Growth Hormone and Synaptogenesis in the Chick Retina

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Abstract

In the chicken embryo, GH gene expression occurs in the neural retina, where it promotes cell survival and induces axonal growth of retinal ganglion cells (RGCs). Neuroretinal GH is therefore of functional importance before the appearance of somatotrophs and the onset of pituitary GH secretion to the peripheral plasma which occurs between embryonic days (ED) 15-17. In this present study we provide evidence that GH is able to promote synaptogenesis in the embryonic chick neuroretina by increasing the expression of SNAP25, PSD95, and GAP43. We have also investigated its effects in the neonatal chick through the use of a kainate treatment model to study GHs effects on damaged retinal synapses. In response to damage, an increase in fluorescently labelled Cy3-GH internalization into RGCs was observed which was correlated with an increase in BDNF and PSD95 expression, suggesting a neuroprotective effect on the dendritic trees of RGCs the inner plexiform layer (IPL). In addition, we observed the presence of PSD95 positive Muller glia, which may suggest GH is having a neuroregenerative effect in damaged retinas. This work puts forth further evidence that GH acts as a synaptogenic modulator in the chick retina and opens a new possibility for the use of GH in retinal regeneration research.

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List of Abbreviations

BBB	Blood-brain barrier
BDNF	Brain derived neurotrophic factor
CNS	Central nervous system
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
Cy3	Cyanine 3
ED	Embryonic day
ERKs	Extracellular-signal-related kinases
FGF2	Fibroblast growth factor 2
GAP43	Growth associated protein 43
GCL	Ganglion cell layer
GDNF	glial cell line derived neurotrophic factor
GH	Growth hormone
GHR	Growth hormone receptor
IGF-1	Insulin-like growth factor
INL	Inner nuclear layer
IPL	Inner plexiform layer
JAK	Janus kinase
KA	Kainic acid
MAPK	MAP kinase

MGPC	Muller glia-derived progenitor cell
NGF	Nerve growth factor
NR	Neuroretina
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
OFL	Optical fiber layer
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OT	Optic tectum
PCR	Polymerase chain reaction
PKC	Protein kinase 3
PSD95	Postsynaptic density protein 95
QNR/D	Quail neuroretina derived
rcGH	Recombinant chicken growth hormone
RGC	Retinal ganglion cell
RPE	Retinal pigmented epithelium
scGH	Small chicken growth hormone
SH2	Src homology 2
SNAP25	Synaptosomal associated protein 25
SOC2	Suppressor of cytokine signalling 2
STAT	Signal transducer and activator of transcription
Trks	Tyrosine kinases

Chapter One

Literature Review

1.1 Extrapituitary GH

1.1.1 Overview

The classical model of growth hormone (GH) action, where circulating GH is produced and released from the pituitary, then acts on its receptor in the liver to induce insulin-like growth factor 1 (IGF-1) synthesis and release, which in turn mediates the somatic effects of GH on cell proliferation and differentiation, has changed in recent years (Isaksson et al., 2001). GH-induced IGF-1 expression in the liver is now is largely considered to function as negative feedback for pituitary GH secretion (Sjögren et al., 2002). The somatic effects of GH are now thought to be directly mediated at the target tissue or from the tissue-specific induction of local IGF-1 expression which then acts in an autocrine/paracrine manner (Hull and Harvey, 2014; Radosavljević et al., 2005).

Furthermore, the widespread expression of GH at extrapituitary sites, independent of pituitary synthesis, has been documented in a large number of different tissues and models including humans (Harvey, 2010a; Harvey et al., 2015; Luna et al., 2014; Pérez-Ibave et al., 2014). The distribution and functions of extrapituitary GH been extensively reviewed (Harvey, 2010). Recently, studies have provided an increased emphasis on the functional importance of extrapituitary GH, particularly during the early stages of development where it may act locally in an autocrine/paracrine manner (Harvey, 2010a; Harvey et al., 2009a). Given that the expression of GH in many of these tissues is seen prior to the ontogeny of the somatotrophs in the pituitary (Harvey et al., 1979), it is likely that extrapituitary GH plays a key developmental role during the early embryonic stages of growth (Harvey et al., 2000).

1.1.2 Distribution

The presence of GH in the nervous system is now well established. While endocrine GH has been reported to cross the blood-brain barrier (BBB) it is not the only source of GH in neural tissue (Pan et al., 2005). The central nervous system (CNS) is commonly considered to be a site of GH synthesis (Harvey, 2010a). GH mRNA has been found in the lateral hypothalamus (Yoshizato et al., 1998) and hippocampus of the adult rat where its expression may be regulated in part by estrogen (Donahue et al., 2006). GH mRNA expression is also present in the hippocampus of Ames mice (Sun et al., 2005) and in the brain of adult trout (Harvey, 2010a). GH immunoreactivity has also been shown to be present in the brains of both rats and Ames mice (Harvey, 2010a). The presence of GH in human cerebrospinal fluid (CSF) has been reported in addition to the expression of GH receptor (GHR) in areas of the human CNS such as the choroid plexus, hippocampus, hypothalamus, and spinal chord (Pérez-Ibave et al., 2014). Presence of GH in the chick nervous system is also well established. Intense staining of the neural tube from which the nervous system develops has been observed from embryonic day three (ED3) throughout neural development (Harvey, 2010a). At ED 14, GH expression in the developing brain is no longer widespread, however it is still present in the molecular and pyramidal layers of the cerebral cortex, in the gray matter of the cerebellum, in the choroid plexus and in the walls of the ventricles, as well as in the pineal gland (Harvey, 2010a). GH has also been detected in cerebellar cells of the chicken (Alba-Betancourt et al., 2011).

GH and GH receptor (GHR) immunoreactivity have been found in many immune tissues including the thymus, spleen, tonsils, lymph nodes, and lymphocytes (Harvey, 2010a). GH immunoreactivity is present in the chicken spleen, bursa of Fabricius, and thymus (Luna et al., 2005). Although this immunoreactivity is mainly associated with a 17kDa moiety (Luna et al.,

2005). GH and GHR mRNA are also present in these tissues, suggesting an autocrine/paracrine route of action (Harvey, 2010a). The human immune system in generally considered to be an extrapituitary site of GH production. GH and GHR are found in the thymus, spleen, tonsil, lymph node, thymoma, lymphomas, and normal T and B lymphocytes (Pérez-Ibave et al., 2014).

Extrapituitary GH has been implicated in reproductive function and is thought to act in an autocrine/paracrine manner. GHR are present in the granular and stromal cells of the uterus, as well as the decidua, and myometrium as well as in testicular tissues and the prostate of the mammalian reproductive system (Harvey, 2010a). Mammary tissues are a site of GH production and function as they also produce GHR. In chicken, GH immunoreactivity is present in the stroma of the ovary and in large and small follicles, where it occurs in a higher concentration in granulosa cells than thecal cells (Harvey, 2010a).

1.1.3 Roles of Extrapituitary GH

GHs action in neural tissues include neuroprotection, neural proliferation, and differentiation which are all thought to be particularly important during development (Harvey and Hull, 2003). GH has been previously shown to increase in cortical pyramidal neurons after focal hypoxic-ischemic injury suggesting a possible neuroprotective role in these cells (Scheepens et al., 2001). Indeed, GHR immunoreactivity has been shown to be increased after ischemic injury to the juvenile rat subventricular zone suggesting the presence of a local endogenous response to damage (Christophidis et al., 2009). This neuroprotective effect of GH is also present in chickens, where GH has also been shown to protect cells of the cerebellum from hypoxic-ischemic injury (Alba-Betancourt et al., 2013). In addition to its neuroprotective effects, GH likely promotes axon growth and has been shown to increase the number and length of neurite outgrowths in cultured N1E-115

neuroblastoma cells (Grimbly et al., 2009). GH is also thought to be involved with neurogenesis in the adult rat hippocampus where it is involved in learning and memory (Harvey, 2010a). Blocking of locally produced GH has been shown to reduce the proliferation and survival of progenitor cells in the subgranular zone of the mouse (Devesa et al., 2014). In addition, GH may be involved with stem cell proliferation after injury as GH treatment has been shown to increase the proliferation of progenitor cells in the rat hippocampus after kainic acid (KA) induced injury (Devesa et al., 2011). Many of the neural functions of GH are conserved in humans as both GH and prolactin promote the proliferation of neural stem cells derived from fetal human forebrains (Pathipati et al., 2011). A relative GH deficiency is often found in the CSF of multiple sclerosis patients, which may indicate that GH is involved in myelination (Pérez-Ibave et al., 2014). Indeed exogenous GH may aid in recovery from other neural diseases and has been used previously as a treatment for traumatic brain injury (Arce et al., 2013).

In the immune system, immunoneutralization of GH was shown to reduce lymphocyte proliferation as well as decrease IGF-1 synthesis (Harvey, 2010a). GH is thought to act in these tissues to promote proliferation and cell survival through the Akt pathway, the transcription factor nuclear factor-kappa b, and through c-Myc and cyclin proteins (Harvey, 2010a). GH is thought to act in an autocrine/paracrine role in the human immune system, where it plays an immunomodulatory role (Pérez-Ibave et al., 2014). In thymocytes GH also promotes the synthesis of IGF-1, which may mediate some of GHs effects in human immune tissue (Pérez-Ibave et al., 2014).

In the reproductive system, GH may act directly on the oocyte to induce its maturation, since GHR mRNA has been detected in the oocytes of many species (Hull and Harvey, 2014). GH promotes uterine growth and may aid in implantation (Hull and Harvey, 2014). GH has also been

implicated in the etiology of uterine and cervical cancers. Indeed, it is thought that aberrant autocrine GH/IGF-1 signalling may promote the formation and progression of certain breast cancers (Waters and Barclay, 2007). GH is involved in lactation from mammary tissues (Hull and Harvey, 2014). In humans, exogenous GH stimulates testosterone production in panhypopituitary patients, and thus it is likely it may function to modulate testosterone biosynthesis and spermatogenesis under normal conditions (Hull and Harvey, 2014; Pérez-Ibave et al., 2014).

GH and GHR has also been shown to be produced in many other sites including, hepatic, pancreatic, salivary, skeletal, muscle, lung, and dermal tissues, where autocrine GH has been implicated in their proper function (Harvey, 2010a). GH is thought to be wildly expressed during development and to be important to proliferation and differentiation of many cells types (Harvey, 2013). Additionally, GH and GHR are expressed in many cancers where its aberrant signalling is thought to drive tumor progression (Harvey, 2010a; Waters and Barclay, 2007).

1.2 GHR

1.2.1 Structure and Function

GHR is a type 1 cytokine receptor which primarily utilizes the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway. A number of other growth factors utilize this signalling mechanism, inducing prolactin, erythropoietin, interleukin 1, 3 and 5, interferon-γ, thrombopoietin, and leptin (Waters and Brooks, 2015). There is therefore a vast number of physiological processes, from a number of growth factors, which are regulated by this signalling mechanism, from lactation to stem cell proliferation.

The GHR monomer is a single pass transmembrane receptor that contains both a high affinity as well as a low affinity GH binding site in its extracellular domain (Brooks et al., 2008). Activation of GHR and signal transduction results from the binding of one GH molecule to the high affinity site on one receptor and the low affinity site on another, resulting in receptor dimerization (Waters and Brooks, 2015). Though GHR may also exist as a dimer on the cell surface in the absence of GH, this dimer is inactive in the absence of a bound GH molecule. Upon GH binding to is receptor, a rotational change in dimer orientation occurs which allows for signal transduction to occur (Brown et al., 2005).

GHR is devoid of enzymatic activity, and therefore signal transduction must utilize external kinases, such as JAK2, which associates with the intracellular domain of the receptor. GH binding induces a rotational change in GHR which results in the transphosphorylation of the associated GHR receptor by JAK2 (Birzniece et al., 2009). The phosphorylation of GHR provides a docking site for Src homology 2 (SH2) domain proteins, such as STAT5 (Brooks et al., 2008). STAT proteins are then phosphorylated by JAK2 which allows for homo- or hetero-dimerization of the protein, translocation into the nucleus, and its binding to STAT responsive elements which result in initiation transcription (Brooks et al., 2008).

GHR is also thought to signal using a number of other signalling pathways, including Akt, cytosolic tyrosine kinases (Trks), cAMP response element binding protein (CREB), MAP kinases (MAPKs), and extracellular-signal-related kinases (ERKs), allowing for the diverse range of physiological processes which are regulated by GH (Birzniece et al., 2009; Sanders et al., 2009a). Though JAK2 is responsible for the majority of STAT and Akt activation, GHR is capable of utilizing other Src proteins for signal transduction (Barclay et al., 2010). For example, GHs activation of the ERK pathways is thought to be independent of JAK2 activity as its GH dependant

upregulation is not downregulated in a non-functional JAK2 knock-in mouse model (Barclay et al., 2010).

There are several mechanisms for the downregulation of GH signalling, however the main pathway is through the induction of suppressor of cytokine signalling 2 (SOCS2) (Brooks et al., 2008). SOCS2 is a STAT5 regulated protein which provides feedback inhibition for GH signalling. Activated STAT5 binds to the promoter for SOCS2, which in turn induces its expression. SOCS2 then binds to the phosphorylated GHR molecule where it inhibits JAK2 and STAT5 phosphorylation and activation (Vesterlund et al., 2011). SOCS2 also regulates cellular GHR levels through the ubiquitin ligase complex, where it directly ubiquitinates GHR, targeting it for proteasomal degradation (Vesterlund et al., 2011).

1.2.2 Autocrine GH and GHR

When GH and GHR are expressed in the same cell, GH binds to GHR in the endoplasmic reticulum (ER), where it may aid in receptor maturation and dimerization (van den Eijnden and Strous, 2007). Mature GHR, which arrives at the cell surface does so with a GH molecule already bound, blocking endocrine GH from binding to the receptor. Internalization and degradation of the GH-GHR complex occurs at a higher rate when bound compared to unbound receptor (van den Eijnden and Strous, 2007). GHR also acts to sequester autocrine GH which would normally be packaged into secretory granules for storage or release (Martinez-Moreno et al., 2015; van den Eijnden and Strous, 2007). In some cancer cells, the overexpression of autocrine GH may result in the sequestration of GHR in the golgi and ER. This limits the possible effectiveness of exogenous

GHR antagonists as a therapeutic option for some GH overexpressing cancers (Nakonechnaya et al., 2013).

Autocrine GH can activate traditional GH signalling pathways, however only when at the cell surface (van den Eijnden and Strous, 2007). It has been suggested that autocrine GH may also activate different signalling pathways inside the cell which may result from sustained activation (Perry et al., 2006). Given that endocrine GH is released from the pituitary in a pulsatile manner (Hull and Harvey, 2014), autocrine GH may regulate the cells sensitivity to endocrine GH and provide a sustained source of GHR receptor activation to promote cell function under normal physiological conditions (Sanders et al., 2009a).

Though the majority of GHR is located in the ER and cell membrane, there is also a nuclear localization of GHR in many cells (Brooks et al., 2008). Nuclear GHR receptor is thought to be a common feature in tissues which are highly proliferative as is thus found in many cancer cells (Conway-Campbell et al., 2007). The expression of autocrine GH is thought to activate nuclear GHR and promote tumorigenesis in many cancer cells (Brooks et al., 2008; Waters and Barclay, 2007).

1.3 Retinal GH

1.3.1 Distribution and Ontogeny

The expression of extrapituitary GH has been widely reported in the visual system of many animals including fish, amphibians, reptiles, birds, rats, mice, bovine, and humans (Ávila-Mendoza et al., 2016; Harvey et al., 2007; Pérez-Ibave et al., 2014). The expression and function of extrapituitary GH in the chick visual system has been mostly studied during embryogenesis.

GH immunoreactivity is detectable in the optic cup of the developing chick as early as ED2 (Harvey et al., 2000). As endocrine GH is not detectable in the serum of the chick until ED17, any GH immunoreactivity detected prior is due to local expression rather than sequestration of endocrine GH (Harvey et al., 1979). GH expression in the retina is often accompanied by staining for GHR which suggests an autocrine/paracrine mechanism of action (Baudet et al., 2009a; Harvey et al., 2000). By ED7, GH immunoreactivity is present throughout the neural retina as well as in the retinal pigmented epithelium (RPE), choroid, sclera, vitreous, cornea, and lens epithelium (Baudet et al., 2003; Harvey et al., 2007). GH and GHR immunoreactivity have been reported in developing retinal ganglion cells (RGCs) at ED7, with staining present in the optical fiber layer (OFL), optic nerve, optic chiasm, and optic tract (Baudet et al., 2007). As development progresses, GH staining appears in both the inner nuclear layer (INL) as well as the inner plexiform layer (IPL) (Harvey et al., 2007). GH is also detectable in the vitreous after secretion from RGCs, where it forms a complex of 60 to 62kDa with the binding protein opticin (Sanders et al., 2003).

GH and GHR staining have also been detected in *in vitro* models. Quail neural retina derived cells (QNR/D, ED7 quail embryonic RGC line) express both GH and GHR (Martínez-Moreno et al., 2014). Additionally, immunopanned RGCs from ED7 chick embryos have been shown to express both GH and GHR (Baudet et al., 2009).

GH and GHR receptor expression has been detected in the neural retina of fetal (ED17), newborn, and adult rats (Harvey et al., 2004). GH mRNA is present in both the newborn and adult mouse, particularly in RGCs of the newborn and in the outer nuclear layer (ONL) and INL of the adult mouse (Harvey et al., 2007). Immunostaining of the adult mouse neuroretina also shows GH expression throughout the neuroretina, though sparsely in the ONL (Harvey et al., 2007). In

humans, 22kDa GH is detectable in the vitreous (Harvey et al., 2009b) and GH immunostaining is present in RGCs (Sanders et al., 2009b).

GH mRNA expression in chick retinal and whole eye extracts share a 100% sequence identity to full-length pituitary GH mRNA (Baudet et al., 2003). However, the translated 24kDa GH monomer is rapidly degraded to a 15kDa moiety in retinal tissues (Harvey, 2010a). In addition, exogenous GH is internalized into RGCs at ED10 in a perinuclear location and is also rapidly degraded into a 15kDa variant (Fleming et al., 2016). This likely reflects the fate of endocrine GH, post somatotroph development as GH has been reported to cross the blood-retinal-barrier (Fleming et al., 2016; Pan et al., 2005).

A small 16kDa variant is also present in the chicken retina (scGH), which is derived from a truncated GH mRNA (Takeuchi et al., 2001). Its presence is detectable throughout the retina except in the OFL, optic nerve head, and the vitreous, as it lacks the signal peptide required for secretion (Harvey, 2010a). It lacks essential residues for GHR binding and is normally dimerized to form a 31kDa protein (Baudet et al., 2007).

1.3.2 Actions of Retinal GH

GH is often co-localized with GHR receptor in the visual system, suggesting an autocrine/paracrine mechanism of action (Baudet et al., 2009a; Harvey et al., 2007). The expression of GH in the ED7 retina is associated with the expression of GHGR-1, a GH responsive gene (Baudet et al., 2007). It is likely that GH plays an important role in RGC differentiation and development as its expression in the ganglion cell layer (GCL) or OFL correlates to a critical time during their respective development (Baudet et al., 2007; Thanos and Mey, 2001). This has been demonstrated *in vitro* where GH was shown to promote axon growth in immunopanned RGCs

(Baudet et al., 2009a). As well, GH and IGF-1 are thought to have a proliferative effect in the developing neuroretina, as their expression during in the fish development is thought to be involved in progenitor cell proliferation (Harvey et al., 2007).

GHs neuroprotective effects in the neuroretina are well documented. GH has been shown to have a neuroprotective effect on RGCs during developmental waves of apoptosis in the chick retina (Harvey et al., 2009a). Local GH expression is important for retinal cell survival and maintenance, as evidenced through siRNA knockdown of local GH, which promotes the formation of apoptotic cells *in vivo* (Sanders et al., 2011). Knockdown of GH in QNR/D cells also results in an increase in apoptotic cells (Sanders et al., 2010), while exogenous GH was able to protect QNR/D cells against glutamate/BSO induced excitotoxicity (Martínez-Moreno et al., 2016). Additionally, the presence of GH in human RGCs has previously been shown to promote their survival (Sanders et al., 2009b).

It is thought that cell death in the neuroretina as a result of GH immunoneutralization is induced through caspase-9 and caspase-3 activation, as well as an increase in PARP-1 activity (Sanders et al., 2008; Sander et al., 2009). Calpain activation is also a major cause of cell death in RGCs (Sanders et al., 2009). GH promotes the survival of RGCs through the upregulation of cAMP and CREB and subsequent activation of Trk and ERK pathways (Sanders et al., 2008; Sander et al., 2009). In addition, GH induced neuroprotection against glutamate/BSO induced excitotoxicity in QNR/D cells was associated with and increase in Bcl-2 expression and STAT5 phosphorylation (Martínez-Moreno et al., 2016).

1.4 Retinal Synaptogenesis

1.4.1 Neuroretinal Anatomy

During development, the retina is derived from the optic vesicle neuroepithelium, which is composed of proliferating retinal neural progenitor cells (Vergara and Canto-Soler, 2012). These progenitor cells give rise to all retinal neurons, which consists of photoreceptor cells, horizonal cells, bipolar cells, amacrine cells and RGCs which are all derived from a common progenitor cell (Nguyen-Ba-Charvet and Chédotal, 2014; Vergara and Canto-Soler, 2012). These cells are contained within the five distinct layers of the retina. The ONL contains the cell bodies of photoreceptor cells; the INL contains cells bodies of horizontal cells, amacrine cells, and bipolar cells; the ganglion cell layer GCL is where cell bodies from RGCs are located as well as displaced amacrine cells. Synapses between these cells occur in two distinct synaptic strata; the outer plexiform layer (OPL) and the IPL. The OPL consists of ribbon synapses between pre-synaptic horizontal cells and photoreceptor cells onto post synaptic bi-polar cells. During proper development, after differentiation, horizontal cells migrate to the outer part of the INL which forms the inner region of the OPL (Nguyen-Ba-Charvet and Chédotal, 2014). The IPL consists of bi-polar and amacrine processes which synapse onto the dendrites of retinal ganglion cells (Nguyen-Ba-Charvet and Chédotal, 2014).

1.4.2 Formation of Retinal Synapses

Synaptogenesis is the process of synapse formation between the axon of a pre-synaptic neuron and a dendrite of a post-synaptic neuron. In the retina, visual signalling begins with the photoreceptor cells and they are essential to the proper formation of the OPL (Specht et al., 2007). Recent electron microscopy data suggests both electrical and conventional synapses in the OPL begin shortly after the formation of the layer, which occurs around ED8 (Drenhause et al., 2007). Bipolar cells and horizontal cells are unable to form synapses in the OPL in the absence of

photoreceptor cells. In mice lacking photoreceptor cells, horizontal cells develop in the ONL instead of the OPL, and bipolar cell dendrites grow along their axons (Specht et al., 2007).

Bipolar cells receive input from photoreceptor cells and project onto RGCs, the output neurons of the retina. Inputs from bipolar cells grow toward the IPL in a guided manner as evidenced by time lapse studies in the zebrafish retina (Mumm et al., 2006). Once guided into place, excitatory synapses between bipolar cells and RGCs occur with even spatial distribution in the retina, with puncta occurring approximately every 1-2 μm in the mouse retina, suggesting synaptogenesis is spatially regulated (Chen and Chiao, 2014). Though the point in development in which synaptogenesis is initiated varies between organisms, in the chick neuroretina synapses onto RGCs from bipolar and amacrine cells begin around ED11/12, reaching adult levels around ED16 (Catsicas et al., 1991). However, more recent transmission electron microscope studies suggest electrical synapses could form as early as ED7 in the IPL with conventional synapses beginning to form around ED 8 (Drenhause et al., 2007).

In addition to bipolar cells, RGCs also receive inputs from amacrine cells, where they contribute the majority of synapses present in the IPL (Maslund 2012; Balasubramanian and Gan, 2014). Inputs from amacrine cells are generally inhibitory, releasing either GABA or glycine (Balasubramanian and Gan, 2014). During development, amacrine and bipolar cells synapse onto RGCs in a coordinated fashion to attain a balance between excitation and inhibition prior to the presence of a visual stimulus (Soto et al., 2011). However, in addition to GABA and glycine, amacrine cells have also release an array of different transmitters and neuromodulators such as acetylcholine (Lee et al., 2010) and dopamine (Hirasawa et al., 2012). While it is thought that dopamine plays a more neuromodulatory role, cholinergic inputs from starburst amacrine cells may have a neuroprotective function in RGCs (Mata et al., 2015; Xiao et al., 2014).

In chicks, axons from RGCs project to the optic tectum which is located in the dorsal mesencephalon (Baudet et al., 2007). These projections reach their target site and synapse around ED6 (Thanos and Mey, 2001). The projection of RGCs axons to their target sites requires the presence of signalling a guidance molecules (Goldberg et al., 2002)

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1.4.3 GH and Retinal Synaptogenesis

In the ED7 chicken embryo, GH and GHR immunoreactivity have been reported in the OFL, optic nerve, optic chiasm, and optic tract (Baudet et al., 2007). In addition to GH and its receptor, the presence of GHGR-1 mRNA (a GH responsive gene) was also observed. GH activity in this area corresponds to the time in which RGC axons synapse with their target sites in the optic tectum (Thanos and Mey, 2001). Furthermore, additional results have shown exogenous GH promotes long axon formation in immunopanned RGCs, while the siRNA knockdown of locally produced GH inhibited the formation of long axons (Baudet et al., 2009a). Given that endocrine GH is not detectable in the chicken circulation until ED17 (Harvey et al., 1979), this suggests locally produced GH maybe be involved with RGC axon growth and synaptogenesis during early development.

GH immunoreactivity has been reported in the IPL of the chicken at ED12 (Baudet et al., 2009a). This is recognized as the onset of synaptogenesis in the chick IPL and coincides with the first detection of SNAP25, a presynaptic SNARE protein involved in synaptogenesis and vesicle release (Catsicas et al., 1991). Therefore, it is possible that GH promotes synaptogenesis in the IPL between axons from amacrine and bipolar cells onto RGC dendrites and induces the expression of SNAP25.

1.5 GH, IGF-1, and Neurotrophins

1.5.1 GH and IGF-1

IGF-1 is classically thought of as the mediator of GH action on target tissues. While it is recognized that GH can function independently of IGF-1, the induction of IGF-1 expression by GH in target tissues often make their actions difficult to differentiate from one another. Indeed, IGF-1 and GH share many of the same signalling pathways and thus it is often not possible to elucidate the differential effects of GH and IGF-1 on retinal neurons (Sanders et al., 2008).

In retinal cells, GH has been shown to increase IGF-1 expression in immunopanned RGCs (Baudet et al., 2009a) and QNR/D cells (Martínez-Moreno et al., 2016). GH has been shown to be neuroprotective against glutamate/BSO induced excitotoxicity in QNR/D cells. Since IGF-1 expression was also increased in this system, some of GHs neuroprotective effects may be mediated through IGF-1 (Martínez-Moreno et al., 2016). Indeed GH and IGF-1 share some of the same signalling pathways which are thought to be responsible for neuroprotection in RGCs (Sanders et al., 2008). IGF-1 alone has previously been shown to be neuroprotective of RGCs against hypoxia-induced apoptosis which occurred by activating Akt and ERK pathways (Yang et al., 2013). Both GH and IGF-1 expression are increased in the green iguana after kainate excitotoxic injury, and IGF-1 expression in this system further increased with exogenous GH treatment. This exogenous GH treatment and subsequent increase in IGF-1 was able to prevent cell loss in the INL and GCL caused by kainic acid (KA) injection (Ávila-Mendoza et al., 2016). Outside of the retina, GH increased the expression of IGF-1 in cells of the chicken cerebellum after hypoxic-ischemic injury, which could account for some of its neuroprotective effects (Alba-Betancourt et al., 2013).

IGF-1 has an important role in the development and differentiation of the retina. Block of the IGF-1 receptor during zebrafish embryogenesis causes improper development and retinal defects (Schlueter et al., 2007). In the developed chick retina, IGF-1 co-administered with fibroblast growth factor 2 (FGF2) promotes the formation and proliferation of progenitor cells in the neuroretina, though IGF-1 had no effect when administered alone (Fischer et al., 2002). In addition, IGF-1 may be responsible for some of the proliferative effects GH has on human neural stem cells (Pathipati et al., 2011). IGF-1 is increased in goldfish RGCs after optic nerve injury and is associated with nerve regeneration (Koriyama et al., 2007). IGF-1 may mediate some of GHs effects on proliferation and differentiation found the nervous system.

1.5.2 GH and other neurotrophins

Though IGF-1 is thought to be the main regulator of GH action, many other neurotrophins are expressed in the neuroretina which have neuroprotective and neurotrophic actions. Many of the actions of these neurotrophins mimic the reported actions of GH in neural tissues (Harvey, 2010a; Weber, 2013). While growth factor specific differences may exist, in general, neurotrophins and their receptors within the retina are mainly localized in RGCs as well as in Muller glia (Weber, 2013). Cultured Muller glia cells respond to glutamate stimulation by upregulating brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and glial cell line derived neurotrophic factor (GDNF) secretion, which could be an endogenous neuroprotective response against excitotoxicity since glutamate did not increase cell death (Taylor et al., 2003). Anterograde transport of BDNF and NT-3 has been reported in RGCs from the optic tectum. It is thought that their synaptic connections to their target site in the optic tectum promotes cell survival during developmental apoptosis through the

anterograde transport of neurotrophins (Baudet et al., 2007). NT-3 and BDNF both promote cell survival in the neuroretina via the activation of Akt and ERK pathways (Weber, 2013), in a similar fashion to GH and IGF-1 (Sanders et al., 2009a). GH has been shown to induce neuroprotection in QNR/D cells (Martínez-Moreno et al., 2016) as well as stimulate the expression of NT-3 (Martínez-Moreno et al., 2014) and BDNF has previously been proposed as a mediator of GH action in neuroretinal cells (Sanders et al., 2008).

BDNF may be responsible for neurogenesis during development as well as after injury. The ontogeny of BDNF in the reptilian retina is similar to that of GH in the developing of the chick retina, suggesting it may play a similar role in RGC development (Harvey et al., 2007, Santos et al., 2011). Neuroregeneration of RGC axons in reptiles involves the expression of BDNF and NT-3 (Santos et al., 2011). In the avian retina, several other growth factors have been implicated in neural regeneration after injury through Muller glia cells including FGF2, CTNF, and BMP4 (Fischer et al., 2004, 2002).

1.6 Experimental Rational

1.6.1 Quantification of Synaptogenesis

Synaptogenesis is an incredibly complex process which involves the interaction of many proteins such as neurotransmitter receptors, secondary messengers, intracellular adhesion molecules, scaffold proteins, and vesicular fusion proteins. Thus, the study synaptogenesis from a molecular point of view is often done so through the observation of select markers of synaptogenesis known to be involved with axon growth and/or synaptogenesis.

Growth associated protein of 43kDa (GAP 43) is a presynaptic, growth cone associated protein which is widely used as a marker for axon growth as its expression is essential for neurite growth during development and regeneration (Chen et al., 2015). GAP 43 is phosphorylated by protein kinase C (PKC) at serine residue 41 which allows for its interaction with actin and the stabilization of F actin during neurite extension (Gauthier-Kemper et al., 2014). Mice over expressing GAP43 have shown to have aberrant fiber growth while mice deficient in GAP43 have shown to have behavioral abnormalities, hippocampal structural abnormalities, and impaired axon growth and synaptogenesis (Benowitz and Routtenberg, 1997; Latchney et al., 2014). GAP 43 expression is also thought to be important in neural repair as its expression is upregulated in neuroprotective models of glaucoma (Stankowska et al., 2015).

Synaptosomal protein of 25kDa (SNAP 25) is a SNARE complex protein which is involved with vesicular fusion events, which are required for both neurotransmitter release and axon elongation (Lawrence et al., 2014). The SNARE complex consists of three proteins; SNAP 25 and syntaxin which are both anchored into the cytosolic side of the plasma membrane, and synaptobrevin, which is anchored in the vesicular membrane (Fang and Lindau, 2014). The interaction between synaptobrevin, syntaxin and SNAP 25 allows for the fusion of the vesicular membrane with the plasma membrane (Fang and Lindau, 2014). Cleavage of these SNARE proteins by certain toxins causes an inhibition of neurotransmitter release showing SNAP25s importance at active synapses (Fang and Lindau, 2014). Previous studies have shown SNAP25 expression in the chick neuroretina (NR) where it is thought to be important in the formation of the synapses in the IPL. In addition to synaptogenesis, SNAP 25 has been implicated in axon growth. SNAP 25 is upregulated in regenerating axons and its overexpression is sufficient to

induce an increase in axon branching in SH-SY5Y cells, while its knockdown negatively affected axonal growth (Wang et al., 2012).

Post synaptic density protein of 95kDa (PSD95) is a post synaptic scaffold protein found at excitatory synapses (Okabe, 2007). PSD95 interacts with a number of proteins in the post synaptic bouton to regulate their localization and function and its expression is associated with synaptic development and organisation (Goyer et al., 2015; Sheng and Hoogenraad, 2007). It interacts with both AMPA and NMDA receptors to modulate glutamatergic signalling as well as trans-synaptic signalling molecules, such as neurexin and neuroligin, where it influences synapse development and formation (Han and Kim, 2008). In mouse models, an increase in PSD95 expression has been associated with enhanced memory and neuroplasticity (Jiang et al., 2015). GH has also been shown to increase PSD95 mRNA expression in the hippocampus of hypophysectomised rats which was associated with an improvement in spatial memory (Le Grevès et al., 2006). Although, chicks lack the gene for PSD95 (DLG4), they still contain genes for the three other DLG group proteins, of which DLG1 shares the closest homology in chickens for the mammalian DLG4 gene (PSD95). PSD95 immunoreactivity is found in chick models and DLG1 has previously been used as synaptogenic marker in chicks (Kumar et al., 2014).

1.6.2 Experimental models

Avian models will be used in this project. The benefits of using the chick embryo to study retinal development have been well documented (Vergara and Canto-Soler, 2012). Avian retinal ganglion cells have been used previously as a model to study axonal growth (Baudet et al., 2009a) and synaptogenesis (Wahlin et al., 2010) by our lab, as well as other groups.

QNR/D cells are embryonic RGCs derived from the ED7 quail. Previous work from our lab has shown QNR/D cells to be a valid model for RGC physiology as they are electrically active, and express both GH and GHR, as well as other important retinal markers (Martínez-Moreno et al., 2014). QNR/D cells have been previously used by our lab to study the neuroprotective effects of GH on RGCs (Martínez-Moreno et al., 2016; Sanders et al., 2010). The use of QNR/D cells in conjunction with ED10 primary retina cells cultures allows for a comprehensive study of the synaptogenic effects of GH in embryonic avian retinal cells.

Chick neuroretinal explants have been used previously by our lab as they allow for the *in vitro* study of the effects of GH on an intact retina (Sanders et al., 2005). Additionally, the *ex ovo* culture of ED10 chick embryos allows for the unobstructed injection of GH into the chick eye, and are thus an excellent *in vivo* model for the study GH during retinal development (Jhanji et al., 2011).

Since retinal synaptogenesis in the chick is thought to be complete prior to hatch, the study of the synaptogenic effects of GH in the post-natal chick is limited. A previous study from our group in the green iguana, has shown KA injection to cause cell loss in the neuroretina which was reversed with exogenous GH treatment (Ávila-Mendoza et al., 2016). The use of NMDA (another glutamatergic agonist) to induce retinal damage in the chick has been used previously in studies which focus on retinal regeneration (Hayes et al., 2007; Todd et al., 2016). KA has also been used in prior experiments to damage neuroretinal cells in the chick (Ehrlich et al., 1990). Excitotoxicity in the retina results in synaptic loss (Baltmr et al., 2010) and SNAP25, PSD95, and GAP43 have all previously been shown to be downregulated under excitotoxic conditions (Hung et al., 2014; Kamat et al., 2016). Thus, the intravitreal injection of KA in the post-hatch chick provides us with

a model to determine the neuroprotective/neuroregenerative effect of GH on retinal synapses through the analysis of the synaptogenic markers SNAP25, PSD95, and GAP43.

1.6.3 GH promotes synaptogenesis in the neuroretina of the embryonic chick

GH has established roles in RGCs differentiation. GH is expressed in RGCs of the developing chick retina during their period of differentiation and synaptogenesis with their target sites in the optic tectum (Baudet et al., 2007; Thanos and Mey, 2001). In addition, immunopanned RGCs respond to exogenous GH by increasing axon length, while block of autocrine/paracrine GH with siRNA inhibited long axon formation (Baudet et al., 2009a). The molecular mechanism of GH induced axon elongation has not previously been accessed. GAP43 is a likely candidate as it is essential for neural growth cone formation and axon elongation (Dijk et al., 2007; Gauthier-Kemper et al., 2014). The effect of GH on GAP43 expression was thus analyzed in the present study.

GHs expression in the IPL corresponds with a time of synaptogenesis between amacrine/bipolar cells with RGCs which occurs around ED 11 (Catsicas et al., 1991). This GH expression also corresponds to the onset of synaptosomal protein of 25kDa (SNAP 25) expressions, which is a pre-synaptic SNARE protein thought to be involved in synaptogenesis (Catsicas et al., 1991). Post-synaptically, PSD95 promotes synaptogenesis through stabilization and development of the post-synaptic bouton (Zhu et al., 2016). Increases in pre-synaptic SNAP25 and post-synaptic PSD95 expression together is associated with an increase in synaptogenesis (Christopherson et al., 2005; Greenlee et al., 2001). The effect of GH on synaptogenesis was therefore analysed in this current study through changes in the expression of SNAP25 and PSD95.

1.6.4 GH promotes synaptogenesis after excitotoxic insult in the post-natal chick

GH is also thought to be neuroprotective in the chick neuroretina and is anti-apoptotic during waves of apoptosis that occur during normal development of the chick retina (Sanders et al., 2011). Furthermore, GH has shown to be neuroprotective against glutamate/BSO induced excitotoxicity *in vitro* in QNR/D cells (Martínez-Moreno et al., 2016). In the green iguana GH was found to be upregulated in experimentally induced excitotoxicity caused by the injection of KA, a glutamatergic agonist (Ávila-Mendoza et al., 2016). Exogenous GH injections were able to significantly reduce KA induced damage and were found to be neuroprotective (Ávila-Mendoza et al., 2016). Whether GH is neuroprotective of synapses in the chicken retina after damage has not previously been studied. GAP43, PSD95, and SNAP25 are important proteins in synapse function and synaptogenesis and have all been shown to be downregulated in excitotoxic conditions (Hung et al., 2014; Kamat et al., 2016). As the neurotrophic effects of GH in the retina have only been studied thus far in embryo, the ability of GH to protect retinal synapses and promote synaptogenesis after neurotoxic insult was examined in this study through its effects on the expression of GAP43, SNAP25, and PSD95.

1.7 Hypothesis and Aims

The hypothesis of this current study is that GH promotes synaptogenesis in the chicken neuroretina, both during development and postnatally, after injury. The aims of this current study are therefore to demonstrate a synaptogenic effect of GH during development through its effect on the expression of specific synaptogenic markers. As well, to demonstrate a

neuroprotective/neuroregenerative effect in the post-natal retina on synaptogenic marker expression after excitotoxic insult.

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Chapter Two

Internalization and synaptogenic effect of GH in retinal ganglion cells (RGCs).

Publication and Author Contributions

The results of this chapter have been published in Fleming, T., Martínez-Moreno, C.G., Mora, J., Aizouki, M., Luna, M., Arámburo, C., Harvey, S., 2016. Internalization and synaptogenic effect of GH in retinal ganglion cells (RGCs). Gen. Comp. Endocrinol. 234, 151-160.

This manuscript was co-written by Thomas Fleming and Dr. Carlos Martinez-Moreno. The experimental design, laboratory techniques (including QNR/D cell culture, neuroretinal explant culture, *ex ovo* chick embryo culture, treatments, western blot analysis, and RNA extraction and RT-PCR), data collection, and interpretation represented in figures 2.6, 2.7, and 2.8 were carried out by Thomas Fleming.

2.1 Introduction

It is well established that growth hormone (GH) is internalized in target cells after binding to the GH receptor (GHR) (Roupas and Herington, 1987; Husman et al., 1988; Govers et al., 1999). Following internalization, GH may be degraded (Husman et al., 1988; van Kerkof and Strous, 2001; Strous and van Kerkof, 2002) and/or translocated to the nucleus (Lobie et al., 1994a, 1994b; Mertani et al., 2003) or to other subcellular compartments (Postel-Vinay et al., 1982; Perret-Vivancos et al., 2006; Ardail et al., 2010). As GHRs are also found in pituitary somatotrophs (Fraser and Harvey, 1992; Hull et al., 1992; Mertani et al., 1994), GH may be similarly processed in GH-secreting cells. This possibility is supported by the fact that after binding to the plasma membrane, GH is internalized into several cellular compartments in the rat pituitary gland (Mertani et al., 1996). Different cellular localizations of GH pools within GH secreting cells could therefore result in different endocrine, paracrine or autocrine roles for GH (Mertani et al., 1996). Indeed, nuclear GH in tumorous tissue and in extrapituitary locations is thought to have autocrine actions distinct from those of pituitary GH (Liu et al., 1997; van den Eijnden and Strous, 2007; Perry et al., 2008; Harvey, 2010).

In addition to the pituitary gland, GH gene expression is now known to occur in many extrapituitary tissues (Harvey 2010), including the neural retina, in which it is abundantly expressed in retinal ganglion cells (RGCs) (Baudet et al., 2003). The possibility that GH may be internalized in these GH-secreting cells has, however, never been investigated and was an aim for the current investigation. This is of particular importance, as retinal GH has been shown to promote neurogenesis in early chicken embryos, prior to the ontogeny of pituitary somatotrophs (Baudet et al., 2009). Early ocular development in the chicken embryo, prior to embryonic day (ED) 15, may thus reflect autocrine or paracrine actions of retinal GH, but the ontogenic appearance of GH

secretory somatotrophs in the chicken pituitary gland, at ED15 (Harvey and Hull, 1997), suggests later embryonic development additionally occurs in the presence of pituitary (endocrine) GH. This possibility was also investigated in the present study.

During ocular development, we have shown that GH stimulates neurogenesis, since siRNA GH knock-down reduces the number and length of RGC neurites outgrowths (Baudet et al., 2009). GH has similarly been shown to increase the number and length of neurite outgrowths in cultured N1E-115 neuroblastoma cells (Grimbly et al., 2009). The possibility that GH may also participate in synaptogenesis during development of the neural retina has not, however, been investigated. The possibility that GH promotes synaptogenesis has therefore been additionally examined in the present study.

2.2 Materials and Methods

2.2.1 Animals

Pathogen-free, fertilized eggs (*Gallus gallus*, White Leghorn) were obtained from Pilgrim's Pride (Querétaro México) for Cy3-GH *in-ovo* injection experiments and were incubated at 38 °C in a humidified air chamber (IAMEX, México). The eggs were rotated one-quarter of a revolution every 50 min during incubation. For *ex-ovo* injection, retinal whole mount, and neuroretinal explant experiments, fertilized eggs were obtained from the University of Alberta Poultry research center (Edmonton, Canada). Chicken embryos were sacrificed, with approval from the University of Alberta animal welfare committee and from the Instituto de Neurobiología (UNAM) bioethical committee.

2.2.2 QNR/D cell culture

QNR/D cells (Pessac et al., 1983) acquired from ATCC (American Type Culture Collection; No. CRL-2532) were cultured in DMEM (Gibco, Grand Island, NY, USA) containing D-glucose (4.5 g/L), L-glutamine and sodium pyruvate (100 mg/L) supplemented with 10% fetal bovine serum (Gibco, Canada) in 5% CO_2 / 95% O_2 at 39 °C in a water jacketed incubator (Forma Scientific). Cells were subcultured from 1 flask into 4 every 5-7 days (at 80-95% confluence). QNR/D cells were used for experiments only between passage 15 and 25 in order to preserve their phenotype. Molecular cells markers for RGCs (Brn3, Thy-1, NTF-3, Islet-1, calretinin and RA4) were evaluated to confirm their RGC identity (Martínez-Moreno et al., 2014). Morphological examinations were performed on a daily basis, and cultures with abnormal growth, lack of neurites and aggregations were discarded. Contamination with other cell lines was not possible since it was the only cell line in the laboratory.

2.2.3 Neuroretinal explant culture

Fertilized chicken eggs were incubated until ED8/10 at 38 °C. Eyes were surgically dissected in Neurobasal (Gibco, Grand Island, NY, USA) medium in the presence of antibiotic and anti-mycotic (Anti-Anti 100x, Gibco, Grand Island, NY, USA). A circular incision was made around the cornea using iridectomy scissors, allowing for the removal of the cornea and vitreous humor. The neuroretina (NR) was then peeled from the pigmented epithelium and radial cuts were made in the neuroretina to allow it to lay flat. NRs were placed onto an explant membrane insert (EMD, Millipore, USA) with the retinal ganglion cell layer contacting the membrane. The insert was placed into corresponding wells containing 0.35 ml of DMEM culture media with 10% FBS and B27 supplement (Sigma). After two days of incubation with 5% CO_2/95% O_2 at 39 °C in a water jacketed incubator, GH treatment was applied. Explant media was replaced with media containing 1 nM GH. Media was also changed in control groups without GH. Explants were

incubated with treatments for 24 h. Media was replaced with new treatments to ensure short-time activation of the JAK/STAT 1 h before collecting the explants for prior analysis.

2.2.4 Treatments

Recombinant chicken growth hormone (rcGH) (Protein Laboratories Rehovot (PLR) Ltd., Rehovot, Israel) treatments were dissolved in bicarbonate buffer and then added to DMEM (Gibco, Grand Island, NY, USA) containing D-glucose (4.5 g/L), L-glutamine and sodium pyruvate (100 mg/L) supplemented with 10% fetal bovine serum (Gibco, Canada). Media was changed after 1h for both rcGH treatments and negative controls without rcGH. For internalization studies QNR/D cells were incubated with Cy3-labeled GH (50 mM) for 30 and 60 minutes. Cy3 conjugation to GH was performed according to Cy3 Mono-Reactive dye pack manufacturer's instructions (GE Healthcare Life Sciences, Amersham, ON, Canada). QNR/D cells were incubated with rcGH (50 nM) and its internalization was determined by western blotting, ELISA and immunofluorescence analysis. STAT5 activation in QNR/D cell cultures was induced by incubations in the presence of rcGH (100 nM) for 30, 60, 120 and 240 min and in embryos injected with Cy3-GH the NR was collected 1 h post-injection. In retinal explant experiments, stabilization media (DMEM with FBS 10%) was replaced with media containing GH (100 nM). Media was also changed in control groups without GH. Explants were incubated with treatments for 24h. In *ex-ovo* injection experiments, at ED10 embryos were intravitreally injected with 3 µl of Cyanine 3 (Cy3)-GH in PBS twice (0.3 µg Cy3-GH/µl), using an insulin syringe with a 30 gauge 5/16" needle. The volume of 3 µl was previously determined by a micropipette and loaded into the syringe from a piece of parafilm. Each embryo was injected in the right eye and the other eye was used as non-injected negative control. Eyes were dissected in cold PBS.

2.2.5 Ex-ovo chicken embryo cultivation and Cy3-GH intravitreal injection

Fertilized eggs were cleaned with 70% ethanol and pre-incubated for 72 h at 38 ºC in an incubator with constant air flow and 60% humidity. Eggs were gently open using a Jig-saw and the contents were carefully transferred into a 100-mm x 20 cell culture dish (Corning Inc, NY, USA). Sterilized ground eggshell of several eggs was added to the albumin as calcium source. At ED10 embryos were intravitreally injected as mentioned in the previous section. Each embryo was injected in the exposed eye and the other eye was used as non-injected negative control. For phospho-STAT5/STAT5 and SNAP25/actin western blotting analysis the retinas were collected 1 and 6 h post-treatments respectively.

2.2.6 In-ovo Cy3-GH systemic injection

White Leghorn hens' eggs were incubated in order to bring them to ED15. Eggs were first cleaned with iodine solution. Using an optic fiber light at maximum intensity, the egg shells were careful windowed (5x5 mm). The chorioallantoic artery was microinjected (*in ovo)* with Cy3-GH (at dose of 150 µg/kg of body weight in 100 µl) using an insulin syringe with 30-gauge needle (Palmer et al., 2011). Negative controls were injected with 100 µl of saline solution. The injection window was closed with micropore tape and incubated for 2 h at 37 ºC. Eyes were dissected and fixed in Zamboni's solution (4% paraformaldehyde and 1.5% picric acid in PBS) % and then immersed in a 20% sucrose solution at 4 °C until eyes stopped floating.

2.2.7 Immunohistochemistry

Immunohistochemistry was performed as previously described (Baudet et al., 2007, 2009; Sanders et al., 2010; Alba-Betancourt et al., 2011). In brief, Zamboni's-fixed retinas and paraformaldehyde-fixed QRN/D cells were incubated overnight at 4 °C with primary antibody

solution (Table 2.1) at 1:1000. The antibodies for cGH (Arámburo et al., 1989; Harvey et al., 2012) and cGHR (Harvey et al., 2012), raised against a synthetic portion (CH17) of the extracellular domain of the cGHR, are specific for their ligands, as detailed elsewhere (Hull et al., 1999; Martínez-Moreno et al., 2014). Normal mouse and rabbit sera at similar dilutions were used as negative controls. After primary antibody solution incubation, samples were then washed with PBS three times and incubated in a dark cold room with 1% non-fat milk/TPBS/secondary antibody solution (all Invitrogen, Burlington, Ontario, Canada). To label cell nuclei, these sections were also counterstained with 300 nM 4,6-diamidino-2-phenylindole (DAPI, Invitrogen) in PBS for 45 min, rinsed three times with TBS and mounted with a fluorescent mounting medium. The slides were analyzed using a Carl Zeiss LSM 780 confocal microscope with lasers at excitation wavelengths of 488 nm, 514 nm, and 561 nm (for Cy3), respectively. A Coherent-XR multiphotonic laser at 350 nm was also employed (DAPI).

2.2.8 Western blotting

Total protein extracts from QNR/D cells and chicken NRs were extracted by sonication and homogenization in presence of protease-inhibitor cocktail (Mini-complete, Roche Diagnostics) in 50 mM Tris-HCl buffer (pH 8.0). Equivalent amounts of protein were added to each well: 40 μg of lysate (for STAT5/pSTAT5 blotting in QNR/D and NR), 40 μg (for GAP-43 in QNR/D), 30 μg (for GAP-43 and SNAP-25 in the NR) and 15 μg (for GH). Samples were boiled for 5 min with 2x sample buffer containing bromophenol blue and 5% β-mercaptoethanol. Samples were loaded in each lane and electrophoresed in a 12.5% polyacrylamide gel using the buffer system of Laemmli (1970). The Bio-Rad Protein Assay (Bio-Rad) was used to ensure equal protein loading of each line. Samples were transferred from the gel onto a nitrocellulose membrane. For immunoblotting, membrane blocking was carried out using 5% non-fat milk (Bio-Rad) in PBS for

1 h. Primary antibodies solutions (Table 2.1) were made in a 1% non-fat milk TPBS solution: rabbit anti-STAT5 (Abcam, Ontario, Canada), rabbit anti-phospho-STAT5 (Abcam), rabbit anti-actin (SantaCruz Biotechnology, CA, USA), rabbit anti-chicken-GH (Harvey et al., 2012), and membranes were incubated overnight at 4 °C with moderate shaking. For staining, secondary antibody solutions were made at a concentration of 1:5000 (IgG-HRP cat anti-goat (SantaCruz Biotechnology), goat anti-rabbit (Bio-Rad), goat anti-mouse (Bio-Rad) in a 1% non-fat milk/TPBS solution, and membranes were incubated for 2 h at RT. Immunoreactive bands were developed by chemoluminescense using ECL reagent (Amersham Biosciences, Montreal) after exposing the membranes to Kodak Biomax ML films which were then processed in an automated film processor. Protein loading was re-checked by labeling lanes for actin after stripping the blots 5 min using Re-Blot striping solution (Chemicon International Inc.). Immunoblots were repeated up to 3 times, scanned, and quantified by densitometric analysis using ImageJ software.

2.2.9 GH ELISA

Growth hormone in samples was quantified by ELISA as described elsewhere (Alba-Betancourt et al., 2011), with minor modifications. In brief, 96-well microtiter plates were coated overnight at 4 °C with recombinant chicken GH (12 ng per well) in carbonate buffer (0.1 M, pH 10.3). ED8, ED10, ED12 and ED15 chicken NR samples (75 µg of protein in each well) and serial dilutions of recombinant chicken GH in 1% non-fat milk/TPBS were incubated overnight (16 h) with rabbit anti-chicken GH primary antibody (Harvey et al., 2012) at a concentration of 1:10000. The 96-well plates were rinsed 3 times with TPBS (300 µl per well) and incubated with 3% non-fat milk/TPBS to block free binding sites. The samples and standards were then added to the coated wells and incubated at room temperature for 2 h. Horseradish peroxidase-anti-rabbit IgG conjugate in 1% non-fat milk/TPBS (1:3000) was incubated at room temperature for 2h. 2,20-amino-di-[3-

ethyl-benzothiazoline sulfate] substrate (ABTS; Roche Diagnostics, Manheim, Germany) was then used to develop bound secondary antibodies. The well plates were read 30 min later using an ELISA microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 405 nm. The assay has a sensitivity of 2 ng/well (20 ng/ml), and the inter-assay and intra-assay coefficients show a variation lower than 4% (Alba-Betancourt et al., 2011).

2.2.10 GAP-43 and SNAP-25 RT-PCR

Total RNA was extracted from QNR/D cell cultures, ED10 chicken NR, and chicken optic tectum (OT). Total RNA was isolated using TRIzol reagent (Invitrogen, Canada Inc., Burlington, Ontario) and RNeasy Mini Kit (Qiagen, Ontario, Canada). Genomic DNA contamination was digested with RNase-Free DNase Set for 20 min at room temperature (DNase I treatment; Qiagen, Ontario, Canada) before washing the column 3 times with the corresponding buffer. A second incubation with DNAse I (Invitrogen, Canada) was performed at 37 ºC for 15 min. Total RNA was quantified in a nanodrop-2000 (Thermo Scientific) and purity (>1.8 ratio of 260/280 nm) was confirmed. Reverse transcription of 3 µg of DNA-free total mRNA using Super Script 3 Reverse Transcriptase (Invitrogen, Canada Inc.) according to manufacturer's instructions and 1 µl of oligo (dT)18-20 was used to synthesize cDNA. 2 µl of cDNA was amplified in the presence of 10 µM sense and antisense primers for GAP-43 or SNAP-25, 10x PCR buffer, 50 mM $MgCl_2$ 10 nM dNTP mix, and 0.2 µM Platinum Taq High Fidelity DNA polymerase. GAP-43 primers included forward (5'-AAAGAGAGTGAGGCTTCCGC-3') and reverse (5'-TGCTGGGCACTTTCAGTAGG-3') primers designed to amplify a 446 bp product (NCBI accession number, NM 001305054.1), and SNAP-25 primers included forward (5'-GGAACGCATCGAGGAAGGAA-3') and reverse (5'-TTCCCGGGCATCGTTTGTTA-3') primers designed to amplify a 259 bp product (NCBI accession number, NM 205458.1). Primers

were designed to amplify avian genes and to cross intron-exon boundaries to control for genomic DNA contamination. PCR mixtures were first denatured at 95 °C for 5 min, then subjected to 30 cycles of 95 °C, 15 sec denaturation; 55 °C, 40 sec annealing; and 70 °C, 40 sec elongation with a final extension of 72 °C for 10 min using a thermal cycler (Techgene, Fisher Scientific, Canada). PCR products were compared with a 1 kb plus DNA ladder (Invitrogen, Canada Inc.).

2.2.11 Statistical analysis.

In all the experiments values are expressed as mean ± SEM. Significant differences between multiple groups were determined by one-way ANOVA with Dunnett's post-hoc test for treatments against controls. Unpaired Student's t-test was used to compare between two groups where appropriate. P-values less than 0.05 were determined to be statistically significant (*, $P< 0.05$; **, $P< 0.01$; ***, $P<0.001$). All the experiments were repeated 3-5 independent times including triplicates. Mean pixel intensities (in arbitrary units) of selected cells were compared within at least 10 images per experimental condition from 3-4 individual experiments. RT-PCRs were repeated 3 times from 3 different tissues and 3 cell cultures.

2.3 Results

The presence of GHR in QNR/D cells was confirmed by western blotting (Fig. 2.1 A). Immunoreactive bands of 66-kDa and 54-kDa were present in extracts of the cells, as in extracts of the NR and (as positive control) in the liver of adult chickens. When incubated in the presence of 50 nM Cy3-rcGH, uptake of labeled GH to QNR/D cells was demonstrated (Fig. 2.1 B), bound to GHR immunoreactivity (Fig. 2.1 C and D). Interestingly Cy3-rcGH (red) was primarily co-

localized within a subcellular compartment close to the nuclei of QNR/D cells (Fig. 2.1 B and 2.2 A, arrows).

The uptake of Cy3-rcGH by QNR/D cells was further shown (Fig. 2.2 A) and the specificity of uptake demonstrated by its loss following its co-incubation with a 10-fold excess of unlabeled rcGH (Fig. 2.2 B) or in the presence of a specific GH antibody (Fig. 2.2 C). For comparative purposes, the uptake of the same dose of unlabeled rcGH was determined by ELISA, which showed that it was significantly increased ($P < 0.05$, by almost 3-fold) after 30 and 60 min (Fig. 2.2 D). Western blotting showed that this reflected an increased presence of monomeric GH (26-kDa under reducing conditions) and a 15-kDa moiety. Immunocytochemical staining showed that the increase in QNR/D GH content (Fig. 2.2 F) was similarly accompanied by an increase in its perinuclear localization (Fig. 2.2 I, arrows), as seen with the localization of Cy3-GH (Fig 2.2 A).

In a similar experiment, the co-existence of exogenous Cy3-GH with endogenous GH in QNR/D cells was shown by immunocytochemistry (Fig. 2.3), which more clearly showed the perinuclear localization of the labeled hormone (Fig. 2.3 A and D, arrow heads). Unlabeled GH immunoreactivity (green) was present in the neurite outgrowths of QNR/D cells (Fig. 2.3 B and E, arrows).

Cy3-GH was next injected into the systemic circulation of ED15 chicken embryos and its uptake into RGCs of the ganglion cell layer shown (Fig. 2.4 A, B and E). Interestingly, Cy3-GH was not taken up by cells in the inner plexiform layer (IPL), nor by RGC axons that compose the optic fiber layer (OFL) (Fig. 2.4 A). Within the RGCs, Cy3-GH was again localized close to the nucleus (Fig. 2.4 A) and associated with GHR immunoreactivity (Fig. 2.4 C and D). Unlabeled GH immunoreactivity (green) was present in the INP and OFL (Fig. 2.4 F).

In the presence of GH, pSTAT5/STAT5-immunoreactivity ratio increased in QNR/D cells after 30, 60 and 240 min (Fig. 2.5). Within QNR/D cells, GAP-43 and SNAP-25 mRNAs were identified by RT-PCR (Fig 2.6 A), and the translation of these genes was shown by western blotting (Fig 2.6 B) and immunocytochemistry (Fig. 2.6 C). The abundance of GAP-43 was significantly increased ($P<0.05$) after a 6 h incubation in 100 nM rcGH (Fig 2.7 A and B), as was the abundance of SNAP-25 immunoreactivity in NR explants (Fig. 2.7 C and D).

When injected intravitreally into the eyes of ED10 embryos, pSTAT5/STAT5 immunoreactivity ratio was also significantly ($P<0.05$) increased (Fig. 2.8 A). This was also accompanied by an increase ($P<0.05$) in the abundance of SNAP-25 immunoreactivity (Fig. 2.8 B).

2.4 Discussion

This study clearly shows that exogenous GH is taken up by GH-secreting RGCs and internalized into a perinuclear compartment. The results of this investigation also demonstrate GHR mediated actions that suggests actions in RGC synaptogenesis during the development of the neural retina.

2.4.1 GH internalization

We have previously shown that QNR/D cells provide an experimental model for investigating the role of GH in retinal ganglion cells (Martinez-Moreno et al., 2014). The demonstration of GHR immunoreactivity in QNR/D cells in this study (Fig. 2.1) supports our earlier findings (Martinez-Moreno et al., 2014) and demonstrates that the GHR immunoreactivity proteins detected (66- and 54-kDa) were of the same size as those previously detected in chicken

hepatic tissues (Hall et al., 1999). The presence of GHRs in these cells, as in the RGCs in the neuroretina (Harvey et a., 2001; Harvey et al., 2003; Baudet et al., 2007) also demonstrates that these GH-secreting cells are like pituitary somatotrophs in that they can take up and internalize exogenous GH (Mertani et al., 1996). Moreover, the perinuclear localization of Cy3-labeled GH inside QNR/Ds and retinal RGCs (Fig. 2.2) is consistent with the subcellular localization of GH in pituitary somatotrophs (Mertani et al., 1996). The metabolism of exogenous monomer GH into a 15-kDa moiety is also consistent with the degradation fate of internalized GH (Postel-Vinay et al., 1982) and with the processing of monomer GH into a 15-kDa moiety in retinal tissue (Harvey et al., 2007) and in the cerebellum (Alba-Betancourt et al., 2011). Furthermore, as 15-kDa GH is thought to have increased biological activity in comparison with the monomer 22-kDa GH (Arámburo et al., 2001; Harvey et al., 2014), this finding is similar to the observation that cleavage of GH by rabbit liver membranes is thought to enhance its biological activity (Schepper et al., 1984).

If the internalized GH in the chick RGCs is biologically active, this would support the possibility that retinal development during the last trimester of the 21-day incubation period would be at least partially due to endocrine (pituitary) GH. Our studies might therefore provide a useful experimental model to further investigate the relative contributions of local (autocrine or paracrine) GH and those resulting from exogenous (pituitary) GH in neuroretinal development.

In this study, Cy3-GH was injected into the chorioallantoic circulation of the chick embryo and it was then internalized into the RGCs of the inner neural retina. This suggests the movement of the tracer through the *pecten oculi* (a modified and specialized blood-brain barrier in birds) and its translocation into the vitreous. This possibility is supported by the finding that the internalization of the tracer following its intravenous administration was identical to its intravitreal

injection. However, while GH produced in the RGCs accumulates in the OFL, it was noticeable that the exogenous tracer did not, suggesting a difference in the processing or trafficking of local and exogenous (pituitary) GH in the neural retina.

2.4.2 GH and synaptogenesis

While actions of GH in neural differentiation are now well established (Turnley, 2005; Baudet et al., 2009; Waters and Blackmore, 2011), there is a paucity of information on the possible involvement of GH in synaptogenesis. In this study, GH treatment was found to increase SNAP-25 synthesis in RGCs. This was probably through the activation of the JAK/STAT signaling pathway, since we observed simultaneous increase of STAT5 phosphorylation and SNAP-25 immunoreactivity in neuroretinal extracts and QNR/D cell cultures. SNAP-25 expression in the chick neural retina is thought to largely occur in amacrine cells and RGCs (Catsicas et al., 1991) and its expression is first detectable at ED11. Interestingly, this is at the time that synaptogenesis with RGCs is initiated (Catsicas et al., 1991) and coincides with peak presence of GH in the OFL of the chicken neural retina (Baudet et al., 2009).

GAP-43 is critical for the development of the nervous system and mice that do not express this protein show marked deficits in axon growth and synaptogenesis (Latchney et al., 2014). In this study, GH treatment increased GAP-43 immunoreactivity coincident with an increase in pSTAT5 immunoreactivity. As increased pSTAT5 occurs after the activation of GHR (Walters and Brooks, 2015) these results suggest that GH acts via a receptor-mediated mechanism to induce GAP-43 and SNAP-25 production and neural development and synaptogenesis in the developing neural retina.

BASP-1 is another marker of nerve regeneration, neurite sprouting and synaptic growth and it is thought to be a GH-responsive gene in the retinal proteome that is involved in synaptogenesis (Baudet et al., 2008). Synuclein is also thought to be involved in synaptogenesis (Finnegan et al., 2008) and synuclein has also been found in the neural retina (Sanders et al., 2009), Furthermore, GH has been shown to co-localize with secretoneurin in QNR/Ds (Martinez-Moreno et al., 2015), which is similarly involved in synaptogenesis (Marksteiner et al., 2002). SNAP-25 has also been found to be co-localized with GH in these cells (Martinez-Moreno et al., 2015) and SNAP-25 is expressed in the chick neural retina (Catsicas et al., 1991). It is therefore possible that GH promotes synapse formation in the neural retina, presumably between the amacrine and bipolar cells that innervate the RGCs.

In summary, GH is internalized in GH-secreting RGCs in the chick neuroretina after binding to the GHR. Activation of RGC GHRs increases STAT5 phosphorylation and the expression of SNAP-25 and GAP-43, suggesting GH involvement in neural development, including an induction of synaptogenesis.

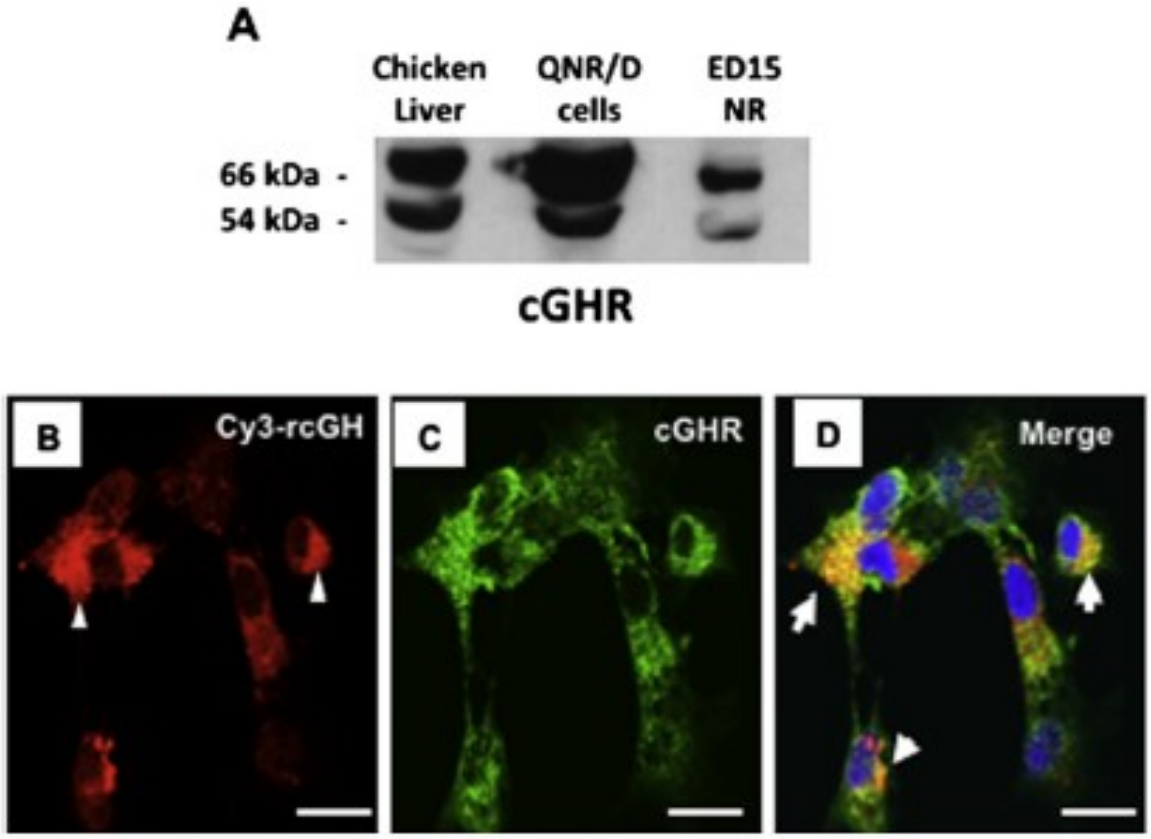

Figure 2. 1 Co-localization of Cy3-labeled rcGH and GHR in QNR/D cells. **A**, western blotting: GHR immunoreactivity is present in the chicken liver, neuroretina, and QNR/D cells. Immunocytochemistry: **B**, Cy3-GH (red) internalization after incubation with 50 nM Cy3-GH. **C**, GHR immunofluorescence in QNR/D cells (green). **D**, Co-localization of internalized Cy3-GH and local GHR (yellow). Scale bars, 10 μm.

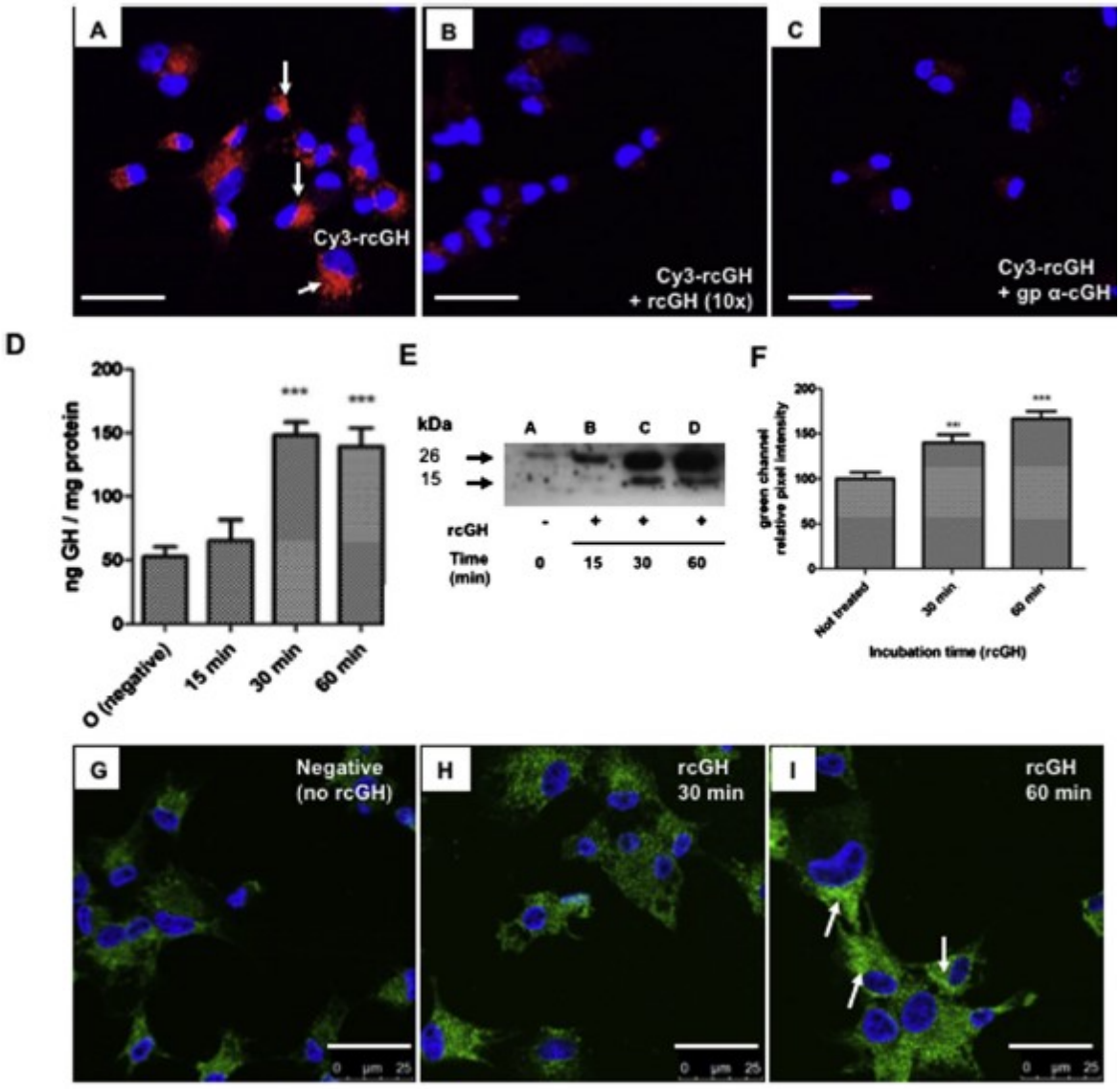

Figure 2. 2 Growth hormone internalization on QNR/D cell cultures. **A**, Cy3-labeled GH (red) in QNR/D cells after 1 h of incubation with 50 nM Cy3-rcGH. Arrows indicate the peri-nuclear localization of GH after its endocytosis. **B**, Cy3-rcGH (red) label internalization decreases after simultaneous co-incubation of Cy3-GH (50 nM) with non-labeled rcGH (500 nM). **C**, Cy3-rcGH immunoneutralization with guinea pig anti-chicken GH antisera (gp α-cGH). **D**, GH quantification by ELISA. **E**, Analysis of GH immunoreactivity (26 kDa and 15 kDa bands) by western blotting. 15 kDa GH-IR immunoreactivity started to appear after 30 min of incubation. **F**, GH-

immunofluorescence quantification increased in a time-dependent manner after incubation with rcGH, as observed in panels **G-I**. Bars represent the mean ± SEM (n=4). Statistical significance was determined by one-way ANOVA, and Dunnett's multiple comparison test. Asterisks (***, **, *) indicate significant differences ($p<0.001$, $p < 0.01$, $p < 0.05$), respectively. Scale bars, 25 μm.

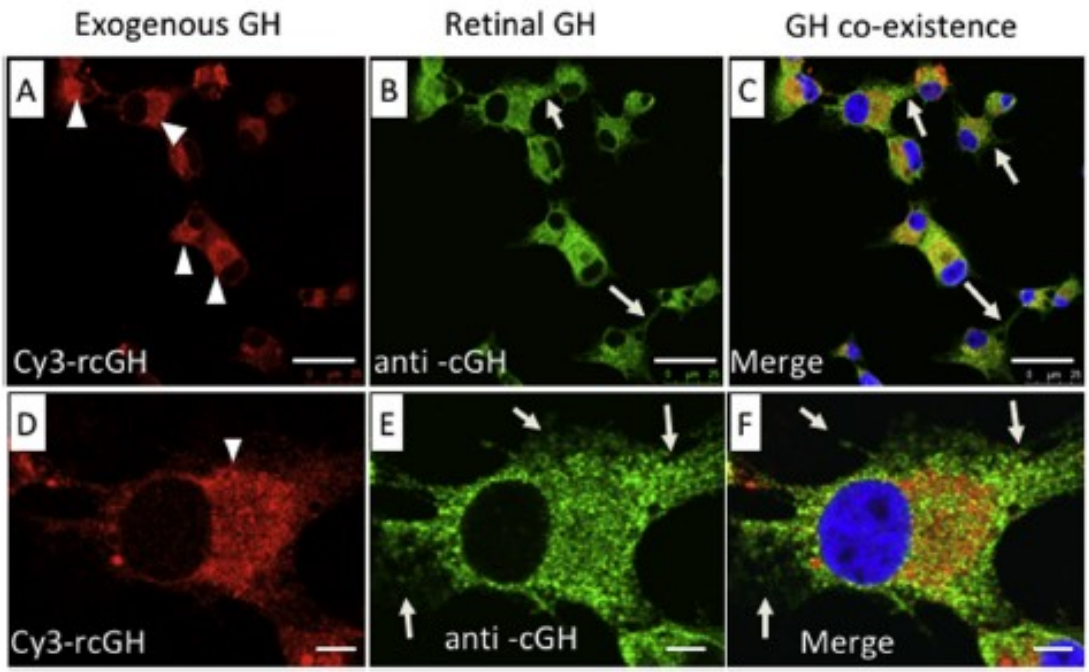

Figure 2. 3 Co-localization of Cy3-labeled GH and retinal (autocrine/paracrine) GH in QNR/D cell cultures. **A-C**, Internalized Cy3-GH (arrowheads, red) and endogenous GH (arrows, green) co-localization (**C**, yellow), in QNR/D cells. Arrows in B and C show GH immunoreactivity in cell outgrowths. **D-F**, Figures correspond to a 5X higher magnification of A, B and C (arrowheads show internalized Cy3-GH and arrows show endogenous GH-immunoreactivity). Scale bars D-F, 5 µm; A-C, 25 µm.

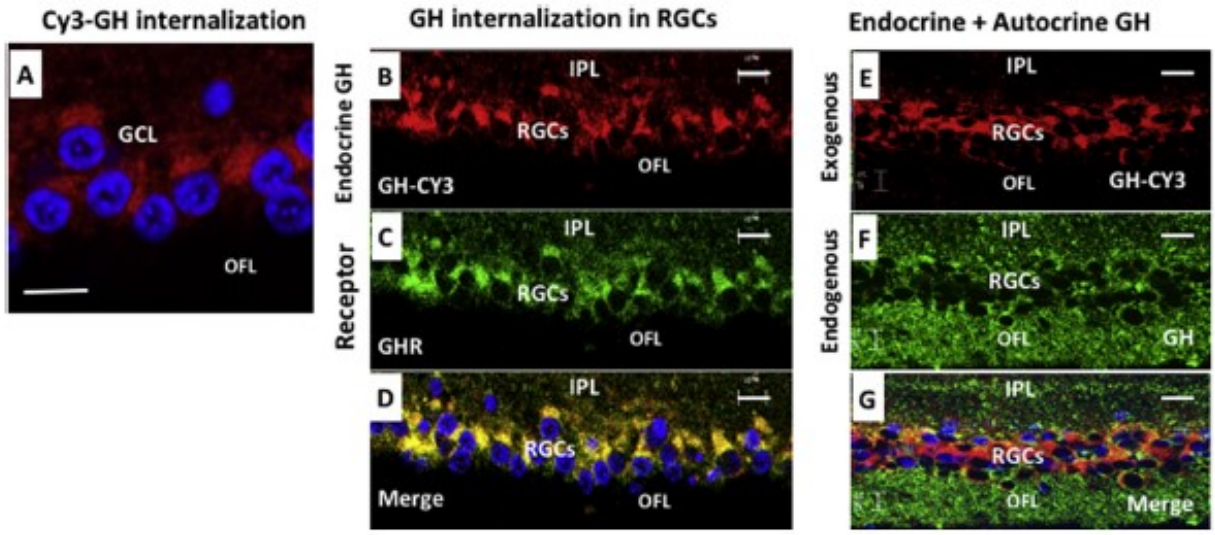

Figure 2. 4 Co-localization of internalized Cy3-labeled GH with GH and GHR in the chicken embryonic neural retina. **A**, Cy3-GH (red) is mainly internalized to the GCL in a perinuclear distribution; **B**, Cy3-GH (red) and GHR-immunofluorescence (**C**, green) co-localize (**D**, yellow) in cell bodies of retinal ganglion cells (RGCs). **E**, whereas Cy3-GH localization is localized mainly in RGCs of the ganglion cell layer (GCL), endogenous GH-immunoreactivity (**F**) is distributed in the OFL, the IPL and in some retinal ganglion cells. **G**, Image overlay of **E** and **F** showing Cy3-GH and endogenous retinal GH co-localization in some RGCs (arrows). Scale bars B-G,10 µm.

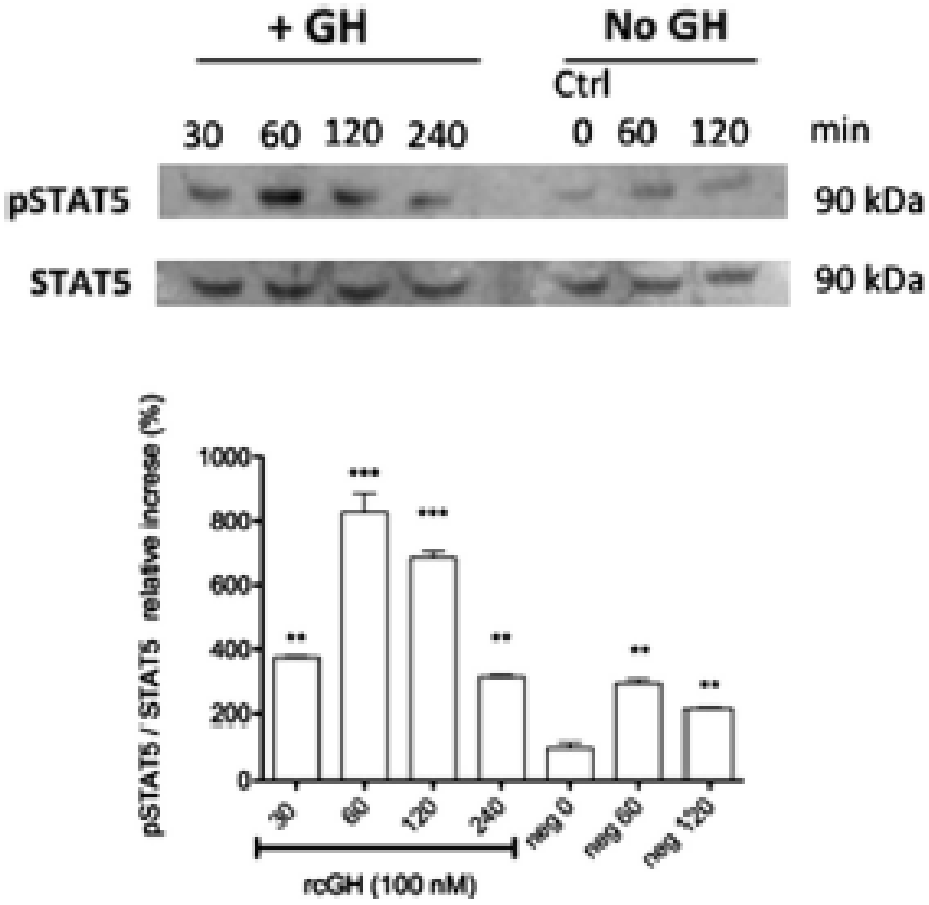

Figure 2. 5 GH induces STAT5 phosphorylation in QNR/D cells. **A**, representative immunoblots of pSTAT5 and STAT5 in QNR/D cells treated with 100 nM of rcGH at 30, 60, 120 and 240 min. Negative controls (neg) were incubated without rcGH. **B**, Relative change (%) in pSTAT5/STAT5 immunoreactivity ratio (results from 3 independent experiments by triplicate). All groups were compared with the negative control at time 0. Bars represent mean ± SEM (n=3) Asterisks show significant differences by one-way ANOVA and Dunnet's multiple comparison test (**, $P<0.01$; ***, $P<0.001$).

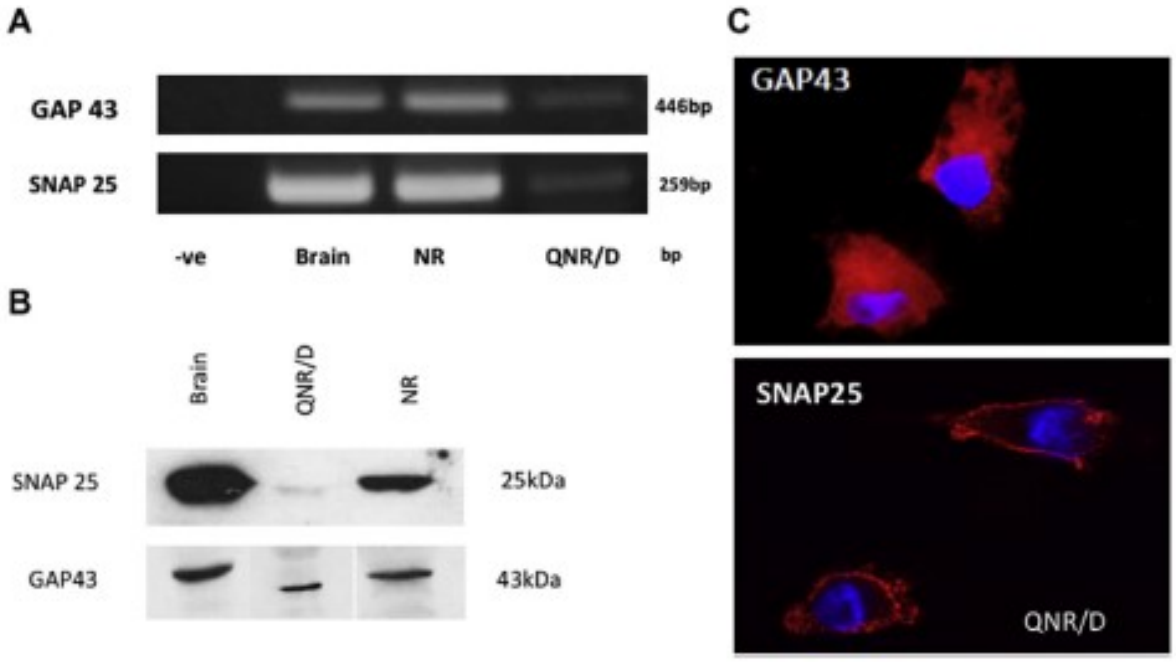

Figure 2. 6 Expression of GAP 43 and SNAP 25 in the chicken NR and QNR/D cell. **A**, PCR products of cDNA from chicken brain, neuroretina (NR), and QNR/D cells amplified in the presence of primers for GAP 43 (446 bp) and SNAP 25 (259 bp) (n=4). **B**, Immunoblots with total protein extract from chicken brain, NR, and QNR/D cells analyzed for SNAP-25 (25kDa) and GAP-43 (43kDa). **C**, Immunohistochemical staining of SNAP-25 and GAP-43 in QNR/D cells. Nuclei were visualized with DAPI (blue). Cells from 3 independent cultures were analyzed (n=3).

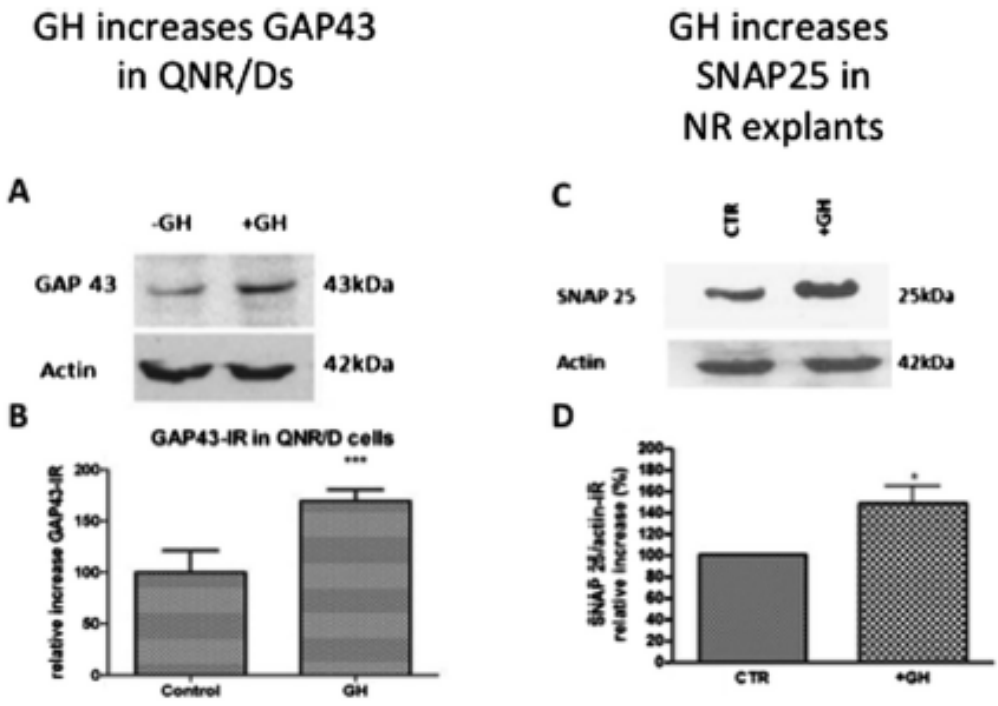

Figure 2. 7 Effect of GH on the expression of GAP43 and SNAP-25 in RGCs. **A**, QNR/D cells were cultured for 6 h in the absence (-GH) or presence of 100 nM recombinant GH (+GH), and analyzed for GAP43 immunoreactivity. Equivalent protein (40 μg) was added to each well. **B**, Quantification of the 43 kDa band in immunoblots in the absence (control) or presence (GH) of GH (n=3). **C**, Effect of GH on SNAP-25 in chick retinal explants (ED 8/10). +GH: 100 nM rcGH and CTR: media without rcGH. Equivalent amounts of protein were added to each well (30 μg). **D**, Quantification of the 25 kDa band/actin ratio in immunoblots (n=9). Bars=mean optical density ± SEM. Asterisks (***, *) indicate significant differences ($P<0.001$, $P<0.05$ respectively). Statistical significance was determined using Student's *T*-test

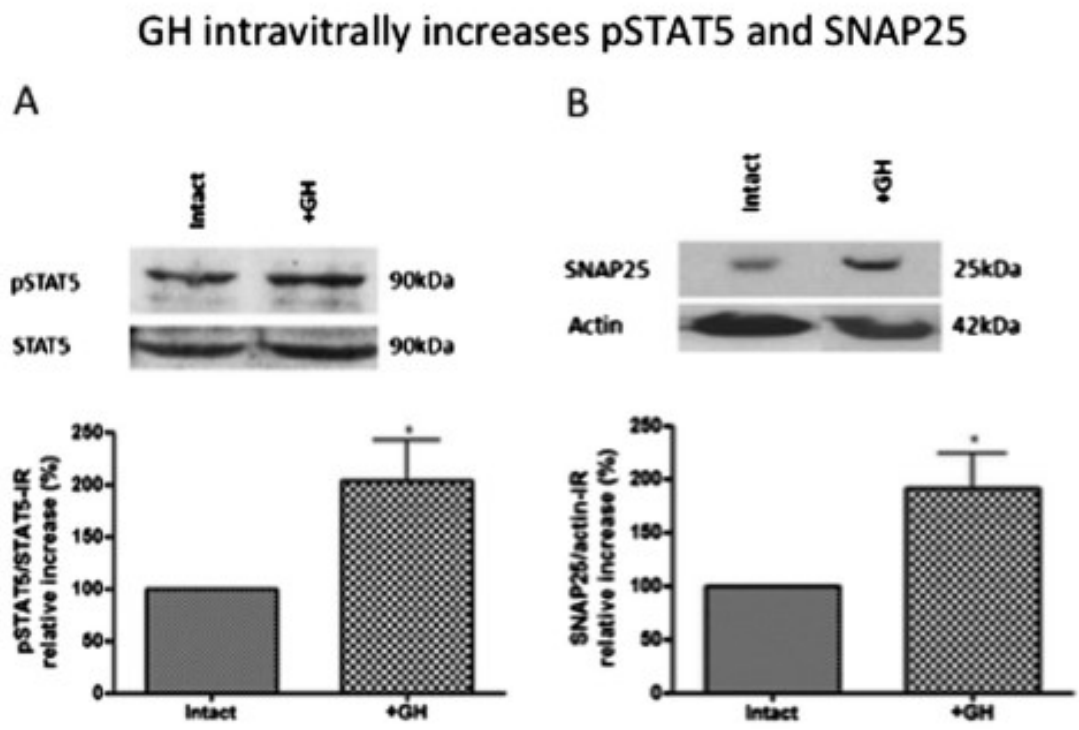

Figure 2. 8 Intravitreal GH injection increases pSTAT5 and SNAP 25 immunoreactivity. **A**, upper panel representative immunoblots of pSTAT5/STAT5 immunoreactivity after GH injection. Equivalent amounts of protein were added to each well (40 µg). Lower panel, relative change (%) of pSTAT5/STAT5 ratio (n=5). **B**, upper panel, immunoblot for SNAP-25. Equivalent amounts of protein (30 µg) were added to each lane. Lower panel, relative change (%) of SNAP-25/actin ratio (n=4). Bars = mean of optical density (%) ± SEM. Asterisks (*) indicate significant differences ($P<0.05$). Statistical significance was determined using Students *T*-test.

Table 2. 1 Antibodies used in IHC and WB

Target	Host/type	Clone/catalog	Dilution	Source
cGH	Rabbit (p)	Non commercial	1:5000 (WB) 1:1000 (IHC)	Harvey et al. (2012)
cGH	Rabbit (p)	CAP-1, Non commercial	1:500 (IHC)	Arámburo et al. (1989)
GAP43	Mouse (m)	GAP-7B10	1:5000 (WB) 1:1000 (IHC)	Sigma, Mi, USA
cGHR	Mouse (m)	Non commercial	1:1000 (WB) 1:1000 (IHC)	Harvey et al. (2012)
pSTAT5	Rabbit (p)	ab36153	1:2500 (WB)	Abcam, Ontario, Canada
SNAP25	Goat (p)	sc-7538	1:2000 (WB) 1:1000 (IHC)	Santa Cruz Biotechnology, USA
Actin	Rabbit (p)	sc-7210	1:1000 (WB)	Santa Cruz Biotechnology, USA

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Chapter Three

Growth hormone protects against kianate induced (*in vitro* and *in vivo*) and induces NT-3 and BDNF expression in neuroretinal cells

Publication and Author Contributions

The results of this paper have been published in Martínez-Moreno, C.G., Fleming, T., Carranza, M., Ávila-Mendoza, J., Luna, M., Harvey, S., Arámburo, C., 2018. Growth hormone protects against kianate induced (*in vitro* and *in vivo*) and induces NT-3 and BDNF expression in neuroretinal cells. Exp. Eye Res. 166: 1-12.

Thomas Fleming was responsible for the data presented in figure 3.6, including experimental design, laboratory techniques (animal handling, treatments, IHC, RNA extraction, RT-PCR, qPCR), data collection, and interpretation. The manuscript was primarily written by Dr. Carlos Martinez-Moreno and edited by Thomas Fleming.

3.1 Introduction

During the development of the CNS, including the neuroretina (NR), GH has well established roles as a neuroprotective factor which exerts pro-survival effects, mainly through the activation of anti-apoptotic pathways (Sanders et al. 2008, 2009; Alba-Betancourt et al., 2012). During embryonic retinal development, GH regulates the physiological equilibrium between cell proliferation and developmental apoptosis through the activation of the PI3K/Akt pathway and promotion of Bcl-2 expression (Harvey et al., 2007, 2007a; Sanders et al., 2009). Prior to somatotroph on-set, locally synthesized GH in the retina has pro-survival actions, evident as gene silencing GH by siRNA results in an increase of apoptotic cells in the chicken embryonic neuroretina at embryonic day 7 (ED7) (Harvey et al., 2009; Harvey, 2010; Sanders et al., 2011).

The neurotrophic actions of GH in the retina include axonal growth in immuno-panned retinal ganglion cells (RGCs) and neuroprotective actions against glutamate-induced cell death in the QNR/D cells (a quail RGC derived cell line) (Baudet et al., 2009; Martinez-Moreno et al., 2016). In embryonic retinal explant cultures, GH decreased LDH release in the retinal ganglion cell layer (GCL) and reduced the number of TUNEL-positive cells after an excitotoxic insult (Martinez-Moreno et al., 2016). *In vivo* results in the green iguana have demonstrated the protective and regenerative actions of GH against kainic acid (KA), a selective agonist for KA-glutamate receptors (Avila-Mendoza et al., 2016). Previous studies from our group were mainly focused on RGCs, however, in this work we have found conclusive evidence that GH has pro-survival effects in cells in the inner nuclear layer (INL) and protective effects on the dendritic trees of the inner plexiform layer (IPL) in the retina.

Most of the studies on neurotrophic actions of GH have been conducted in the embryonic retina of the chick. However, an analysis of *post-mortem* human retinas showed that 100% of

apoptotic retinal ganglion cells did not contain GH while RGCs positive for GH were healthy (Sanders et al., 2009). Indeed, the positive effects of GH in the nervous systems are not limited to the retina, as there is increasing evidence to support the use of GH administration in patients as a novel therapeutic factor to treat neurodegenerative diseases since it promotes neuroprotection, neuro-regeneration, stem cell renewal, cell plasticity and tissue repair (Devesa et al., 2014; Arce et al., 2013). Despite the fact of a clear GH therapeutic potential, side effects such as tumorigenesis, oncogenesis, cancer progression, abnormal angiogenesis and metabolic dysregulation need to be considered and studied (Perry et al., 2017). However, recent clinical studies strongly suggest that growth hormone (GH) administration in patients with neural damage might have positive effects in disease prevention, prognosis, progression, and recovery (Nyberg et al., 2013; Nylander et al., 2016; Devesa et al., 2015, 2016; 2017).

The objective of this current study was to determine if the pro-survival and anti-apoptotic effects of GH observed during development are conserved in functional neuroretinas challenged with experimental excitotoxicity. This work provides *in vitro* and *in vivo* evidence showing the neuroprotective actions of GH in the retina, in both the embryonic and postnatal neuroretina. For the first time, we have shown that exogenous GH induces brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) gene expression in the retina, which suggests the neuroprotective effect of GH in neural tissues might be mediated by a complex cascade of neurotrophins and growth factors involved in damage prevention and retinal tissue repair.

3.2 Materials and Methods

3.2.1 Animals

Pathogen-free, fertilized eggs (*Gallus gallus*, White Leghorn) were obtained from Pilgrim's Pride (Querétaro, México) and were incubated at 39 °C in a humidified air chamber

(IAMEX, Mexico). The eggs were rotated one-quarter of a revolution every 50 min. Eggs were incubated from embryonic day 7 (ED7) until ED10 for cell culturing and for *in vivo* experiments, chickens were incubated until hatch. The neonatal chickens were kept under controlled conditions in the Institute's vivarium, with a photoperiod of 13L-11D and fed *ad libitum*. All experimental animals were sacrificed by decapitation complying with the regulations established by the Institute of Neurobiology's Bioethical Committee, which are in accordance with the Mexican official regulation (NOM). Animals were anaesthetized with subcutaneous xylazine (1 mg/kg) and ketamine (2.2 mg/kg) for intravitreal injections.

3.2.2 Primary retinal cell culture

Chicken embryos (ED10) were anesthetized in ice for 5 min and then sacrificed by decapitation. Eyes were enucleated, the vitreous was removed and neuroretinas were micro-dissected. Using a stereoscopic microscope, pigment epithelium contamination was removed. For cell digestion, the neuroretinas (10 retinas from 5 embryos) were incubated in 2 ml of an enzymatic cocktail of papain, collagenase and cysteine in DMEM for 20 min at 37 °C (Gibco, Grand Island, NY, USA). The cells were finally mechanically triturated with a pipette and passed through a 50 µm pore filter. Cells (5.5×10^5) were counted using trypan blue and hemocytometer, and plated in 24-well plates. Cell cultures were stabilized in DMEM + 10% FBS (Gibco, Grand Island, NY, USA) in a humidified incubator at 39°C (chicken body temperature) and 5% CO_2 for 24h prior to treatments.

3.2.3 QNR/D cell culture

Quail derived neuroretinal cells (QNR/D) (Pessac et al., 1983) acquired from ATCC (American Type Culture Collection; No. CRL-2532) were cultured in DMEM (Gibco, Grand

Island, NY, USA) containing D-glucose (4.5 g/L), L-glutamine and sodium piruvate (100 mg/L), supplemented with 10% fetal bovine serum (Gibco) in 5% CO_2 / 95% O_2 at 39 °C in a water jacketed incubator. Cells were sub-cultured at 90-95% confluency from one flask into 4 flasks every 5-7 days. QNR/D cells were used for experiments only between passage 15 and 25 to preserve their phenotype. Molecular cell markers for RGCs (Brn3, Thy-1, NT3, Islet-1, calretinin and RA4) (Martinez-Moreno et al., 2014) were evaluated on a regular basis to avoid phenotype changes in QNR/D cells. Morphological examinations were performed on a daily basis, and cultures with abnormal growth, lack of neurites, and cell aggregations, were discarded.

3.2.4 Treatments

Experimental excitotoxicity was induced by incubating the cell cultures with kainic acid (KA) (Sigma, Saint Louis, MO, USA) at concentrations of 50, 100, 200, 300, 500 or 750 µM (Ferreira et al., 1998; Fischer and Reh, 2002). For *in vitro* experiments, the KA was diluted in DMEM supplemented with 1 or 3% FBS (long- and short-term respectively). Recombinant growth hormone (rcGH) (Revholt, PRL, Israel) at 1, 10 or 100 nM was administered to the cultures for short-term incubations (1-4 h) in DMEM with 1% FBS or for long-term incubations (24 h) in DMEM with 10% FBS. For *in vitro* long-term incubations (24 h) a single dose of rcGH (1, 10 or 100 nM) was applied. For short-time incubations, to induce neuroprotection, cultures were pre-treated with rcGH (10 nM) for 1 h in order to activate the anti-apoptotic pathways according to previous time frames reported by Luna-Acosta et al. (2016) and Alba-Betancourt et al. (2013). Treatments with KA (50 to 500 µM) were applied together with GH (10 nM) for 2h in order to induce excitotoxic damage by a short-term exposure. Finally, KA was removed and cells were treated with GH at 10 nM for 24 h. For intravitreal injections, KA (20 µg) and rcGH (300 ng and 150 ng) were diluted in injectable water (re-suspended prior to injections). *In vivo* injections

followed the same strategy of our *in vitro* model: a prior 300 ng GH dose (1 h before damage), simultaneous KA (20 µg) + GH (150 ng) and post-injury GH (300 ng) injection (24 h after damage). According to Fischer and Reh (2002), Todd et al. (2015, 2016) and Ritchey et al. (2012), the induction of growth factors expression, neuroprotection and regeneration in the avian retina requires pre- and post- treatments.

To analyze the effect of GH treatment upon expression of BDNF and NT3 during the neuroprotective response following retinal injury, the chickens were injected with GH (300 ng) prior to damage and also during damage (KA [20 µg] + GH [300 ng]). Later, GH (300 ng) intravitreal injections were administered at 24 and 48 h post-injury.

3.2.5 Histochemistry and immunohistochemistry

For histochemical analysis, eyes were fixed with Davidson fixative for 48 h, dehydrated in ethanol, and embedded in paraffin. Sections (5 µm) were cut with a microtome and mounted on pretreated glass slides (Fisherbrand, Fisher Scientific, USA). Paraffin was removed from the slides with Citrisolv (Fisher, Fairlawn, NJ, USA). Sections were rehydrated in absolute alcohol (100 %), ethanol (95, 70, 50 and 30 %) and distilled water; finally, the retinal sections were equilibrated with PBS for 1h. Slides were stained with hematoxylin and eosin according to Avila-Mendoza et al. (2016) and mounted with entellan (Merck, Darmstandt, Germany). For immunohistochemical analysis, right after rehydration, tissues were boiled in citrate buffer (10 nM sodium citrate, 0.05 % Tween 20; pH 6.0) for 20 min to unmask epitopes. BNDF and III-beta-tubulin were both determined with rabbit polyclonal antibody diluted 1:500 in TPBS with 1 % non-fat dry milk (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Negative controls were performed by substituting the primary antibodies for normal rabbit or omitting it completely. After washing (3 x 10 min) in TPBS, sections were incubated for 2 h with Alexa Fluor 488 goat anti-rabbit IgG antibody

(Invitrogen Molecular Probes, Eugene, Oregon, USA) at a concentration of 1:1000 in TPBS with 1 % non-fat dry milk (Bio-Rad). After washing in TPBS (3 x 10 min), the sections were stained with DAPI and mounted with Vectashield antifade mounting medium (Vector laboratories, Burlingame, CA, USA).

3.2.6 Determination of cell survival by MTT

Cell survival in QNR/D cell cultures was determined by the MTT assay using a Vybrant assay kit (Molecular probes, Eugene, OR, USA). In this assay, a decrease in the number of living cells results in a decrease in the total metabolic activity of the cultures. This decrease directly correlates with the amount of formazan crystals, as monitored by absorbance (Vistica et al., 1991). Cell survival was determined after treatments to induce excitotoxicity with KA. The cells (7.5 x 10^5) were grown in 35 mm culture dishes (Costar Corning, NY, USA) with a final volume of 2 ml of DMEM (10 % FBS) per well in humidified atmosphere at 39 °C. After the treatments, cultured media was substituted with 1 ml DMEM media without phenol red (pre-warmed to 40 °C). The MTT labeling reagent (100 µl) was added to each dish (at a final concentration of 0.5 mg/ml) and then incubated for 4 h in humidified atmosphere. The resulting formazan crystals were solubilized using 1 ml of the solubilization solution (1 g SDS/ml in 0.01 HCl) and the plates were then incubated overnight in a humid atmosphere. Aliquots (200 µl) of solubilized formazan product were placed in a 96-well plate and then spectrophotometrically quantified at a wavelength of 570 nm in a microplate reader (Bio-Rad, Hercules, CA, USA).

3.2.7 RT-PCR for BDNF and NT3

Total RNA was extracted from each well by adding 1 ml of TRIzol according to the manufacturer's recommendations. RNA was purified from cellular lysate using the Zymo Direct-

zol purification kit according to instructions (Zymo Research Corp. Irvine, CA, USA). Genomic DNA contamination was removed by DNase I treatment (Invitrogen, Waltham, MA, USA) for 15 min at 37 ºC. First strand cDNA was synthesized from 2 µg of total RNA using 100 U of Superscript III Reverse Transcriptase (Life Technologies, Invitrogen, USA) and 1 mM dNTPs for 60 min at 42 ºC. 2 µl of cDNA was amplified in the presence of 10 µM sense and antisense primers, 50 mM MgCl2 10 nM dNTP mix, and 0.2 µM Platinum Taq High Fidelity DNA polymerase (Invitrogen). Primers were designed to amplify avian BDNF and NT3 (Table 3.1) and to cross intron-exon boundaries to control for genomic DNA contamination. PCR mixtures were first denatured at 95 °C for 5 min, then subject to 32 cycles of 95 °C, 15 sec denaturation; 55 °C, 40 sec annealing; and 70 °C, 40 sec elongation with a final extension of 72 °C for 10 min using a thermal cycler. PCR products were compared with a 1 kb plus DNA ladder (Invitrogen, Ontario, Canada).

3.2.8 Quantitative PCR for GH, IGF-1, BDNF and NT3

The cDNA synthesis was done as described above (2 wells per experimental condition) and the IGF-1, BDNF and NT3 mRNAs expression was quantified by real time PCR in a sequence detection system ABI-PRISM 7900HT (Applied Biosystems, Foster, CA, USA) and using SYBR Green (Roche, Mannheim, Germany) in 10 µl final volume containing: cDNA 3 µl (dilution 1:5) and 1 µl of each specific primer (0.5 µM). Primer sets used were: for GH, cGHf-cGHr; for BDNF, cBDNFf-cBDNFr, for NT3, cNT3f-cNT3r; for IGF1, cIGF1qf-cIGF1qr; for SOD, cSODqf-cSODqr, for 18s, c18sqf-c18sqr (Table 3.1). Primers were designed to amplify avian mRNAs. Reactions were performed under the following conditions: initial denaturation at 95 ºC for 10 min, followed by 45 cycles of 95 ºC for 15 sec, 60 ºC for 15 sec and 72 ºC for 15 sec. Dissociation curves were included after each qPCR experiment to ensure primer specificity. Relative abundance

of GH, IGF1, BDNF and NT3 mRNAs were calculated using the comparative threshold cycle (Ct) method and employing the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) where the quantification is expressed relative to the geometric mean of SOD and 18S mRNA (Vandesompele et al., 2002).

3.2.9 Vector construction

The construction of the plasmid pSPGH for GH over-expression was previously reported by Martínez-Moreno et al. (2016). This system (pCAG by Addgene, Cambridge, MA, USA) has a strong constitutive promoter (CMV) and a chicken actin enhancer. For controlled over-expression, the regulated expression mammalian system (Promega, Madison, WI, USA) was implemented and it was fully functional in avian species. This construction was made using pituitary cDNA as template to amplify by PCR the full-length chicken GH cDNA using the forward AAAGCGATCGCCATGGCTCCAGGCTCGTGGTTTTCTCC and reverse AAAGTTTAAACTCAGATGGTGCAGTTGCTCTCTCC primers. The DNA fragment was directionally cloned in the pF12A vector (Promega) at the *SgfI-PemI* sites as was described previously (Martínez-Moreno et al., 2016). In the constructed vector pfSPGH, the GH expression is induced by a dimeric transactivator that interacts with an operator site in the minimal CMV promoter. The transactivator is constitutively expressed in cells transfected with the plasmid pReg-Neo (Promega) and it is dimerized in presence of coumermycin (Sigma, St Louis, MO, USA).

3.2.10 QNR/D cell line transfection

QNR/D cells were plated at 1 x 10^6 cells in 35 mm dishes. After 16 h of stabilization, the cells were co-transfected with 3 µg of DNA plasmid in a ratio 1:1 pfSPGH:pReg-Neo using 3 µl of Lipofectamine 3000 (Invitrogen) per µg of DNA. In addition, as positive control, other cultures were transfected with the plasmid pSPGH, which constitutively express GH under the CMV

promoter. Four-hours post transfection the cells were treated with different doses of coumermycin (1, 5, 10 and 50 nM, diluted in M199) and 24 h later they were harvested and lysed by sonication (30 sec in ice) in presence of 200 µl RIPA lysis buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a cocktail of protease inhibitors (Complete Mini, Roche Mannheim, Germany) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). GH expression was analyzed resolving 50 µg of proteins extracts in a SDS-PAGE followed by Western-blot. Each condition was evaluated in triplicate. Coumermycin (Sigma) was re-suspended in DMSO and diluted to 100 nM in M199 media (Gibco, NY, USA). Concentrations of 10 or 50 nM of coumermycin were added for 24h to the QNR/D cell cultures to induce over-expression since these were the concentrations that showed to be more effective (Figure 3.8 A and B).

3.2.11 SDS-PAGE and western blot analysis

Total proteins in primary NR cell cultures were extracted using homogenization buffer (HCl-Tris 0.05 M, pH 9.0) in the presence of a protease inhibition cocktail (Mini-complete, Roche). Equivalent amounts of extracted proteins (35 µg) were separated by 12.5 % SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Nitrocellulose-free binding sites were blocked with 5% non-fat milk (Bio-Rad, Hercules, CA, USA) in PBS (Gibco, NY, USA) for 2 hours at room temperature. Then, membranes with protein samples were incubated overnight at 4 ºC with the following antibodies (Table 2): anti-chicken-GH, anti-phospho Akt antibody, anti-bcl-2 or anti-GADPH diluted in TPBS (1 x PBS with 0.05% tween (v/v)). After washing the membranes with TPBS (2 x 5 min) they were then incubated for 2 h with the corresponding HRP conjugated secondary antibody. Bands were visualized using ECL Blotting Detection Reagent

(Amerasham-Pharmacia, Bickinghanshire, UK) on autoradiography film (Fujifilm, Tokio, Japan). Kaleidoscope molecular weight markers (Bio-Rad) were used as reference for mass determination.

3.2.12 Neurite length and retinal thickness analysis

For neurite length measurements, the cell culture images were captured in phase contrast using and inverted microscope. Lumanera capture software was used for imaging. Neurite length was traced using the Simple Neurite Tracer (Fiji freeware; Schindelin et al., 2012) and Image J (NIH freeware). Approximately 50-100 neurites were analyzed by field of view (image), and 3 fields were analyzed per group from 3 independent experiments (n=9 images per condition). Retinal thickness measuring by H/E staining was determined in 4 microscope fields per eye and 6-7 individual eyes were analyzed, for this analysis fields with the same position were selected. Image Pro software was used for measurements. Central retinal measurement was performed in the same area for equivalent group comparison, the criteria of ~1-3 mm from the head of the optic nerve was applied for image capturing.

3.2.13 Statistical analysis

In all the experiments values are expressed as mean ± SEM. Significant differences between groups or treatments were determined by unpaired student's t-test or by one-way ANOVA where appropriate. LSD Fischer provided a *post-hoc* test when one-way ANOVA was used to compare experimental group with controls. P-values less than 0.05 were determined to be statistically different (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Over-expression was corroborated by real-time quantitative PCR (qPCR) and western blotting from 3 different transfections, including at least 3 cell cultures per group. MTT assays were performed in triplicate from 3-5 independent experiments. Changes in pAkt/GADPH and bcl-2/GADPH activation were

determined from 3 experiments in duplicate by western blotting. BDNF, NT3 and IGF-1 mRNA expression statistical analysis was determined from 3-4 different experiments by duplicate (including housekeeping genes).

3.3 Results

3.3.1 Protective effect of GH in neuroretinal (NR) cultures under long-term KA exposure

Long-term incubations (24 h) of embryonic NR primary cell cultures with KA induced excitotoxic cell death in a dose-response manner (Fig. 3.1 A). Cell survival significantly decreased in cultures treated with KA at 200, 300, 500, and 1000 µM in comparison to controls without KA, as determined by MTT assay. To determine if GH has a positive effect on cell survival during chronic KA exposure, cells were co-incubated with KA (300 µM) and chicken recombinant GH at different concentrations (1, 10 or 100 nM) for 24 h (Fig. 1B). Excitotoxic cell death induced by KA decreased cell viability (49.28 %) in comparison with cells maintained in media without KA. Long-term incubations with exogenous GH at 10 and 100 nM induced an increase in cell survival ($P < 0.05$) by 12.09 % and 10.98 % respectively in comparison with cells treated only with KA.

3.3.2 Protective/regenerative effect of GH on neurites under long-term KA exposure

To determine the protective and regenerative effect of exogenous GH on the dendritic trees of neuroretinal cultures, the cells were incubated with GH (1 or 10nM), with KA (500 or 750 µM), or with GH 10 nM + KA 500 µM (Fig. 2). GH, at both 1 nM and 10 nM, increased the average neurite length in comparison with control (Fig. 3.2 B, C and H) due to an increase in the proportion of large neurites (>30 µm), and at 10 nM decreased the proportion of small outgrowths (<10 µm) (Fig. 3.2 G). Cell cultures incubated with KA at 500 or 750 µM, decreased in number, showed a severe loss and damage of neurites and evident morphological changes including perikarya shrinkage (Fig. 3.2 D and E). The KA significantly decreased the number of medium and long

neurites (>10 µm) and increased the number of short neurites (<10 µm) (Fig. 3.2 G). The average length of all outgrowths was also significantly decreased in cultures treated with KA in comparison with the untreated control (Fig. 3.2 H). Co-incubations of GH (10 nM) and KA (500 µm) for 24h resulted in a protective/regenerative effect on medium and long neurites (Fig. 3.2 F). The proportion of small neurites also decreased in the KA+GH group in comparison with the KA group (Fig. 3.2 G). Cell cultures co-treated with KA and GH showed an increase in the average length in comparison with the group treated with KA (Fig. 3.2 H).

3.3.3 Protective effect of GH in NR cultures against short-time KA exposure

Short-term incubations (2 h) of embryonic NR primary cell cultures with KA (50, 100, 200, 300 or 500 µM) induced cell death in a dose-response manner (Fig. 3.3). To determine the protective effect of GH, the cells were incubated in presence of KA or GH and KA, while untreated cells were used as control. The cultures treated with GH and KA were pre-incubated with GH (10 nM) for 1 h and then co-incubated with KA at the aforementioned concentrations together with GH (10 nM) for 2 h. KA and KA+GH was replaced with GH (10nM) and incubated for 24 h. Cell cultures treated with KA (at all doses) showed a significant decrease in cell viability compared to their corresponding KA+GH group. Co-incubations with KA and GH showed no significant difference when compared against untreated control except for KA (500 µM) with GH.

3.3.4 Neuroprotective effect of GH in the retina (in vivo)

Neonatal chickens (at postnatal day 1; P1) were injected with a single dose of KA (20 µg per eye) and to determine the effect of GH as a neuroprotective factor in the postnatal chicken retina, the eyes were pre-treated twice with GH (300 ng per eye), 1 day and 1 h before respectively, and finally KA was also co-injected with GH (150 ng). KA induced excitotoxic-damaged tissue in the neuroretinal epithelium was clearly observed 48h post injection (Fig. 3.4). Morphological

damage was observed in all the retinal layers (optic fiber layer, OFL; ganglion cell layer, GCL; inner plexiform, IPL; inner nuclear layer INL; outer plexiform layer, OPL and outer nuclear layer, ONL) in both, the central and peripheral retina (Figure 3.4 D-F). A reduction in the eosin staining showed decreased synaptic tree integrity in the IPL in chicken eyes with neurotoxic damage. This damage was reduced when retinas were treated with GH, though we still observed displaced cells in the IPL (arrows; Fig 3.4 G and 4H).

Neuroretinal thickness was significantly reduced (by 58.8 μm; $P < 0.01$) after KA treatment and GH reduced this damage to 23.9 μm ($P < 0.05$) in relation to KA group (Fig. 3.5 A). The neurotoxic damage was mostly located in the IPL and INL (>50%); from 24.14 ± 2.3 to 13.12 ± 2.8 μm and 62.16 ± 4.8 to 29 ± 6.9 μm respectively (Fig. 3.5 D and E). A neuroprotective effect of GH was observed in both layers since hormone treated groups (23.5 ± 3.9 and 39.45 ± 4.7 μm respectively for IPN and INL) were significantly different from KA groups (Fig. 3.5 D and E). GH showed no neuroprotective effect against KA in OFL or GCL as determined by thickness analysis (Fig. 3.5 B and C). The OPL and ONL showed no changes in thickness in response to KA or GH and KA (Fig. 3.5 F and G). However, cell arrangement as determined by H/E and III-ß-tubulin showed changes in cytoarchitecture in these layers (Fig. 3.4). Cell number (in 0.1 mm retinal width) significantly decreased in the INL and GCL ($P < 0.001$ and $P < 0.05$ respectively) after KA treatments. KA-induced cell death was reduced by GH (by 20.1 % and 26.6 % respectively) in comparison with KA group ($P < 0.05$) (Fig. 3.5 H and I).

3.3.5 Effect of GH on BDNF and NT3 expression after KA injury

The presence of BDNF and NTF3 in postnatal neuroretinas (NR), QNR/D cells, and brain was confirmed by RT-PCR (Fig. 3.6 A). No other bands were observed and the negative control without template (NTC) showed no band. BDNF immunofluorescence was localized in the cellular

body of RGCs in the postnatal neuroretina (Fig. 3.6 B; arrows). BNDF and NT3 mRNA expression levels were analyzed in retinas extracted from eyes injected with KA and/or GH (Fig. 3.6 C and D). Eyes were injected over 4 consecutive days with 300 ng of GH (at P1, 2, 3 and 4) and showed similar levels of both, BDNF and NT3 in comparison to sham group. Experimental excitotoxicity induced by KA injection (P2, 20 µg) significantly decreased BDNF and NT3 mRNA levels ($P < 0.05$) at 96 h post-injury. Multiple injections of GH prevented a decrease in BDNF mRNA induced by a single dose of KA (Fig. 3.6 C). NT3 mRNA levels in the GH+KA group were not significantly different than levels of the KA treated group (Fig. 3.6 D).

3.3.6 Exogenous GH induces IGF-1, BDNF and NT3 mRNA expression in NR cultures

BDNF and NT3 gene expression was significantly increased by GH treatment (10 and 100 nM) in NR cell cultures after a short-incubation (4 h), whereas IGF-1 was increased only with the 100 nM GH dose (Fig. 3.7 A-C). However, long-term incubations (24 h) with GH at 10 or 100 nM both significantly decreased IGF-1 gene expression, while BDNF and NT3 mRNA levels were not significantly different in comparison with control.

3.3.7 Regulated GH over-expression induces BDNF and NT3 in QNR/D cells

GH over-expression was induced in QNR/D using two plasmid systems; a regulated vector (pfSPGH) that is regulated by coumermycin and a vector with a strong and constitutive promoter (pSPGH). GH mRNA expression and its corresponding immunoreactivity were over-expressed with both vectors (Fig. 3.8 A and B). The highest GH expression observed in cells transfected with the regulated expression system (pfSPGH) resulted from treatment with 10 and 50 nM coumermycin. However, the constitutive expression provided by the pSPGH plasmid produced the maximum GH expression and synthesis. GH over-expression resulted in increased BDNF and NT3 mRNA levels (Fig. 3.8 C and D). BDNF mRNA levels were significantly increased ($P < 0.05$)

only in the cultures transfected with the constitutive expression plasmid pSPGH (Fig. 3.8 C). Cell cultures transfected with inducible GH expression plasmids did not show significant difference in BDNF expression in groups stimulated with coumermycin at 10 and 50 nM in comparison with the control (Fig. 3.8 C). NT3 mRNA was increased ($P < 0.05$) only in the group treated with 50 nM of coumermycin (Fig. 3.8 D).

3.3.8 GH increases pAkt and Bcl-2 in NR cultures

Bcl-2 (27-kDa) and phospho-Akt (60-kDa) immunoreactive bands were observed in neuroretinal extracts (Fig. 3.9). As expected, cell cultures incubated for (30 min) with exogenous GH (at 1 or 10 nM) significantly increased Akt phosphorylation and the anti-apoptotic protein Bcl-2. GH treatment at a concentration of 100 nM did not show significant difference in pAkt or Bcl-2 immunoreactivity in comparison with untreated control.

3.4 Discussion

This study provides evidence that exogenous GH protects against excitotoxic damage in the neuroretina, both *in vitro* and *in vivo*. The protective actions of GH include a significant increase of cell survival and neurite growth in embryonic neuroretinal cell cultures and the preservation of the cytoarchitecture of the postnatal neural retina after a KA intravitreal injection. The neuroprotective actions of GH in neuroretina were mainly observed in the IPN and INL, however protection was also detected in the GCL. Our findings have also demonstrated the involvement of BDNF and NT3 in GH-induced neuroprotection.

The pro-survival actions of GH in the chicken neural retina during eye development in early and late embryonic stages are well established (Sanders et al., 2005, 2010; Harvey et al 2016; Harvey and Martinez-Moreno et al., 2016). In the cerebellum and embryonic retina, the anti-

apoptotic effects of GH involve the activation of the JAK/STAT, PI3K/Akt pathways and Bcl-2 (Sanders et al., 2006; Alba-Betancourt et al., 2013; Avila-Mendoza et al., 2016; Fleming et al., 2016; Martinez-Moreno et al., 2016). Our findings demonstrated that GH activates the PI3K/Akt pathway and increases Bcl-2 in the neuroretina; these results are similar to previous reports in the chicken cerebellum and embryonic retina, indicating a conserved neuroprotective action within the nervous system. This work has also corroborated Akt pathway activation and Bcl-2 increase in NR primary cell cultures by GH. We have previously demonstrated in organotypic cultures of chicken embryonic retinas that GH acts as a neuroprotective factor since it decreases LDH release and the number of TUNEL-positive cells (Martinez-Moreno et al., 2016). Neuroprotective effects of GH against glutamate have also been previously shown in QNR/D cells, an experimental model of embryonic avian RGC physiology (Martinez-Moreno et al., 2014, 2016). Moreover, a single intravitreal dose of GH prevented the damage progression induced by a KA injection in the retina of the green iguana (Avila-Mendoza et al., 2016). Until now, most studies of the neuroprotective actions of GH were focused mainly in embryonic RGCs through *in vitro* models (Harvey et al., 2016). This work shows, for the first time, a robust effect of GH protecting against experimental excitotoxicity induced by KA in postnatal chicken neuroretina (*in vivo*).

The glutamate receptor subunits GluK1 and Gluk2 (kainate receptors) in the avian retina are highly expressed in the INL, particularly in the bipolar cells (BCs) located in the outer half compared to the BCs of the inner half (Atoji, 2015). The GluK1 and Gluk2 subunits were also expressed in horizontal and amacrine cells in the INL, and in RGCs of the GCL (Atoji et al., 2015). In concordance with the KA receptor distribution reported by Atoji, we observed drastic damage in the IPL and INL induced by intravitreal injections of KA. In addition, explant retinal cultures incubated in the presence of glutamate/BSO showed the presence of a high number of TUNEL-

positive cells in the IPL (Martinez-Moreno et al., 2016). This demonstrates that chicken bipolar cells contained in the INL are particularly susceptible to experimentally induced excitotoxicity.

Neuroretinal primary cell cultures exposed to KA in range of 50 to 750 μM for short- and long-term (2 and 24 h, respectively) provided an *in vitro* model to determine pro-survival actions of GH. In many neural tissues including the retina, KA treatments are an accepted model to induce neurodegeneration, epilepsy and neurotoxicity (Fischer and Reh, 2002; Zheng et al., 2011). The administration of recombinant GH protected in both short- and long-term exposures to KA. However, the extended over-activation of KA-glutamate receptors reduced the protective effect of GH in comparison with a short-time KA exposure. Interestingly, short time incubations with KA combined with a preventive pretreatment of GH, resulted almost in a complete blockade of cell death. Short-time KA exposure resembles the glutamate release pattern during excitotoxicity in neural tissues, which occurs in peaks that expose the cells to short periods of GluRs over-activation enough to trigger cell death and to suppress pro-survival signaling (Zheng et al., 2011; Zhou et al., 2013).

Apoptotic and necrotic cell death are not the only consequences of retinal excitotoxicity, synaptic damage/loss is another of the many non-lethal negative and early effects induced by glutamate receptor overstimulation (Baltmr et al., 2010). Synaptic loss induces neurodegeneration and it triggers a cascade of aversive cellular effects that have been described as trans-synaptic degeneration, which is present in different diseases such as Alzheimer and in experimental and human glaucoma (Osborne et al., 1999; Casson, 2006; Gupta and Yucel, 2007; Cheung et al., 2008). Isolated embryonic RGCs increase axon length from GH stimulation (Baudet et al., 2009) and the blockade of local GH expression using siRNA significantly reduces the number and length of axons in culture. However, the ability of GH to protect the length and number of neurites in

postnatal retinas has not been demonstrated until now. We observed a significant increase in the average length of neurites in primary neuroretinal cell cultures treated with physiological concentrations of exogenous GH. In co-incubations of GH with KA, a significant protective effect was observed in neurite average length and in the number of long-neurites (>29 μm). KA incubations increased the number of short-neurites (<10 μm) which clearly was associated with neurite damage. These short-neurites formations significantly decreased in cells treated with KA and GH. Our results unfortunately do not differentiate if neurites re-grew or if they were protected against damage. However, there is a clearly a positive effect of GH in the outgrowths of neuroretinal cells treated with or without KA.

Neurotrophins are secreted growth factors that have an important role in retinal development, neural cell survival and renewal, differentiation, homeostasis, and vision physiology. Neurotrophins, such as NT3 and BDNF, are produced in both neuroretinal and glial cells (Hu et al., 2011). Neurotrophin privation compromises survival and induces apoptosis (Berkelaar et al., 1994; Johonson et al., 2009; Sipll et al., 2011) and the administration of BDNF and/or NT3 induces retinal neuroprotection and neuroregeneration in different pathophysiological experimental models (Johnson et al., 1986; Hohn et al., 1990; Berkemeier et al., 1991; Baltmr et al., 2010; Gauthier and Liu, 2016). BDNF as a mediator of neuroprotective actions of GH in the chicken embryo retina has been suggested by Sanders et al. (2008). This work provides evidence for the induction of BDNF by GH in the retina for the first time, similarly to the effect of GH upon BDNF expression in the injured rat brain and its positive impact on cognitive function (Zhang et al., 2014). We corroborated the presence of both, BDNF and NT3 in the chicken neuroretina, brain, and QNR/Ds. We also observed the presence of BDNF immunoreactivity in RGCs of the postnatal chick. Exogenous GH induced a significant increase in BDNF expression in primary retinal

cultures and the over-expression of GH using a system of antibiotic-inducible plasmids in QNR/D cells corroborated the responsiveness of BDNF expression to GH. Previous reports from our group showed that QNR/D cells incubated with GH induced NT3 expression (Martinez-Moreno et al., 2014). As expected, NR cell cultures incubated with GH increased NT3 expression as well in QNR/D cells transfected with plasmids for GH-overexpression. In KA injected neuroretinas, the gene expression of NT3 and BDNF was significantly decreased, although multiple intravitreal injections of GH restored BDNF expression to control levels. This suggests that after 96 h post excitotoxic injury, GH-induced BDNF plays a role in retinal healing. However, these results were not mirrored in NT3, which showed no significant increase in expression when compared with KA injected eyes. Thus, NT3 could be acting outside this time frame or not acting in this model of neuroprotection/neuroregeneration. The GH injections (without KA injury) did not increase NT3 nor BDNF. However, our *in vitro* experiments showed that GH is likely to induce a transient response, which is not maintained over time in neuroretinal cells without an excitotoxic insult.

The classical mediator of GH actions in growth, homeostasis, and metabolism is IGF-1. We have previously demonstrated that over-expression or administration of GH in QNR/D cells induce IGF-1 gene expression (Martinez-Moreno et al., 2014, 2016), and in immunopanned RGCs obtained from chicken embryos exogenous GH also increases IGF-1 mRNA expression (Baudet et al., 2009). This study demonstrates that GH induces a brief increase IGF-1 expression followed by a significant decrease after 24h, which is possibly due to a negative feedback induced by the high levels of IGF-1 in the culture media. Our data suggest that GH-induced neuroprotection is mediated in part through the expression of growth factors such as IGF-1, BDNF and NT3. The local production of GH in the retina and the influence of endocrine GH implies a complex network of autocrine, paracrine and endocrine peptides that participate in retinal physiology and

physiopathology. There are other growth factors such as CNTF, PEDF, BMP4 and FGFs, which have key roles in retinal development, and also promote homeostasis, protection and regeneration (Fischer et al., 2004; Thanos and Emerich, 2005; Johonson et al., 2011; Kimura et al., 2016; Todd et al., 2016). Thus, it is possible that GH could also work as a positive regulator of different of neurotrophic peptides in retinal development, neuroprotection and neuroregeneration.

Despite the significant amount of evidence supporting GH as a neuroprotective factor with strong neurotrophic actions, it has been associated with side effects that need to be studied and controlled. It is clear now that dysregulation in the levels of GH during development and adulthood are associated with several retinopathies in humans (as revised in Harvey and cols., 2007 and Harvey and Martinez-Moreno, 2016). One of the challenges for ocular GH therapy involves the modulation of angiogenic actions, which could disrupt the extremely controlled balance of pro- and anti-angiogenic factors which regulate the retinal microvasculature (Rymaszewski et al., 1991; Hikino et al., 2001). In humans, GH has been implicated in the anomalous cell growth and pathological neovascularization observed in patients with proliferative diabetic retinopathy (PRD) (Wilkinkson-Berka et al., 2006). In addition, patients with PDR have elevated concentrations of vitreal GH (Ziaei et al., 2009; Saric et al., 2015). The actions of GH on VEGF and its receptor, among many other angiogenic factors, remains unknown. However, it has been proposed that during development VEGF, IGF-1 and GH are stored in the vitreous to promote normal retinal growth and vascularization (Modanlou et al., 2006). It has also been demonstrated, in the chick embryo chorio-allantoic membrane, that GH has strong angiogenic activity (Gould et al., 1995). Regulated and cell-specific GH gene expression or specific delivery into retinal cell layers will be the approach for future research in order to target specific cells in controlled time frames. Regardless of the potential side effects, GH has demonstrated to exert beneficial effects in

patients with brain trauma, neurodegenerative processes or cognitive deficiencies (Zhang et al., 2014; Devesa et al., 2016a)

3.5 Conclusions

GH acts as a neutrophic factor promoting survival of neuroretinal cells, as well as neurite growth and regeneration. Intravitreal injections of GH protect the cells contained in the INL and GCL from excitotoxic insult in addition to decreasing the KA-induced damage in the dendritic trees that form the IPL. GH was able to induce BDNF expression in NR cultures and, *in vivo*, was also able to restore its expression 96 h after excitotoxic injury. GH administration only induced NT3 increase in NR cultures. Our data corroborates previous findings that suggest that the Akt pathway and the Bcl-2 protein are involved in retinal neuroprotection. This work is an important step towards the use of GH as a therapeutic factor to treat neurodegenerative retinal diseases.

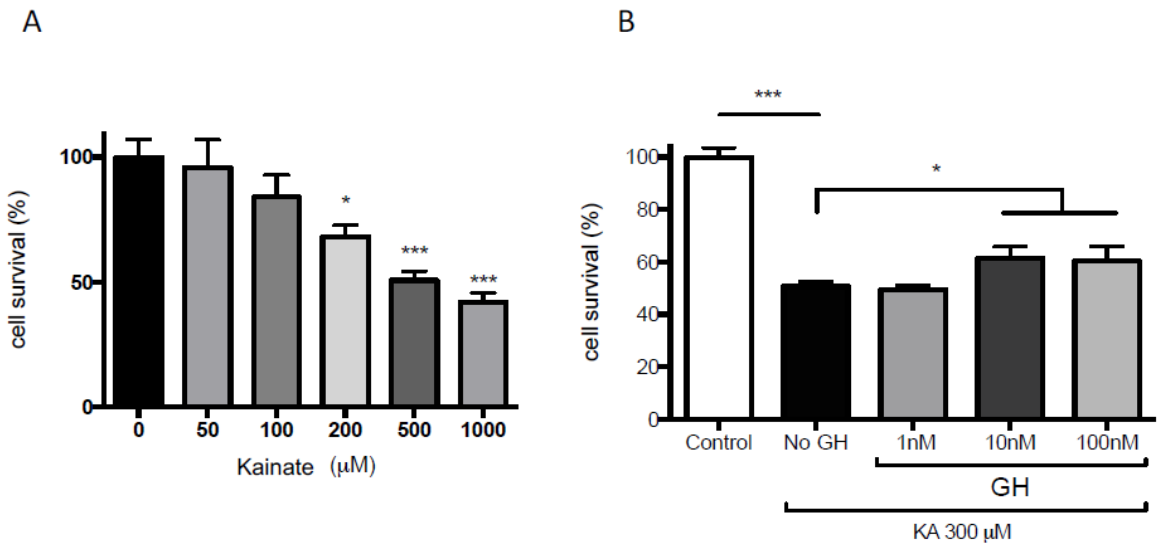

Figure 3. 1 GH protection against kainic acid (KA) injury after long-term (24 h) incubations in neuroretinal cultures. **A**, KA cell death induction (dose-response). KA at 50, 100, 200, 300, 500 and 1000 μM was applied in DMEM with FBS 3 % for 24 h. **B**. Increased cell survival by recombinant chicken GH (1, 10 and 100 nM) in the KA cell death assay; cell death was induced with 300 μM KA. Control group was incubated only in DMEM + FBS 3%. Bars represent mean ± SEM (from 3-4 independent experiments in triplicate; n=9-12 cultures per group). Cell survival was determined by MTT. Asterisks indicate significant difference (**, $P < 0.01$; ***, $P < 0.001$) as determined by one-way ANOVA and LSD Fischer as *post-hoc* test.

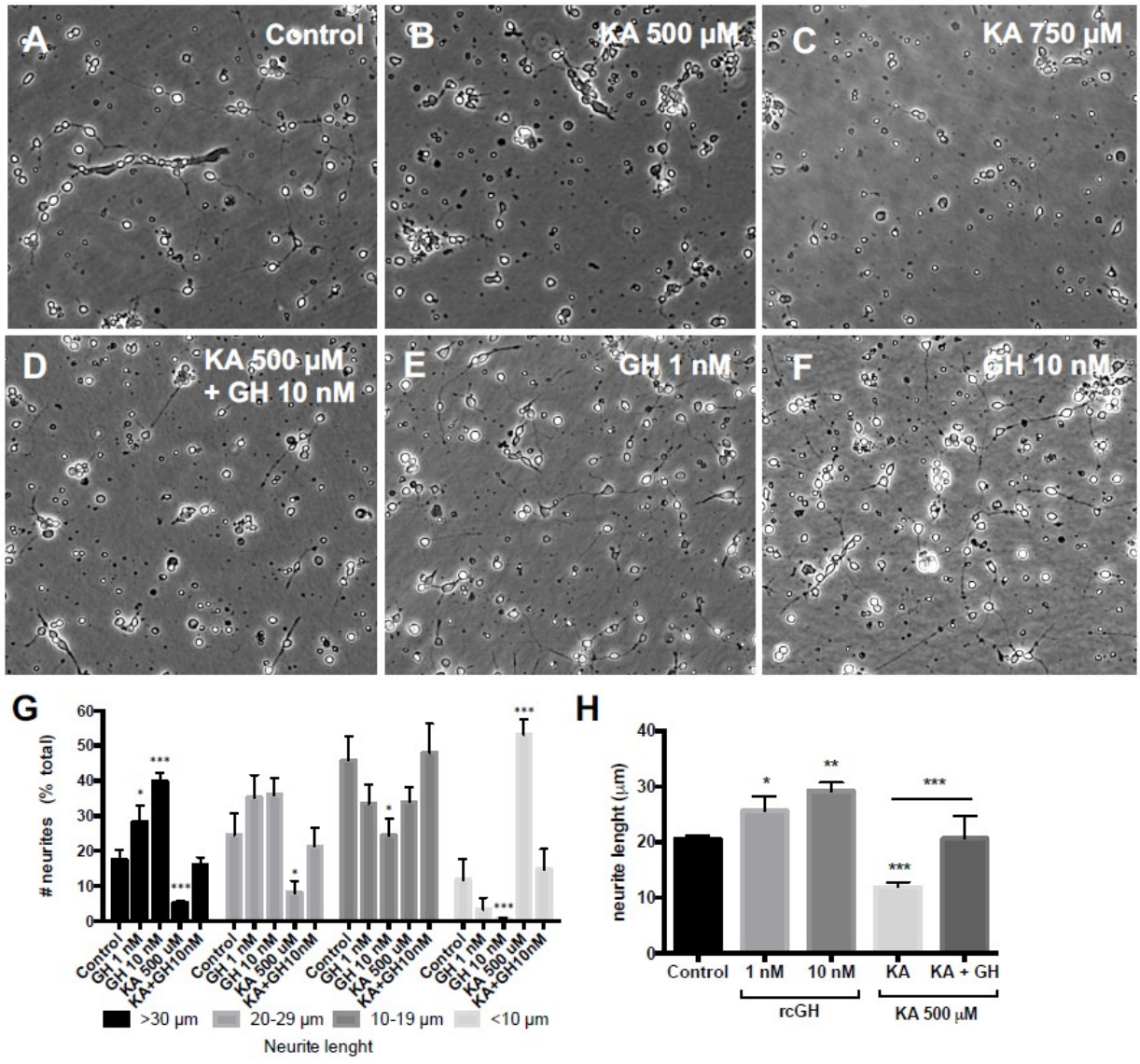

Figure 3. 2 Protective and neurotrophic effect of GH in neurites (*in vitro*) (long-term incubations; 24 h). **A-F.** Phase contrast micrographs from neuroretinal cultures. (A) Intact control; (B and C) experimentally induced-excitotoxic damage controls (incubated with KA at 500 and 750 µM); (D) protective effect of GH in NR cultures; (E and F) Increase of outgrowths by GH treatments (1 and 10 nM). **G.** Relative proportion (%) of axon length in NR cultures treated with GH and/or KA. **F.** Increased neurite length by recombinant chicken GH (1 and 10 nM) in the KA cell death assay; cell death was induced with 300 µM KA. Bars represent percentage ± SEM from total neurites.

Asterisks indicate significant difference (**, $P < 0.01$; ***, $P < 0.001$) as determined by one-way ANOVA for multiple comparisons and LSD Fischer as *post-hoc* test.

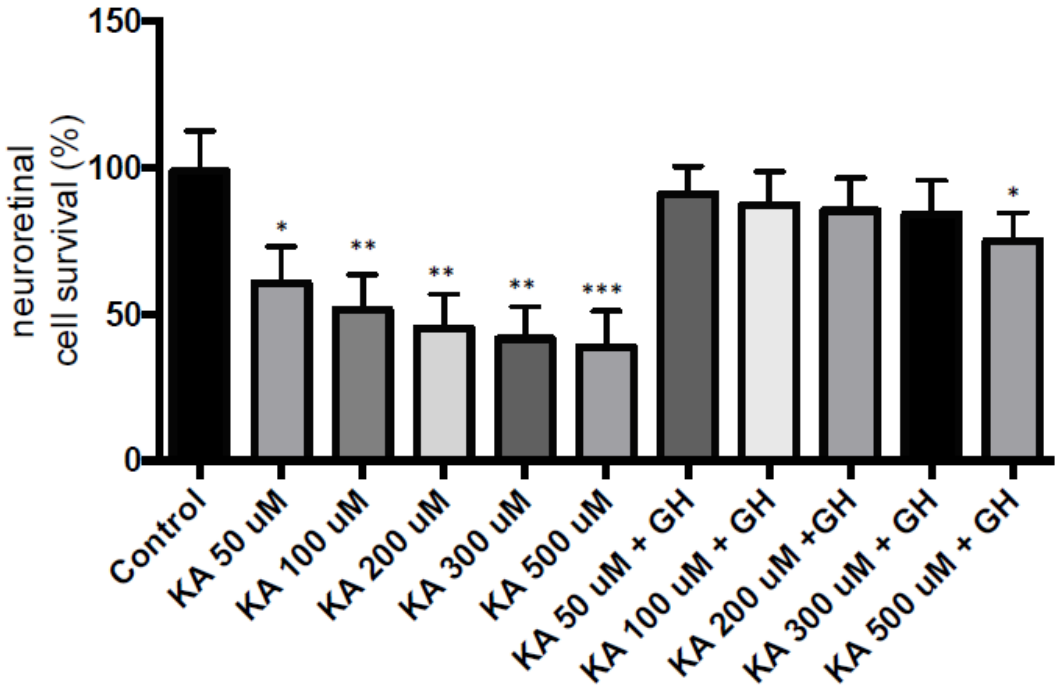

Figure 3. 3 GH protection against short-term (2h) incubations with kainic acid (KA) in neuroretinal cultures. KA cell death induction (dose-response); KA at 50, 100, 200, 500 or 1000 µM was applied in DMEM with FBS 1% for 24 h. GH treatments (10 nM) were applied to different doses of kainate (GH + KA 50-500 µM). Cells were pretreated with 300 ng prior to KA+GH co-incubations. Bars show mean ± SEM of survival percentage (%) as determined by MTT (representative of 3 independent experiments in triplicate, 9 cultures per group). Asterisks indicate significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) as determined by one-way ANOVA for multiple comparisons and LSD Fischer as *post-hoc* test.

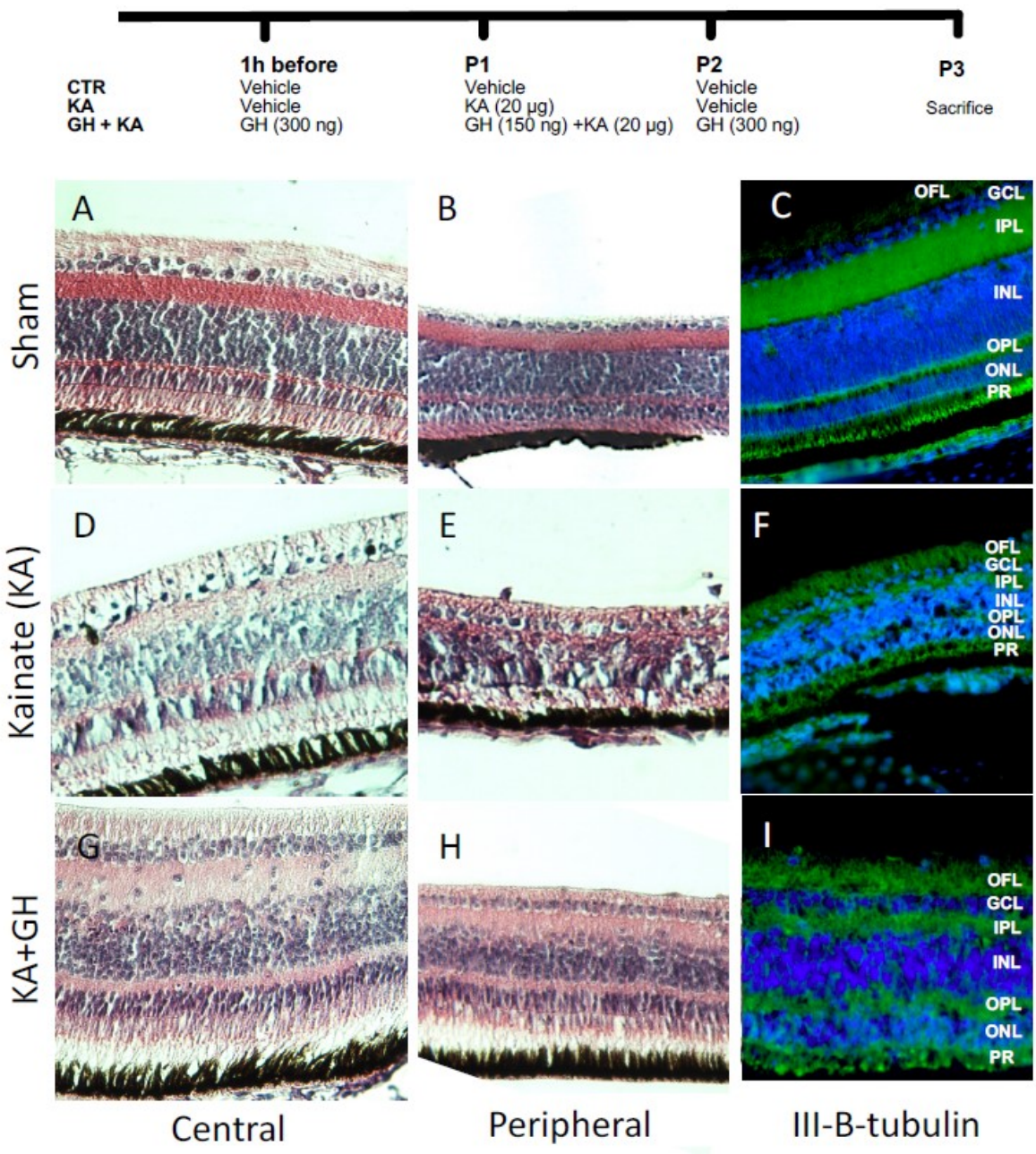

Figure 3. 4 Cytostructural effect of cGH against KA intravitreal injections. **A-C**, sham group. **D-F,** kainate damaged group. **G-I,** GH and KA treated group. First column stained with hematoxylin and eosin (H/E) (A, D and G) shows central retina; second column stained with H/E (B, E and H) shows a peripheral retinal area; third column, IHC against III-ß-tubulin and DAPI (C, F and I) shows central retina. Arrows show displaced cells in the IPL. Representative of 6-7 animals per

group. CTR; control group; KA, kainate group, GH+KA, growth hormone + kainate group; P, postnatal day. OFL; optic fiber layer; GCL, retinal ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PR, photoreceptors. Units in nanograms (ng) and micrograms (μg).

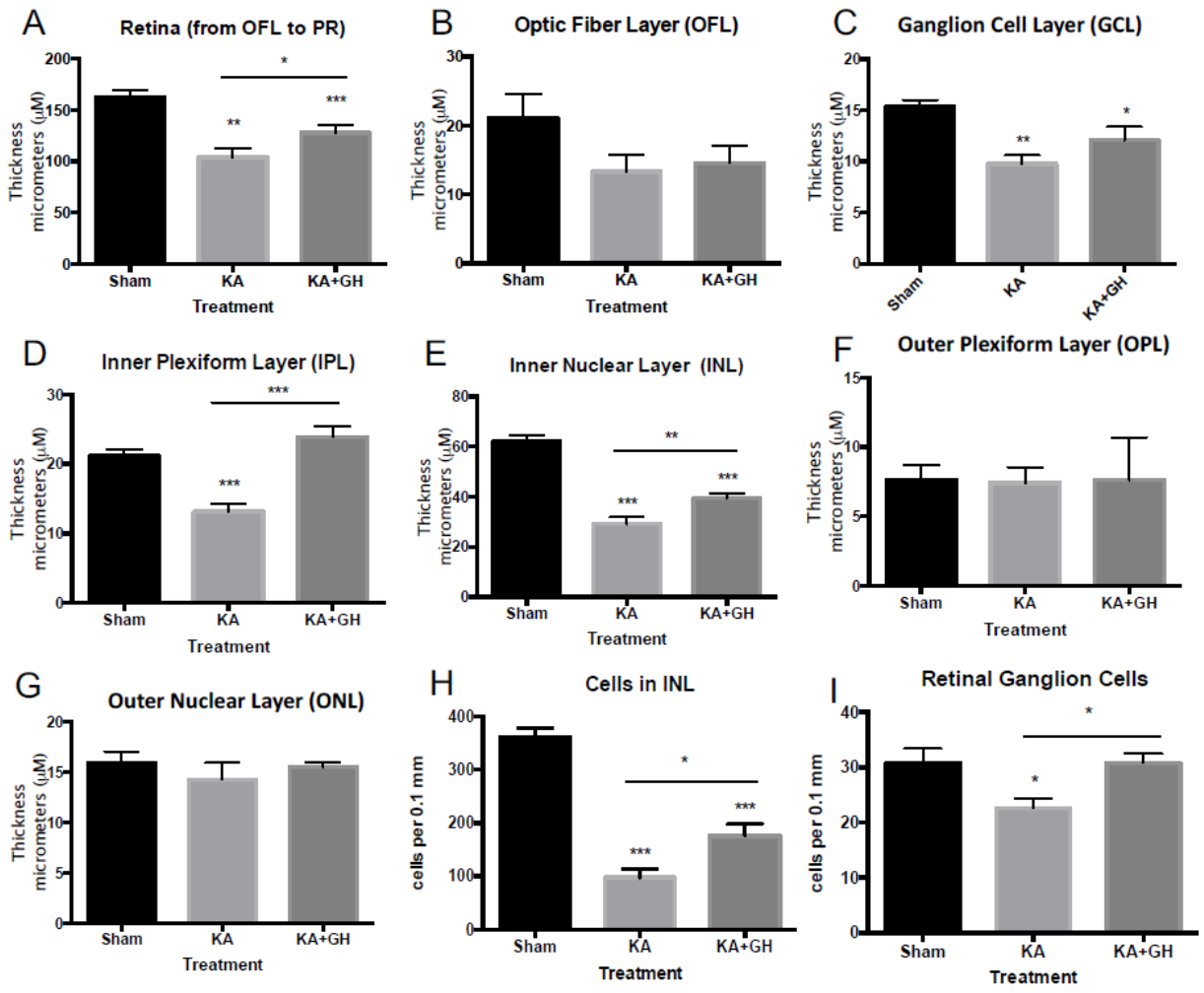

Figure 3. 5 Effect of GH on retinal thickness after a KA intravitreal injection. **A**. Whole retinal thickness from photoreceptors (PR) to optic fiber layer (OFL). Protective effect of GH on the thickness of: **B,** OFL; **C,** ganglion cell layer (GCL); **D,** inner plexiform layer (IPL); **E,** inner nuclear layer (INL); **F,** outer plexiform layer (OPL); and **E,** outer nuclear layer (ONL). Protective effect of GH in the number of cells in the INL (**H**) and in the RGCs (**I**). Units are expressed in micrometers (µm); bars show mean ± SEM from 6-7 animals per group (3 micrographs were analyzed per animal). Asterisks indicate significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P <$

0.001) as determined by one-way ANOVA for multiple comparisons and student's t-test (bar) was used to compare 2 groups (KA vs KA+GH; in A, H and E).

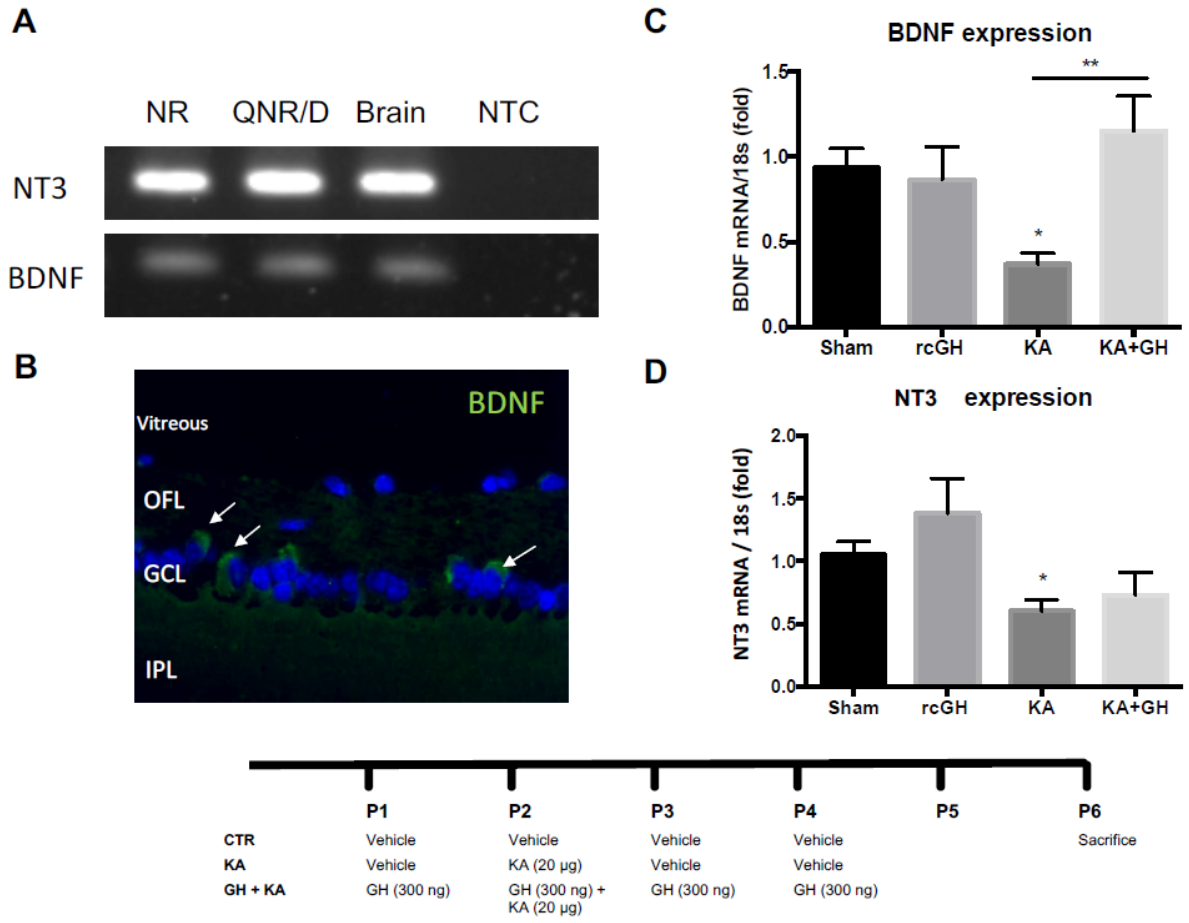

Figure 3. 6 BDNF and NT3 levels after treatments of GH and KA in the neuroretina. **A.** End-point RT-PCR for BDNF and NT3 in cDNA obtained from postnatal neuroretina (NR), QNR/D cells and neonatal chicken brain. Negative control without template (NTC). **B**. Immunohistochemistry for BDNF in neuroretina. Arrows show immunoreactivity (green). OFL; optic fiber layer, GCL; ganglion cell layer; inner plexiform layer (IPL). **C.** BDNF mRNA expression levels determined by qPCR. **D.** NT3 mRNA expression levels determined by qPCR. qPCR values were corrected by ΔΔCt. Ribosomal RNA 18s was used as housekeeping gene. CTR; control group; KA, kainate group, GH+KA, growth hormone + kainate group; P, postnatal day. Bars show mean fold ± SEM (n=4). Asterisks indicate significant difference (**, $P < 0.01$; ***, $P < 0.001$) as determined by one-way ANOVA for multiple comparisons and LSD Fischer as *post-hoc* test.

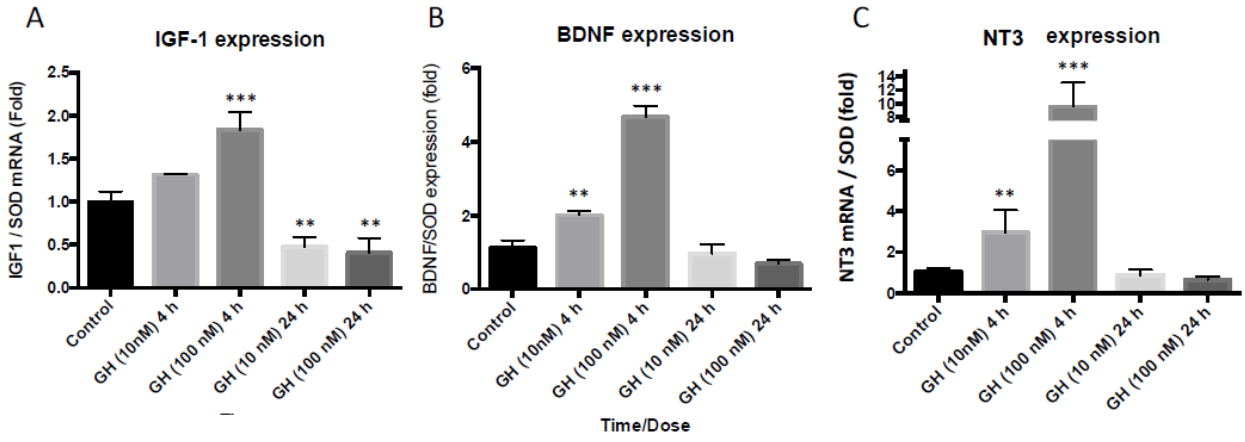

Figure 3. 7 Effect of short (4 h) and long (24 h) incubation of neuroretinal cultures with GH on the expression of IGF-1, BDNF and NT3. Cell cultures were incubated with GH at 1 or 10 nM. Expression levels were determined for: (**A**) IGF-1 mRNA, (**B**) BDNF mRNA and (**C**) NT3 mRNA. qPCR values were corrected by ΔΔCt. Superoxide dismutase (SOD) mRNA was used as housekeeping gene. Bars show mean fold ± SEM (from 3 independent experiments in duplicate). Asterisks indicate significant difference (**, P<0.01; ***, P<0.001) as determined by one-way ANOVA for multiple comparisons and LSD Fischer as *post-hoc* test.

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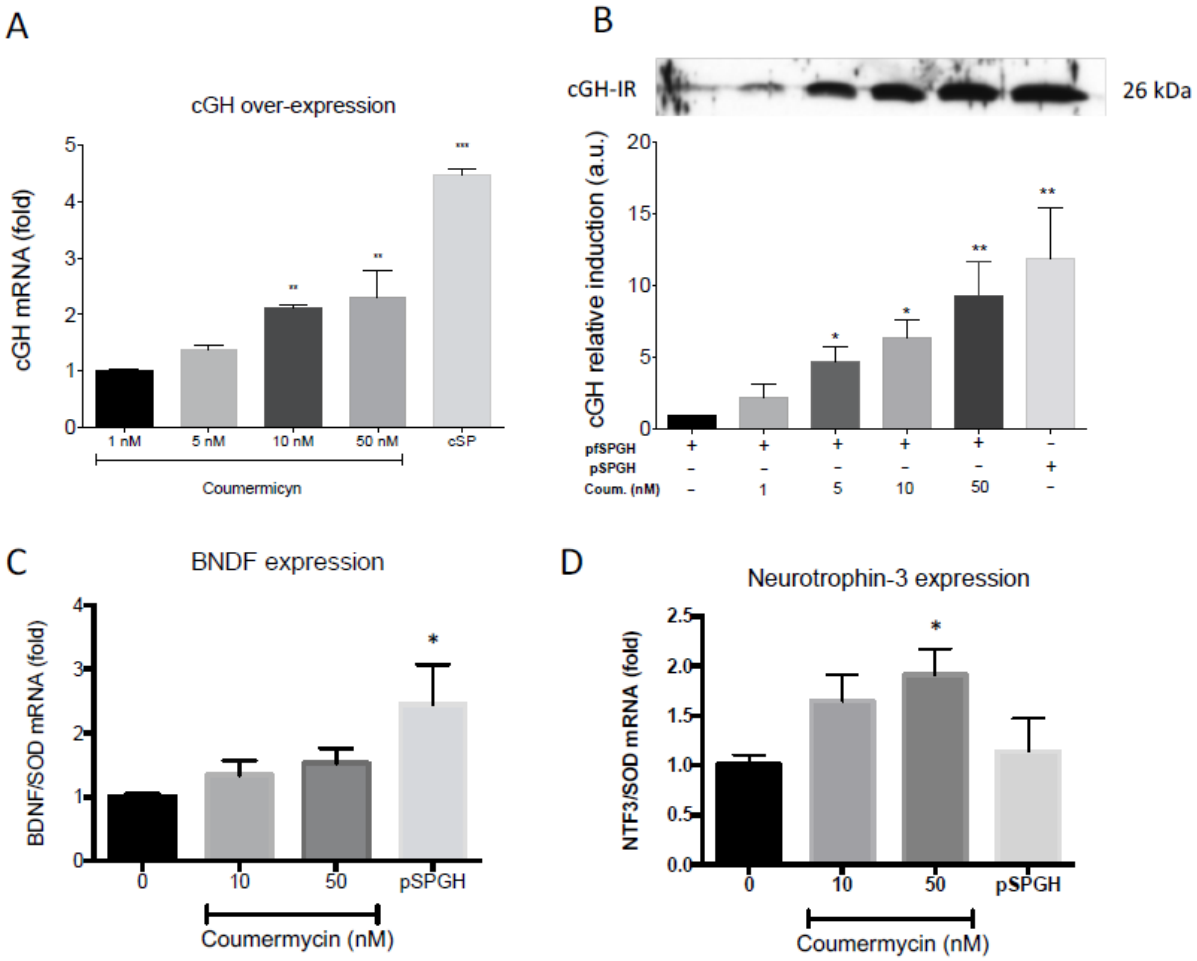

Figure 3. 8 Effect of GH over-expression on BDNF and NT3 mRNA levels. **A.** GH mRNA qPCR in QNR/D cultures transfected with plasmids for GH-overexpression. **B.** Representative luminogram and densitometry of GH-immunoreactivity (GH-IR) of transfected QNR/D cells; cGH relative induction is expressed in arbitrary units (a.u.). (**C**) BDNF mRNA levels and (**D**) neurotrophin-3 (NT3) mRNA levels in QNR/D cells transfected with plasmids for GH-overexpression. pfSPGH; plasmidic system with coumermycin-inducible GH over-expression. pSPGH; plasmidic system with constitutive promoter (CMV) and chicken b-actin enhancer for GH over-expression. Coumermycin (nM) was used to induce GH expression. qPCR values were corrected by ΔΔCt. Superoxide dismutase (SOD) mRNA was used as housekeeping gene. Bars

represent mean ± SEM (n=3; from 3 independent experiments analyzed in duplicate). Asterisks indicate significant difference (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$) as determined by one-way ANOVA for multiple comparisons and LSD Fischer as *post-hoc* test.

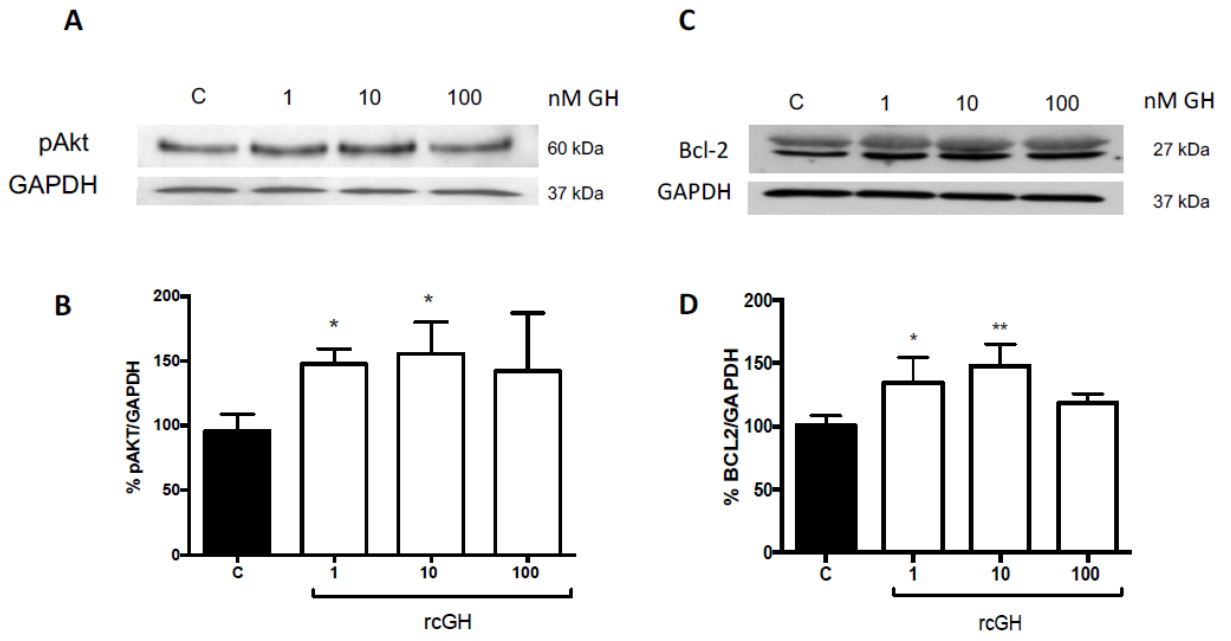

Figure 3. 9 Akt phosphorylation and Bcl-2 expression are increased after GH treatments in neuroretinal cell cultures. **A**, Phospho-Akt (pAkt) immunoreactivity (representative luminogram) in NR cells treated with GH at 1, 10 or 100 nM. GAPDH (37 kDa) re-blotting was performed as loading control. **B**, Densitometric analysis was used to determine the ratio of pAkt/GAPDH n=3 from independent experiments performed by triplicate (3 dishes per condition). **C**, Bcl-2-IR (representative luminogram) in the NR treated with GH at 1, 10 or 100 nM as determined by western blotting. **D**, Densitometry of immunoreactive of Bcl-2 bands. Bcl-2/GAPDH ratio was determined from 3 independent experiments performed in triplicate. Bars represent mean of relative change (%) ± SEM. Asterisks show statistical differences (*, $P < 0.05$; **, $P < 0.01$) as determined by one-way ANOVA for multiple comparisons and LSD Fischer as *post-hoc* test. kDa, kilodaltons.

Table 3. 1 Oligonucleotide primer sequences

Target	Primer	Sequence (5'-3')	Size	Accession #
cBDNF	Fwd	AGCAGTCAAGTGCCTTTGGA	167 bp	NM_001031616
	Rev	TCCGCTGCTGTTACCCACTCG		
cNT3	Fwd	AGGCAGCAGAGACGCTACAAC	248 bp	NM_001109762
	Rev	AGCACAGTTACCTGGTGTCCT		
cGH	Fwd	CGCACCTATATTCCGGAGGAC	128 bp	NM_204359
	Rev	GGCAGCTCCATGTCTGACT		
cIGF1	Fwd	TACCTTGGCCTGTGTTTGCT	170 bp	NM_001004384
	Rev	CCCTTGTGGTGTAAGCGTCT		
cSOD	Fwd	TTACAGCTCAGGTGTCGCTTC	146 bp	NM_204211
	Rev	ACCAAAGTCACGTTTGATGGC		
c18s	Fwd	CTCTTTCTCGATTCCGTGGGT	100 bp	M59389
	Rev	TTAGCATGCCAGAGTCTCGT		

Table 3. 2 Antibodies

Target	Host/Type	Dilution	Source	Cat. No.
BDNF	rabbit/polyclonal	1:100	Pepro Tech Inc.	500-P84
Phospho-Akt	mouse/monoclonal	1:750	Cell Signaling	92715
Bcl-2	rabbit/polyclonal	1:1000	Invitrogen	138800
Chicken GH	rabbit/antiserum	1:5000	Not commercial	CAP-1
GAPDH	goat/polyclonal	1:2000	Cell Signaling	21182
III-ß-tubulin	rabbit/polyclonal	1:1000	Sigma	T8578

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Chapter Four

Growth hormone promotes synaptogenesis and protects neuroretinal dendrites against KA induced damage

Publication and Author Contributions

The results of this paper have been submitted for publication in Fleming, T., Martinez-Moreno, C.G., Carranza, M., Luna, M., Harvey, S., Arámburo, C. Growth hormone promotes synaptogenesis and protects neuroretinal dendrites against KA induced damage. Gen. Comp. Endo.

Thomas Fleming and Dr. Carlos Martinez were responsible for all data presented in this paper. The manuscript was primarily written by Thomas Fleming.

4.1 Introduction

Growth hormone (GH) is produced in the pituitary and has many documented effects throughout the body, particularly effects on cell differentiation, proliferation, and survival (Oberbauer, 2015). However, in addition to its production in the pituitary, GH is now known to have many extra-pituitary sites of production (Harvey, 2010b). Of particular importance is its production in the neuroretina (NR), where it is expressed together with GH receptor (GHR), suggesting a local autocrine/paracrine mode of action (Harvey et al., 2015).

Several studies from our group have suggested critical roles for extrapituitary GH in retinal development and function (Harvey et al., 2007). For instance, GH involvement in axon growth and synaptogenesis has been demonstrated in immunopanned retinal ganglion cells (RGCs), which express both GH and its receptor as well as respond to exogenous GH by increasing axon length, whereas knockdown of endogenous GH expression with small interfering RNA (siRNA) resulted in a block of long axon formation (Baudet et al., 2009a). Additionally, exogenous GH treatments were able to increase GAP43 immunoreactivity in QNR/D cells (a quail RGC line) and SNAP25 immunoreactivity in chicken neuroretinal explants *in vitro* as well as increase SNAP25 immunoreactivity *in vivo* after the injection of exogenous GH into the vitreous of ED 10 chick embryos (Fleming et al., 2016). Extrapituitary GH is also neuroprotective in the chick neuroretina where its local expression is anti-apoptotic during waves of apoptosis that occur during normal development of the chick retina (Sanders et al., 2011). Furthermore, GH has shown to be neuroprotective against glutamate/BSO induced excitotoxicity *in vitro* in QNR/D cells (Martínez-Moreno et al., 2016).

Recent evidence in the chicken and green iguana suggests GH is a neuroprotective factor against injury caused by the injection of kainic acid (KA), a glutamatergic agonist (Ávila-Mendoza

et al., 2016; Martinez-Moreno et al., 2018). In the green iguana, locally produced GH was shown to be upregulated in response to KA treatment and exogenous GH was able to prevent cell loss in the inner nuclear layer (INL) and ganglion cell layer (GCL) caused by KA injection (Ávila-Mendoza et al., 2016). Similar results were found in the chick, where the neuroprotective effects of GH were mainly located in the INL and inner plexiform layer (IPL) (Martinez-Moreno et al., 2018). While these results show that GH is able to protect the cytoarchitecture of the retina after excitotoxic damage, its ability to protect/regenerate retinal synapses after KA treatment have not previously been reported.

The objective of this study was to determine if GH acts as a synaptogenic modulator by examining its protective effect on synapses of the retina under experimentally induced excitotoxicity with KA. This work provides further evidence showing GH promotes synaptogenesis *in vitro* as well protects dendrites of the neuroretina after excitotoxic insult.

4.2 Materials and Methods

4.2.1 Animals

Pathogen-free, fertilized eggs (*Gallus gallus*, White Leghorn) eggs were obtained from the University of Alberta Poultry research center (Edmonton, Canada) for use in primary cell culture experiments. For post-hatch experiments, eggs were obtained from Pilgrim's Pride (Querétaro México). Eggs were incubated at 38 °C in a humidified air chamber and were rotated one-quarter of a revolution every 50 min during incubation. Chicken embryos and post-hatch chicks were sacrificed with approval from the University of Alberta animal welfare committee and from the Instituto de Neurobiología (UNAM) bioethical committee.

2.2 Primary Retinal cell culture

Chicken embryos (ED10) were anesthetized in ice for 5 min and then sacrificed by decapitation. Eyes were enucleated, the vitreous was removed and neuroretinas were micro-dissected. Using a stereoscopic microscope, pigmented epithelium contamination was removed. For cell disaggregation, the neuroretinas were incubated in 70 U/ml collagenase (Sigma, Saint Louis, MO, USA) in DMEM (Gibco, Grand Island, NY, USA) for 40 min at 37 °C. The cells were vortexed and mechanically triturated with a pipette every 10 minute to aid in disaggregation. Cells were then pelleted and washed with DMEM + 10% FBS (Gibco, USA) twice. Cells were resuspended in DMEM + 10 % FBS (Gibco, USA) and plated on 35 x 10 mm poly-l-lysine coated (Sigma, Saint Louis, MO, USA) plates (Corning, New York, USA). Cells (5.5×10^5) were counted using trypan blue and hemocytometer, and plated in 24-well plates. Cell cultures were stabilized in a humidified incubator at 39 °C (chicken body temperature) and 5 % CO_2.

4.2.2 Treatments

For *in vitro* primary cell culture experiments, recombinant chicken growth hormone (rcGH) (Revholt, PRL, Israel) was dissolved in bicarbonate buffer and administered in DMEM with 10% FBS (Gibco, Grand Island, NY, USA) at a final concentration of 1 (PSD95) or 10 (SNAP25) nM for 24h. Negative controls were incubated without rcGH. For Cy3-GH experiments, 300ng of Cy3-GH was injected either 1h before KA injection (20µg) or 1 or 24h after then incubated for 24h. Cy3-GH pre-incubated with a GH antibody at room temperature for 24h was used for control. Cy3 conjugation to GH was performed according to Cy3 Mono-Reactive dye pack manufacturer's instructions (GE Healthcare Life Sciences, Amersham, ON, Canada). For intravitreal injections, KA (20 µg) and rcGH (300 ng and 150 ng) were diluted in injectable water (re-suspended prior to injections). *In vivo* injections for analysis by Western blot and qPCR were

administered into the left eye using the following strategy: a prior GH dose (300 ng, 24 h before damage), simultaneous KA (20 μg) + GH (300 ng), and two injections at 24 h and 48 h post-injury (GH, 300 ng). Eyes were stabilized for 24 h and were then collected. Injections for eyes analyzed by IHC were injected as follows: GH (300 ng) 24 h prior, GH (150 ng) and KA, and GH (300 ng) 24 after after-damage. The opposite eye (right) was injected with vehicle as a negative control. GH injections pre-and post-injury were used according to Fischer and Reh (2002), Todd et al. (2015, 2016) and Ritchey et al. (2012) to all for the induction of growth factor expression neuroprotection in the avian retina.

4.2.3 Immunohistochemistry

For immunohistochemical analysis, eyes were fixed with Davidson fixative for 48 h, dehydrated in ethanol, and embedded in paraffin. Sections (5 μm) were cut with a microtome and mounted on pretreated glass slides (Fisher Scientific, Hampton, HS, USA). Paraffin was removed from the slides with Citrisolv (Fisher Scientific, Hampton, HS, USA). Sections were gradually rehydrated in absolute alcohol (100 %), ethanol (95, 70, 50 and 30 %), distilled water, then lastly samples were equilibrated in PBS for 1h. Tissues were then boiled in citrate buffer (10 nM sodium citrate, 0.05 % Tween 20; pH 6.0) for 20 min to unmask epitopes. PSD95 and GAP43 were both used at a dilution of 1:500 in TPBS with 1 % non-fat dry milk (Bio-Rad Laboratories, Inc., Hercules, CA). Negative controls were performed in the absence of primary antibody. After washing (3 x 10 min) in TPBS, sections were incubated for 2 h with Alexa Fluor 488 goat anti-mouse (GAP43; Invitrogen Molecular Probes, Eugene, Oregon, USA) or Alexa Fluor 594 donkey anti-rabbit IgG antibody (PSD95; Invitrogen, USA) at a concentration of 1:2000 in TPBS with 1% non-fat dry milk (Bio-Rad). After washing in TPBS (3 x 10 min), the sections were stained with

DAPI and mounted with Vectashield antifade mounting medium (Vector laboratories, Burlingame, CA, USA).

4.2.5 Cryosectioning

Cy3-GH eyes were extracted and soaked for 24h in a 30% sucrose solution. After 24 h, the cornea and vitreous were removed and allowed to incubate for an additional 24 h. Eyes were then fixated in a 4 % PFA solution for 24 h. Eyes were then cryosectioned into 5 µm sections with a microtome, stained for DAPI and visualized using a confocal microscope Carl Zeiss LSM-510 (Carl Zeiss Inc., Thornwood, NJ).

4.2.6 Western blot analysis

Total protein extracts from primary cell cultures and whole chicken NRs were extracted by sonication and homogenization in presence of protease-inhibitor cocktail (Mini-complete, Roche Diagnostics, Basel, Switzerland) in 50 mM Tris-HCl buffer (pH 8.0). Samples were boiled for 5 min with 2x sample buffer containing bromophenol blue and 5% β-mercaptoethanol. Equivalent amounts of protein were added to each lane (40 µg) and electrophoresed in a 12.5% polyacrylamide gel using the buffer system of Laemmli (1970). The Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA) was used to ensure equal protein loading of each lane. Protein samples were transferred from the gel onto a nitrocellulose membrane. For immunoblotting, membrane blocking was carried out using 5% non-fat milk (Bio-Rad Laboratories, Inc., Hercules, CA) in PBS for 1 h. Primary antibodies solutions (Table 4.1) were made in a 1% non-fat milk TPBS solution, added to membranes and were incubated overnight at 4 °C with moderate shaking. Secondary antibody solutions were made at a concentration of 1:5000 with IgG-HRP cat anti-goat (Santa Cruz Biotechnology, CA, USA), goat anti-rabbit (Bio-Rad

Laboratories, Inc., Hercules, CA), or goat anti-mouse (Bio-rad) in a 1% non-fat milk/TPBS solution, and membranes were incubated for 2 h at RT. Immunoreactive bands were developed by chemoluminescense using ECL reagent (Amersham Biosciences, Montreal, Canada) after exposing the membranes to Kodak Biomax ML films which were then processed in an automated film processor. Protein loading was re-checked by labeling lanes for GAPDH after stripping the blots 5 min using Re-Blot striping solution (Chemicon International Inc. Fischerbrand, Hampton, HS, USA). Immunoblots were repeated 3 times, scanned, and quantified by densitometric analysis using ImageJ software.

4.2.7 RT-PCR

Total RNA was extracted from chicken NR using TRIzol reagent (Invitrogen, Canada Inc., Burlington, Ontario) Zymo Direct-zol purification kit according to instructions (Zymo Research Corp. Irvine, CA, USA). Genomic DNA contamination was digested with RNase-Free DNase Set for 20 min at room temperature (DNase I treatment; Qiagen, Ontario, Canada) before washing the column 3 times with the corresponding buffer. A second incubation with DNAse I (Invitrogen, Carlsbad, CA, USA) was performed at 37 ºC for 15 min. Total RNA was quantified in a nanodrop-2000 (Thermo Scientific, Waltham, MA, USA) and purity (>1.8 ratio of 260/280 nm) was confirmed. Reverse transcription of 3 µg of DNA-free total mRNA using Super Script 3 Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions and 1 µl of oligo (dT)18-20 was used to synthesize cDNA. 2 µl of cDNA was amplified in the presence of 10 µM sense and antisense primers for SNAP25, DLG1, or GAP43 (Table 4.2), 10x PCR buffer, 50 mM $MgCl_2$ 10 nM dNTP mix, and 0.2 µM Platinum Taq High Fidelity DNA polymerase. Primers were designed to amplify avian genes and to cross intron-exon boundaries to control for genomic DNA contamination. PCR mixtures were first denatured at 95 °C for 5 min, then

subjected to 30 cycles of 95 °C, 15 sec denaturation; 55 °C, 40 sec annealing; and 70 °C, 40 sec elongation with a final extension of 72 °C for 10 min using a thermal cycler (Techgene, Fisher Scientific, Canada). PCR products were compared with a 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

4.2.8 Quantitative PCR

Total RNA was extracted from each retina (opposite eye as control) by adding 500 ml of TRIzol according to the manufacturer's recommendations. RNA was purified from cellular lysate using the Zymo Direct-zol purification kit according to instructions (Zymo Research Corp. Irvine, CA, USA). First strand cDNA was synthesized from 1.5 µg of total RNA. Retrotranscription was performed with 100 U of Superscript III reverse transcriptase (Invitrogen) and 1 mM dNTPs for 60 min at 42 ºC. The DLG1, SNAP25, GAP43, GH, and GHR mRNA quantification was carried out by real time PCR in a sequences detection system ABI-PRISM 7900HT (Applied Biosystems, Foster, CA, USA) and using SYBR Green (Roche Diagnostics, Basel, Switzerland) in 10 µl final volume containing: cDNA 3 µl and 0.5 µM of each specific primer (Table 4.2). Reactions were performed under the following conditions: initial denaturation at 95 ºC for 10 min, followed by 45 cycles of 95 ºC for 15 sec, 60 ºC for 15 sec and 72 ºC for 15 sec. Dissociation curves were included after each qPCR experiment to ensure primer specificity. Relative abundance of DLG1, SNAP25, GAP43, and GH mRNA were calculated using the comparative threshold cycle (CT) method and employing the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) where the quantification is expressed relative to the geometric mean of 18S mRNA (Vandesompele et al., 2002).

4.2.9 Statistical analysis

In all the experiments values are expressed as mean ± SEM. Significant differences between multiple groups were determined by either one-way ANOVA (for GH + KA experiments) or two-way ANOVA (for KA damage over time experiments) with LSD Fischer *post-hoc* test. Unpaired Student's t-test was used to compare between two groups where appropriate. P-values less than 0.05 were determined to be statistically significant (*, P< 0.05; **, P< 0.01; ***, P<0.001). All the experiments were repeated 3-5 independent by duplicate. RT-PCR and Western blots from *in vivo* experiments were repeated 3-4 times from 3 different tissues.

4.3 Results

4.3.1 GH increases SNAP25 and PSD95 in chick embryonic neuroretinal primary cell cultures

The synaptogenic effect of GH on ED10 neuroretinal cell cultures was determined through its effect on SNAP25 (presynaptic) and PSD95 (postsynaptic) immunoreactivity (Fig. 4.1 A). Cultures were incubated for 24 h with 10nM (for SNAP25 analysis) or 1nM (for PSD95 analysis) GH. Cultures incubated in the absence of GH were used for control. Both SNAP25 (Fig. 4.1 B) and PSD95 (Fig. 4.1 C) showed a significant increase in immunoreactivity over control ($P < 0.05$ and 0.01, respectively).

4.3.2 KA treatment damages retinal synapses and decreases local GH abundance

Specificity of our primers for SNAP25 (185bp), DLG1 (PSD95; 162bp), and GAP43 (178bp) was confirmed by RT-PCR (Fig. 4.2 A). Single bands were observed at the predicted size which were not present in the absence of template.

To analyze the effect of excitotoxicity on synaptogenic gene expression in the chicken NR, 1-day post-hatch (P1) chicks were injected with 20 µg of KA in their left eye. Right eyes were

injected with vehicle (injection water) and used as sham control. Eyes were collected at 2, 24, 48, and 96 h post KA injection and analyzed for SNAP25, DLG1, GAP43, GH, and GHR expression by qPCR (Fig. 4.2). SNAP25 gene expression was significantly decreased at 2, 24 and 96 h post injection (Fig. 4.2 B; $P < 0.001$ and 0.05, respectively). DLG1 gene expression was decreased at 2 and 24 h post injection (Fig. 4.2 C; $P < 0.01$ and 0.05, respectively). GAP43 gene expression increased at 24 h post injection (Fig 4.2 D; $P < 0.05$). GH gene expression was decreased compared to control at 48 and 96 h post injection (fig. 4.2 E, $P < 0.001$). GHR gene expression increased at 48h post KA treatment over in comparison to sham control (Fig. 4.2 F, $P < 0.01$).

The effect of KA excitotoxicity in SNAP25, PSD95, GAP43, and GH immunoreactivity was determined by western blot (Fig. 4.3 A). SNAP25 IR was significantly decreased at 2, 24, 48 and 96 h post injection (Fig. 4.3 B; $P < 0.01$ and 0.001). PSD95 IR was decreased at 2h and 24h post injection (Fig. 4.3 C, $P < 0.01$ and 0.05 respectively). GAP43 IR showed no significant change. GH IR was decreased at 96 h post KA injection (Fig. 4.3 E, $P < 0.001$).

4.3.3 Exogenous Cy3-GH is internalized in the GCL after KA treatment

To determine if GH was internalized into the chick retina after damage by kainate treatment, Cy3-labelled GH was intravitreally injected into P1 chicks. Chicks were injected with 300 ng of Cy3-GH 1 h before, 1h after, or 24 h after a single injection of 20 µg of KA (Fig. 4.4 A-D). Cy3-GH injected eyes were incubated for 2 h then collected. Eyes injected with Cy3-GH preabsorbed with a GH antibody were included as control (Fig. 4.4 E). Eye sections were also counterstained for DAPI (Fig. 4.4 F-J). Cy3-GH was internalized into the GCL in a perinuclear location (Fig. 4.4 K). An increase in Cy3-GH uptake was seen in KA treated retinas when injected with Cy3-GH 1h before or 1h after damage (Fig. 4.4 M and N). However, perinuclear

internalization was absent when Cy3-GH was administered 24h after KA injury (Fig. 4.4 N). No Cy3 staining was observed in the GH pre-absorbed control (Fig. 4.4 O).

4.3.4 GH preserves PSD95 expression in KA damaged retinas

SNAP25, DLG1, and GAP43 mRNA expression levels were analyzed in retinas extracted from eyes injected with KA and/or GH (Fig. 4.5 A-C). Eyes were injected over 4 consecutive days with 300 ng of GH (at P1, 2, 3 and 4). KA injections (20 μg) were performed on P2. Eyes were then collected at P6. GH injected groups showed similar levels of expression to control groups in all genes analyzed. KA induced excitotoxicity caused a significant decrease in SNAP25 (Fig. 4.5 A) and DLG1 (Fig. 4.5 B) expression ($P < 0.05$ and 0.01, respectively) while GAP43 showed no significant change in expression. GH and KA treatment showed a significant increase in DLG1 expression in comparison to retinas treated with KA alone (Fig. 4.5 B, $P < 0.001$). SNAP25 and GAP43 expression in GH and KA treated retinas were not significantly different than levels in KA treated retinas (Fig. 4.5 B and C).

The effect of KA excitotoxicity on SNAP25, PSD95, and GAP43 immunoreactivity was determined by western blot (Fig. 4.6 A). KA treatment caused a significant decrease in both SNAP25 (Fig. 4.6 B) and PSD95 (Fig. 4.6 C) immunoreactivity ($P < 0.05$ and 0.01, respectively). GH and KA was significantly decreased compared to control and showed no change in SNAP25 immunoreactivity in comparison to KA treated retinas (Fig. 4.6 B). GH treatment prevented a decrease in PSD95 immunoreactivity seen in KA treated retinas (Fig. 4.6 C, $P < 0.001$). No change was seen in GAP43 immunoreactivity in comparison to KA or GH + KA treatment (Fig. 4.6 C).

4.3.5 GAP43 and PSD95 immunohistochemical analysis during GH neuroprotection

Changes in PSD95 and GAP43 immunofluorescence were observed in the retina after KA or KA and GH treatment. P1 chickens received an injection of GH (300ng) 1h prior to receiving

a co-injection of KA and GH (20 µg and 150 ng, respectively), then received a final GH (300 ng) injection 24 h after. Eyes were incubated for 24 h then collected. PSD95 and GAP43 immunofluorescence was strongly observed in the IPL under control conditions (Fig 4.7 A and B). Upon KA induced damaged, the pattern of PSD95 immunofluorescence was disrupted and the appearance of PSD95-postive muller cell-like structures throughout out the retina was observed (white arrows, fig 4.7 C). GH treatment resulted in intense PSD95 immunofluorescence in Müller-like cells (Fig. 4.7 E).

KA treatment relocated GAP43 immunofluorescence closer to the INL (white arrows, Fig 4.7 D). GH treatment caused GAP43 immunofluorescence to be more present throughout the IPL, however in a more fragmented manner than seen in control conditions (white arrows, Fig. 4.7 F).

4.4 Discussion

The effects of GH on neural differentiation are now well established and our data presents further evidence to support GHs involvement in embryonic retinal synaptogenesis. GH has been implicated in RGC development both *in vivo* (Baudet et al., 2007) and *in vitro* (Baudet et al., 2009a; Fleming et al., 2016). GH is able to promote embryonic synaptogenesis through the increase of PSD95 and SNAP25 expression in primary retinal cell cultures. In addition, the synaptogenic effect of GH in the post-hatch chick was determined under experimentally induced excitotoxicity from KA treatment. We show for the first time a neuroprotective effect on the dendritic trees of RGC as evidenced through a preservation of PSD95 expression in the neuroretina after GH treatment. This neuroprotective effect was associated with an internalization of Cy3-GH in the GCL. Our results may also implicate GH in the neuroregenerative process which occurs in the chicken retina after damage.

PSD95 is a MUGUK family scaffold protein located in the post synaptic density, where it interacts with a number of other scaffold proteins, receptors, and extracellular adhesion molecules to regulate their localization and function in the post synaptic bouton (Okabe, 2007). PSD95, along with the other DLG proteins (DLG1, DLG2, and DLG3) are found at excitatory synapses and are essential to glutamatergic synaptic signalling (Zhu et al., 2016). GH has previously been shown to increase PSD95 mRNA expression in the hippocampus of hypophysectomised rats which was associated with an improvement in spatial memory (Le Grevès et al., 2006). Although, chicks lack the gene for PSD95 (DLG4), they still contain genes for the three other DLG group proteins. In this current study we analyzed DLG1, the closest homolog in chickens for the mammalian DLG4 gene (PSD95), which has been used previously as a synaptogenic marker in birds (Kumar et al., 2014). Our results show, for the first time, the ability for GH to regulate PSD95 expression and post-synaptic function in both the embryonic and post-hatch chick. The increase in post-synaptic PSD95 and pre-synaptic SNAP25 suggests that GH is promoting synaptogenesis in our primary cell cultures. Changes in pre- and post-synaptic protein reflect functional modifications by direct correlation with synaptic formation during development, neuroregeneration and neurogenesis (Christopherson et al., 2005; Eroglu et al., 2009).

Glutamatergic kainate receptor subunits GluK1 and GluK2 have been shown to be expressed in the pigeon retina, particularly in bipolar cells in the peripheral retina (Atoji, 2015). However, they are also expressed in bipolar cells of the central retina, as well as amacrine and horizontal cells in the INL, and RGCs in the GCL (Atoji, 2015). Previous studies on the effects of KA in the chicken retina have found that damage mirrors this distribution, with the majority of structural damage occurring in the INL and IPL (Martinez-Moreno et al., 2017). Excitotoxicity in the retina is associated with synaptic loss (Baltmr et al., 2010), thus it is possible to suggest that

the decrease in SNAP25 and PSD95 expression seen in our system is from damage to the synapses in the IPL. GH has been previously shown to be preventative of excitotoxic cell death in QNR/D cells (Martínez-Moreno et al., 2016) and importantly, GH was shown to protect neuroretinal dendritic trees against kainate induced damage (Martinez-Moreno et al., 2017). Our results showed that GH restored the expression of PSD95 after excitotoxic damage and it also increases PSD95 immunofluorescence throughout the neuroretina. This suggests GH is neuroprotective of dendrites of the IPL from KA induced excitotoxicity.

Growth associated protein of 43kDa (GAP 43) is a presynaptic, growth cone associated protein which is widely accepted as a marker for axon growth and is thought to have an important role in axon development and synaptogenesis (Chen et al., 2015). Previous results from our group have demonstrated GHs ability to promote axon growth during development in RGCs (Baudet et al., 2009a), which is likely to involve the induction of GAP43 expression (Fleming et al., 2016). In addition, an upregulation of GAP43 was associated with increased dendritic arborisation after glutamatergic excitotoxicity in cultured rat neurons (Hung et al., 2014). Fish and lower vertebrates have the ability to increase GAP43 expression and regenerate axons after injury which is lost in higher vertebrates (Becker and Becker., 2007; Diekmann et al., 2015). GAP43 expression is essential for both developmental and regenerative axon growth, however these stages do not share the same regulatory mechanism (Udvadia et al., 2001). Therefore, the inability of GH to increase GAP43 expression in our experimental model could be due divergence of the mechanism regulating developmental axon growth versus regenerative axon growth in the chicken, which is present in the zebrafish (Kusik et al., 2010) as well as the presence of growth cone inhibition found in the central nervous system (McKerracher and Rosen, 2015).

SNAP25 is a pre synaptic SNARE protein essential for vesicle synaptic fusion and increases in its expression are associated with active synaptogenesis (Catsicas et al., 1991; Lawrence et al., 2014; Wang et al., 2012). There is increasing evidence that suggests GH is a positive regulating factor on SNAP25 expression during retinal embryonic development which implies a role in developmental synaptogenesis in the neural retina (Fleming et al., 2016). Previous studies from our group have shown that exogenous GH is able to increase SNAP25 immunoreactivity in the embryonic chick retina at ED10, both *in vitro* and *in vivo*, which was correlated with an increase in STAT5 phosphorylation (Fleming et al., 2016). This is corroborated by the current study where we show an increase in SNAP25 immunoreactivity in ED10 primary cell cultures in response to GH treatment. In the postnatal chicken retina, KA induced damage by decreasing SNAP25 mRNA expression and its protein immunoreactivity, however, GH was not able to compensate loss at 96h post-injury. We observed that GH was not internalized into the INL after damage which could account for the absence of a neuroprotective effect on the expression of pre-synaptic SNAP25.

In our experimental model for KA-induced excitotoxicity, both GH mRNA expression and immunoreactivity decreased, reflecting the retinal damage observed in cells of the GCL and INL (Ehlrich et al., 1990; Martinez-Moreno et al., 2018). This is contrary to results observed in reptiles, where KA intravitreal injections increased GH immunoreactivity in the adult green iguana (Ávila-Mendoza et al., 2016). This discrepancy of GH response to an excitotoxic injury could be partially explained by the capacity of reptiles to fully regenerate retinal tissue, since this capacity in birds is restricted to early neonatal stages (Alunni and Bally-cuif, 2016). Interestingly, GH expression is increased in chicken cerebellum cells (Alba-Betancourt et al., 2013) and in cortical pyramidal neurons (Scheepens et al., 2001) in response to hypoxic-ischemic injury, which clearly suggest the

existence of GH endogenous protective mechanisms in neuronal tissue. Therefore the loss of GH expression in our system could expedite the damage caused by kainate treatment as it has previously been shown that immunoneutralization of GH increases apoptosis in neuroretinal cells (Sanders et al., 2009a). In addition, a decrease in GH immunoreactivity observed in this work suggests that endocrine GH does not increase its permeation through the blood-brain-barrier and internalization into neuroretinal cells in response to damage (Pan et al., 2005; Fleming et al., 2016). The drastic loss in GH expression is likely to be related to the high concentration of KA used in our experimental model which ensures severe retinal damage. Future studies with lower doses of a glutamatergic agonist to mimic the normal physiological progression of excitotoxicity are required.

Despite the decrease of GH in response to excitotoxic damage in our model, there is an increase in GHR expression 48h after insult, suggesting a cellular response to the KA injury. GHR functionality in damaged retinas is supported by previous studies which showed that exogenous GH protected neuroretinal cells and their neurites after KA treatment in both the post-hatch chicken (Martinez-Moreno et al., 2018) and the green iguana (Ávila-Mendoza et al., 2016).

Cy3-GH injected intravitreally was internalized into the GCL, as has been previously reported by our group (Fleming et al., 2016). Interestingly, Cy3-GH treatments administer either 1h prior or 1h after KA treatment showed an increase in the amount of Cy3-GH internalized. However, Cy3-GH internalization was absent when administered 24h after KA insult, suggesting there may be a brief window for GH treatment post-injury.

The administration of 300 ng of Cy3-GH was mainly internalized into the GCL, which is the same dose used to induce the neuroprotective effects of GH seen in other retinal layers. It is possible that GHs neuroprotective effects observed in other layers in the retina may be due to

paracrine interactions of neurotrophins released from RGCs in response to GH stimulation (Ávila-Mendoza et al., 2016; Martinez-Moreno et al., 2017; Weber, 2013). Previous results from our lab have shown that GH induces the expression neurotrophin-3 in primary retinal cell cultures and in QNR/D cells (Martínez-Moreno et al., 2014). IGF-1, a classic mediator of GH, has been shown to increase its expression in response to GH in immunopanned RGCs (Baudet et al., 2009a), QNR/D cells (Martínez-Moreno et al., 2016) and retinal primary cell cultures (Martinez-Moreno et al., 2018). GH has also recently been shown to regulate BDNF expression in primary neuroretinal cultures and restore its expression after KA injury (Martinez-Moreno et al., 2018). These growth factors could be acting as secondary mediators and regulators induced by GH stimulation in the retina, as they also have well established neuroprotective actions in the neuroretina (Mead et al., 2014; Taylor et al., 2003).

Of particular interest is the appearance of PSD95 immunofluorescence in Muller glia-like structures after KA treatment, which increased when combined with GH. This is likely from the formation of Muller glia-derived progenitor cells (MGPCs), which form in the avian retina in response to damage (Fischer et al., 2004). After retinal damage Müller glial cells can re-enter the cell cycle and re-differentiate into a progenitor cell phenotype which are able to give rise to new retinal neurons (García and Vecino, 2003). PSD95 is expressed in stem cells and its immunoreactivity increases as they differentiate into new neurons (Yoshimura et al., 2016). However, in the green iguana, GHR immunofluorescence is absent in Muller glia (Ávila-Mendoza et al., 2016). IGF-1 and FGF2 treatments has been shown to increase the formation of MGCP's in the chick retina (Gallina et al., 2014) and IGF-1 is regulated by GH in the chick retina (Sanders et al., 2011). In addition, GH has been shown to regulate other neurotrophins such as neurotrophin-3 and BDNF both associated with the Muller cells transdifferentiation and proliferation of neural

stem cells in the chick retina (Fisher and Reh, 2002; Santos et al., 2011; Martinez-Moreno et al., 2017). Outside of the retina, GH treatment has been shown to increase the proliferation of progenitor cells in the rat hippocampus after KA induced injury (Devesa et al., 2011) and blocking locally produced GH has been shown to reduce the proliferation and survival of progenitor cells in the subgranular zone of the mouse (Devesa et al., 2014). GH has been implicated in the proliferation of human neural stem cells (Pathipati et al., 2011) and its neuroprotective and behavioral actions are well established (Harvey and Hull, 2003; Nyberg and Hallberg, 2013). GHR immunoreactivity has been shown to be increased after ischemic injury to the juvenile rat subventricular zone (Christophidis et al., 2009). As we also reported an increase in GH receptor expression 48h after injury, this suggest an endogenous response after injury in an environment of active neurogenesis such as the injured retina (Gallina et al., 2014). These results present an interesting possible use of GH for future research in retinal regeneration.

In summary, GH is a synaptogenic modulator in the avian retina, which promotes synaptogenesis through the increased expression of pre- and post-synaptic proteins. After excitotoxic damage to the synapses of the retina with kainate, an increase in internalization of exogenous GH is seen into the GLC. This increase in internalization is associated with a protective effect on the dendrites of the IPL, as evidenced through the perseveration of PSD95 expression. This work provides evidence that suggests GH involvement in the regeneration of the retina after damage.

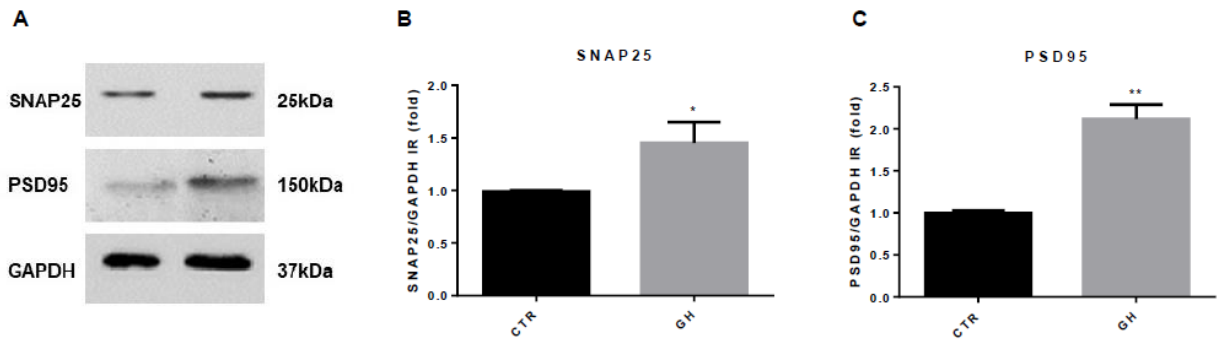

Figure 4. 1 GH increases the PSD95 and SNAP25 immunoreactivity in chick primary neuroretinal cultures. Chicken ED10 primary neuroretinal cell cultures were incubated with either 10nM (SNAP25) or 1nM (PSD95) rcGH for 24h. (A) Representative immunoblot for SNAP25 and PSD95. GAPDH reblotting was performed as a loading control. (B) Densitometry analysis of immunoblots for SNAP25 (B) and PSD95 (C) corrected against GAPDH immunoreactivity. Bars show mean fold ± SEM (n=3). ED, embryonic day. Asterisks indicate significant difference (*, $P < 0.05$; **, $P < 0.01$) as determined using students t-test.

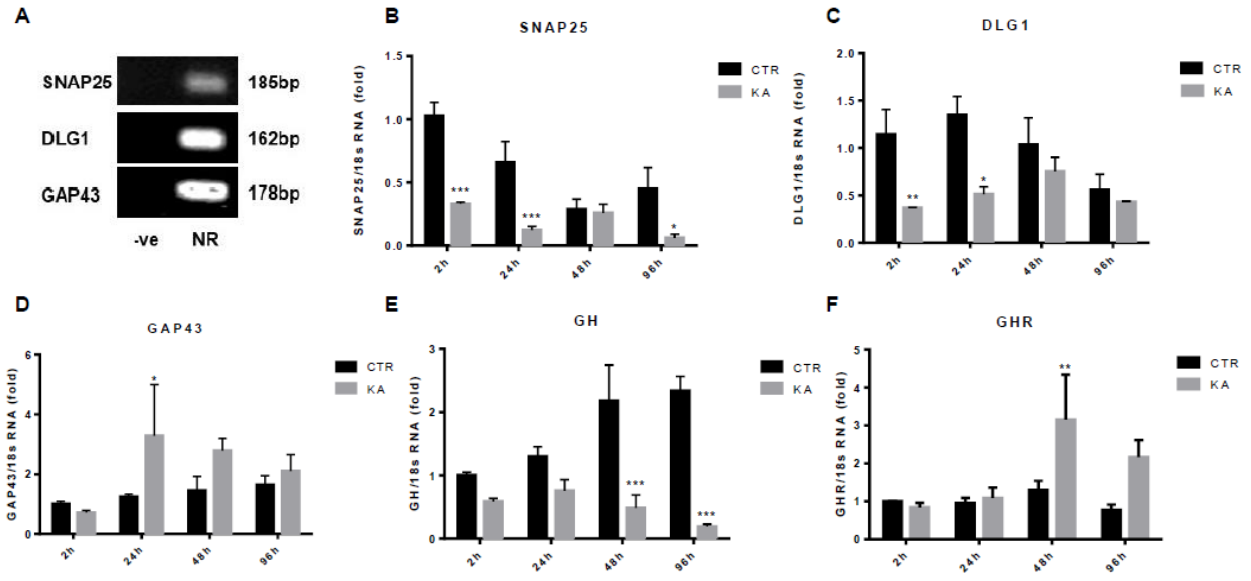

Figure 4. 2 The effect of KA treatment on synaptogenic genes in the neuroretina. A) End point RT-PCR for SNAP25. DLG1, and GAP43 in chicken neuroretinal cDNA. Negative was performed in the absence of cDNA. mRNA expression after 2h, 24h, 48h, or 96h in either KA injected eye (left, 20µg) or control eye (right, vehicle) for B) SNAP25, C) DLG1, D) GAP43, E) GH, and F) GHR. Bars show mean fold ± SEM (n=3). qPCR values were corrected by ΔΔCt. Ribosomal RNA 18s was used as a housekeeping gene. Asterisks indicate significant difference between KA and control conditions at their respective time (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) as determined by two-way ANOVA for multiple comparisons and LSD Fischer as *post-hoc* test.

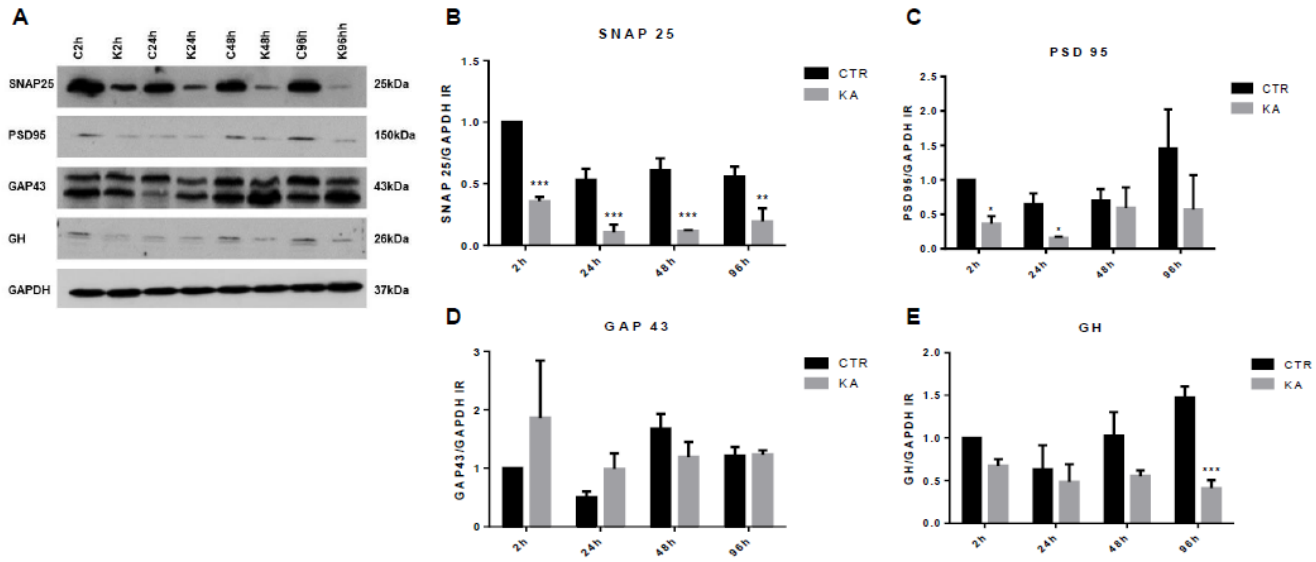

Figure 4. 3 The effect of KA treatment on immunoreactivity of synaptogenic proteins in the neuroretina. A) Representative immunoblot for SNAP25, PSD95, and GAP43, and GH immunoreactivity in chicken neuroretina 2h, 24h, 48h, or, 96h after intravitreal injection of KA (left eye, 20µg) or vehicle (right eye, control). GAPDH reblotting was performed as a loading control. Densitometry analysis of immunoblots for B) SNAP25, C) PSD95, D) GAP43, and E) GH corrected against GAPDH immunoreactivity. Bars show mean fold ± SEM (n=3). Asterisks indicate significant difference between KA and control conditions at their respective time (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) as determined by two-way ANOVA for multiple comparisons and LSD Fischer as post-hoc test.

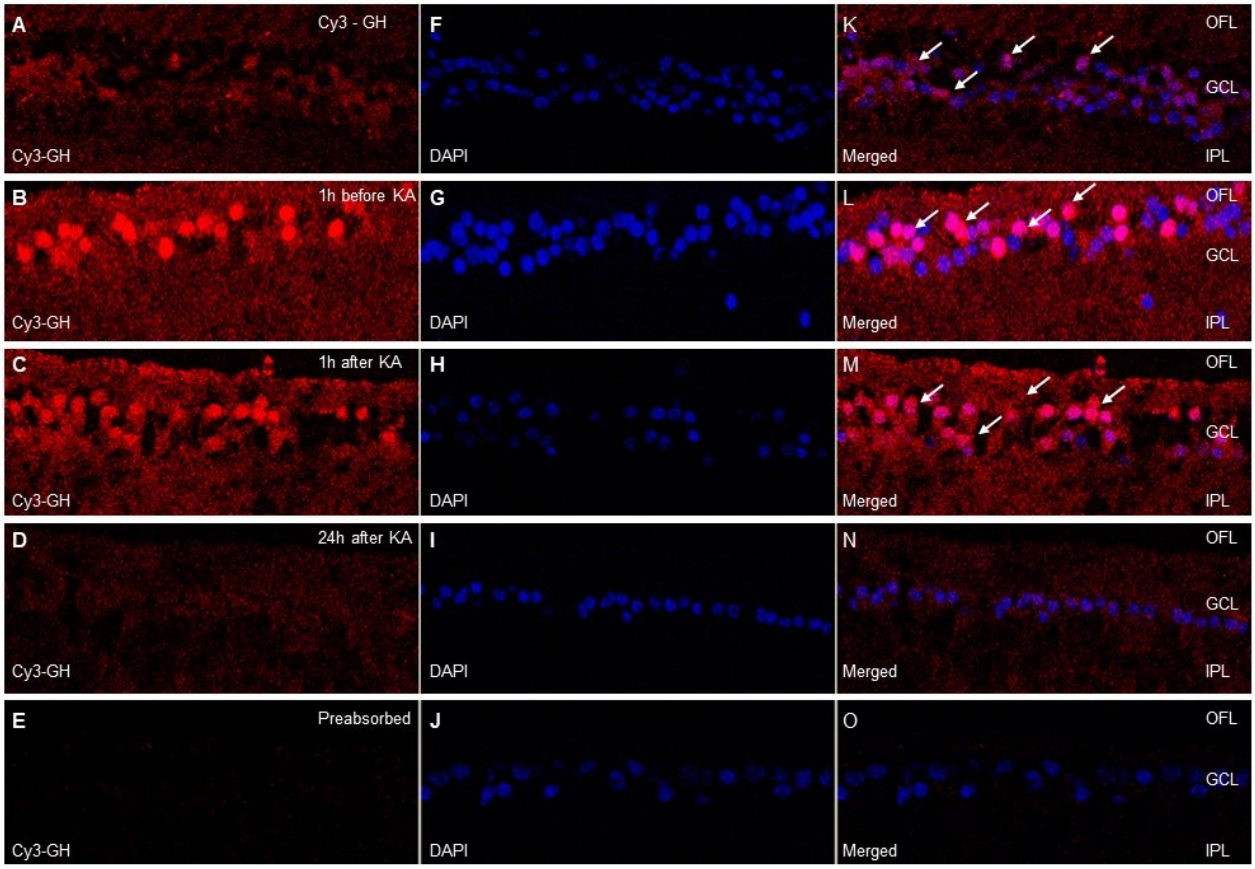

Figure 4. 4 Internalization of Cy3-labelled GH in the ganglion cell layer after KA treatment. P1 post hatch chicks injected with 300ng of Cy3-GH and/or 20µg of KA. Eyes were incubated for 2h after GH, collected and fixed. Samples were analyzed by confocal microscopy. Cy3-GH injections were performed in the absence of (A), 1h before (B), 1h after (C), or 24h after (D) KA treatment. Cy3-GH preabsorbed with a GH antibody was used as control (D). Samples were counter stained for DAPI (F-J). Cy3-GH was internalised in the ganglion cell layer in a perinuclear location (white arrows; panels K-M) which was absent in 24h post KA (N) and control groups (O). P, postnatal day. Images representative of 3 animals per group.

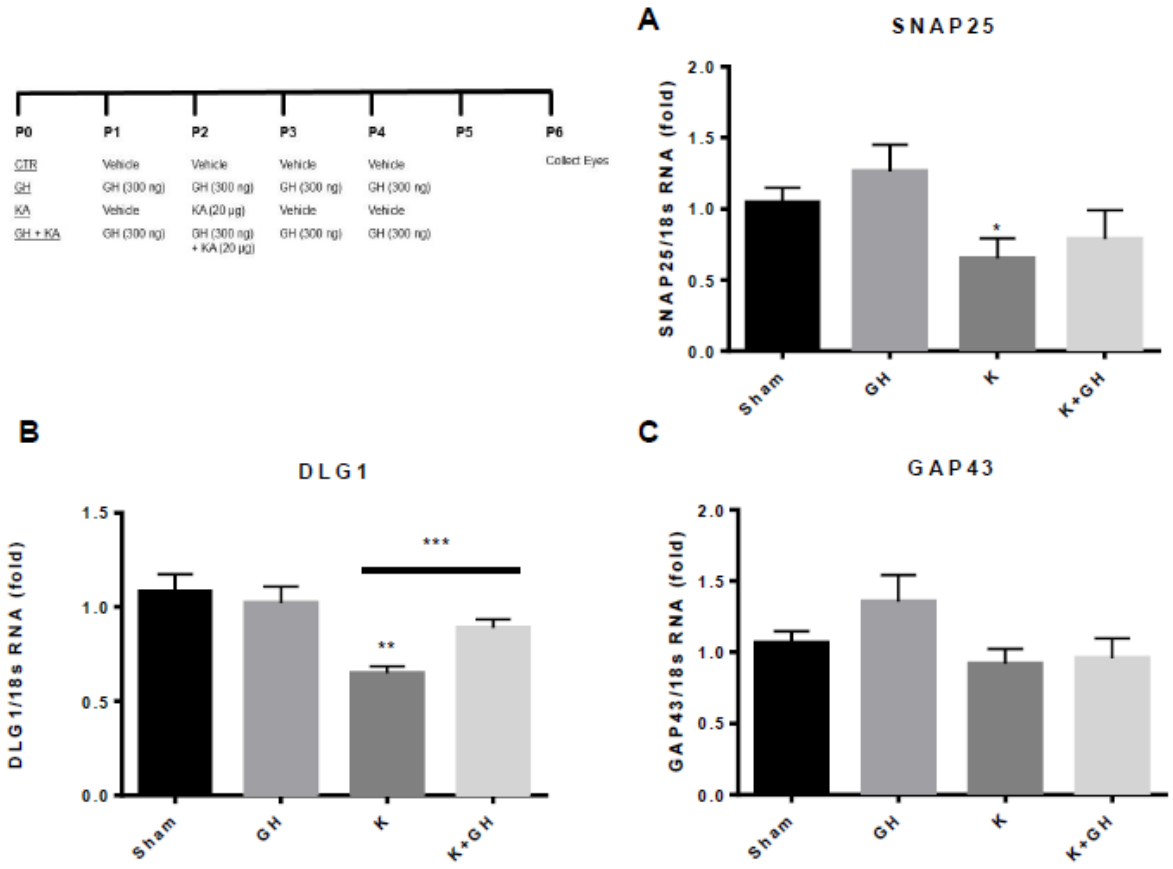

Figure 4. 5 Neuroretinal SNAP25, DLG1, and GAP43 mRNA expression after KA and/or GH treatments. Chickens were intravitreally injected with either vehicle (CTR, right eye), KA and/or GH (left eye). SNAP25 (A), PSD95 (B), and GAP43 (C) mRNA expression levels determined by qPCR. qPCR values were corrected by ΔΔCt. Ribosomal RNA 18s was used as a housekeeping gene. Bars show mean fold ± SEM (n=4). P, postnatal day. Asterisks indicate significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) as determined by one-way ANOVA for multiple comparisons and LSD Fischer as post-hoc test.

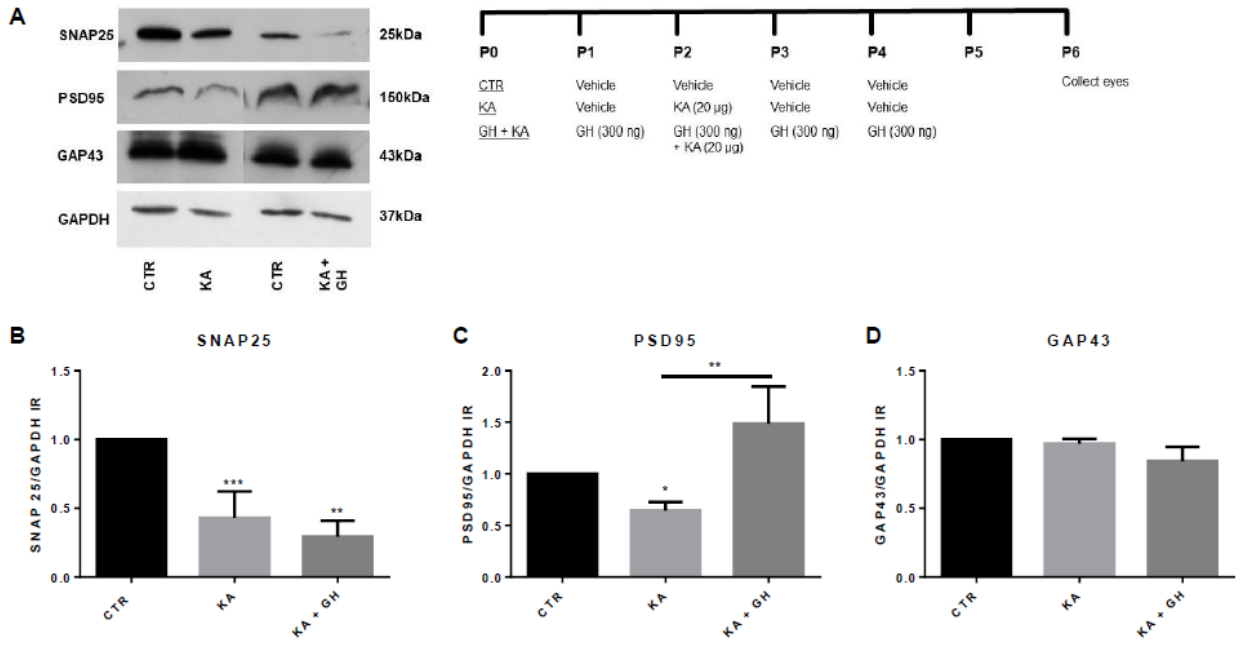

Figure 4. 6 Neuroretinal SNAP25, PSD95, and GAP43 immunoreactivity after KA and GH treatments. Chickens were intravitreally injected with either vehicle (CTR, right eye), KA or GH and KA (left eye). (A) Representative immunoblot for SNAP25, PSD95, and GAP43. GAPDH reblotting was performed as a loading control. (B) Densitometry analysis of immunoblots for SNAP25 (B), PSD95 (C), and GAP43 (D) corrected against GAPDH immunoreactivity. Bars show mean fold ± SEM (n=3). P, postnatal day. Asterisks indicate significant difference (*, $P < 0.05$; **, $P < 0.01$) as determined by one-way ANOVA for multiple comparisons and LSD Fischer as post-hoc test.

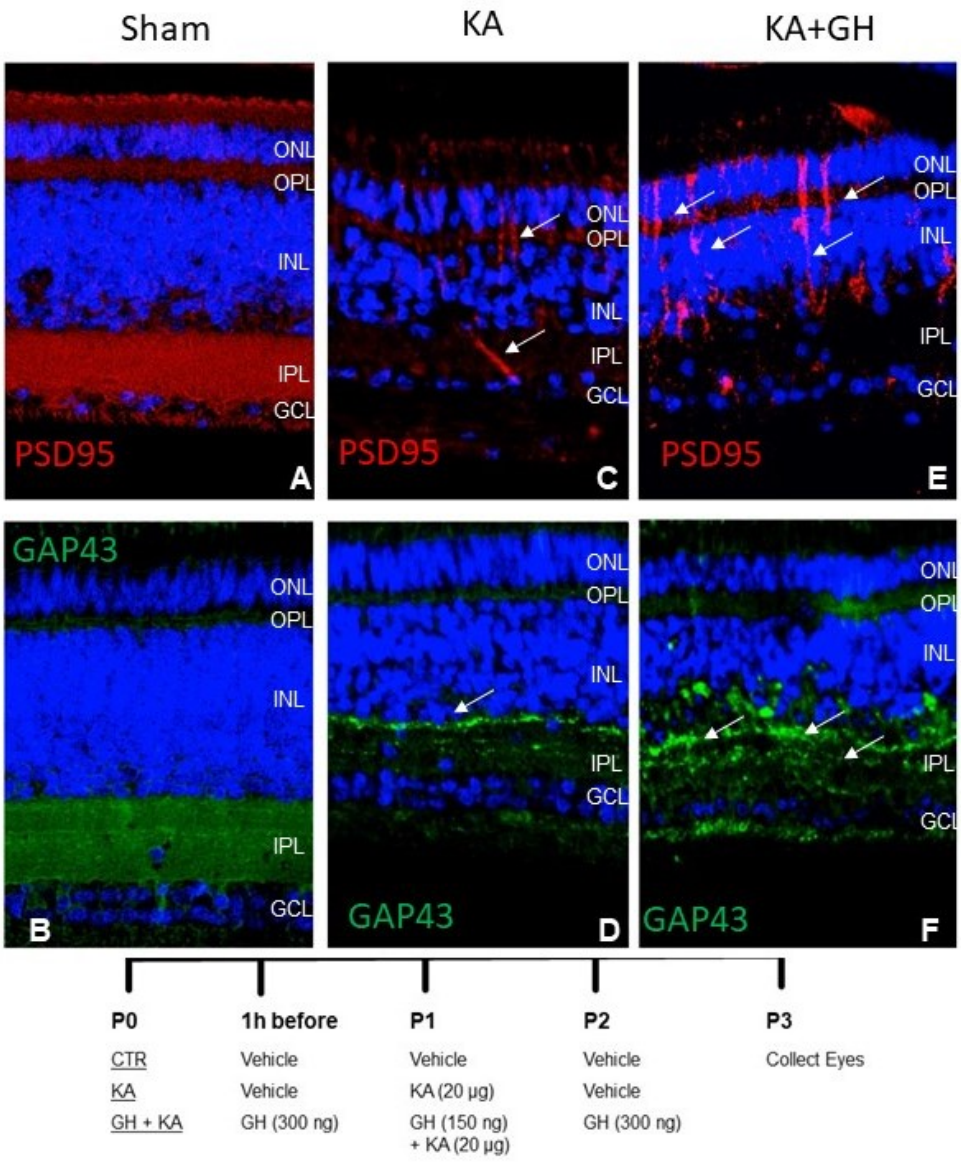

Figure 4. 7 GH/KA treatment changes GAP43/PSD95 localization in the chick retina. A and B, sham group. C and D**,** kainate damaged group. E and F**,** GH and KA treated group. A, C and E stained for PSD95. Arrows note appearance of Muller cell-like structures in KA and KA+GH groups. B, D, and F stained for GAP43. Arrows show changes in localization for GAP43 in KA group and GH + KA group. Representative of 6-7 animals per group.

Table 4. 1 Antibodies

Target	Host/Type	Clone/catalog	Dilution	Source
SNAP 25	Goat/polyclonal	sc-7538	1:2000 (WB)	Santa Cruz Biotechnology, CA, USA
PSD95	Rabbit/polyclonal	ab18258	1:5000 (WB) 1:500 (IHC)	abcam1, MA, USA
GAP 43	Mouse/monoclonal	GAP-7B10	1:5000 (WB) 1:500 (IHC)	Sigma, Mi, USA
cGH	Rabbit/polyclonal	Non Commercial	1:5000	Harvey et al. (2012)
GAPDH	Goat/polyclonal	21182	1:2000	Cell singalling, MA, USA

Table 4. 2 Oligonucleotide primer sequences

Gene	Forward Primer	Reverse Primer	Sequence
SNAP25	GCC TGC CCG TGT GGT AGA T	TCT GGC GAT TCT GTG TGT CG	185
DLG1	ACC AGC CAG AAG AGA TCC CT	TGG AGT TAC CTG CCG TGC TT	162
GAP43	AGG AGC CTA AAC AAG CCG AC	TGC TGG GCA CTT TCA GTA GG	178
cGH	CGC ACC TAT ATT CCG GAG GAC	GGC AGC TCC ATG TCT GAC T	128
cGHR	ACT TCA CCA TGG ACA ATG CCT A	GGG GTT TCT GCC ATT GAA GCT C	181
18s	CTC TTT CTC GAT TCC GTG GGT	TTA GCA TGC CAG AGT CTC GT	100

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Chapter Five

General Discussion

5.1 Overview

While the extrapituitary expression of GH in many tissues is well established, its functional relevance in these tissues remains less clear. In the retina, GH has an established role during embryonic neurogenesis as a neuroprotective modulator, however its effects on synaptogenesis post-natally and during development have not been studied. In this work we provide evidence for the first time that GH promotes synaptogenesis during development by increasing the expression of the synaptogenic proteins SNAP25, PSD95, and GAP43. In addition, we show for the first time a neuroprotective effect of GH on the dendritic trees of RGC as evidenced through a preservation of PSD95 expression in the neuroretina after KA treatment. This neuroprotective effect was associated with an internalization of Cy3-GH in the GCL and an increase in BDNF expression. We have also implicated GH in the neuroregenerative process after damage in the chick neuroretina, through an increase of PSD95-postive progenitor-like cells.

5.2 GH promotes retinal synaptogenesis in the chick embryo

We have demonstrated the involvement of GH in synaptogenesis in the developing chick retina for the first time. Previous results from our group have implicated GH in axon growth and development. Importantly, immunopanned RGCs respond to exogenous GH by increasing axon length, while knockdown of locally produced GH with siRNA resulted in a block of long axon formation (Baudet et al., 2009). Similar results have also found in N1E-115 neuroblastoma cells, where exogenous GH promotes axon growth (Grimbly et al., 2009). Here, we show the ability of GH to increase GAP43 expression in QNR/D cells for the first time. GAP43 is a presynaptic, growth cone associated protein which is widely used as a marker for axon growth and is thought

to have an important role in axon development and synaptogenesis (Chen et al., 2015). In addition, its expression if essential for proper optic nerve formation, both during development and after injury (Diekmann et al., 2015; Kusik et al., 2010). Given that GH is actively expressed in the OFL during RGCs development (Baudet et al., 2007), these results may provide a mechanism for GHs actions in RGCs during development. Local GH expression may promote the expression of GAP43 which in turn aids the developing RGCs in axonal development and synaptogenesis with their target site in the optic tectum (Baudet et al., 2007; Thanos and Mey, 2001).

SNAP25 is a pre synaptic SNARE protein essential for vesicular synaptic fusion, neurotransmitter release and increases in its expression are associated with active synaptogenesis (Catsicas et al., 1991; Lawrence et al., 2014; Wang et al., 2012). In the developing chick retina, the onset of SNAP25 expression coincides with the appearance of GH staining in the IPL (Baudet et al., 2009b; Catsicas et al., 1991). The hypothesis that GH induces SNAP25 expression was therefore examined by testing the effect of exogenous GH on both *in vitro* in neuroretina primary cell cultures and explants as well as *in vivo* in ED10 chicks cultured *ex ovo*. We found that exogenous GH was able to increase SNAP25 expression both *in vitro* as well as *in vivo* and this increase in expression was correlated with an increase in STAT5 phosphorylation. These results suggest the expression of extrapituitary GH in the IPL of the chick during development may promote synaptogenesis through the induction of SNAP25 expression.

PSD95 is a scaffold protein located in the glutamatergic excitatory post synaptic density, where it interacts with a number of other scaffold proteins, receptors, and extracellular adhesion molecules to regulate their localization and function in the post synaptic bouton (Okabe, 2007). GH has previously been shown to increase PSD95 mRNA expression in the hippocampus of hypophysectomised rats which was associated with an improvement in spatial memory (Le Grevès

et al., 2006). While previous results from our lab have implicated GH in pre-synaptic development, through GAP43 and SNAP25 expression (Fleming et al., 2016), its effects on post synaptic function have not previously been accessed. We analyzed the expression of both SNAP25 and PSD95 expression in primary retinal cultures from the ED10 chick since increases in both pre- and post-synaptic proteins have been previously used infer synaptogenesis (Christopherson et al., 2005; Eroglu et al., 2009), and increases in PSD95 expression promote post-synaptic function (Goyer et al., 2015). Our results show, for the first time, the ability for GH to regulate PSD95 expression and post-synaptic function in embryonic chick neuroretinal cells. In addition, the increase in SNAP25 expression which accompanied the increase in PSD95 suggests that GH is promoting synaptogenesis in our primary cell cultures. Indeed, GH may promote post-synaptic development in the IPL though the promotion of PSD95 expression.

5.3 The effect of GH in KA damaged retina

5.3.1 KA damage model

Our intravitreal KA injection model was successful in causing both structural as well as synaptic damage in the chick retina. Glutamatergic kainate receptors are expressed in the avian retina, particularly in bipolar cells of the peripheral retina as well as in the in bipolar cells of the central retina, amacrine and horizontal cells in the INL, and RGCs in the GCL (Atoji, 2015). The expression of glutamatergic KA receptors mirrors the structural damage that our group has previously found in response to KA treatment (Martinez-Moreno et al., 2018). Excitotoxicity in the retina is associated with synaptic loss (Baltmr et al., 2010), thus our findings suggest the structural damage induced by KA injection is also accompanied by synaptic loss as evidenced

through the relative loss of both SNAP25 and PSD95 expression in our model. Our results support the use of the KA-induced excitotoxicity model for future use in the study of synaptogenesis and neural repair in the post-natal chick with GH and other growth factors.

5.3.2 GH expression in KA model

In response to KA treatment, both GH mRNA expression and immunoreactivity saw a decrease in relative abundance. This is contrary to results observed in reptiles, where KA intravitreal injections increased GH immunoreactivity in the adult green iguana (Ávila-Mendoza et al., 2016). This discrepancy of GH response to an injury could be partially explained by differences in the regenerative response in retiles as compared to birds, which is relatively more limited in capacity (Alunni and Bally-cuif, 2016). Outside of the retina, GH expression is increased in the chicken cerebellum cells (Alba-Betancourt et al., 2013) and in cortical pyramidal neurons (Scheepens et al., 2001) in response to hypoxic-ischemic injury. This clearly indicates that GH is involved in the endogenous protective response to injury in neural cells. Therefore the loss of GH expression in our system could expedite the damage caused by kainate treatment has it has previously been shown that immunoneutralization of GH increases apoptosis in neuroretinal cells (Sanders et al., 2009a). The drastic loss in GH expression is likely to be related to the high concentration of KA used in our experimental model which ensures severe retinal damage. The loss in GH expression may be due to the concentration of KA used in our experiments and therefore future studies with lower doses of a glutamatergic agonist are required.

We have previously shown the capacity of GH to cross the BBB into the retina in chicks, suggesting endocrine GH may be involved in the systems response to injury (Fleming et al., 2016; Pan et al., 2005). However, the decrease in GH immunoreactivity observed in this work suggests

that endocrine GH does not increase its permeation through the BBB into neuroretinal cells in response to excitotoxic damage.

Despite the decrease of GH in response to excitotoxic damage in our model, there is an increase in GHR expression 48h after insult, suggesting a cellular response to the KA injury. This corroborates data from other groups which showed GHR immunoreactivity was increased after ischemic injury to the juvenile rat subventricular zone (Christophidis et al., 2009). GHR functionality in damaged retinas is supported by previous studies which showed that exogenous GH protected neuroretinal cells and their neurites after KA treatment in both the post-hatch chicken (Martinez-Moreno et al., 2018) and the green iguana (Ávila-Mendoza et al., 2016). As well, in our current study, we show Cy3-GH treatments administer either 1h prior or 1h after KA treatment showed an increase in the amount of Cy3-GH internalized. This Cy3-GH was internalized into the GCL, as has been previously reported by our group (Fleming et al., 2016). This indicates that GH treatment may be beneficial as intravitreal GH injections were able to prevent cytostructural retinal damage in our model as well as others (Ávila-Mendoza et al., 2016; Martinez-Moreno et al., 2018). However, Cy3-GH internalization was absent when administered 24h after KA insult, suggesting there may be a brief window for GH treatment post-injury.

5.3.3 GH and Neurotrophins

Neurotrophins are secreted growth factors that have an important role in retinal development, neural cell survival and renewal, differentiation, homeostasis, and vision physiology, and their absence during development compromises neuroretinal cell health and development (Johonson et al., 2009; Sipll et al., 2011). Neurotrophins, such as NT3 and BDNF, are produced in both neuroretinal and glial cells (Hu et al., 2011). Similar to GH, the

administration of BDNF and/or NT3 induces retinal neuroprotection and neuroregeneration in different pathophysiological experimental models (Hohn et al., 1990; Berkemeier et al., 1991; Gauthier and Liu, 2016). We corroborated the presence of both, BDNF and NT3 in the chicken neuroretina, brain, and QNR/Ds. In KA injected neuroretinas, the gene expression of NT3 and BDNF was significantly decreased compared to control. Multiple intravitreal injections of GH were able to restore BDNF expression to control levels but not NT3. NT3 is GH responsive in retinal cells however, as previous reports from our group showed that QNR/D cells incubated with GH induced NT3 expression (Martinez-Moreno et al., 2014) and neuroretinal primary cell cultures incubated with GH increase NT3 expression (Martinez-Moreno et al., 2018). Thus, NT3 may not be involved in the neuroprotective response elicited by GH.

BDNF as a mediator of neuroprotective actions of GH in the chicken embryo retina has been suggested by Sanders et al. (2008). This work provides evidence for the induction of BDNF by GH in the retina for the first time. We also observed the presence of BDNF immunoreactivity in RGCs of the postnatal chick. As we also observed the internalization of Cy3-GH into RGCs after KA injury and thus it is likely that exogenous GH is acting directly on RGCs to induce BDNF expression and possibly other neurotrophic factors. It is likely that this GH mediated increase in BDNF expression is at least partially responsible for the cytostructural neuroprotective effects of GH seen in the IPL and INL, as direct GH internalization was not observed in those retinal layers.

Our data suggest that GH-induced neuroprotection is mediated in part through the expression of growth factors such as IGF-1, BDNF and NT3. It is likely the down regulation of GH in our system in response to damage may have also resulted in the loss NT-3 and BDNF expression and other neuroprotective factors as well (Martinez-Moreno et al., 2018). The local production of GH in the retina and the influence of endocrine GH implies a complex network of

autocrine, paracrine and endocrine peptides that participate in retinal physiology and physiopathology. Future studies are required to understand the relationship between GH and neurotrophic peptides in retinal development, neuroprotection and neuroregeneration.

5.3.4 GH and synaptogenesis in KA damaged retina

Our results show, for the first time, the ability for GH to regulate PSD95 expression and post-synaptic function in the post-natal chick. GH treatment restored the expression of PSD95 after excitotoxic damage. This increase was also associated with an internalization of Cy3-GH into the GCL. Since dendrites of the IPL originate from RGCs, this could suggest a direct action from GH. This corroborates our *in vitro* results which showed that GH treatment was able to increase PSD95 immunoreactivity in embryonic neuroretinal primary cell cultures. Thus, GHs effects on post synaptic function may not be limited to early development, as it may be neuroprotective of the dendrites of the IPL after excitotoxic injury.

We have previously demonstrated that GH is a positive regulating factor on SNAP25 expression during retinal embryonic development which implies a role in developmental synaptogenesis in the neural retina (Fleming et al., 2016). We have shown that exogenous GH is able to increase SNAP25 immunoreactivity in the embryonic chick retina at ED10, both *in vitro* and *in vivo*, as well an increase in SNAP25 immunoreactivity in ED10 primary cell cultures in response to GH treatment. The effect of GH on SNAP25 expression seen during development was not observed in post-natal KA treated retinas. Though GH treatment was able to protect the IPL and INL from structural damage (Martinez-Moreno et al., 2018), no recovery in pre-synaptic SNAP25 expression was observed. However, we observed that GH was not internalized into the

INL after damage, from where presynaptic axons of the IPL originate and could account for the absence of a neuroprotective effect on the expression of SNAP25.

Our previous results demonstrated GHs ability to upregulate GAP43 expression in embryonic chick models, which suggests a mechanism for GHs actions on axon growth (Baudet et al., 2009; Grimbly et al., 2009). However, we did not observe an effect on GAP43 expression from damage or GH treatment. This could be due to differences in GAP43 regulation in development versus neuroregeneration. Though GAP43 expression is essential for both developmental and regenerative axon growth, they do not share the same regulatory mechanism (Udvadia et al., 2001). Fish and lower vertebrates have the ability to increase GAP43 expression to regenerate axons after injury which is lost in higher vertebrates such as chickens (Becker and Becker., 2007; Diekmann et al., 2015). Therefore, the inability of GH to increase GAP43 expression in our current model could be due to a difference in the mechanism regulating developmental axon growth versus regenerative axon growth in the chicken (Kusik et al., 2010). Experiments using a zebrafish model may be of use for future studies as they retain the ability to upregulate GAP43 in response to injury to regenerate axons (Becker and Becker., 2007; Diekmann et al., 2015; Udvadia et al., 2001).

Of particular interest in our study is the appearance of PSD95 immunofluorescence in Muller glia-like structures after KA treatment, which increased when combined with GH. This is likely a neuroregenerative response in the retina, which results in the formation of Muller glia-derived progenitor cells (MGPCs) (Fischer et al., 2004). After retinal damage, Müller glial cells can re-enter the cell cycle and re-differentiate into a progenitor cell phenotype which are able to give rise to new retinal neurons (García and Vecino, 2003). PSD95 is expressed in stem cells and its immunoreactivity increases as they differentiate into new neurons (Yoshimura et al., 2016).

However, in the green iguana, GHR immunofluorescence has been shown to be absent in Muller glia (Ávila-Mendoza et al., 2016). We have shown an increase in GHR expression 48h post injury which has been corroborated by other models as GHR immunoreactivity has been shown to be increased after ischemic injury to the juvenile rat subventricular zone (Christophidis et al., 2009). Indeed GH treatment has been shown to increase the proliferation of progenitor cells in the rat hippocampus after KA induced injury (Devesa et al., 2011) and blocking locally produced GH has been shown to reduce the proliferation and survival of progenitor cells in the subgranular zone of the mouse (Devesa et al., 2014). This suggests that there is an endogenous response involving GH, though its effects may not be directly mediated. As we have shown the ability of GH to regulate BDNF in this study, GHs effects may be mediated through the induction of other neurotrophic factors after internalization into the RGCs. IGF-1 and FGF2 have been shown to increase the formation of MGCP's in the chick retina (Gallina et al., 2014) and IGF-1 has been shown to be regulated by GH in the chick retina (Sanders et al., 2011). These results implicate GH in the physiology of neural repair after damage though its main role may be the induction of other growth and pro-neural factors. These results present an exciting potential for GH in future use in retinal regeneration research.

5.4 Conclusions

In summary, GH is a synaptogenic modulator in the avian retina, which promotes the synaptogenesis through the increased expression of pre- and post-synaptic proteins. After excitotoxic damage GH is neuroprotective of the dendrites of the IPL as evidences through PSD95 expression. This neuroprotective effect may be directly mediated or via the induction of other

growth factors in RGCs, such as BDNF. This work also provides evidence that suggests GH involvement in the regeneration of the retina after damage.

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