvent lier les récepteurs Trks et induire la survie des CGRs lorsque $p75^{NTR}$ est bloqué. Cependant, une autre hypothèse est évoquée par Harada et ses collaborateurs, qui ont démontré que l'inhibition de $p75^{NTR}$ sur les cellules de Müller prévenait l'apoptose des photorécepteurs durant la dégénérescence provoquée par la lumière, en raison d'une augmentation de la synthèse du facteur de survie des photorécepteurs, le *basic fibroblast growth factor* (bFGF) (Harada et al., 2000b). Dans cette étude, l'action du NGF sur les cellules de Müller semble diminuer la production de bFGF, lequel est impliqué dans la survie des photorécepteurs. De plus, l'absence de $p75^{NTR}$ prévient de façon significative la mort des photorécepteurs induite par la lumière, tout comme nous l'avons montré dans notre modèle de mort des CGRs. Le bFGF, aussi connu comme étant le FGF-2, permet de soutenir de façon très limitée la survie des CGRs, tout comme la plupart des membres de cette famille (Sapieha et al., 2003b). Cependant, il est tout-à-fais envisageable qu'un mécanisme similaire intervienne dans notre modèle, où l'action du NGF sur le $p75^{NTR}$ pourrait inhiber la production d'un facteur neurotrophique capable de réguler la survie des CGRs axotomisées.

Une expérience toute simple nous a cependant permis d'en arriver à une tout autre hypothèse explicative. Nous nous sommes intéressés à la production endogène du NGF et de sa forme précurseur, le proNGF. Nous avons ainsi démontré que, contrairement au NGF, le niveau de proNGF semble augmenter par suite de l'axotomie. Comme le proNGF lie le $p75^{NTR}$ et non les récepteurs Trks et qu'il semble être un ligand pro-apoptotique (Lee et al., 2001), il est fort probable que la survie suivant l'inhibition de $p75^{NTR}$ passe par l'inhibition de la signalisation du proNGF dans les cellules de Müller. À l'appui de cette hypothèse, plusieurs groupes de recherche ont démontré un déséquilibre à la hausse du niveau de proNGF autant dans des cerveaux âgés (Fahnestock et al., 2001) que des cas de maladie d'Alzheimer (Peng et al., 2004; Pedraza et al., 2005). De plus,

plusieurs études ont démontré une capacité des neurones corticaux (Bruno and Cuello, 2006), des neurones sympathiques (Hasan et al., 2003), de la microglie (Srinivasan et al., 2004a) et des astrocytes (Domeniconi et al., 2007) à sécréter le proNGF. Des données non publiées nous laissent croire que cette production de proNGF provient d'un sous-type de cellules amacrines exprimant le transporteur-1 de la glycine. Dans un modèle expérimental de glaucome, où la survie est mesurée ± 21 jours après le traitement comparativement à 1 et 2 semaines dans notre modèle, l'inhibition de $p75^{NTR}$ n'est pas en mesure d'induire de neuroprotection des CGRs (Shi et al., 2007b). Dans ce modèle, la mort des cellules ganglionnaires est plus progressive, et il est probable que l'inhibition de $p75^{NTR}$ puisse transitoirement bloquer l'action de protéines telles que le proNGF, mais que l'activation de TrkA soit ultimement requise pour soutenir la neuroprotection.

5.2.4. L'inhibition de $p75^{NTR}$ et l'activation de TrkA, un effet synergique.

La co-injection du D3 avec l'inhibiteur du $p75^{NTR}$ a permis de révéler un effet synergique des deux actions (Chapitre 2, Figure 7). Notre étude a montré, pour la première fois, qu'une régulation ciblée des récepteurs des neurotrophines, à la fois sur les cellules gliales et sur les neurones, pouvait augmenter l'effet neuroprotecteur. Ces données démontrent une mort cellulaire non-autonome des CGRs axotomisées, qui implique les cellules de Müller, en plus des mécanismes autonomes de mort classique préalablement décrits (Garcia-Valenzuela et al., 1995a). Effectivement, la section du nerf optique entraîne la production de proNGF, qui semble exacerber la mort des CGRs de façon indirecte, en agissant sur le récepteur $p75^{NTR}$ des cellules de Müller. L'hypothèse de la mort causée par le proNGF dans la rétine adulte a servi de prémice au second projet de cette thèse, qui vient fournir d'autres données à l'appui de cette théorie.

5.3. Le proNGF induit la mort des cellules ganglionnaires rétiniennes en agissant sur les cellules de Müller.

Notre première étude a démontré, qu'après axotomie du nerf optique, il y a une régulation à la hausse de la production de proNGF. Cependant, une question restait en suspens, à s'avoir si le proNGF pouvait induire la mort des neurones rétiniens *in vivo* ? Bien que la réputation du proNGF le relègue la plupart du temps du côté des mauvais

garçons, bien peu d'études ont démontré de façon définitive sa capacité d'induire la mort neuronale *in vivo*, d'où une certaine controverse sur ses propriétés neurotoxiques. Une étude a même décrit une capacité neurotrophique du proNGF dans des cellules en culture (Fahnestock et al., 2004). En injectant du proNGF dans l'humeur vitrée de rats et de souris, nous avons démontré de façon simple et catégorique que le proNGF peut induire la mort neuronale *in vivo*. Dans le même sens, une autre équipe a montré, que par suite d'une blessure des neurones corticospinaux, il y a production de proNGF, et que l'inhibition ou l'absence de $p75^{NTR}$ sur ces neurones induit leur survie chez l'adulte (Harrington et al., 2004b). Grâce à la technique d'immunoprécipitation, cette étude démontre clairement que le proNGF peut lier le $p75^{NTR}$ *in vivo,* et conclut qu'en situation pathologique le proNGF est un ligand physiologique qui induit la mort neuronale dans le cerveau lésé.

En cherchant à mieux comprendre les acteurs impliqués dans la signalisation du proNGF dans la rétine adulte *in vivo*. Nous avons constaté, en confirmant par microscopie confocale, que le patron d'expression du $p75^{NTR}$ était limité aux cellules de Müller chez la souris et le rat, et que l'inhibition ou l'absence de $p75^{NTR}$ bloquait la mort induite par le proNGF. Dès lors, une hypothèse majeure s'imposait : celle d'une mort cellulaire non-autonome des CGRs axotomisées induite par le proNGF. Le rôle du récepteur $p75^{NTR}$ comme récepteur de mort est bien établi, puisqu'il a été démontré que ce récepteur participait à la mort neuronale développementale des neurones sympathiques et des neurones rétiniens (Majdan et al., 1997b; Frade and Barde, 1999b; Harada et al., 2006). Le $p75^{NTR}$ est aussi connu pour favoriser l'apoptose de neurones corticaux, de l'hippocampe, d'oligodendrocytes et des photorécepteurs en plus d'être impliqué dans plusieurs lésions affectant le SNC (Roux and Barker, 2002b). De façon générale, le rôle fonctionnel de $p75^{NTR}$ dans la mort neuronale est mieux compris dans les neurones qui expriment ce récepteur de façon endogène. Cependant, dans notre étude, nous avons identifié un nouveau mécanisme par lequel le $p75^{NTR}$ des cellules gliales peut grandement influencer la mort neuronale. En se basant sur les informations connues au niveau neuronal, nous avons testé les différents partenaires possibles du $p75^{NTR}$ dans la signalisation du proNGF.

5.3.1. Le p75^{NTR}, la sortiline et NRAGE sont essentiels pour induire la mort neuronale dépendante du proNGF.

Nykjaer et ses collaborateurs ont récemment montré que la co-expression de la sortiline avec le récepteur p75^{NTR} est nécessaire à l'induction de l'apoptose par le pro-NGF (Nykjaer et al., 2004a). De façon similaire, le pro-BDNF induit aussi l'apoptose des neurones des ganglions cervicaux supérieurs en culture, grâce à la formation d'un complexe entre le récepteur p75^{NTR} et la sortiline (Teng et al., 2005). Dans le même sens, l'expression de la sortiline ayant récemment été constatée dans les cellules de Müller (Xu et al., 2009b), nous avons confirmé l'implication de la sortiline dans notre modèle. En fait, la présence à la fois de p75^{NTR} et de sortiline est essentielle, puisque les souris *knockout* pour chacun de ces gènes ont montré une résistance à l'injection du proNGF. Comment la sortiline affecte-t-elle la signalisation des pro-neurotrophines dans les neurones? La question est toujours d'actualité, mais quelques faits suggèrent qu'elle puisse réguler la reconnaissance des pro-neurotrophines par le p75^{NTR}; la sortiline lierait la portion immature et présenterait la portion mature au p75^{NTR} (Willnow et al., 2008). De plus, d'autres mécanismes sont probablement facilités par la présence de la sortiline. Plusieurs signaux secondaires à l'activation de p75^{NTR} sont issus du complexe ligand-récepteur à la surface cellulaire. D'autres voies de signalisation dépendantes de p75^{NTR} nécessitent l'internalisation du récepteur dans des vésicules à clathrine et le transport entre les endosomes précoces et les endosomes de recyclage, à partir desquels des signaux peuvent être émis (Bronfman et al., 2003). Comme la sortiline a d'abord été connue pour son rôle dans le transport vésiculaire, il est fort probable qu'elle puisse jouer un rôle dans l'internalisation et le trafic de p75^{NTR}.

Quelles voies de signalisation découlent de l'activation du complexe p75^{NTR}-sortiline? Les réponses restent obscures. Cependant, quelques protéines sont connues pour induire une apoptose p75^{NTR}-dépendante. La protéine adaptatrice NRAGE est l'une d'elles et participe à l'apoptose développementale; les souris *knockout* pour cette protéine démontrent des propriétés similaires à celles des *knockout* pour p75^{NTR} (Bertrand et al., 2008b). Par exemple, tout comme les *knockout* pour p75^{NTR}, les souris *knockout* pour NRAGE présentait une résistance à l'apoptose développementale des neurones sympathiques du ganglion cervical supérieur (Bertrand et al., 2008b). Dans notre modèle,

les souris *knockout* pour NRAGE présentait la même résistance que les souris *knockout* pour le $p75^{NTR}$ et la sortiline face au proNGF, révélant pour la première fois une implication de NRAGE dans la mort dépendante du proNGF. La voie de signalisation en cause reste à démontrer. La transfection de NRAGE dans des cellules qui ne l'expriment pas de façon constitutive induit une augmentation de l'apoptose $p75^{NTR}$-dépendante (Salehi et al., 2000). Cette augmentation apparaît dûe à l'activation de la JNK, conduisant à une augmentation de l'activation de la voie des caspases (Salehi et al., 2002). De plus, après activation par le récepteur $p75^{NTR}$, NRAGE induit une augmentation de l'expression et de l'activation de la protéine p53 dans les cellules PC12 (Salehi et al., 2000; Wen et al., 2004). Puisque nos données subséquentes ont démontré qu'il y a production de TNFα, il serait intéressant d'examiner si p53 ou JNK pourraientt être impliqués dans la signalisation reliant le recrutement de NRAGE à la production subséquente de TNFα. La production et l'implication du TNFα dans ce modèle seront discutées dans le prochain paragraphe.

5.3.2. Une mort cellulaire non-autonome impliquant le TNFα.

Palmada et ses collaborateurs ont démontré que l'ajout de cycloheximide, un inhibiteur de synthèse protéique, empêche la mort dépendante de $p75^{NTR}$ des neurones sympathiques, ce qui suggère qu'une synthèse protéique pourrait être nécessaire à celle-ci. On peut aussi envisager un rôle de la transcription dans la signalisation de $p75^{NTR}$ puisque plusieurs protéines qui s'associent au domaine intracellulaire de $p75^{NTR}$ sont retrouvées dans le noyau, où elles sont fortement suspectées de réguler la transcription. Effectivement, sur la surface cellulaire comme sur les endosomes, le $p75^{NTR}$ peut subir une protéolyse intramembranaire par la γ-sécrétase. Dans des cultures de neurones sympathiques, le proBDNF induit le clivage de $p75^{NTR}$, ce qui permet la relâche du domaine intracellulaire (ICD) du récepteur (Kenchappa et al., 2006). Ce domaine peut lier différentes protéines telles que NRIF, et provoquer sa translocation au noyau, où cette protéine qui lie l'ADN a été identifiée comme étant impliquée dans l'apoptose-dépendante de $p75^{NTR}$ (Casademunt et al., 1999; Linggi et al., 2005a). Au cours de notre étude, nous n'avons pas testé l'implication de NRIF, bien qu'il soit intéressant de savoir si elle est impliquée. Même si NRAGE n'est elle-même pas connue comme protéine

pouvant être transloquée, il est probable qu'une signalisation en aval de NRAGE puisse influencer la transcription.

Le proNGF induit une mort cellulaire non-autonome des CGRs. En partant de l'hypothèse que la transcription dans les cellules de Müller induite par la signalisation de $p75^{NTR}$ peut aboutir à la production d'un facteur pouvant influencer la survie des CGRs, nous avons identifié le TNFα comme un candidat potentiel. Le TNFα est pro-apoptotique en aval de $p75^{NTR}$ dans la mesure où $p75^{NTR}$ active NF-κB, lequel peut induire la production de TNFα (Shakhov et al., 1990 ; Hiscott et al., 1993b; Mori and Prager, 1996). En effet, les cellules de Müller activées peuvent avoir un effet cytotoxique en sécrétant des facteurs solubles comme la cytokine pro-inflammatoire TNFα (Tezel and Wax, 2000a; Yuan and Neufeld, 2000). De plus, il a été décrit que l'ajout exogène de TNFα induit la mort des CGRs (Tezel and Wax, 2000a). Nous avons démontré de façon pharmacologique et génétique que l'inhibition du TNFα réduit considérablement la mort des CGRs, révélant ainsi un rôle crucial du TNFα dans la perte neuronale causée par le proNGF. La capacité de NRAGE d'influencer la transcription n'est toujours qu'une hypothèse qui reste à vérifier. Cependant, le facteur de transcription NF-κB fait partie de la signalisation de $p75^{NTR}$ et peut induire la production de TNFα (Shakhov et al., 1990 ; Hiscott et al., 1993b). De plus, dans notre troisième projet, nous avons démontré que le NF-κB est responsable de la production de TNFα lors de l'excitotoxicité causée par le NMDA (Chapitre 4, Figure 5). Par contre, des résultats plutôt mitigés non publiés n'ont pas permis, jusqu'à maintenant, d'établir l'implication du facteur de transcription dans ce modèle. L'utilisation des souris transgéniques rapporteuses de l'activation de NF-κB (Chapitre 4, Figure 3) ont démontré une activation locale de NF-kB daprès du site d'injection, dans la partie supérieure de la rétine ; par contre la co-injection d'inhibiteurs de NF-κB (SN50, PS1145) avec les proNGF n'a pas été en mesure d'induire de survie des CGRs. De plus amples études seront donc nécessaires pour confirmer notre hypothèse.

La voie classique d'activation de la mort neuronale par le TNFα implique la liaison de la cytokine à son récepteur TNFR1, qui induit ensuite l'apoptose par clivage de la pro-caspase-8 en caspase-8 (Thorburn, 2004b). Cependant, des résultats non publiés ont démontré que la co-injection d'un inhibiteur de la caspase-8 et du proNGF n'a pas

d'effet sur la survie des CGRs. Nous nous sommes donc tournés vers d'autres hypothèses. Une série d'expériences *in vitro* menée par le groupe du Dr. Tezel a révélé qu'en plus de l'activation des caspases, un dysfonctionnement mitochondrial accompagne la mort des CGRs induite par le TNFα (Tezel et al., 2004a). Cette étude, tout comme la nôtre, démontre que l'inhibition de l'activité des caspases n'est pas suffisante pour bloquer la mort des CGRs dans des cultures primaires exposées au TNFα. Ces auteurs ont aussi démontré que les CGRs exposées au TNFα accumulent des composés réactifs à l'oxygène et qu'un traitement chélateur de radicaux libres combiné avec l'inhibition des caspases permet d'augmenter de façon substantielle la survie des CGRs (Tezel et al., 2004a). De tels résultats laissent croire qu'en plus de la cascade d'activation des caspases induite par le récepteur TNFR1, la mort des CGRs par le TNFα implique aussi une composante caspase-indépendante via la cascade de signalisation des mitochondries et du stress oxydatif. Cette hypothèse pourrait être testée dans notre modèle, pour expliquer pourquoi notre inhibiteur de la caspase-8 n'a pas donné de résultats probants. Par exemple, un traitement avec un chélateur de radicaux libres comme le TEMPOL (4-hydroxytetramethylpiperidine-1-oxyl) pourrait être co-injecté avec le proNGF. De plus, le dysfonctionnement des mitochondries pouvant éventuellement mener à l'activation d'autre caspases comme la caspase-3, il serait intéressant de tester un inhibiteur de cette enzyme dans notre modèle.

Finalement, les conclusions de notre troisième article permettent de fonder une autre hypothèse. Dans notre modèle d'excitotoxicité causé par le NMDA, le TNFα sécrété par les cellules de Müller est également responsable de la mort des CGRs. Dans ce modèle, le TNFα induit la relocalisation de récepteurs AMPA à la surface cellulaire. Ces récepteurs n'ayant pas la sous unité GLUR2 pourraient exacerber la perte des neurones par augmentation de l'entrée calcique. On peut imaginer que l'entrée de Ca^{2+} et de Zn^{2+}, potentiellement toxique pour les neurones, puisse conduire à leur mort. Cette hypothèse pourrait être vérifiée en reproduisant l'expérience d'insertion de cobalt utilisée dans le chapitre 4 (Figure 6). Nous reviendrons plus en détail sur ce mécanisme de relocalisation de récepteur AMPA dépendant du TNFα, mais, grâce à l'utilisation d' inhibiteurs des récepteurs AMPA (Chapitre 4, Figure 6), il serait intéressant de vérifier si

l'entrée excessive de calcium ou de Zn^{2+} dans les CGRs via ces récepteurs AMPA pourrait aussi faire partie de l'explication dans ce modèle.

5.4. Les cellules gliales de Müller induisent la mort des neurones rétiniens lors de dommages excitotoxiques causés par le NMDA.

Dans notre dernière étude, nous avons démontré une fois encore le rôle joué par les cellules de Müller dans la mort des CGRs, cette fois lors de dommages excitotoxiques causés par le NMDA. Cette étude justifie quatre conclusions majeures. Premièrement, que l'exposition de NMDA dans la rétine adulte cause une importante activation du NF-κB dans les cellules de Müller, et non dans les neurones. Deuxièmement, que la mort des cellules amacrines et des CGRs est nettement atténuée lorsque l'activation du NF-κB dans les cellules de Müller est bloquée. Troisièmement, que le NF-κB induit une forte production de TNFα par les cellules de Müller, cette production de TNFα étant requise pour induire la perte neuronale. Finalement, que l'augmentation du niveau de récepteurs AMPA perméables au calcium provoquée par le TNFα dérivé des cellules de Müller est le mécanisme prédominant qui rend les neurones rétiniens encore plus sensibles à l'excitotoxicité et accélére la mort neuronale. Cet article forme un ensemble assez complet en raison de la complexité des mécanismes qu'il évoque, je me permettrai d'insister sur quelques uns de ses résultats.

5.4.1. Une mort neuronale dépendante des récepteurs NMDA.

L'activation de NF-κB dans les cellules de Müller par le NMDA est largement bloquée par le MK-801, lequel induit par le fait même une forte survie des CGRs, ce qui démontre le rôle des récepteurs NMDA dans cette réponse. Les cellules ganglionnaires expriment elles-mêmes les récepteurs NMDA (Brandstätter et al., 1994; Watanabe et al., 1994; Gründer et al., 2000), mais nous allons revenir sur ce sujet dans la section suivante. Par contre, une question demeure : les cellules de Müller expriment-elles des récepteurs NMDA? La question semble simple au premier abord, mais, comme d'autres, nous nous y sommes bûtés. Nous avons déjà mentionné que plusieurs autres ont démontré la présence de ces récepteurs dans des cultures de cellules de Müller dissociées de l'humain ou du poulet (Puro et al., 1996b; Lamas et al., 2005a). Cependant, chez les rongeurs le

mystère persiste. En effet, les résultats négatifs obtenus par immunohistochimie ou par des techniques d'électrophysiologie ne permettent pas de se prononcer. Des récepteurs NMDA fonctionnels ont déjà été décrits dans la microglie du SNC, les astrocytes et les oligodendrocytes (Conti et al., 1996; Conti et al., 1999; Karadottir et al., 2005). Cependant, les récepteurs NMDA de la glie et des neurones sont fonctionnellement et structurellement différents. Par exemple, dans les cellules gliales de Bergmann, le NMDA induit un courant mesurable, mais insensible à la glycine, alors que ces cellules restent imperméables au Ca^{2+} (Müller et al., 1993). Plusieurs autres caractéristiques particulières ont été identifiées dans différentes populations gliales, mais celle-ci revient pour tous les récepteurs NMDA de la glie est d'être très peu sensibles, voir même insensibles au blocage par le Mg^{2+} extracellulaire (Ziak et al., 1998; Karadottir et al., 2005; Lalo et al., 2006). Cette caractéristique n'est pas surprenante puisque les cellules gliales ne peuvent produire de dépolarisation suffisante pour se libérer du blocage par le magnésium. En fait, l'absence de blocage par le Mg^{2+} accentue la sensibilité des cellules gliales au NMDA (Ziak et al., 1998; Karadottir et al., 2005; Lalo et al., 2006). Ces cellules peuvent réagir immédiatement au NMDA à leur potentiel membranaire de repos, comparativement aux neurones qui doivent être préalablement dépolarisés pour lever l'inhibition par le Mg^{2+}. On peut imaginer que dans notre modèle, où la stimulation par le NMDA est massive et rapide, l'activation des récepteurs NMDA de la glie soit la première initiée et que cette réponse puisse masquer en partie l'activation subséquente des récepteurs NMDA des neurones. Ceci n'est qu'une hypothèse, mais qui exige une démonstration de la présence de récepteurs NMDA sur les cellules de Müller. D'ici là, une expérience d'imagerie calcique pourrait être menée, afin de vérifier si l'injection de NMDA est suivie d'une augmentation de la concentration intracellulaire de calcium dans les cellules de Müller.

5.4.1.1. Les récepteurs NMDA sur les cellules ganglionnaires et l'excitotoxicité.

Deux possibilités découlent du fait que les CGRs et les cellules amacrines exprimant des récepteurs NMDA (Brandstätter et al., 1994; Watanabe et al., 1994; Gründer et al., 2000; Matsui et al., 2001). Premièrement, il est fort probable que

l'activation directe de ces récepteurs sur les CGRs et les cellules amacrines joue un rôle dans la mort de ces neurones, même si celui-ci semble modeste selon nos observations. Deuxièmement, on peut supposer que l'activation des récepteurs NMDA sur les CGRs et amacrines induise de façon indirecte l'activation du NF-κB dans les cellules de Müller. Cette deuxième hypothèse pourrait être testée *in vitro* en vérifiant l'activation du NF-κB dans des cellules rMC-1 (Sarthy et al., 1998), une lignée de cellules de Müller de rat, que l'on mettrait en présence de CGRs purifiées préalablement traitées avec le NMDA. Si tel était le cas, il serait raisonnable de croire qu'un produit d'activation des récepteurs NMDA comme le NO (Dawson et al., 1991; Girouard et al., 2009), connu pour pouvoir induire l'activation du NF-κB (von Knethen et al., 1999), puisse diffuser vers les cellules de Müller. Pour revenir à la première hypothèse, de nombreuses études ont rapporté une vulnérabilité des CGRs face à l'excitotoxicité du glutamate et du NMDA *in vivo*, *in vitro* et *ex vivo* (Mosinger et al., 1991; Izumi et al., 1995; Sucher et al., 1997). Pourquoi donc l'activation des récepteurs NMDA sur les CGRs par le NMDA ne serait-elle pas la cause principale de leur mort excitotoxique dans notre modèle? La question est légitime et la réponse reste de façon très humblement à mes yeux un peu obscure. Comme nous l'avons déjà mentionné, la réponse rapide des récepteurs NMDA de la glie masque peut-être la réponse neuronale, mais ceci n'est qu'une hypothèse. Il y a quelques années le groupe du Dr. B.A. Barres a démontré que des CGRs de rat purifiées en culture sont résistantes à de fortes concentrations de NMDA et de glutamate, bien qu'elles expriment des récepteurs NMDA fonctionnels (Ullian et al., 2004). Dans les mêmes conditions, les cellules amacrines et les neurones de l'hippocampe meurent très rapidement par excitotoxicité (Ullian et al., 2004). Ces auteurs en concluent que le NMDA cause une mort rapide des cellules amacrines, laquelle entraîne une mort subséquente des CGRs sans nécessairement impliquer l'excitotoxicité. Les conclusions de cet article sont très controversées. Premièrement, la mort neuronale est constatée uniquement après une heure d'exposition au NMDA, ce qui ne donne pas un intervalle de temps suffisant pour justifier les conclusions. D'autant plus, la méthode de mesure de la mort neuronale est uniquement basée sur une analyse morphologique des cellules et des noyaux par microscopie, alors qu'un changement morphologique visuellement évident ne pourrait apparaître qu'après un temps d'exposition plus long. De plus, la contribution des cellules

gliales est adressée très sommairement et seulement avec des astrocytes, et ce, sans montrer les résultats. Il n'en serait pas moins intéressant de vérifier l'effet du MK-801 sur la survie des cellules amacrines dans notre étude. Il se pourrait que l'effet neuroprotecteur soit plus grand sur ces cellules que celui que nous avons observé sur les CGRs (Chapitre 4, Figure 2). Par contre, l'inhibition du NF-κB dans les cellules de Müller supportant leur survie, il est peut probable que, contrairement aux CGRs, les cellules amacrines soit davantage affectées de façon directe par le NMDA. Nos résultats favoriserait plutôt le concept que les CGRs et les cellules amacrines ne soient pas invulnérables au NMDA, mais que la mort excitotoxique qui les affecte passe principalement par une activation robuste du NF-κB dans les cellules de Müller.

5.4.2. Une mort neuronale dépendante de l'activation de NF-κB dans les cellules de Müller.

Le NF-κB, présent à la fois dans les neurones et la glie (Kaltschmidt et al., 2005), est un régulateur à double tranchant favorisant une réponse protectrice ou dégénérative en fonction de la situation et du type cellulaire. Sa capacité d'engendrer une réponse pro-apoptotique a été démontrée dans plusieurs expériences *in vivo* et *in vitro* (Perkins and Gilmore, 2006). Une théorie assez bien acceptée est que toute perturbation du niveau physiologique d'activation de NF-κB peut entraîner un état pathologique où l'action du NF-κB devient problématique (Kaltschmidt et al., 2005). Dans notre cas, l'injection de NMDA engendre une activation robuste et très rapide du NF-κB (Chapitre 4, Figure 3), probablement équivalente à une situation pathologique pour les cellules de Müller. Plusieurs études ont démontré une activation du NF-κB par les récepteurs NMDA (Kaltschmidt et al., 1995; Shen et al., 2002; Schölzke et al., 2003), mais peu d'entre elles ont tenté d'identifier la cascade de signalisation en cause. Cependant, le groupe du Dr. Schwaninger a rapporté quelques observations pertinantes. Premièrement, dans des cultures primaires de neurones, le glutamate induit, de façon dépendante des récepteurs NMDA, une diminution rapide du niveau de IκB et la translocation de p65 au noyau (Schölzke et al., 2003). Deuxièmement, l'inhibition de la protéase calpaine, dépendante du Ca^{2+}, bloque complètement la dégradation de IκB induite par l'activation des récepteurs NMDA, ce qui réduit la translocation de p65 au noyau (Schölzke et al., 2003).

L'entrée de Ca^{2+} provoquée par l'activation des récepteurs NMDA active la calpaine qui vient dégrader la protéine inhibitrice du NF-κB, un phénomène qui pourrait être testé dans notre modèle en utilisant la calpeptine, un inhibiteur de la calpaine. Une fois activé, le NF-κB induit la transcription de plusieurs gènes dont le TNFα.

5.4.3. Le TNFα produit par les cellules gliales induit la mort des cellules ganglionnaires dans un modèle d'excitotoxicité.

Plusieurs données suggèrent que le TNFα pourrait jouer un rôle dans des maladies oculaires tel que le glaucome (Lin et al.; Tezel and Carlo Nucci, 2008), mais la signalisation activée par le TNFα qui conduit à la mort des CGRs reste jusqu'à maintenant obscure. Comme dans le modèle de mort induite par le proNGF, la mort induite par le TNFα dans cette maladie ne semble pas passer par l'activation de la caspase-8 puisque la co-injection d'un inhibiteur de cette caspase avec le NMDA ne protège pas les CGRs (Chapitre 4, Figure supplémentaire 2). Pourtant, nous avons clairement démontré par la technique de TUNEL que des mécanismes apoptotiques sont en cause dans ce modèle d'excitotoxicité. Comme nous l'avons mentionné plus haut, il est fort probable que la signalisation du TNFα comporte une partie caspase-indépendante activant plutôt la voie mitochondriale de mort cellulaire. Cette voie vient ultimement activer la caspase-3 qui propulse les neurones dans le cycle apoptotique. Il n'est donc pas surprenant d'observer des neurones positifs par la technique de TUNEL sans avoir détecté aucun effet neuroprotecteur de l'inhibiteur de la caspase-8. De plus, nous avons montré que les récepteurs AMPA sont largement responsables de la mort des CGRs, même si, les inhibiteurs des récepteurs AMPA n'induisent pas une survie complète à 1 semaine après la blessure (~75%). Cette voie alternative pourrait aussi être responsable, dans une certaine mesure, de la mort des CGRs.

La principale nouveauté de notre étude a été de démontrer un mécanisme alternatif de mort excitotoxique induite par le NMDA, impliquant une augmentation de récepteurs AMPA à la surface neuronale, elle-même causée par la production de TNFα dans les cellules gliales. En fait, il semble bien qu'il s'agisse d'une relocalisation des récepteurs à la surface et non une sur-production puisqu'une expérience d'immunobuvardage non publiée a confirmé qu'il n'y avait pas de changement des

différentes sous-unités des récepteurs AMPA entre la rétine témoin et la rétine traitée au NMDA. Par contre, l'insertion de cobalt par les récepteurs AMPA est nettement supérieure dans la rétine traitée au NMDA. Il y a quelques années, le TNFα a été identifié comme un facteur relâché par les cellules gliales et capable, entre autres, d'augmenter l'expression des récepteurs AMPA à la surface cellulaire (Beattie et al., 2002a). Par la suite, les études du Dr. Stellwagen, notre collaborateur dans cette étude, ont démontré que le TNFα agissait par son récepteur TNFR1 sur les neurones pour induire l'exocytose préférentielle des récepteurs AMPA dépourvus de la sous-unité GLUR2. En fait, la sous-unité GLUR2 a une argine positivement chargée orientée vers le pore du récepteur qui empêche la perméabilité au calcium (Kwak and Weiss, 2006a). Le Dr. Stellwagen a aussi démontré que ce processus est dépendant de l'activation de la PI3-Kinase. Il serait donc intéressant de vérifier l'implication de cette enzyme dans notre modèle en co-injectant un inhibiteur de la PI3-Kinase tel que le LY294002 et le NMDA, et d'utiliser la technique d'insertion de cobalt (Chapitre 4, Figure 6) pour établir la présence de récepteurs AMPA. Comme nous l'avons mentionné, ces récepteurs AMPA sont responsables de l'exacerbation de l'excitotoxicité en laissant entrer le Ca^{2+} (Kwak and Weiss, 2006a). L'augmentation de calcium peut induire la mort neuronale de bien des manières, comme l'activation excessive d'enzymes, et entraîner des réactions mitochondriales amenant à la relâche par les mitochondries de facteurs pro-apoptotiques (Lipton, 2006). Ces réactions sont déjà bien connues. Cependant, en plus du calcium, les récepteurs AMPA sans la sous-unité GLUR2 peuvent aussi servir d'entrée pour le Zn^{2+}, qui peut être relâché avec le glutamate par certaines synapses (Kwak and Weiss, 2006a). Ces récepteurs sont en fait fortement perméables au Zn^{2+} (Kwak and Weiss, 2006a) qui s'avère aussi toxique pour les neurones. Comme le Ca^{2+}, le Zn^{2+} semble induire des dommages par plusieurs mécanismes incluant l'activation d'enzymes et la production de composés réactifs à l'oxygène (Kim and Koh, 2002a). Il pourrait même être plus efficace que le calcium pour entraîner des dysfonctionnements mitochondriaux (Kwak and Weiss, 2006a). Finalement, les récepteurs AMPA n'ayant pas la sous-unité GLUR2 sont tout désignés pour induire des effets excitotoxiques. Contrairement, aux récepteurs NMDA, ils ne sont pas sujets à un blocage voltage-dépendant par le Mg^{2+} et ne nécessitent pas de forte dépolarisation synaptique. De plus, la forte perméabilité au Zn^{2+} ne s'applique pas aux récepteurs

NMDA, qui sont bloqués par le Zn^{2+} (Smart et al., 2004). Nos données révèlent donc une nouvelle façon d'approcher les différents traitements contre l'excitotoxicité dans les différentes maladies neurodégénératives où l'implication des cellules gliales devra être prise en compte. De plus, alors que les récepteurs AMPA étaient quelque peu laissés pour compte dans les approches contre les dommages excitotoxiques, nous avons démontré qu'ils pouvaient jouer un rôle primordial.

5.5. Conclusion générale

Alors que des études antérieures avaient démontré une incapacité du NGF à induire la survie des CGRs, nous avons découvert que l'inhibition du récepteur $p75^{NTR}$ sur les cellules de Müller est suffisante pour rétablir de façon convaincante le pourvoir neuroprotecteur du NGF sur les CGRs axotomisées. Cette première étude a démontré que la signalisation de $p75^{NTR}$ dans les cellules gliales de Müller pouvait être néfaste pour les neurones rétiniens en agissant de manière indirecte sur leur survie. De plus, nos données ont montré que l'action ciblée des différents récepteurs des neurotrophines à la fois sur les neurones et les cellules gliales pouvait avoir un effet synergique sur la neuroprotection (Fig.14A). Cette première étude a révélé une production de proNGF par suite d'une blessure du nerf optique. Notre deuxième projet a poursuivi dans cette veine en explorant l'hypothèse que le proNGF puisse être impliqué dans la mort des neurones rétiniens lors de conditions pathologiques. Nous avons démontré que la mort induite par le proNGF dans la rétine adulte passait par l'activation du récepteur $p75^{NTR}$ et la production de TNFα par les cellules gliales de Müller (Fig.14B). Les conclusions de ces deux études soulèvent la possibilité que des mécanismes cellulaires non-autonomes soient une caractéristique générale de l'apoptose cellulaire dépendante de $p75^{NTR}$ *in vivo*.

Les cellules de Müller sont grandement responsables de l'homéostasie des neurones rétiniens. Elles exécutent un recyclage actif des neurotransmetteurs tout en exprimant leurs récepteurs. Pourtant, peu d'études ont tenté d'établir leur rôle dans les mécanismes excitotoxiques affectant les CGRs. Pour cette raison, nous avons cherché à savoir si les cellules de Müller étaient impliquées dans la mort des CGRs causée par l'injection intravitréenne de NMDA. Cette étude a révélé que la mort par excitotoxicité des neurones rétiniens se produit en raison d'une signalisation provenant des cellules de

Müller. Nous avons montré que, par suite de l'injection de NMDA, il y a production de TNFα par les cellules de Müller de façon dépendante de l'activation du NF-kB. Finalement, nous avons montré que le TNFα produit par la glie induit la mort des neurones par l'augmentation des récepteurs AMPA perméables au Ca^{2+} à la surface cellulaire (Fig. 14C).

Dans cette thèse, nous avons décrit trois modèles de mort des cellules ganglionnaires incriminant les cellules gliales de Müller. Alors que les cellules de Müller sont plutôt connues pour maintenir la survie des neurones rétiniens, nos résultats ont démontré qu'en situation pathologique les cellules de Müller peuvent venir exacerber les mécanismes neurodégénératifs. En conclusion, nos données démontrent que les réactions gliales face aux blessures représentent un enjeu majeur dans l'établissement de thérapies pour les maladies neurodégénératives.

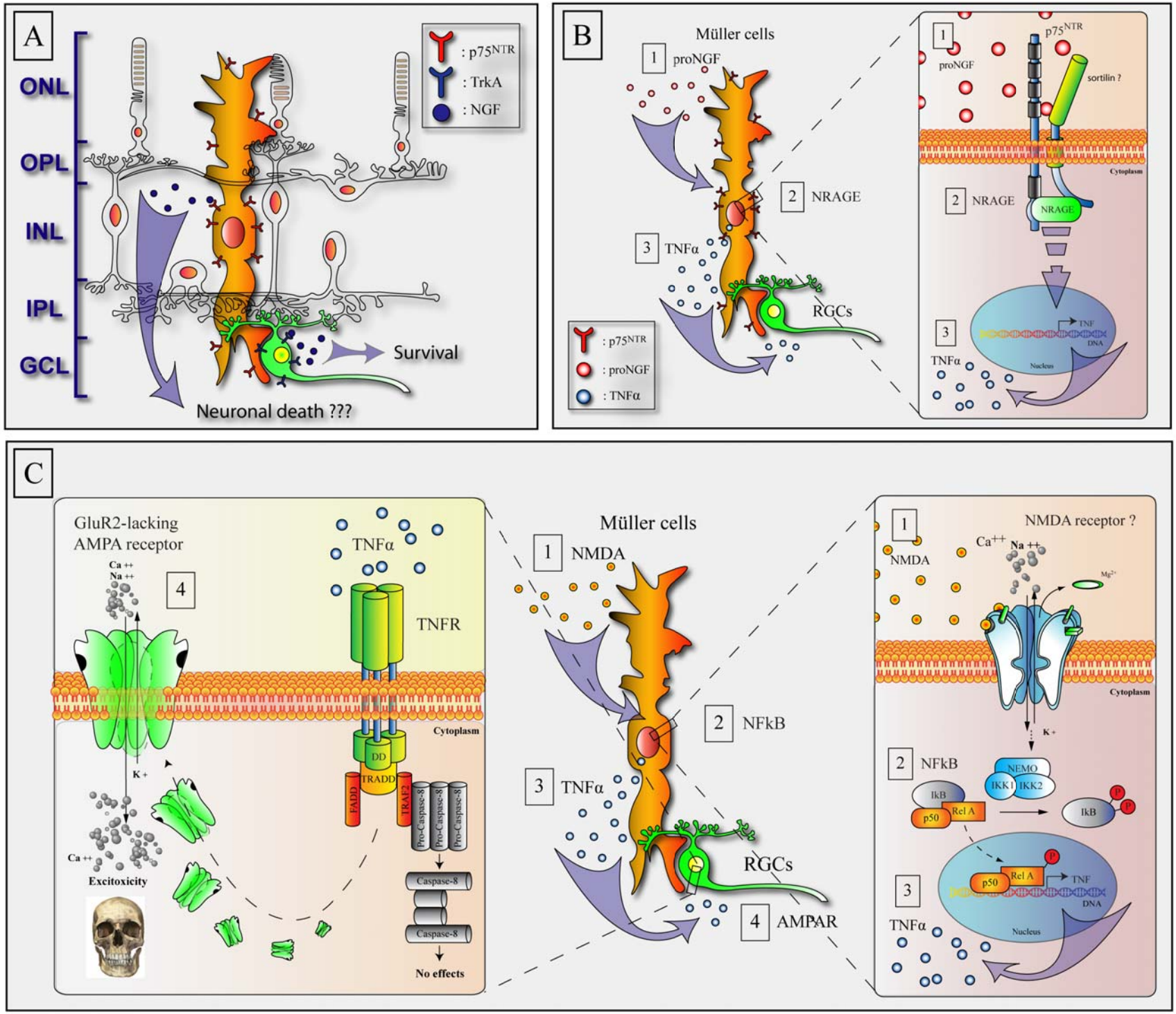

A
ONL
OPL
INL
IPL
GCL
: p75NTR
: TrkA
: NGF
Survival
Neuronal death ???
B
Müller cells
1 proNGF
2 NRAGE
3 TNFα
RGCs
: p75NTR
: proNGF
: TNFα
p75NTR
sortilin ?
Cytoplasm
NRAGE
TNF
DNA
Nucleus
C
GluR2-lacking
AMPA receptor
Ca ++
Na ++
4
K+
Ca ++
Excitoxicity
TNFα
TNFR
Cytoplasm
DD
TRADD
FADD
TRAF2
Pro-Caspase-8
Caspase-8
No effects
Müller cells
1 NMDA
2 NFkB
3 TNFα
RGCs
4 AMPAR
NMDA receptor ?
Mg2+
NEMO
IKK1
IKK2
IkB
p50
Rel A
P

Figure 14. Trois modèles de mort des cellules ganglionnaires par un mécanisme cellulaire non-autonome. (A) La signalisation du récepteur $p75^{NTR}$ des cellules de Müller et la signalisation de TrkA dans les cellules ganglionnaires de la rétine (CGRs) ont deux effets opposés sur la survie des CGRs axotomisées : la signalisation de $p75^{NTR}$ ést néfaste et celle de TrkA bénéfique. Le NGF seul n'ayant pas d'effet de survie, l'inhibition de $p75^{NTR}$ permet de révéler son effet neuroprotecteur sur les CGRs. L'inhibition de $p75^{NTR}$ combinée à l'activation de TrkA exerce un effet synergique sur la neuroprotection des CGRs. (B) Le proNGF induit la mort des CGRs en activant le complexe de récepteur $p75^{NTR}$/sortiline et la protéine adaptatrice NRAGE dans les cellules de Müller, ce qui induit la production de TNFα. Le proNGF active un mécanisme cellulaire non-autonome qui cause la mort des CGRs de façon dépendante du TNFα. (C) Le NMDA induit la mort des CGRs et des cellules amacrines par la production de TNFα par les cellules de Müller de façon dépendante de l'activation du NF-κB. La mort neuronale est principalement provoquée par une augmentation de récepteurs AMPA perméables au Ca^{2+}, dépendante du TNFα, à la surface des CGRs.

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ANNEXE A

MOLECULAR AND CELL-BASED APPROACHES FOR NEUROPROTECTION IN GLAUCOMA

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Abstract

A hallmark of glaucomatous optic nerve damage is retinal ganglion cell (RGC) death. RGCs, like other central nervous system neurons, have a limited capacity to survive or regenerate an axon following injury. Strategies that prevent or slow down RGC degeneration, in combination with intraocular pressure management, may be beneficial to preserve vision in glaucoma. Recent progress in neurobiological research has led to a better understanding of the molecular pathways that regulate the survival of injured RGCs. Here we discuss a variety of experimental strategies including intraocular delivery of neuroprotective molecules, viral-mediated gene transfer, cell implants and stem cell therapies, which share the ultimate goal of promoting RGC survival following optic nerve damage. The challenge now is to assess how this wealth of knowledge can be translated into viable therapies for the treatment of glaucoma and other optic neuropathies.

1. Introduction

During glaucoma, axons in the optic nerve are damaged leading to retinal ganglion cell (RGC) degeneration (Nickells, 1996; Quigley, 1999a; Schwartz et al., 1999). RGCs have cell bodies located in the inner retina within the eye and project axons along the optic nerve to visual targets in the brain. Because of this special cytoarchitecture, RGCs are the sole neurons that convey visual information from the retina to the brain. The structural and functional integrity of RGCs is therefore critical for visual perception. The primary mechanism of RGC damage in glaucoma is not well understood, but several putative culprits have been proposed including neurotrophin deprivation, mechanical compression, ischemia, reactive astrocytosis, excitotoxicity, and oxidative stress, among others (Wax and Tezel, 2002). Independently of the cause, the death of RGCs underlies the irreversible loss of vision experienced by glaucoma patients. RGCs, like other central nervous system neurons, have a limited abilty to survive or regenerate an axon after optic nerve injury. Thus, the development of strategies that prevent or slow down RGC loss in glaucoma is essential to save vision.

Progress in neurobiological research has led to a better understanding of the molecular pathways that stimulate survival of injured central nervous system neurons, including RGCs. In this review, we will focus on recent molecular approaches to promote RGC neuroprotection or neuroregeneration. Briefly, here we refer to neuroprotection as any therapy that prevents the death of existing RGCs following injury, while neuroregeneration refers to any strategy that stimulates regrowth of an injured RGC axon. We discuss laboratory evidence based on a variety of experimental strategies that include intraocular delivery of neurotrophic factors and related neuroprotective molecules, viral-mediated gene transfer, cell implants and stem cell therapies.

2. Neurotrophic Factors

2.1. What is the evidence for neurotrophic factor deprivation in glaucoma?

During development of the nervous system there is overproduction of neurons, which are then eliminated by a default intrinsic death program known as apoptosis(Raff, 1992). This self-destructive program is activated in all neurons and only those that are exposed to the appropriate neurotrophic factors can escape death (Figure 1). This process

forms the basis of the "neurotrophic factor hypothesis" in which developing neurons compete for limited amounts of target-derived neurotrophic factors that are essential for survival and differentiation. Neurons that successfully compete for optimal amounts of these factors survive, whereas less competitive neurons die. It is now clear that neurotrophic factors act by suppressing the intrinsic apoptotic process and by activating survival signals(Raff et al., 1993).

One family of neurotrophic factors, named the neurotrophins, has received special attention due to its important role in the complex process of shaping the nervous system. Neurotrophins are small, secreted peptides with a potent survival effect on developing and adult neurons. They are a family composed of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Segal and Greenberg, 1996; Huang and Reichardt, 2001b). The biological effects of neurotrophins are mediated by two cell surface receptor systems: the tropomyosin-related kinase (Trk) family and the p75 neurotrophin receptor ($p75^{NTR}$) (Figure 2). The Trk receptors contain a large extracellular ligand binding domain, a single trans-membrane domain, and a cytoplasmic tyrosine kinase catalytic domain. TrkA is the receptor for NGF; TrkB, the receptor for BDNF and NT-4; and TrkC, the receptor for NT-3 (Kaplan et al., 1991; Klein et al., 1991; Lamballe et al., 1991). $p75^{NTR}$ is related to the tumor necrosis factor receptor, and binds all neurotrophins with equal affinity (Rodriguez-Tebar et al., 1990; Rodriguez-Tebar et al., 1992) . Of interest, $p75^{NTR}$ has been shown to promote the demise of neurons following injury or when they fail to reach their appropriate targets (Roux and Barker, 2002a; Nykjaer et al., 2005).

There is substantial evidence that deprivation of neurotrophins, mainly BDNF and NGF secreted by targets in the brain, leads to apoptotic death of RGCs during visual system development (Nurcombe and Bennett, 1981; Thoenen et al., 1987; Chau et al., 1992; Rabacchi et al., 1994). Nevertheless, the role of target-derived neurotrophic factors in the survival of adult RGCs, particularly in the context of glaucomatous damage, is not clear. It has been proposed that blockade of axonal transport during intraocular pressure elevation, a major risk factor in glaucoma, leads to neurotrophin deprivation and neuronal death(Quigley, 1999b). Indirect evidence for this hypothesis is provided by the observation that both anterograde and retrograde axonal transport in the optic nerve is blocked in experimental glaucoma (Hayreh et al., 1979)' (Gaasterland et al., 1978)'

(Anderson and Hendrickson, 1974). Consistent with this, increased immunolabeling for BDNF and its receptor TrkB was found at the optic nerve head following intraocular pressure increase in a rat glaucoma model (Pease et al., 2000).

Local sources of neurotrophins within the retina may also play a prominent role in adult RGC survival. Evidence for this idea stems from studies where RGC target tissue was ablated in adult animals, but neuronal loss was not detected until several months post-lesion (Carpenter et al., 1986b; Pearson and Stoffler, 1992a; Pearson and Thompson, 1993b). This finding suggests that retina-derived neurotrophic factors support the survival of RGCs that have been disconnected from their targets. Hence, target-derived factors may act to compensate for a deficiency in local trophic support (Murphy and Clarke, 2006a). Of interest, neurotrophin receptor TrkB gene expression is markedly downregulated in RGCs after axotomy or chronic elevation of intraocular pressure (Cheng et al., 2002a; Jia et al., 2004) indicating that injured RGCs may have impaired ability to respond to neurotrophins produced locally within the retina.

Collectively, these results suggest that deficiencies in neurotrophic factors and/or their receptors, derived either from the target or the retina, may play a role in RGC death in glaucoma. Consequently, exogenous administration of neurotrophic factors has been extensively studied as a potential neuroprotective strategy to prevent or delay the death of injured RGCs.

2.2. Neurotrophic factor supplementation

There is substantial evidence that exogenous neurotrophic factors have a powerful survival effect on injured RGCs. Several groups have investigated the effect of intraocular injection of BDNF in models of RGC death. For example, injection of BDNF protein in the superior colliculus has been shown to markedly reduce developmental RGC death (Ma et al., 1998b). In the adult visual system, a single intravitreal injection of BDNF protein or adenovirus-mediated BDNF gene transfer promoted robust survival of RGCs after axotomy (Mey and Thanos, 1993b; Mansour-Robaey et al., 1994b; Peinado-Ramon et al., 1996a; Di Polo et al., 1998b; Klöcker et al., 2000; Chen and Weber, 2001b). Similarly, intraocular delivery of BDNF protein (Ko et al., 2001) or gene transfer using adeno-associated virus (AAV) (Martin et al., 2003a) led to RGC neuroprotection in experimental glaucoma induced by chronic intraocular pressure elevation.

Long-term studies of RGC survival have demonstrated that the effect of exogenous BDNF is temporary: it delays, but does not prevent, the onset of RGC death (Mansour-Robaey et al., 1994b; Di Polo et al., 1998b). Administration of BDNF by repeated intravitreal injections or osmotic minipumps failed to extend the time-course of RGC neuroprotection(Mansour-Robaey et al., 1994b; Clarke et al., 1998). Delivery of BDNF by adenovirus-infected Müller cells provided a sustained source of neurotrophin, but led to only transient rescue of RGCs (Di Polo et al., 1998b). Why might this happen? BDNF exerts its biological effect on RGCs upon binding to its cognate receptor TrkB, therefore a reduction in receptor levels may limit the intrinsic capacity of these neurons to respond to neurotrophic factor stimulation. Consistent with this hypothesis, TrkB mRNA and protein levels are markedly downregulated in adult RGCs following axotomy close to the eye(Cheng et al., 2002b; Cui et al., 2002a) and in experimental glaucoma(Jia et al., 2004). These results suggest that reduced TrkB expression in injured RGCs contributes to their desensitization to exogenous, and possibly endogenous, BDNF. Based on this, Cheng et al. (Cheng et al., 2002b) developed a neuroprotective strategy involving upregulation of endogenous TrkB in RGCs by AAV-mediated gene transfer. This study demonstrated a marked increase in the duration and level of BDNF-induced neuroprotection of axotomized RGCs upon upregulation of TrkB (Cheng et al., 2002b). Specifically, TrkB gene transfer into RGCs, combined with exogenous BDNF administration, increased survival by 76% at two weeks after axotomy, a time when >90% of these neurons are lost without treatment. This strategy substantially extended RGC survival compared to the shorter effect of BDNF protein alone (Mansour-Robaey et al., 1994b; Peinado-Ramon et al., 1996b; Clarke et al., 1998; Di Polo et al., 1998b).

In addition to BDNF, other neurotrophic factors have shown efficacy in models of RGC injury. For example, exogenous ciliary neurotrophic factor (CNTF) protein enhanced RGC survival after axotomy (Mey and Thanos, 1993b), during elevated intraocular pressure (Ji et al., 2004b), and in acute autoimmune optic neuritis (Maier et al., 2004). Of interest, adenovirus-mediated CNTF gene transfer was reported to increase retinal CNTF and CNTFRα levels, which correlated with increased survival of axotomized RGCs(van Adel et al., 2005). Lentivirus-mediated CNTF gene transfer was also reported to enhance survival of axotomized RGCs (van Adel et al., 2003). More

recently, AAV-mediated expression of CNTF stimulated RGC survival as well as axon regeneration after optic nerve crush or peripheral nerve graft transplantation(Leaver et al., 2006).

A novel approach to deliver CNTF *in vivo* involves the use of encapsulated cell intraocular implants. Cells transfected with the human *CNTF* gene are sequestered within capsules that can then be surgically implanted into the vitreous chamber of the eye. A semi-permeable membrane in the implant allows CNTF to diffuse out and nutrients to diffuse in, while preventing immune cells from destroying CNTF-producing cells. A Phase I safety clinical trial of this technology in patients with retinal degeneration was recently completed without apparent ocular or systemic complications (Sieving et al., 2006). Thus, the use of encapsulated cell implants is a novel approach for retinal delivery of neurotrophic factors that may have future applications for glaucomatous and other optic neuropathies as well as other retinal diseases.

The effect of glial cell line-derived neurotrophic factor (GDNF) on the survival of injured RGCs *in vivo* has also been examined. Intraocular injection of GDNF or neurturin was neuroprotective for axotomized RGCs, albeit with less efficacy than BDNF (Klocker et al., 1997; Koeberle and Ball, 1998; Yan et al., 1999; Koeberle and Ball, 2002). Of interest, combined treatment of BDNF and GDNF resulted in increased RGC survival compared to independent administration of each neurotrophic factor (Yan et al., 1999; Koeberle and Ball, 2002). Similarly, GDNF gene transfer using adenovirus or electroporation conferred protection of RGCs after optic nerve transection(Straten et al., 2002; Ishikawa et al., 2005). More recently, intraocular injection of slow-release poly(DL-lactide-co-glycolide) (PLGA) microspheres containing GDNF was shown to promote RGC survival in DBA/2J mice, a model of inherited glaucoma (Ward et al., 2007).

3. Peptidomimetic ligands

The use of neurotrophic factors for clinical applications has been limited because these molecules have pleiotropic effects that lead to non-specific signaling, toxicity and short half-lives *in vivo* (Barinaga, 1994b; Verrall, 1994a; Jonhagen, 2000b). Moreover, neurotrophic factors diffuse poorly in target tissues and do not cross the blood brain

barrier. These restrictions may be overcome by designing small molecules that mimic the biological activity of neurotrophic factors without undesirable effects. Peptidomimetic ligands are small, proteolytically stable molecules that mimic the binding and activation properties of native ligands. They interact specifically with their appropriate receptor and act as high affinity agonists or antagonists(Saragovi and Gehring, 2000). The pharmacophore of a mimetic molecule is the active part required for optimal interaction with a biological target (e.g. neurotrophic factor receptor) to stimulate or antagonize a biological response. The specific domain of a receptor that triggers a biological response upon binding of a ligand would be the receptor hot spot (Saragovi et al., 1992a). Peptidomimetic ligands have several pharmacological advantages over native ligands including lack of immunogenicity, low molecular mass, good pharmacokinetic profiles and high receptor specificity.

Peptidomimetic ligands of neurotrophins, with high affinity for specific neurotrophin receptors, have been developed. Recently, a novel peptidomimetic agonist of the neurotrophin receptor TrkA was tested as a neuroprotective therapy for degenerating cholinergic neurons in cognitively impaired aged rats. This study demonstrated that treatment with this TrkA peptidomimetic ligand afforded significant and long-lasting protection of cholinergic neurons and resulted in improvement of cognitive ability (Bruno et al., 2004). More recently, this compound was shown to promote RGC neuroprotection in experimental glaucoma (Shi et al., 2007a). In summary, small, proteolytically stable ligands with selective agonist activity have good therapeutic potential for glaucoma and other neurodegenerative diseases.

4. Anti-apoptotic signaling

Neurotrophic factors act by suppressing an intrinsic self-destruction process known as apoptosis(Raff et al., 1993). There is substantial evidence that neurotrophic factors can suppress apoptosis by activating intracellular survival signals. Upon binding to their cognate tyrosine kinase receptors, most neurotrophic factors stimulate two primary signaling pathways: including the extracellular signal-regulated kinase 1/2 (Erk1/2) and the phosphatidylinositol-3 kinase (PI3K) pathways (Kaplan and Miller, 2000b). Some factors, such as CNTF, can also activate the janus kinase/signal transducer

and activator of transcription 3 (JAK/STAT3)(Peterson et al., 2000b). Both PI3K and Erk1/2 can activate the cAMP-response-element-binding protein (CREB), a nuclear factor that promotes transcription of several pro-survival genes, including Bcl-2 and Bcl-XL(Walton and Dragunow, 2000) (Figure 3). In addition, Akt, the direct downstream target of PI3K(Segal and Greenberg, 1996), can inactivate the pro-apoptotic protein BAD(Datta et al., 1997b) and preserve mitochondrial function (Majewski et al., 1999). Endogenous activation of Erk1/2 and PI3K has been reported in RGCs in response to several neurotrophic factors, including BDNF, insulin growth factor and tumor necrosis factor alpha; and pharmacological inhibition of these molecules effectively blocks their neuroprotective effect (Kermer et al., 2000a; Diem et al., 2001b; Cheng et al., 2002b).

Different neurotrophic factors may promote RGC survival via different signaling mechanisms. For example, BDNF promotes RGC survival mainly via the Erk1/2 pathway (Cheng et al., 2002b; Pernet et al., 2005a), while CNTF promotes RGC survival and regeneration via protein kinase A and the JAK/STAT3 pathway (Ji et al., 2004a; Park et al., 2004b). Therefore, selective activation of signaling intermediaries is an attractive strategy to inhibit apoptosis and promote RGC survival in glaucoma. Zhou et al. recently demonstrated that AAV-mediated gene transfer of constitutively active MEK1, the obligate upstream activator of Erk1/2, resulted in robust survival of RGCs in an ocular hypertension rat model of glaucoma(Zhou et al., 2005b). Specifically, MEK1 gene transfer into RGCs increased neuronal survival to 1,366 ± 70 RGCs/mm^2 (mean ± S.E.M.) at five weeks after ocular hypertension surgery, a time when only 680 ± 86 RGCs/mm^2 of these neurons remained in control eyes. These data provide proof-of-principle that selective stimulation of key intracellular signaling pathways can be an effective strategy to delay or prevent RGC death in glaucoma.

Other strategies have focused on blocking apoptotic events that occur further downstream, including modulation of Bcl-2-type proteins and inactivation of caspases. Overexpression of the anti-apoptotic protein Bcl-2 in transgenic mice results in sustained RGC survival after axotomy (Bonfanti et al., 1996). Moreover, Bcl-XL gene transfer led to sustained neuroprotection of axotomized RGCs (Malik et al., 2000). A recent study demonstrated RGC soma were preserved in Bax deficient DBA/2J mice, a model of inherited glaucoma, indicating that BAX is required for RGC death in this model (Libby et

al., 2005). Interestingly, however, the same study showed that lack of BAX did not prevent axonal degeneration.

Suppression of apoptosis using caspase inhibitors is an approach that has been investigated with modest success (Bilsland and Harper, 2002). In adult rats, active caspase immunoreactivity has been shown to increase in the retina after optic nerve transection and in glaucoma (Hanninen et al., 2002b; McKinnon et al., 2002b), and axotomy-induced RGC death is reduced by intraocular administration of specific caspase inhibitors (Kermer et al., 1998b; Chaudhary et al., 1999b; Kermer et al., 2000c). As mentioned above, intravitreal administration of BDNF also delays RGC death after axotomy and in glaucoma, a neuroprotective effect that has been partly associated with the suppression of caspase 3 activity (Klöcker et al., 2000). BIRC4, a member of the inhibitors of apoptosis (IAPs) family, is a direct inhibitor of several caspases including caspases 3, 7 and 9(Deveraux et al., 1997a). Overexpression of BIRC4 in RGCs by means of an AAV viral vector promoted RGC axon protection in a chronic ocular hypertension model of rat glaucoma (McKinnon et al., 2002c). Mitochondrial dysfunction, which marks the point of no return during apoptosis, occurs even in the presence of caspase inhibition leading to impairment in ATP production and increased levels of reactive oxygen species (Chang et al., 2002). Thus, strategies that halt the commitment to die prior to irreversible mitochondrial dysfunction are likely to contribute to a better functional outcome.

Calcium (Ca^{+2})-dependent apoptotic pathways have also been shown to participate in RGC death during glaucomatous damage. Ca^{+2}-induced neuronal apoptosis is regulated by calcineurin, a Ca^{+2}-dependent phosphatase. Calcineurin can dephosphorylate Bad, a pro-apoptotic member of the Bcl-2 family. Bad dephosphorylation results in its translocation from the cytoplasm to the mictochondria, where it can bind to Bcl-2 or Bcl-XL. Formation of these protein complexes leads to increased permeability of the mitochondrial membrane, cytochrome C release and neuronal apoptosis(Wang et al., 1999). A recent study demonstrated that increased intraocular pressure in experimental glaucoma correlated with the formation of constitutively active calcineurin in the retina(Huang et al., 2005). In this study, ocular hypertension was also associated with Bad dephosphorylation, cytochrome C release and RGC death. Furthermore, calcineurin inhibition with the immunosuppressant FK506 inhibited Bad dephosphorylation and promoted RGC survival in experimental glaucoma.

5. Stem cell-based therapies

In recent years, stem cells have attracted tremendous attention as a potential source for cell replacement therapy in blinding diseases such as glaucoma and age-related macular degeneration. Two cardinal features of stem cells are their ability to divide indefinitely (self-renewal) and their capacity to differentiate into multiple cell types. Based on this, the concept behind stem cell-based therapy for these neurodegenerative diseases is relatively simple. Stem cells can be isolated, induced to differentiate into a specific cell type and then transplanted into the retina, where they will replace a lost RGC or photoreceptor. This strategy is not classical neuroprotection, in which the goal is to protect existing neurons from further damage and death, but rather aims to replace lost cells with new, healthy ones. Although there has been substantial progress in our understanding of the biology and properties of stem cells derived from ocular tissues, we will discuss important hurdles that need to be overcome before this approach can be applied in a clinical setting.

Several potential sources of stem cells for transplantation to the retina have been identified. Neural stem cells, for example, can self-renew and differentiate into neurons, astrocytes, and oligodendrocytes both *in vitro* and *in vivo* (Gage, 2000). There is evidence that neural stem cells can integrate into the retina following transplantation, but they fail to differentiate into retinal phenotypes (Takahashi et al., 1998a; Young et al., 2000). Retinal-derived stem cells, on the other hand, can differentiate into multiple retinal phenotypes (Chacko et al., 2000) , but appear to be unable to integrate within the host adult retina. This observation suggests that the environmental cues that drive successful migration, integration and synaptogenesis are different between the developing and mature retina. Recently, McLaren et al. showed that when retinal precursor cells are isolated at a time coincident with the birth of rod photoreceptors, these cells can successfully differentiate into rods, form synapses and improve visual function in experimental models of retinal degeneration (MacLaren et al., 2006). Another recent study demonstrated that human embryonic stem cells can be manipulated in culture to generate photoreceptor progenitor cells, which successfully integrate into isolated mouse retinas (Lamba et al., 2006). Photoreceptor progenitors generated in this fashion could potentially serve as a source for transplantation into degenerating retinas. These results

are undoubtedly promising; however, the use of embryonic stem cells for human retinal degenerative diseases has inherent ethical and bureaucratic limitations. In addition, there are well known risks associated with allogeneic transplantation and tumorigenesis that need to be carefully considered.

A major breakthrough in the field has been the identification of stem cells in the adult mouse and human ciliary epithelium (Ahmad et al., 2000; Tropepe et al., 2000; Coles et al., 2004). Single, pigmented cells from the mature ciliary margin epithelium have been shown to proliferate *in vitro* and to differentiate into retina-specific cell types including photoreceptors, bipolar cells and Müller glia (Ahmad et al., 2000; Tropepe et al., 2000). Furthermore, stem cells isolated from human ciliary epithelium successfully integrated into the host retina. Interestingly, RGCs were generated when stem cells were injected early during retinal development while photoreceptors were formed when stem cells were transplanted at later developmental stages (Coles et al., 2004). These findings raise the possibility of using autologous stem cells from adult ciliary epithelium for transplantation, eliminating the risks associated with allogeneic transplantation and ethical issues surrounding the use of human embryonic cells. Many challenges remain, however, to achieve efficient methods for expansion of stem cells derived from adult ciliary epithelium *in vitro*, and to optimize the differentiation of these stem cells into specific retinal cell types (RGCs or photoreceptors). There may also be great variability in the intrinsic properties of these stem cells between patients, particularly in their ability to proliferate and differentiate, which may hinder the establishment of standardized protocols for treatment.

When considering the use of stem cells to replace lost RGCs in glaucoma and to repair the optic nerve, one is faced with additional challenges. These include how to stimulate the newly transplanted RGCs to extend axons along the damaged optic nerve, to find their way to the appropriate targets in the brain and, finally, to establish functional synaptic connections with brainstem target neurons to restore vision. Successful stem cell therapies for glaucoma, whether based on cell transplantation or reprogramming of endogenous stem cells, will require a better understanding of the normal pathways that guide RGC differentiation, process extension and wiring.

6. Summary and future directions

Recent studies have led to a better understanding of the molecular pathways that stimulate the survival of adult, injured RGCs. The roles of key neurotrophic factors, signaling intermediaries and anti-apoptotic molecules have been characterized and validated using a variety of strategies in experimental models of optic nerve injury and glaucoma. While other neuroprotective strategies may reduce RGC death by targeting known mechanisms involved in neuronal injury (e.g. excitotoxic and oxidative damage, nitric oxide synthesis, etc), the molecular approaches discussed here boost RGC survival by modulating conserved signaling pathways. Thus, these strategies are likely to promote neuroprotection following a wide range of insults and, as such, may be applicable to a variety of retina and optic nerve diseases independently of the primary cause of damage. The challenge now is to assess how this wealth of knowledge can be translated into potential therapies for the treatment of ocular neurodegenerative diseases. The use of stem cells for optic nerve repair holds promise, but is likely to require an in-depth understanding of the extrinsic and intrinsic mechanisms that direct RGC differentiation and connectivity. It is conceivable that neuroprotective strategies may be required as part of a successful cell replacement therapy to ensure that new RGCs survive and differentiate appropriately.

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8. FIGURES LEGENDS

Figure 1. The neurotrophic factor hypothesis is based on the observation that neurons compete for limited amounts of target-derived neurotrophic factors that are required for their survival. Neurons that successfully compete for these factors survive, whereas less competitive neurons die. In glaucoma, neurotrophic deprivation caused by obstruction of retrograde axonal transport may be involved in RGC death.

Figure 2. The biological effects of neurotrophins are mediated by Trk receptors: TrkA is the receptor for NGF; TrkB is the receptor for BDNF and NT-4; and TrkC is the receptor for NT-3. The p75 receptor ($p75^{NTR}$), related to the tumor necrosis factor receptor, binds NGF, BDNF, NT-3 and NT-4 with equal affinity.

Figure 3. Upon binding to TrkB, BDNF stimulates receptor autophosphorylation and activation of multiple signaling pathways, including the extracellular signal-regulated kinases 1/2 (Erk1/2) and the phosphatidylinositol-3 kinase (PI3K)/Akt pathways. Both PI3K and Erk1/2 can activate the cAMP-response-element-binding protein (CREB), a nuclear factor that promotes transcription of several pro-survival genes, including Bcl-2 and Bcl-XL. Akt, the direct downstream target of PI3K, can inactivate the pro-apoptotic protein BAD and preserve mitochondrial function.

FIGURE 1

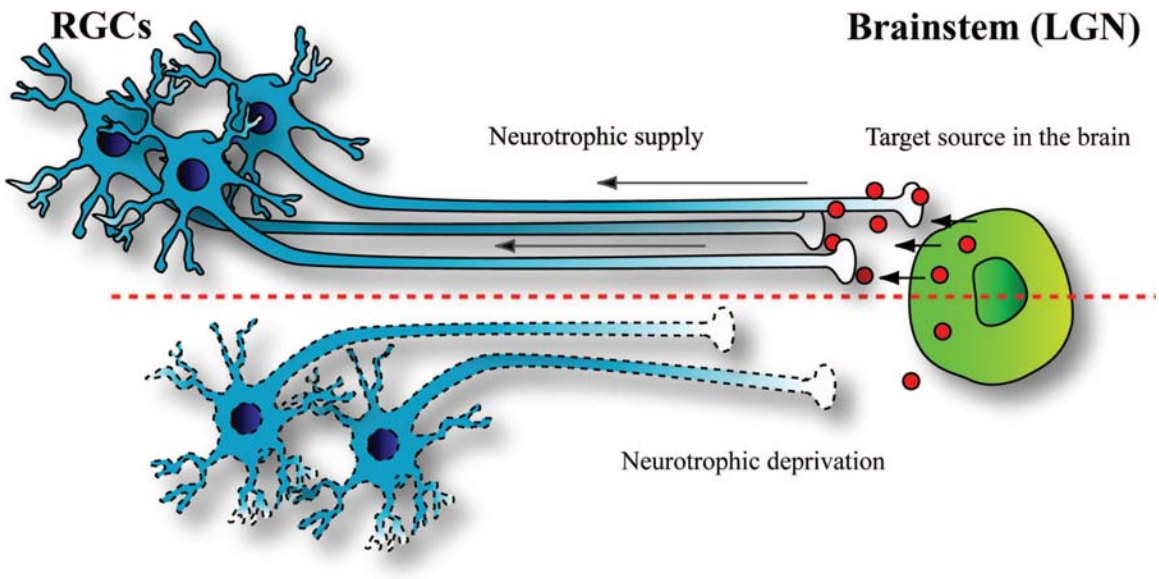

RGCs
Brainstem (LGN)
Neurotrophic supply
Target source in the brain
Neurotrophic deprivation

FIGURE 2

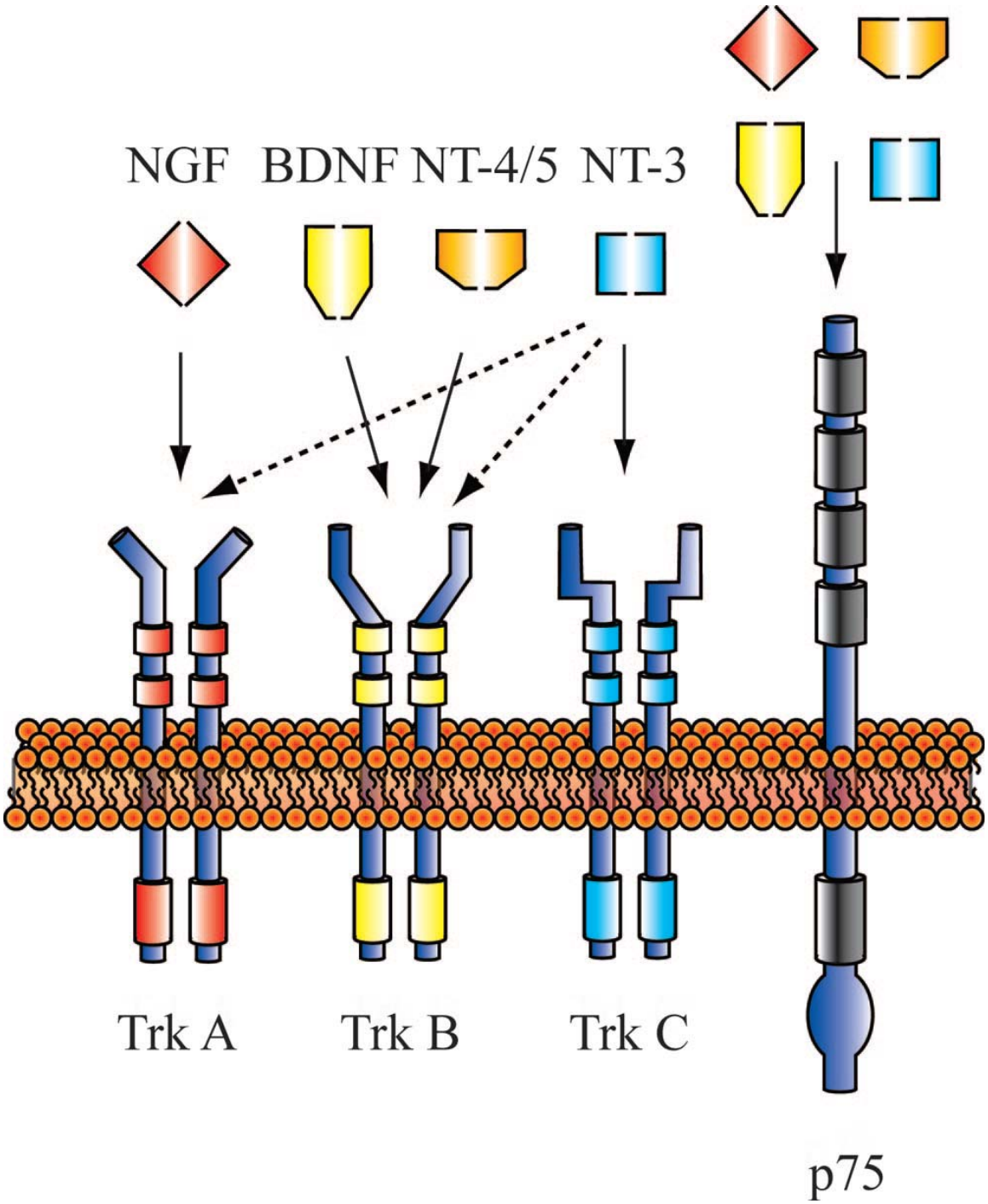
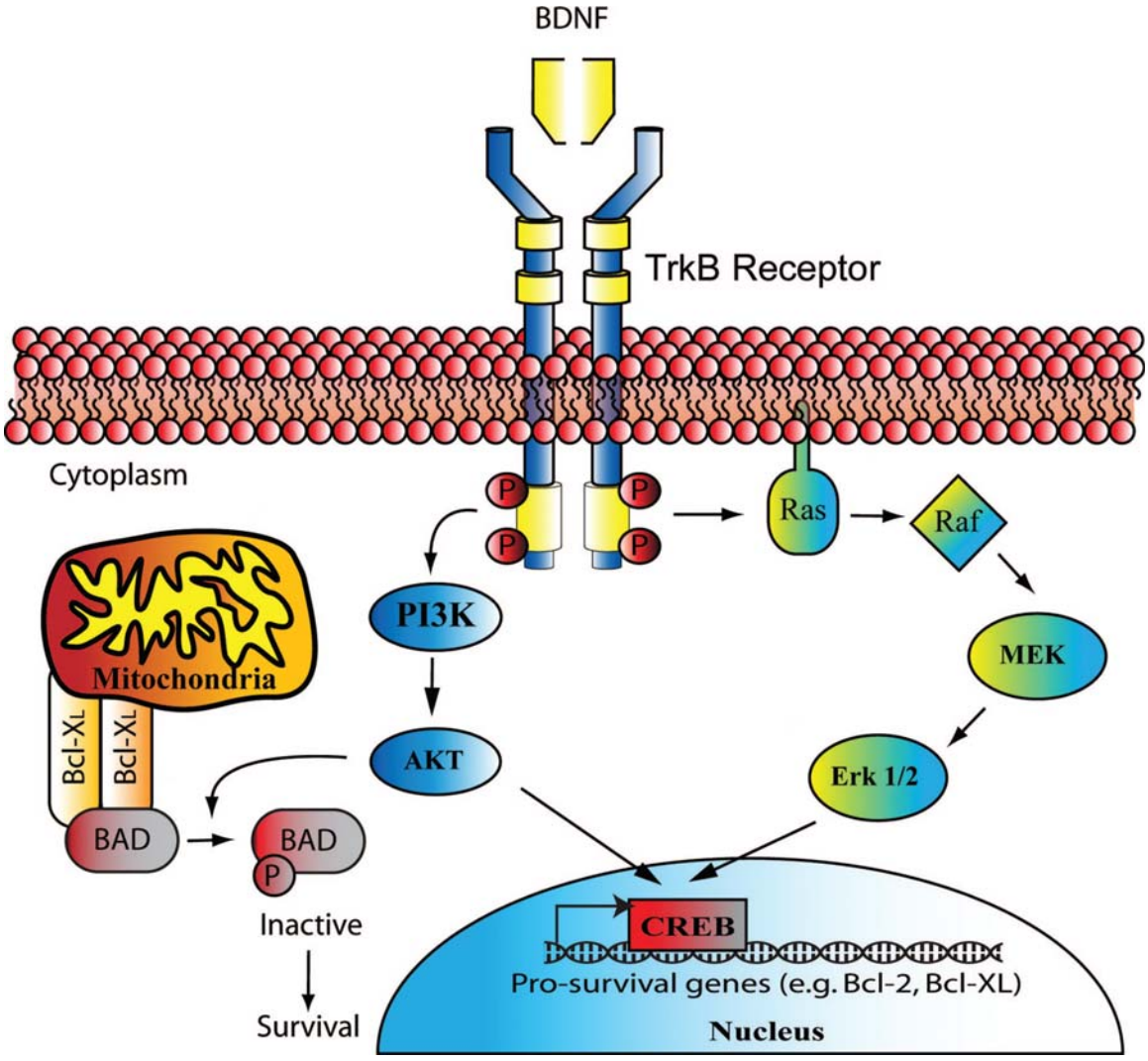


FIGURE 3


BDNF
TrkB Receptor
Cytoplasm
P
P
P
P
Ras
Raf
PI3K
MEK
Mitochondria
Bcl-XL
Bcl-XL
AKT
Erk 1/2
BAD
BAD
P
Inactive
Survival
CREB
Pro-survival genes (e.g. Bcl-2, Bcl-XL)
Nucleus

ANNEXE B

NEUROPROTECTIVE SIGNALING PATHWAYS IN GLAUCOMA

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1. Introduction

Glaucoma is an optic neuropathy characterized by progressive death of retinal ganglion cells (RGCs) and optic nerve degeneration leading to irreversible vision loss. Elevated intraocular pressure is a major risk factor in glaucoma. Open angle and angle-closure glaucoma, the most common forms of the disease, are often associated with high intraocular pressure. The current standard therapy for glaucoma is to lower eye pressure by medication (e.g. prostaglandin therapy) or surgery. However, there are risks and adverse side effects associated with these treatments(Lee and Higginbotham, 2005). More importantly, a significant number of patients continue to experience visual loss in spite of responding well to pressure lowering medications(Harbin et al., 1976; Caprioli, 1997; Georgopoulos et al., 1997; Leske et al., 2003). Therefore, current therapeutic strategies for glaucoma are insufficient and new approaches to slow disease progression are urgently needed.

The primary mechanism of RGC damage in glaucoma is not well understood, but there is convincing evidence that neuronal loss in this disease occurs by apoptosis (Nickells, 1999; Quigley, 1999b). This self-destructive, genetically driven death program is activated in all neurons. It is now widely accepted that neurotrophic factors promote neuronal survival by inhibiting default apoptotic pathways(Raff et al., 1993). For example, there is strong direct evidence that deprivation of neurotrophins, mainly brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), secreted by targets in the brain, leads to apoptotic death of RGCs during development (Nurcombe and Bennett, 1981; Thoenen et al., 1987; Chau et al., 1992; Rabacchi et al., 1994) (Figure 1). In contrast, the role of endogenous target-derived factors in the maintenance of adult RGCs in glaucoma is not clear. It has been proposed that blockade of axonal transport during IOP elevation leads to neurotrophin deprivation and neuronal death (Quigley, 1999b). Indirect evidence for this idea is provided by the observation that both anterograde and retrograde axonal transport in the optic nerve is blocked in experimental glaucoma (Hayreh et al., 1979)' (Gaasterland et al., 1978)' (Anderson and Hendrickson, 1974). In addition, increased immunolabeling for BDNF and its receptor TrkB was found behind the optic nerve head following IOP increase in a glaucoma rat model (Pease et al., 2000).

Local sources of neurotrophins within the retina may also play a prominent role in adult RGC survival (Figure 2). Evidence for this premise comes from studies where RGC target tissue was ablated in adult animals and neuronal loss was not detected until several months after the lesion(Carpenter et al., 1986b; Pearson and Stoffler, 1992a; Pearson and Thompson, 1993b). This finding suggests that retina-derived neurotrophic factors, acting in a paracrine or autocrine fashion, support the survival of RGCs that have been disconnected from their targets. Thus, it has been proposed that target-derived factors may act to compensate for a deficiency in local trophic support (Murphy and Clarke, 2006a). Of interest, neurotrophin receptor TrkB gene expression is markedly downregulated in RGCs after axotomy or chronic IOP elevation (Cheng et al., 2002a; Jia et al., 2004) indicating that injured RGCs may have impaired ability to respond to neurotrophins produced locally within the retina.

Collectively, these results suggest that deficiencies in neurotrophic factors and/or their receptors, derived either from the target or the retina, may play a role in RGC death in glaucoma. Consequently, exogenous administration of neurotrophic factors has been extensively studied as a potential strategy to prevent or delay the death of injured RGCs. In this chapter, we will focus on neurotrophic factors with demonstrated efficacy for keeping RGCs alive in different models of optic nerve injury, including chronic IOP elevation. In addition, the intracellular signaling pathways that mediate neurotrophic-factor RGC survival and/or regeneration, as well as novel compounds that lead to highly specific activation of neuroprotective signaling will be discussed.

2. The neurotrophins

Neurotrophins are diffusible trophic molecules that exert a potent survival effect on adult central nervous system (CNS) neurons undergoing degeneration induced by a broad variety of stimuli. They are a family of small, secreted peptides that include NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) in mammals (Segal and Greenberg, 1996; Huang and Reichardt, 2001b). Neurotrophin-6 (NT-6) and 7 (NT-7) are two family members that have been identified exclusively in fish (Gotz et al., 1994; Nilsson et al., 1998). In addition to survival, neurotrophins mediate several key cellular responses in the developing and mature CNS including proliferation, differentiation, axon

growth, as well as dendrite and synapse formation (Huang and Reichardt, 2001b; Miller and Kaplan, 2003; Lu, 2004).

The biological effects of neurotrophins are mediated by cell surface receptors of the tropomyosin-related kinase (Trk) family comprising TrkA, the receptor for NGF; TrkB, the receptor for BDNF and NT-4; and TrkC, the receptor for NT-3 (Kaplan et al., 1991; Klein et al., 1991; Lamballe et al., 1991). These receptors share in common a large extracellular ligand binding domain, a single trans-membrane domain, and a cytoplasmic tyrosine kinase catalytic domain (Figure 3). In addition, the p75 receptor ($p75^{NTR}$), related to the tumor necrosis factor receptor, binds all the neurotrophins with equal affinity (Rodriguez-Tebar et al., 1990; Rodriguez-Tebar et al., 1992). Both receptors can be present on the same cell population, however, activation of Trk receptors has been typically associated with cell survival, while $p75^{NTR}$ receptor can stimulate both survival and apoptotic signals(Chao, 1994; Miller and Kaplan, 2001). Developing and adult RGCs have been shown to express high levels of the BDNF receptor TrkB(Jelsma et al., 1993; Pérez and Caminos, 1995; Rickman and Brecha, 1995). Importantly, adult RGCs do not express $p75^{NTR}$ (Hu et al., 1998; Hu et al., 1999) and a newly identified receptor named TROY has been shown to mediate some of the functions originally attributed to $p75^{NTR}$ (Shao et al., 2005a).

2.1. Brain-derived neurotrophic factor

Originally discovered and isolated from pig brain in 1982 by Yves Barde and collaborators(Barde et al., 1982b), BDNF was initially found to promote robust survival of primary sensory neurons (Lindsay et al., 1985; Davies et al., 1986). The first evidence of the neuroprotective effect of BDNF on central neurons came from studies demonstrating BDNF-induced survival of cultured RGCs (Johnson et al., 1986a). Since then, it has become clear that BDNF is the most potent neurotrophin for developing (Ma et al., 1998b), (Weibel et al., 1995) and adult RGCs following optic nerve injury (Mansour-Robaey et al., 1994b),(Takano et al., 2002),(Watanabe et al., 1997). BDNF is strongly expressed in the superior colliculus (Hofer et al., 1990), (Wetmore et al., 1990) and is retrogradely transported by RGC axons to the retina (Herzog and von Bartheld, 1998b; Ma et al., 1998b). Within the retina, BDNF is mainly produce by cells in the

ganglion cell layer and by some cells of the inner nuclear layer (Cohen-Cory and Fraser, 1994; Pérez and Caminos, 1995; Vecino et al., 1998).

Many groups have investigated the effect of intraocular injection of BDNF in models of RGC death. For example, injection of BDNF protein in the superior colliculus has been shown to markedly reduce developmental RGC death (Ma et al., 1998b). In the adult rat and cat visual system, a single intravitreal injection of BDNF promoted robust survival of RGCs after axotomy (Mey and Thanos, 1993b; Mansour-Robaey et al., 1994b; Peinado-Ramon et al., 1996a; Di Polo et al., 1998b; Klöcker et al., 2000; Chen and Weber, 2001b). Similarly, intraocular delivery of BDNF protein (Ko et al., 2001) or gene transfer using adeno-associated virus (AAV) led to RGC neuroprotection in experimental glaucoma induced by chronic IOP elevation (Martin et al., 2003a).

Long-term studies of RGC survival have demonstrated that the effect of exogenous BDNF is temporary: it delays, but does not prevent, the onset of RGC death (Mansour-Robaey et al., 1994b; Di Polo et al., 1998b). Administration of BDNF by repeated intravitreal injections or osmotic minipumps failed to extend the time-course of RGC protection(Mansour-Robaey et al., 1994b; Clarke et al., 1998). Moreover, delivery of BDNF by adenovirus-infected Müller cells provided a sustained source of neurotrophin but resulted in transient RGC rescue(Di Polo et al., 1998b). Why might this happen? The biological effect of BDNF is exerted upon binding to its cognate receptor TrkB. There is now evidence that the levels of expression of these receptors in RGCs varies following injury, in some cases limiting the intrinsic capacity of these neurons to respond to neurotrophic factor stimulation. *In vitro* studies using highly purified RGCs have shown that TrkB internalization is likely to contribute to loss of trophic responsiveness after axotomy (Meyer-Franke et al., 1998; Shen et al., 1999). *In vivo*, TrkB mRNA and protein levels are markedly downregulated in adult RGCs following axotomy close to the eye(Cheng et al., 2002b; Cui et al., 2002a) and in experimental glaucoma(Jia et al., 2004). Together, these results suggest that reduced TrkB expression in injured RGCs contributes to their desensitization to exogenous, and possibly endogenous, BDNF. Consistent with this, a strategy that involved upregulation of TrkB in RGCs by AAV-mediated gene transfer markedly enhanced the duration and level of BDNF-induced neuroprotection after optic nerve axotomy (Cheng et al., 2002b). It would

be relevant to assess whether such neuroprotective strategy is effective in experimental glaucoma.

2.2 Neuroprotective signaling pathways downstream of the BDNF/TrkB complex

There are several important limitations to applying exogenous factors as neuroprotective therapy for RGCs. The survival effect of diffusible factors is transient, as discussed above, and it may also be non-specific. BDNF, for example, will likely affect other retinal cells that express the receptor TrkB, including amacrine cells, Müller glia and cone photoreceptors (Pérez and Caminos, 1995; Rohrer et al., 1999; Di Polo et al., 2000). This pleiotropic, non-specific effect may also be applicable to other neurotrophic factors. In addition, exogenous neurotrophic factors may also produce adverse side effects. It is known that BDNF leads to upregulation of nitric oxide synthase activity(Klocker et al., 1999b) and suppression of heat shock proteins (Krueger-Naug et al., 2003), which may limit its neuroprotective action. Therefore, increasing effort is being invested to understand and target intracellular signaling pathways that lead to RGC survival, bypassing the use of exogenous peptide factors.

Upon binding to the TrkB receptor, BDNF stimulates multiple signaling pathways, including the extracellular signal-regulated kinases 1/2 (Erk1/2) and the phosphatidylinositol-3 kinase (PI3K)/Akt pathways (Kaplan and Miller, 2000b) (Figure 4). Until recently, studies on the participation of these pathways in neurotrophin-induced survival had been limited to neurons in culture (Xia et al., 1995; Bartlett et al., 1997; Dudek et al., 1997; Crowder and Freeman, 1998; Meyer-Franke et al., 1998; Skaper et al., 1998; Bonni et al., 1999b; Dolcet et al., 1999; Atwal et al., 2000; Orike et al., 2001) or at early developmental stages *in vivo* (Anderson and Tolkovsky, 1999; Hetman et al., 1999; Hee Han and Hotlzman, 2000). We demonstrated that both the Erk1/2 and the PI3K pathways are stimulated in adult RGCs following TrkB activation *in vivo* (Cheng et al., 2002b). Furthermore, we showed that pharmacological inhibition of the obligate upstream activator of Erk1/2, the mitogen activated protein (MAP) kinase kinase 1 (MEK1), blocked the survival effect produced by AAV-mediated TrkB overexpression, while PI3K inhibition did not alter this neuroprotective effect (Cheng et al., 2002b).

Collectively, these results indicate that activation of the Erk1/2 pathway, but not the PI3K pathway, mediates TrkB-induced RGC survival.

More recently, we tested the hypothesis that selective stimulation of the Erk1/2 pathway would promote survival of injured RGCs. For this purpose, we used recombinant AAV for *in vivo* gene delivery of constitutively active (ca) or wild-type (wt) MEK1 into RGCs. The AAV vector system was selected based on our finding, and that of others, that RGCs are the primary cellular target for AAV transduction upon intravitreal virus administration (Cheng et al., 2002b; Martin et al., 2003b; Sapieha et al., 2003a). Our results demonstrated that *in vivo* stimulation of Erk1/2 by overexpression of a constitutively active MEK1 mutant potentiated RGC survival after optic nerve axotomy (Pernet et al., 2005b) and in experimental glaucoma(Zhou et al., 2005a). Interestingly, however, MEK1 activation did not enhance of RGC axon regeneration within the axotomized optic nerve(Pernet et al., 2005b). Collectively, these data indicate that the Erk1/2 pathway plays a key role in the regulation of adult RGC survival after traumatic injury and in glaucoma. Future studies will be essential to elucidate the specific signaling components that mediate RGC axon growth *in vivo*.

3. Ciliary neurotrophic factor

CNTF is a member of a neurotrophic factor family that includes leukemia inhibitory factor (LIF), interleukin-6 (IL-6), cardiotrophin 1 (CD-1), and oncostatin M (OSM) (Grotzinger et al., 1999; Senaldi et al., 1999; Shi et al., 1999). Cloning and sequencing of CNTF revealed that it is unrelated to the neurotrophins, (Segal and Greenberg, 1996) but structurally similar to cytokines (Bazan, 1991). CNTF is a cytoplasmic protein that lacks consensus sequence for secretion and has been postulated to act as an injury-activated factor under pathological conditions (Lin et al., 1989; Stockli et al., 1989). Early *in vitro* studies identified CNTF as a survival factor for embryonic chick ciliary ganglion neurons in the eye (Adler et al., 1979), (Lin et al., 1989), (Stockli et al., 1989), followed by work demonstrating that, in fact, CNTF is neuroprotective for a wide variety of neuronal cell types (Adler et al., 1979),(Barbin et al., 1984). CNTF binds a trimer receptor complex containing the CNTF-specific α-receptor (CNTFRα), glycoprotein 130 (gp130) and leukemia inhibitory factor receptor (LIFR) (Davis et al.,

1991)(Ip and Yancopoulos, 1992). In the adult rat retina, CNTF is primarily localized in Müller glia, whereas retinal neurons such as RGCs, horizontal, and amacrine cells express its high affinity CNTFRα receptor (Kirsch et al., 1997). Of interest, CNTF and CNTFRα expression have been shown to increase in the rat retina after axotomy (Ju et al.; Wen et al., 1995; Kirsch et al., 1997; Chun et al., 2000; Weise et al., 2000) and ischemic insult (Ju et al., 1999; Ju et al., 2000), suggesting that these molecules play a role in the endogenous response of retinal neurons to injury.

The neuroprotective effect of CNTF has been tested in different models of optic nerve lesion. For example, exogenous CNTF protein induced survival of purified adult RGCs in culture (Jo et al., 1999), after axotomy (Mey and Thanos, 1993b) and elevated IOP (Ji et al., 2004b), as well as in acute autoimmune optic neuritis (Maier et al., 2004). Of interest, adenovirus-mediated CNTF gene transfer was reported to increase retinal CNTF and CNTFRα levels, which correlated with increased survival of axotomized RGCs(van Adel et al., 2005). Thus, the effect of adenovirus-CNTF may be related to glial changes that contribute to RGC survival(van Adel et al., 2005). Lentivirus-mediated CNTF gene transfer was also reported to enhance survival of axotomized RGCs (van Adel et al., 2003). More recently, AAV-mediated expression of CNTF stimulated RGC survival as well as axon regeneration after optic nerve crush or peripheral nerve graft transplantation(Leaver et al., 2006).

A novel approach to deliver CNTF *in vivo* involves the use of encapsulated cell intraocular implants. In this technology, cells transfected with the human *CNTF* gene are sequestered within capsules that can be surgically implanted into the vitreous chamber of the eye. A semi-permeable membrane in the implant allows CNTF to diffuse out and nutrients to diffuse in, but prevents immune cells from removing CNTF-producing cells. Recently, the first Phase I safety clinical trial in retinal degeneration patients was successfully completed, and the results were encouraging in that no systemic or ocular complications ensued (Sieving et al., 2006). Thus, the use of encapsulated cell implants is a novel approach for delivery of neurotrophic factors to the retina that may have future applications for glaucomatous and other optic neuropathies.

3.1 Signaling cascades activated by CNTF

Signal transduction by CNTF requires binding of this neurotrophic factor to the glycosyl phosphatidylinositol (GPI)-linked receptor subunit CNTFRα, which then facilitates the recruitment of gp130 and LIFR forming a tripartite receptor complex (Vergara and Ramirez, 2004). CNTF-induced heterodimerization of these components initiates a signaling cascade by activation of the JAK/TYK tyrosine kinases (Stahl et al., 1994). Once activated, these tyrosine kinases phosphorylate each other as well as gp130 and LIFR, forming a docking site for the signal transduction activator of transcription (STAT3). JAK kinases can phosphorylate STAT3 in response to CNTF binding to its receptor complex. Upon activation, STAT3 forms a dimer that translocates to the nucleus where it can activate transcription of target genes (Ihle, 2001) (Figure 5).

CNTF-induced neuronal survival in the CNS has been associated with activation of the JAK/STAT-dependent pathway(Dolcet et al., 2001) . Consistent with this, intravitreal injection of CNTF (Peterson et al., 2000a), or adenoviral-mediated CNTF delivery (van Adel et al., 2005) increased activation of STAT3 in RGCs suggesting that this pathway is implicated in CNTF-mediated survival. A recent study on the signaling mechanisms used by CNTF demonstrated that this neurotrophic factor activates and uses the JAK/STAT3, PI3K/Akt and Erk1/2 pathways, but not the PKA pathway, to promote RGC survival and axon regeneration in the adult rat (Park et al., 2004a). Furthermore, endogenous STAT3 is transiently activated in experimental glaucoma, and intravitreal CNTF administration extends activation of STAT3 for up to 2 weeks following elevated IOP (Ji et al., 2004b). Additional studies are required to unequivocally demonstrate that these pathways mediate CNTF-induced neuroprotection of RGCs in experimental glaucoma.

4. Basic fibroblast growth factor

The fibroblast growth factor (FGF) family of neurotrophic factors play multiple roles in the development of the brain (Eckenstein, 1994; Abe and Saito, 2001), including the retina (Barnstable, 1991; Hicks, 1998). The biological activity of FGFs is primarily mediated through receptor tyrosine kinases (FGFRs), of which four distinct families have been identified (FGFR-1-4) (Klint and Claesson-Welsh, 1999; Powers et al., 2000). Members of the FGF and FGFR families are expressed in the developing and adult retina

of many species, including human (Gao and Hollyfield, 1992; Bugra et al., 1993; Song and Slack, 1994; Kinkl et al., 2002). *In vitro*, FGFs stimulate survival and neurite outgrowth of embryonic, early post-natal(Lipton et al., 1988; Bahr et al., 1989; McFarlane et al., 1995) or adult RGCs(Kinkl et al., 2003). *In vivo*, FGF-2 (basic FGF), a well-characterized member of the large FGF family, has been shown to be a potent stimulator of axon growth for developing RGCs (Dingwell et al., 2000). Consistent with this, expression of a dominant negative FGFR in *Xenopus* RGC axons significantly impaired their ability to grow along the developing optic tract (McFarlane et al., 1996). Moreover, FGFR function was shown to be required for the orderly projection of developing RGC axons (Brittis et al., 1996).

Endogenous FGF-2 is abundantly expressed by astrocytes in the ganglion cell layer as well as cells in the inner and outer nuclear layers, but not by adult RGCs (Gao and Hollyfield, 1992; Kostyk et al., 1994; Ohsato et al., 1997). Of interest, FGF-2 is upregulated after injury in cells of the inner nuclear layer and ganglion cell layer of the adult retina (Wen et al., 1995; Cao et al., 1997; Wen et al., 1998), as well as in the optic nerve (Eckenstein et al., 1991) and in the optic tract (Kostyk et al., 1994) suggesting that this neurotrophic factor may be involved in RGC survival. We tested this hypothesis using AAV-mediated FGF-2 to evaluate the effect of this neurotrophic factor on the survival and regeneration of axotomized RGCs. Our results demonstrated that FGF-2 gene transfer led to a 10-fold increase in the number of axons that extended past the lesion site compared to control nerves(Sapieha et al., 2003b). The response to FGF-2 upregulation was supported by our finding that FGF receptor-1 (FGFR-1) and heparan sulfate (HS), known to be essential for FGF-2 signaling, were expressed by adult rat RGCs. Intriguingly, FGF-2 transgene expression led to modest protection of injured RGCs, thus the effect of this neurotrophic factor on axon extension could not be solely attributed to an increase in neuronal survival. These data support the hypothesis that key factors involved in axon outgrowth during neural development, such as FGF-2, may promote axon regeneration of adult, injured RGCs. The role of FGF-2 in RGC neuroprotection and regeneration in experimental glaucoma has yet to be examined.

4.1 Signaling mechanisms involved in FGF-2-induced RGC axon regeneration

The robust regenerative growth mediated by FGF-2 suggests that this factor activates unique downstream signaling molecules required for adult RGC axon growth. The obvious next question is: what are the molecular mechanisms by which FGF-2 stimulates regeneration? The biological activity of FGF-2 is primarily mediated through its high affinity receptor tyrosine kinase FGFR1(Klint and Claesson-Welsh, 1999; Powers et al., 2000), which is abundantly expressed by adult RGCs(Sapieha et al., 2003a). Ligand binding leads to FGFR1 dimerization, tyrosine autophosphorylation and subsequent activation of extracellular signal-regulated kinases 1/2 (Erk1/2), phosphoinositide 3-kinase (PI3K) and phospholipase Cγ (PLCγ)(Ullrich and Schlessinger, 1990). We recently demonstrated that FGF-2 strongly activates retinal Erk1/2 *in vivo*, and that pharmacological inhibition of Erk1/2 blocked FGF-2-induced regeneration (Sapieha et al., 2006). Thus, Erk1/2 is a required intermediary for FGF-2-induced RGC axon regeneration *in vivo*. Surprisingly, we also demonstrated that gene transfer of a constitutively active form of MEK1, the obligate upstream activator of Erk1/2, did not stimulate RGC axon outgrowth (Pernet et al., 2005a). These results indicate that Erk1/2 activation is not sufficient to promote regeneration *in vivo*, and suggest that other, yet undefined, signaling cues switch RGCs to an axon growth mode in response to FGF-2.

5. Glial cell line-derived neurotrophic factor

Glial cell line-derived neurotrophic factor (GDNF) was purified and characterized in 1993 as a survival factor for midbrain dopaminergic neurons (Lin et al., 1993). GDNF and its relatives: neurturin, artemin and persephin, have now been identified as neuroprotective factors for many cell types including sympathetic, parasympathetic, sensory and enteric neurons (Airaksinen and Saarma, 2002). Outside the nervous system, GDNF has been shown to play critical roles in kidney development (Sariola and Saarma, 1999) and spermatogenesis (Meng et al., 2000). The biological response to GDNF is mediated via a receptor complex composed of the GPI-linked ligand-binding subunit known as GDNF family receptor α1 (GFRα1) and the RET receptor tyrosine kinase (Jing et al., 1996). Abundant protein and mRNA levels of GDNF and its receptors have been reported in RGCs throughout development, with expression

declining with maturation and after optic nerve injury(Lindqvist et al., 2004; Kretz et al., 2006).

The effect of GDNF on the survival of injured RGCs *in vivo* has been examined by several groups using different models of optic nerve injury. Intraocular injection of GDNF or neurturin was neuroprotective for axotomized RGCs, albeit with less efficacy than BDNF (Klocker et al., 1997; Koeberle and Ball, 1998; Yan et al., 1999; Koeberle and Ball, 2002). Of interest, combined treatment of BDNF and GDNF resulted in increased RGC survival compared to independent administration of each neurotrophic factor (Yan et al., 1999; Koeberle and Ball, 2002). Similarly, GDNF gene transfer using adenovirus or electroporation conferred protection of RGCs after optic nerve transection(Straten et al., 2002; Ishikawa et al., 2005). More recently, intraocular injection of slow-release poly(DL-lactide-co-glycolide) (PLGA) microspheres containing GDNF was shown to promote RGC survival DBA/2J mice, a model of inherited glaucoma (Ward et al., 2006).

In terms of activation of downstream signaling components, GDNF first forms a high-affinity complex with GFRα1 which then brings two molecules of RET together triggering trans-phosphorylation of tyrosine residues in the RET tyrosine kinase domains (Airaksinen and Saarma, 2002). Like other receptor tyrosine kinases, RET can activate various pathways including Erk1/2, PI3K/Akt, p38 mitogen activated protein kinase and c-Jun N-terminal kinase (JNK) pathways (Takahashi, 2001). Interestingly, however, the GPI anchor of the GFRα1 receptor may localize the receptor complex to lipid rafts of the plasma membrane, and GDNF signal transduction may ultimately depend on the co-localization of RET and GFRα1 in these lipid rafts. It is known that GDNF may trigger different signaling pathways depending on whether the RET and GFRα1 receptor complex is inside or outside the rafts. For example, activated RET interacts with the lipid-anchored adaptor protein FRS2 (fibroblast growth factor receptor substrate 2) when it is inside the rafts, while it interacts with soluble Shc (Src-homologous and collagen-like protein) when it is outside the rafts (Paratcha et al., 2001), which may lead to differential activation of downstream pathways. The role of these GDNF-stimulated adaptor proteins and/or downstream signaling components in the survival of injured RGCs remains unknown.

6. Peptidomimetic ligands

The use of neurotrophic factors for clinical application may be difficult because of their pleiotropic effects leading to non-specific signaling, their possible toxicity and their short half-lives *in vivo* (Barinaga, 1994b; Verrall, 1994a; Jonhagen, 2000b). In addition, neurotrophic factors have limited diffusion in target tissues, undergo rapid enzymatic degradation and do not cross the blood brain barrier. A possible solution to these limitations is to develop highly selective peptidomimetic ligands of neurotrophic factor receptors.

Peptidomimetic ligands are small, proteolytically stable molecules that mimic the binding of the original ligand and interact specifically with their appropriate receptor acting as agonists or antagonists(Saragovi and Gehring, 2000). The pharmacophore of the mimetic analogues is the active part necessary to ensure optimal interactions with a specific biological target (e.g. neurotrophic factor receptor) and stimulate or antagonize a biological response. The target region of these compounds is called “hot spots” (Saragovi et al., 1992a) (Figure 6). For example, the specific domain of a receptor causing a biological response when in contact with the ligand would be the receptor hot spot. However, hot spots can also be activated through conformational or allosteric changes without direct contact(Saragovi et al., 1992a). Pharmacological advantages of peptidomimetic ligands include lack of immunogenicity, low molecular mass, good pharmacokinetic profiles and high receptor specificity (Saragovi et al., 1992a).

Peptidomimetic ligands of neurotrophins, with high affinity for specific neurotrophin receptors, have been developed. Recently, a novel peptidomimetic agonist of the neurotrophin receptor TrkA (D3) was tested as neuroprotective therapy for degenerating cholinergic neurons in cognitively impaired aged rats. This study demonstrated that treatment with D3 afforded significant and long-lasting protection of cholinergic neurons and resulted in improvement of cognitive ability (Bruno et al., 2004). Therefore, small, proteolytically stable ligands with selective agonist activity have good therapeutic potential for neurodegenerative diseases. The evaluation of the efficacy of this type of compounds in experimental models of RGC death, including glaucoma and other optic neuropathies, is an important area of future investigation.

7. Anti-apoptotic signaling

There is now little doubt that apoptosis is a central mechanism of RGC death in glaucoma(Garcia-Valenzuela et al., 1995b; Quigley et al., 1995a; Nickells, 1996; Kerrigan et al., 1997; Hosking, 1998a; Nickells, 1999; Quigley, 1999b). Apoptosis involves the activation of a genetic program and *de novo* synthesis of proteins that play crucial roles during this process. A major class of intracellular regulators of apoptosis is the Bcl-2 family of proteins, which is evolutionarily conserved from worms to humans(Adams and Cory, 1998). Several Bcl-2 family members have been identified in mammals: some that inhibit apoptosis (e.g. Bcl-2, Bcl-XL) and others that promote it (e.g. Bax, Bad and Bid). Bcl-2 proteins have a hydrophobic tail that enables them to bind to the outside surface of the mitochondria and, therefore, they function mainly on these organelles(Tatton et al., 2001). For example, Bcl-2 can inhibit apoptosis by blocking the release of cytochrome C from the mitochondria and consequent activation of proteases involved in the dismantling of the cell (Figure 7). Consistent with this, Bcl-XL overexpression has been shown to protect RGCs following optic nerve transection(Malik et al., 2005). Bax, on the other hand, acts as a pro-apoptotic molecule by binding to mitochondria, where it can increase membrane permeability and cytochrome C release. A recent study demonstrated that RGCs are preserved in Bax deficient DBA/2J mice, a model of inherited glaucoma, indicating that BAX is required for RGC death in this model (Libby et al., 2005). Interestingly, however, the same study showed that lack of BAX did not prevent axonal degeneration.

Caspases are a family of proteases that execute the dismantling and demolition of cells undergoing apoptosis. Caspases 8 and 9 have been shown to be activated in experimental glaucoma(Hanninen et al., 2002b; McKinnon et al., 2002b). Suppression of apoptosis using caspase inhibitors is an approach that has been explored with modest success (Bilsland and Harper, 2002). In adult rats, active caspase immunoreactivity has been shown to increase in the retina after optic nerve transection and in glaucoma (Hanninen et al., 2002b; McKinnon et al., 2002b). In addition, axotomy-induced RGC death is reduced by intraocular administration of specific caspase inhibitors (Kermer et al., 1998b; Chaudhary et al., 1999b; Kermer et al., 2000c).

Inhibitors of apoptosis proteins (IAPs) are potent regulators of apoptotic cell death from baculovirus to humans(Clem and Miller, 1994; Hay et al., 1995; Rothe et al.,

1995; Roy et al., 1995a; Duckett et al., 1996; Uren et al., 1996). In mammals, there are several members of the IAP family: the X-linked inhibitor of apoptosis protein (XIAP), neuronal apoptosis-inhibitory protein (NAIP), human inhibitor of apoptosis protein (HIAP), cIAP 1 and 2 and survivin (Deveraux et al., 1997b) (Deveraux et al., 1998) (Roy et al., 1995b) (Tamm et al., 1998). IAPs are defined by the BIR (baculovirus IAP repeat), which is critical for apoptosis inhibition (Takahashi et al., 1998b). IAP can participate in the pro-survival signaling triggered by neurotrophins (Kaplan and Miller, 2000a). The human XIAP, cIAP-1, and cIAP-2 proteins can also bind to and directly inhibit specific caspases, including caspases-3, -7, and -9 (Roy et al., 1997) (Deveraux et al., 1997b) (Deveraux et al., 1998). Interestingly, adenoviral-mediated delivery of XIAP to the axotomized rat optic nerve has been shown to delay RGC death after axotomy (Kugler et al., 2000)' (Straten et al., 2002). Furthermore, overexpression of BIRC4 in RGCs by means of an AAV viral vector promoted RGC axon protection in a chronic ocular hypertension model of rat glaucoma (McKinnon et al., 2002c). Mitochondrial dysfunction, which marks the point of no return during apoptosis, occurs even in the presence of caspase inhibition leading to impairment in ATP production and increased levels of reactive oxygen species (Chang et al., 2002). Thus, strategies that halt the commitment to die prior to irreversible mitochondrial dysfunction are likely to contribute to better functional outcome.

8. Summary and future directions

Neurotrophic factors such as BDNF, CNTF, FGF-2 and GDNF, have been widely investigated for their ability to promote survival of injured RGCs in several models of optic nerve injury. These factors exert their biological function by binding to high-affinity, trans-membrane receptors that, in turn, lead to activation of neuroprotective signaling pathways within these neurons. In spite of their clear neuroprotective effect, the use of exogenous neurotrophic factors may entail limitations including inadequate diffusion in target tissue, short half-life, inability to cross blood-retinal barrier, and non-specific or adverse effects. Because of these restrictions, there has been great interest in identifying the specific intracellular signaling components, activated by neurotrophic factors, which stimulate RGC survival and regeneration. Although this field is still in its infancy, a desirable goal is to selectively activate a neuroprotective signaling pathway in

RGCs, therefore bypassing the use of diffusible neurotrophic factors. The pursuit of this goal will go hand in hand with the development of novel compounds, small molecules and delivery vectors that will enhance receptor activation specificity and targeted stimulation of neuroprotective signaling components. Although there is now little doubt that RGC death in glaucoma occurs by apoptosis, the precise molecular mechanisms that lead to RGC loss remain undefined. Therefore, the elucidation of the intracellular signaling pathways that regulate RGC survival and death will be paramount for the design of effective neuroprotective strategies applicable to glaucoma and other optic neuropathies.

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11. Figure legends

Figure 1. In the "neurotrophic factor hypothesis" neurons compete for limited amounts of target-derived neurotrophic factors that are required for survival. Neurons that successfully compete for these factors survive, whereas less competitive neurons die. In glaucoma, neurotrophic deprivation caused by obstruction of the axonal transport may be involved in RGC death.

Figure 2. Local sources of endogenous neurotrophic factors within the retina may play a prominent role in adult RGC survival. Diffusible factors secreted by supporting Müller glia or retinal pigment epithelium (RPE) may act in paracrine fashion to promote the survival of injured RGCs. Factors secreted by retinal neurons, including RGCs, may act in autocrine or paracrine fashion to stimulate neuronal survival after injury. PS: photoreceptor segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; FL: fiber layer.

Figure 3. The biological effects of neurotrophins are mediated by Trk receptors: TrkA is the receptor for NGF; TrkB is the receptor for BDNF and NT-4; and TrkC is the receptor for NT-3. The p75 receptor ($p75^{NTR}$), related to the tumor necrosis factor receptor, binds all the neurotrophins with equal affinity.

Figure 4. Upon binding to TrkB, BDNF stimulates receptor autophosphorylation and activation of multiple signaling pathways, including the extracellular signal-regulated kinases 1/2 (Erk1/2) and the phosphatidylinositol-3 kinase (PI3K)/Akt pathways. These pathways lead to transcriptional activation of pro-survival genes.

Figure 5. Signal transduction by CNTF requires binding of this neurotrophic factor to the GPI-linked receptor subunit CNTFRα, followed by the recruitment of LIF and gp130. This complex activates downstream signaling components including the JAK/STAT3 pathway.

Figure 6. Receptor activation by neurotrophic factors occurs by contact of a specific ligand domain with the receptor 'hot spot', rather than by interaction of the whole protein surface with the binding site. Small peptidomimetic ligands can mimic the region of the protein needed to activate the receptor hot spot.

Figure 7. Pro-survival Bcl-2 family members can inhibit apoptosis by blocking the release of cytochrome C from the mitochondria and consequent activation of proteases involved in the dismantling of the cell.

FIGURE 1

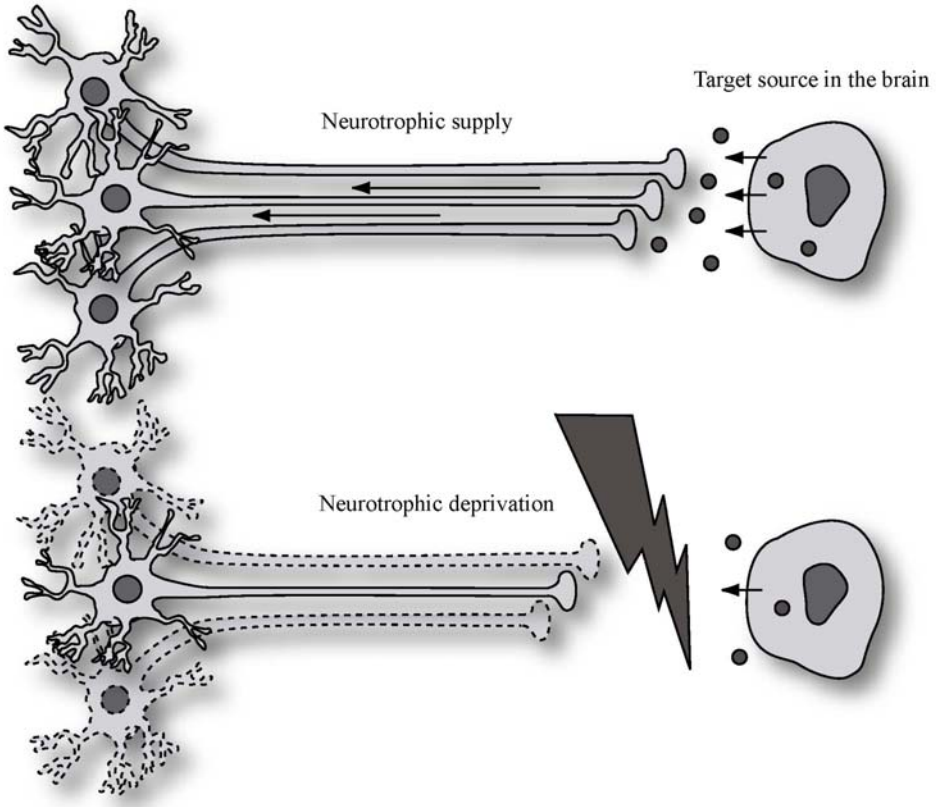

Target source in the brain
Neurotrophic supply
Neurotrophic deprivation

FIGURE 2

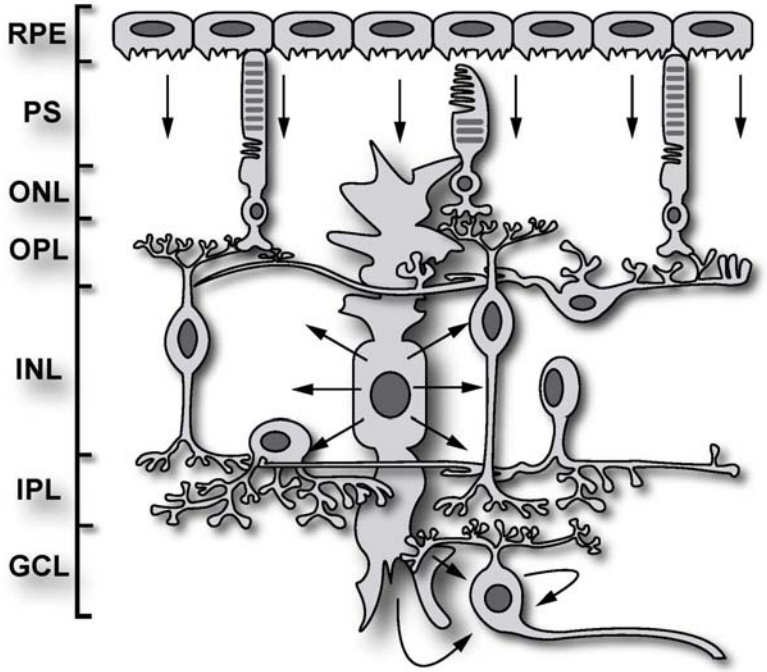


FIGURE 3

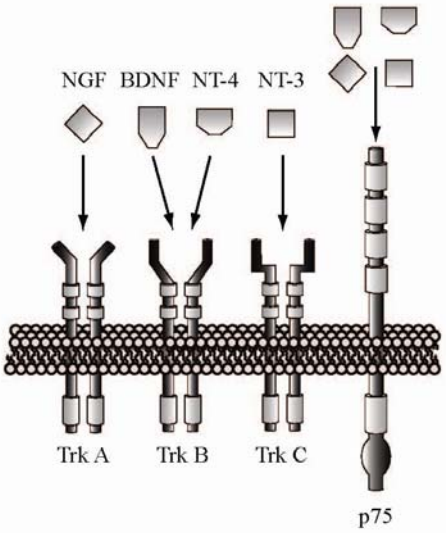


FIGURE 4

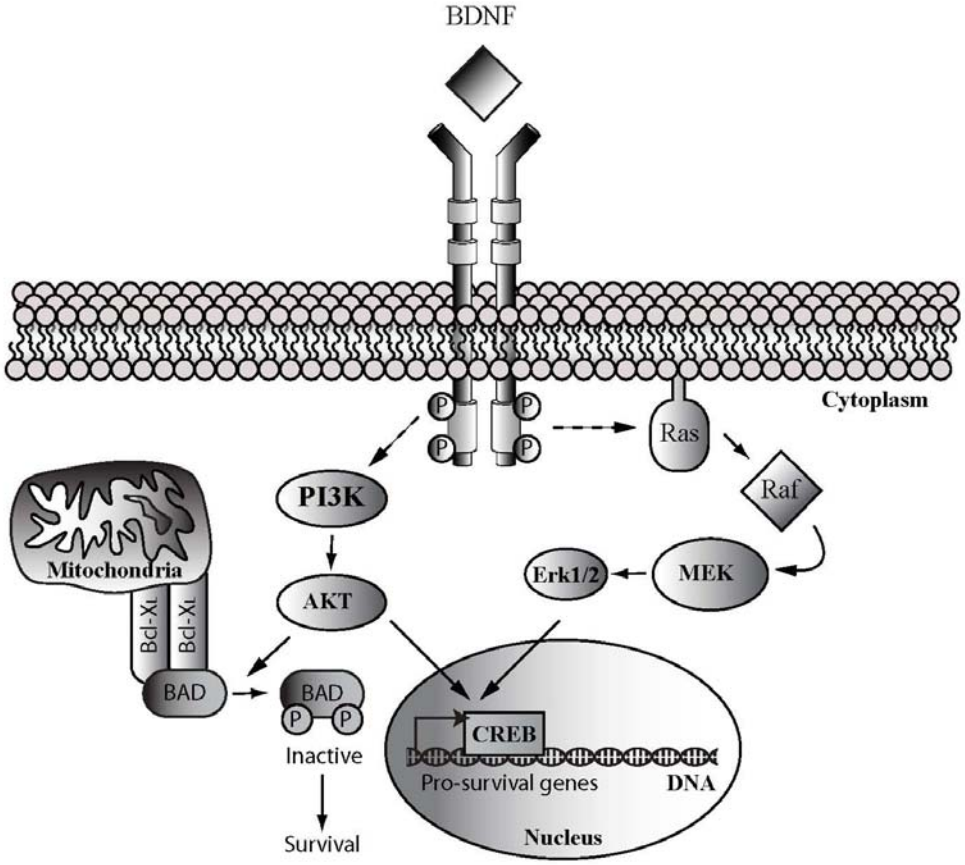

BDNF
Cytoplasm
P
P
P
P
Ras
Raf
PI3K
Mitochondria
Erk1/2
MEK
AKT
Bcl-Xl
Bcl-Xl
BAD
BAD
P
P
Inactive
CREB
Pro-survival genes
DNA
Nucleus
Survival

FIGURE 5

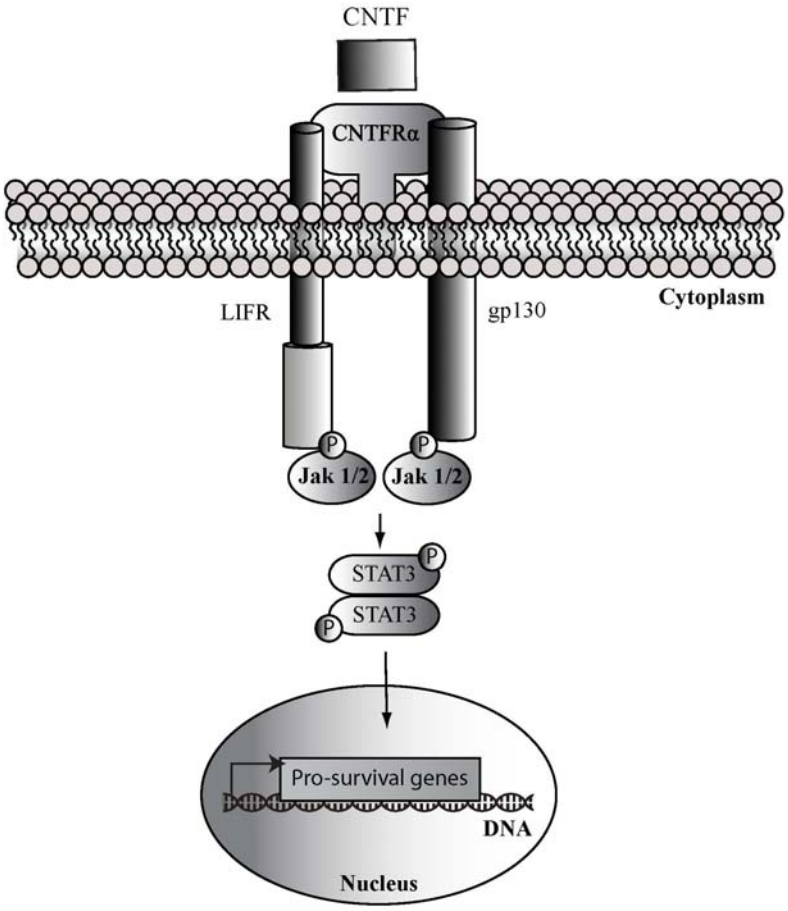

CNTF
CNTFRα
LIFR
gp130
Cytoplasm
P
Jak 1/2
P
Jak 1/2
STAT3
P
STAT3
P
Pro-survival genes
DNA
Nucleus

FIGURE 6

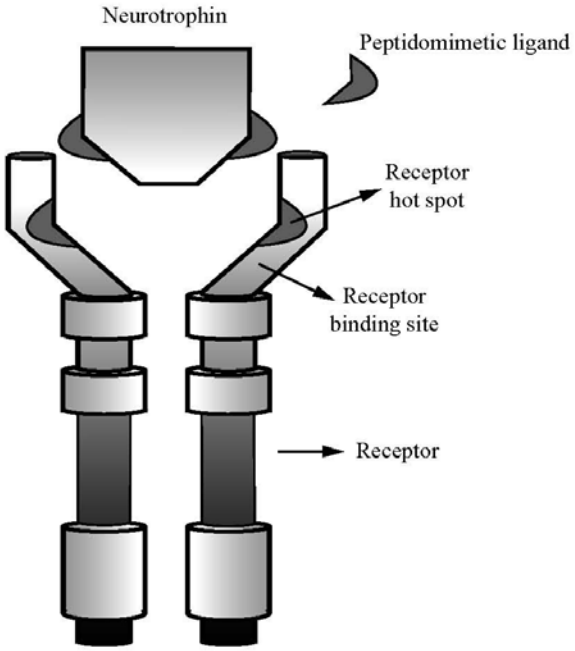
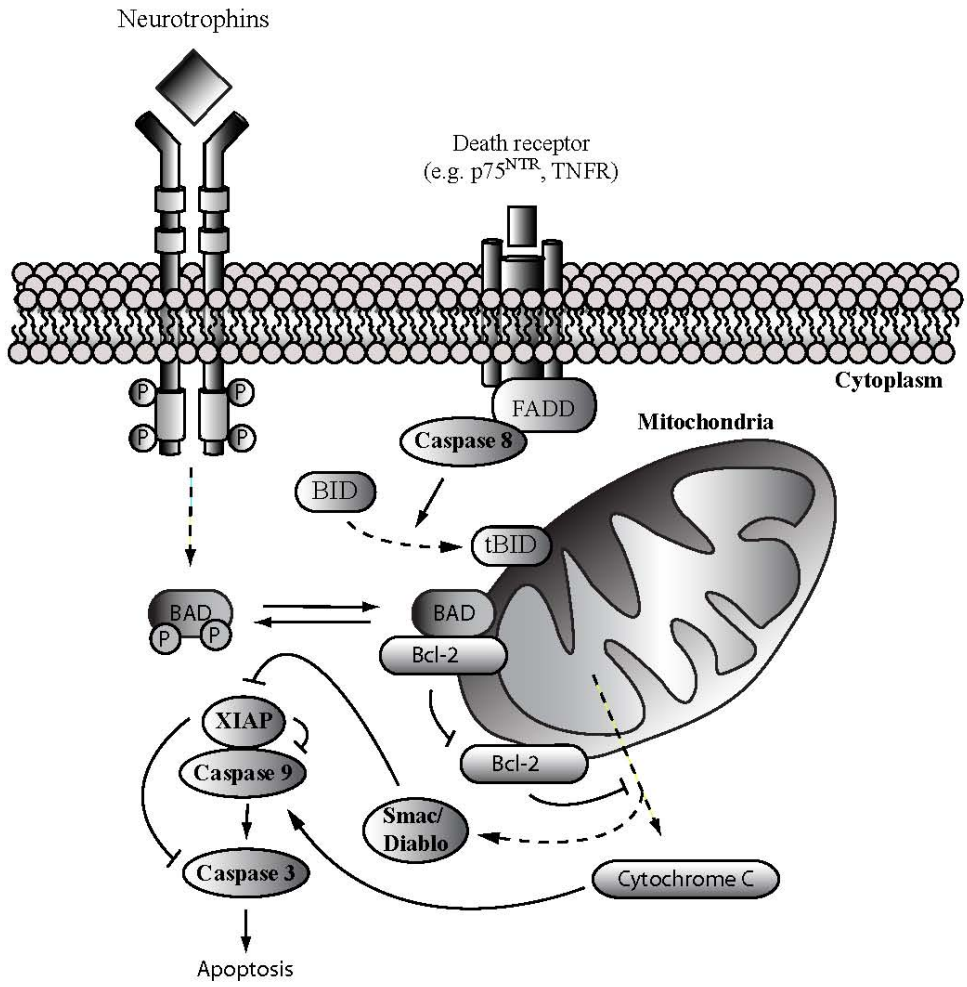

Neurotrophin
Peptidomimetic ligand
Receptor
hot spot
Receptor
binding site
Receptor

FIGURE 7


Neurotrophins
Death receptor
(e.g. p75NTR, TNFR)
Cytoplasm
FADD
Caspase 8
Mitochondria
BID
tBID
BAD
P
P
BAD
Bcl-2
XIAP
Caspase 9
Bcl-2
Smac/
Diablo
Caspase 3
Cytochrome C
Apoptosis

ANNEXE C

CONTRIBUTIONS AUX ARTICLES

Pour l'article "**Inhibition of p75(NTR) in glia potentiates TrkA-mediated survival of injured retinal ganglion cells"** (Chapitre 2 de la thèse)**,** j'ai réalisé la totalité des expériences à l'exception des axotomies sur les souris $p75^{NTR}$ *knockout* et de quelques immunobuvardages. Le Dr. Saragovi a fournit les composés peptidomimétiques ayant été utilisés dans cette article. J'ai analysé l'ensemble des données et j'ai rédigé l'article en collaboration avec ma directrice de recherche, la Dre Adriana Di Polo.

Pour l'article **"ProNGF Induces TNFα-Dependent Death of Retinal Ganglion Cells Through a p75NTR Non-Cell-Autonomous Signaling Pathway"** (Chapitre 3 de la thèse), j'ai réalisé la totalité des manipulations expérimentales à l'exception des immunobuvardages. J'ai participé à l'analyse des donnés et à la rédaction du manuscrit en collaboration avec le Dr. Philip A. Barker, le Dr. Mathieu Bertrand et ma directrice de recherche, la Dre Adriana Di Polo.

Pour l'article "**Excitotoxic death of retinal neurons in vivo occurs via a non-cell-autonomous mechanism"** (Chapitre 2 de la thèse), j'ai réalisé la grande majorité des manipulations expérimentales en collaboration avec la Dre. Laure Duplan. Quelques chirurgies ont été faites par d'anciens membres du laboratoire (Pernet, V. et Sapieha, P.). De plus, plusieurs expériences ont été le fruit de quelques collaborations extérieures. Les expériences de détections des récepteurs AMPA par coloration au cobalt ont été faites en collaboration avec le Dr. Bowie et son étudiante (Osswald I.). J'ai participé à l'analyse des donnés et à la rédaction du manuscrit en collaboration avec la Dre. Laure Duplan, le Dr. Philip A. Barker et ma directrice de recherche, la Dre Adriana Di Polo.

ANNEXE D

Autorisation des co-auteurs des articles