The roles of glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor and nerve growth factor during the final stage of folliculogenesis: a focus on oocyte maturation

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Abstract

Neurotrophic factors were first identified to promote the growth, survival or differentiation of neurons and have also been associated with the early stages of ovarian folliculogenesis. More recently, their effects on the final stage of follicular development, including oocyte maturation and early embryonic development, have been reported. Glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), which are expressed in numerous peripheral tissues outside of the CNS, most notably the ovary, are now known to stimulate oocyte maturation in various species, also enhancing developmental competence. The mechanisms that underlie their actions in antral follicles, as well as the targets ultimately controlled by these factors, are beginning to emerge. GDNF, BDNF and NGF, alone or in combination, could be added to the media currently utilized for *in vitro* oocyte maturation, thereby potentially increasing the production and/or quality of early embryos.

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Introduction

Considerable insights have been gained into understanding the factors and mechanisms that regulate the assembly, growth and development of ovarian follicles, from the initial differentiation of primordial follicles to the proliferation of granulosa cells and the ability of later stage follicles to respond to FSH, which are the topics reviewed elsewhere (Dierich *et al.* 1998, Kezele *et al.* 2002, Skinner 2005, Craig *et al.* 2007). Over the last several decades, neurotrophic factors, originally identified as affecting cells of the CNS, have also been recognized to play important roles in peripheral tissues, including the highly innervated ovary. Ovarian nerve fibres not only connect with blood vessels but also associate with follicles (Malamed *et al.* 1992), and it has been shown that ectopic transplantation of the ovary results in rapid in-growth of nerve fibres (Lara *et al.* 1991). Local ovarian production of neuronal growth-promoting factors, coupled with the initial insensitivity of developing embryonic ovaries to gonadotrophins, suggested that early events during folliculogenesis could, at least in part, be under neurotrophic control (Lara *et al.* 1991, Malamed *et al.* 1992). This notion was supported by the finding that neurotrophins and their receptors are expressed in the ovary before the acquisition of

gonadotrophin responsiveness (Dissen *et al.* 1995, 2009). It is now evident that neurotrophins, together with other growth factors and hormones, exert a substantial influence on both somatic cells and germ cells in the embryo during the early stages of folliculogenesis (Dissen *et al.* 2009), with developing oocytes and granulosa cells maintaining a bidirectional relationship similar to the communication existing between neurons and glial cells (Davies & Wright 1995).

The effects of neurotrophins on developing ovarian follicles have been investigated in the context of follicular survival, assembly and growth (Paredes *et al.* 2004, Dole *et al.* 2008, Dissen *et al.* 2009, Kerr *et al.* 2009, Dorfman *et al.* 2011) and have been reviewed elsewhere (Ojeda *et al.* 2000, Dissen *et al.* 2002, 2009). However, studies investigating their role in antral follicles during the final stage of folliculogenesis, marked by oocyte maturation and the resumption of meiosis, which is concomitant with extrusion of the first polar body, as well as developmental competence of the early embryo, have not been reviewed in detail. The acquisition of developmental competence or the ability to support preimplantation embryo development following fertilization is linked to both cytoplasmic and nuclear maturation of the oocyte and requires that it be

able to adequately transmit signals derived from endocrine, autocrine and paracrine stimuli within the cumulus–oocyte complex (COC). The maintenance of this coordinated crosstalk and the complex signalling network within antral follicles are essential to drive two closely linked processes, oocyte developmental competence and cumulus cell expansion. After the preovulatory LH surge, cumulus cells expand, which corresponds with the resumption of meiosis and also serves as an indirect marker of COC maturation. It has become evident that neurotrophins are present within oocytes and somatic cells comprising antral follicles, as well as in follicular fluid. As recent findings suggest that neurotrophins play a key role during oocyte maturation, the expression and effects of glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) and their respective receptors will be discussed. In addition, emerging evidence regarding the possible mechanisms underlying neurotrophin-mediated oocyte maturation and regulation of ovarian expression will be presented, as well as the potential clinical significance of these neurotrophic factors.

Expression of neurotrophic factors in the postnatal ovary

Glial cell line-derived neurotrophic factor

GDNF, a distant member of the TGFB superfamily, is related to a subset of neurotrophic ligands that also includes neurturin, artemin and persephin (Takahashi 2001). First identified as promoting the survival and differentiation of several classes of peripheral and central neurons (Lin *et al.* 1993, Tomac *et al.* 1995), GDNF is now known to play diverse roles outside of the CNS. Interestingly, a comparative analysis of its mRNA levels by *in situ* hybridization in a panel of murine tissues revealed *Gdnf* to be most prominently expressed in the ovary, where it was found to localize to the urogenital system throughout various stages of embryonic development and in the adult ovary (Golden *et al.* 1999), primarily within the granular layer of developing follicles (Widenfalk *et al.* 2000). Murine *Gdnf* mRNA levels have been reported to be low in maturing follicles, increasing significantly in those closest to maturity (Golden *et al.* 1999). In addition to its transcript, murine GDNF protein localizes to oocytes as well as granulosa and stromal cells (Aravindakshan *et al.* 2006). A DNA microarray analysis conducted by Kawamura *et al.* (2008) also identified *Gdnf* as an important ovarian transcript in the mouse, with follow-up experiments revealing that this factor is expressed by ovarian somatic cells, including cumulus, granulosa and theca cells (Kawamura *et al.* 2008). In rats, *Gdnf* mRNA is present in the neonatal and adult ovary (Choi-Lundberg & Bohn 1995, Trupp *et al.* 1995), and in the ovaries of newborns, GDNF protein

localizes to the cytoplasm of oocytes in follicles representing all developmental stages, and to cumulus, granulosa and theca cells within antral follicles (Dole *et al.* 2008).

GDNF elicits its responses through a receptor complex composed of a glycosylphosphatidylinositol-linked ligand-binding subunit, the GDNF family co-receptor alpha 1 (GFRA1), and its signalling component, the rearranged during transfection (RET) tyrosine kinase receptor (Trupp *et al.* 1995, Jing *et al.* 1996, Baloh *et al.* 2000). RET does not bind directly to GDNF (Treanor *et al.* 1996), requiring the recruitment of GFRA1 for its transactivation, thereby initiating intracellular signalling (Sariola & Saarma 2003). An ovarian *Ret* mRNA signal has been detected in mice (Widenfalk *et al.* 2000), and *Gfra1* and *Ret* are co-expressed in the developing urogenital system (Golden *et al.* 1999). In rats, GFRA1 protein was shown to localize to oocytes within primordial and primary follicles, while in antral follicles, the receptor was present in granulosa and theca cells (Dole *et al.* 2008). Consistent with studies conducted in rodents, work in our laboratory revealed that in the pig, GDNF and both its receptors are expressed in COCs at the mRNA and protein levels during the final stages of folliculogenesis and that GDNF is present in porcine follicular fluid derived from COCs isolated from both small, less mature and larger, more mature antral follicles (Linher *et al.* 2007). Recently, GDNF and its receptors were detected at the mRNA and protein levels in oocytes and granulosa cells from human fetuses collected from terminated pregnancies at 21–35 weeks of gestation and in girls and women 5–39 years of age who underwent ovarian laparoscopies (Farhi *et al.* 2010). Furthermore, single-cell screening for growth factors that could potentially enhance the maturation of cumulus-free, denuded oocytes isolated from patients receiving ICSI treatment revealed that *GDNF* mRNA levels were significantly higher in cumulus cells than in oocytes, while *GFRA1* appeared to be exclusively expressed in oocytes (McElroy *et al.* 2010). Expanding on its presence in the human ovary during the later stages of folliculogenesis, GDNF protein localized to unstimulated human granulosa cells obtained from women undergoing *in vitro* maturation (IVM) procedures (Zhao *et al.* 2011). Cumulatively, paired with data obtained in other mammals, these expression patterns, which are also summarized in Table 1, suggest that GDNF influences the oocyte and cumulus cells in both a paracrine and a autocrine manner within the adult ovary.

Brain-derived neurotrophic factor

The neurotrophin BDNF is a member of a family of neurotrophic factors that includes NGF as well as neurotrophin 3 (NTF3), NTF4/5 and NTF6 (Bibel & Barde 2000, Lu *et al.* 2005). Each of these ligands is required for the survival and differentiation of central and

Table 1 Expression profile and function of GDNF in the ovary.

Expression	Species	Reference	Function	Species	Reference
Adult ovary, granular layer of developing follicles	Mouse	Golden <i>et al.</i> (1999) and Widenfalk <i>et al.</i> (2000)			
Oocytes, ovarian stromal cells	Mouse	Aravindakshan <i>et al.</i> (2006)			
Ovarian follicles, cumulus, granulosa and theca cells; oviducts and uteri of pregnant females	Mouse	Kawamura <i>et al.</i> (2008)	↑ Cyclin D1 levels ↑ Extrusion of 1st polar body ↑ Development of two-cell stage embryos into blastocysts	Mouse	Kawamura <i>et al.</i> (2008)
Neonatal and adult ovary	Rat	Choi-Lundberg & Bohn (1995) and Trupp <i>et al.</i> (1995)			
Primarily in oocytes, to a lesser extent in cumulus, granulosa and theca cells	Rat	Dole <i>et al.</i> (2008)			
COCs (oocytes, cumulus cells), granulosa and theca cells, follicular fluid	Pig	Linher <i>et al.</i> (2007)	↑ Cyclin D1 levels ↑ Extrusion of 1st polar body ↑ Developmental competence of oocytes from less mature follicles to reach the blastocyst stage	Pig	Linher <i>et al.</i> (2007)
Oocytes and granulosa cells from fetuses, girls and women	Human	Farhi <i>et al.</i> (2010)			
Oocytes, cumulus cells	Human	McElroy <i>et al.</i> (2010)	In combination with a cocktail of other factors: ↑ Oocyte nuclear maturation ↑ Formation of parthenogenetic blastocysts	Human	McElroy <i>et al.</i> (2010)
Granulosa cells	Human	Zhao <i>et al.</i> (2011)	↑ Total number of oocytes reaching MI	Human	Zhao <i>et al.</i> (2011)
Follicular fluid	Human	Sadeu <i>et al.</i> (2012)			

peripheral neurons and signals through distinct high-affinity transmembrane receptor tyrosine kinases, as well as through a common, more widely expressed, low-affinity neurotrophin receptor (NGFR), also known as p75 (Raffioni *et al.* 1993, Snider 1994, Davies 2000). Transcripts for these neurotrophins and their receptors have been detected in the rat ovary before the neonatal phase of development (Dissen *et al.* 1995), indicating a role during ovarian differentiation and the initiation of folliculogenesis in the embryo. BDNF binds to and activates either neurotrophic tyrosine kinase receptor 2 (NTRK2), also known as TRKB, or p75 (Klein *et al.* 1991), which can amplify (Hantzopoulos *et al.* 1994) or inhibit (Kohn *et al.* 1999) the actions mediated through the different NTRK receptors. A role for BDNF in the ovary was established *in vivo* using knockout mouse models of all *Ntrk2* receptor isoforms or their ligands *Bdnf/Ntf4/5* (Paredes *et al.* 2004), revealing defects in murine early follicular development by impeding the growth of follicles beyond the primary stage. *Ntrk2* mRNA has been detected in oocytes and granulosa cells in a manner dependent on the stage of ovarian development (Dissen *et al.* 1995, Anderson *et al.* 2002, Paredes *et al.* 2004). In the neonatal mouse ovary, BDNF and NTRK2 are initially present in oocytes, with their expression switching to granulosa cells following the assembly of primordial follicles and further development into primary follicles

(Paredes *et al.* 2004). In the neonatal rat ovary, both *Bdnf* and its receptors are expressed at the mRNA level before the onset of folliculogenesis, before the formation of primordial follicles (Dissen *et al.* 1995), which follows a similar temporal pattern as described in the human fetal ovary (Anderson *et al.* 2002). Seifer *et al.* (2002a, 2002b) demonstrated by immunocytochemistry that NTRK2 is expressed in a majority of murine oocytes (Seifer *et al.* 2002a), which was corroborated by a separate study demonstrating its exclusive expression in murine oocytes (Kawamura *et al.* 2005). Bovine cumulus cells and oocytes were reported to express BDNF and NGFR at both the mRNA and the protein levels (Martins da Silva *et al.* 2005). However, contrary to the results observed in rodents, bovine *NTRK2* mRNA was detected only in cumulus cells (Martins da Silva *et al.* 2005). Porcine COCs express mRNA for *BDNF* and its receptors in both follicular somatic cells and oocytes, while *NTRK2* was not detected in oocytes (Lee *et al.* 2007). In women undergoing IVF, BDNF was present in follicular fluid, with the source of BDNF identified to be derived exclusively from cumulus cells, as neither cultured oocytes stripped of cumulus cells, mural granulosa cells or embryos released any appreciable levels of BDNF into the culture medium (Seifer *et al.* 2002a). In addition, BDNF is also present in follicular fluid from normally cycling women (Seifer *et al.* 2003). In a recent

study, it was shown that BDNF protein localizes to unstimulated human granulosa cells isolated from patients undergoing IVM (Zhao *et al.* 2011). Similar to GDNF, these ovarian patterns of expression support that BDNF mediates aspects of both the early and the later stages of folliculogenesis. Although there appear to be considerable species differences with regard to the localization of its expression within a follicle (Table 2), BDNF most likely influences the maturing oocyte in a paracrine manner.

Nerve growth factor

NGF signals through NTRK1, also known as TRKA (Raffioni *et al.* 1993), as well as through NGFR. One of the first studies demonstrating that NGF is locally produced in the immature rat ovary showed the presence of an mRNA similar to the mature *Ngf* mRNA detected in the murine submaxillary gland, an established source of its production (Lara *et al.* 1990). Furthermore, NGF protein was also confirmed to be present in neonatal rat ovaries (Lara *et al.* 1990). A temporal analysis of *Ngf* and *Ntrk1* mRNA levels revealed that in the rat ovary, both the ligand and the receptor are present during fetal development and decrease in the neonate, remaining

low until the onset of puberty, at which time the expression of both rises (Dissen *et al.* 1995). In particular, *Ntrk1* mRNA levels increased concomitantly with the LH surge, and it has therefore been suggested that NGF may play a role in controlling ovulation (Dissen *et al.* 1996). NTRK1 also localizes to theca cells in large antral follicles in the rat (Dissen *et al.* 1996), supporting this notion. In sheep, NGF was present in the follicular fluid, and NTRK1 localized exclusively to cumulus cells, as oocytes were found to be negative for this receptor in ovine follicles (Barboni *et al.* 2002). NGF, NTRK1 and NGR were expressed in porcine oocytes, granulosa cells and theca cells throughout an oestrous cycle, with NGF staining more predominantly in large, antral follicles than in follicles of smaller diameter (Jana *et al.* 2011), suggesting that it plays an important role during oocyte maturation. During the oestrous cycle of the golden hamster, NGF, NTRK1 and NGFR were reported to localize to oocytes, granulosa cells and theca cells of follicles representing various stages of folliculogenesis and their presence was also detected in interstitial and luteal cells (Shi *et al.* 2004). In humans, NGF was present in normally cycling women (Seifer *et al.* 2003), and it has also been quantified in the follicular fluid of women undergoing IVF and ovulation induction for fertility

Table 2 Expression profile and function of BDNF in the ovary.

Expression	Species	Reference	Function	Species	Reference
Granulosa cells of primordial and primary follicles	Mouse	Paredes <i>et al.</i> (2004)			
Secreted by cumulus and granulosa cells	Mouse	Kawamura <i>et al.</i> (2005)	↑ Extrusion of 1st polar body ↑ <i>In vitro</i> development of zygotes into preimplantation embryos ↑ Maturation of IVM oocytes Improved meiotic spindle configuration and localization/distribution of cortical granules at MI	Mouse	Kawamura <i>et al.</i> (2005)
Neonatal rat ovary before primordial follicle formation	Rat	Dissen <i>et al.</i> (1995)			
Oocytes and cumulus cells	Cow	Martins da Silva <i>et al.</i> (2005)	↑ Blastocyst rate of parthenogenetically activated embryos ↑ Percentage of MII oocytes	Cow	Martins da Silva <i>et al.</i> (2005)
Oocytes and follicular somatic cells	Pig	Lee <i>et al.</i> (2007)	↑ Extrusion of 1st polar body ↑ Developmental competence to reach the blastocyst stage	Pig	Lee <i>et al.</i> (2007)
Follicular fluid	Human	Seifer <i>et al.</i> (2002a, 2002b, 2003) and Sadeu <i>et al.</i> (2012)	↑ Extrusion of 1st polar body	Mouse	Seifer <i>et al.</i> (2002a, 2002b)
Granulosa cells	Human	Zhao <i>et al.</i> (2011)	↑ Total number of MII oocytes	Human	Zhao <i>et al.</i> (2011)
Oocytes and cumulus cells	Human	Anderson <i>et al.</i> (2010)	Blocking antibodies against BDNF ↑ number of MII oocytes ↑ Failure to cleave when included in IVM media	Human	Anderson <i>et al.</i> (2010)

treatment (Seifer *et al.* 2002a, Buyuk & Seifer 2008, Sadeu *et al.* 2012). Its mRNA and protein are expressed in human granulosa cells and oocytes isolated from fetal ovaries collected at 19–33 weeks of gestation and in ovarian samples derived from girls and women aged 13–39 years, while NTRK1 was primarily detected in oocytes (Abir *et al.* 2005). NGF and NTRK1 were also detected in human preantral and antral follicle-derived granulosa cells and in theca cells of antral follicles (Salas *et al.* 2006). The cumulative ovarian expression profile of NGF (Table 3) supports both a paracrine and an autocrine influence on oocytes and cumulus cells.

Regulation of ovarian function by neurotrophins

Although GDNF promotes diverse functions in several different tissues, its potential role in the ovary has, until recently, remained relatively unexplored. It has been suggested that GDNF may contribute to the onset of ovarian tumorigenesis in ageing mutant mice carrying a disruption in the FSH receptor (*Fshr*) gene (Aravindakshan *et al.* 2006). In addition, a role for GDNF in promoting primordial follicle development in 4-day-old rats has been described, demonstrating that ovaries maintained in organ culture and treated with GDNF contain significantly more developing follicles compared with untreated controls (Dole *et al.* 2008). Regarding its influence on the later stages of follicular development, particularly oocyte maturation, our laboratory has reported that GDNF stimulates oocyte nuclear and cytoplasmic maturation as well as cumulus cell expansion in the pig, also enhancing the developmental competence of porcine oocytes derived from small, less mature antral follicles (Linher *et al.* 2007).

More specifically, we observed GDNF to promote nuclear maturation of oocytes as measured by first polar body extrusion, signifying meiotic progression to MII, and addition of GDNF to the IVM media also increased oocyte cyclin B1 protein levels (Linher *et al.* 2007). These effects were mediated by GFRA1, as a blocking antibody reversed the functional effects of GDNF. Our work further demonstrated that GDNF preferentially enhances the competence of small follicle-derived antral oocytes to support preimplantation embryo development to the blastocyst stage, increasing the percentage of blastocysts to levels obtained for untreated large, more meiotically competent antral follicle-derived oocytes (Linher *et al.* 2007). Similar studies on mouse revealed that, following treatment of COCs with GDNF, murine oocytes underwent enhanced extrusion of the first polar body, and cyclin B1 protein levels were also significantly increased (Kawamura *et al.* 2008). While inclusion of GDNF in the IVM media does not enhance preimplantation development in the mouse, treatment of cultured early murine embryos at the two-cell stage with GDNF specifically enhances the percentage that reaches the expanded and hatched blastocyst stages, as use of a GDNF-neutralizing antibody blocked this effect (Kawamura *et al.* 2008). Of particular interest, during these early stages of mouse preimplantation embryo development, GDNF was found to be expressed in the oviducts and uteri of pregnant mice, and both its co-receptors were expressed in embryos, demonstrating that this factor not only supports oocyte maturation and blastocyst formation *in vitro* but it also has a physiologically relevant role to support early embryonic development *in vivo* (Kawamura *et al.* 2008).

Table 3 Expression profile and function of NGF in the ovary.

Expression	Species	Reference	Function	Species	Reference
Fetal, neonatal and adult ovary	Rat	Lara <i>et al.</i> (1990) and Disen <i>et al.</i> (1995, 1996)	Possible role during ovulation	Rat	Disen <i>et al.</i> (1996)
			↑ Formation of parthenogenetic pronuclei	Mouse	Fedorushchenko <i>et al.</i> (1996, 1999)
Follicular fluid	Sheep	Barboni <i>et al.</i> (2002)	↑ Resumption of meiosis	Sheep	Barboni <i>et al.</i> (2002)
Oocytes, granulosa cells and theca cells	Pig	Jana <i>et al.</i> (2011)	↑ Resumption of meiosis		
Oocytes, granulosa cells, theca cells, interstitial and luteal cells	Golden hamster	Shi <i>et al.</i> (2004)	↑ Cumulus cell expansion		
			↑ Germinal vesicle breakdown	Xenopus	Nebreda <i>et al.</i> (1991)
Follicular fluid	Human	Seifer <i>et al.</i> (2002a, 2002b, 2003), Buyuk & Seifer (2008) and Sadeu <i>et al.</i> (2012)	Activation of maturation-promoting factor		
Oocytes, granulosa cells and theca cells	Human	Abir <i>et al.</i> (2005) and Salas <i>et al.</i> (2006)			

In further support of our initial findings using a porcine model and studies conducted in rodents, it has recently been shown that in women undergoing IVM, GDNF enhances the total number of human oocytes that reach MII (Zhao *et al.* 2011). Furthermore, inclusion of GDNF in IVM medium containing a defined mixture of other important ovarian factors (BDNF, insulin-like growth factor 1, oestradiol, fibroblast growth factor 2 (FGF8), and leptin) resulted in a greater percentage of human oocytes that attained nuclear maturation as well as enhanced parthenogenetic blastocyst formation (McElroy *et al.* 2010). It is therefore becoming increasingly apparent that GDNF, which is normally present in follicular fluid, has distinct, stage-dependent effects on oocyte maturation beyond its previously recognized roles. These findings support the notion that GDNF acts as an important intra-ovarian regulator during follicular development (Table 1).

BDNF is also able to promote oocyte maturation. Obtaining follicular aspirates from women undergoing IVM, Seifer *et al.* (2002a) were the first to show that BDNF is secreted by cumulus cells into follicular fluid. Due to the constraints of using human COCs, their study also utilized murine oocytes as a model to evaluate a possible role for BDNF during oocyte maturation, revealing that this neurotrophin significantly enhances the percentage of oocytes extruding a first polar body (Seifer *et al.* 2002a). Results of a DNA microarray analysis of ovarian transcripts led Kawamura *et al.* (2005) to identify BDNF as a factor stimulated by the LH surge, also demonstrating that it is secreted by murine granulosa and cumulus cells (Kawamura *et al.* 2005). Furthermore, with NTRK2 expressed exclusively in oocytes, BDNF acted as a paracrine factor that enhanced the extrusion of the first polar body, also promoting the developmental competence of preimplantation embryos (Kawamura *et al.* 2005). Both these BDNF-mediated effects were specifically blocked using a NTRK2 receptor inhibitor. In a more detailed study using immunofluorescence and confocal laser microscopy, Zhang *et al.* (2010a, 2010b) demonstrated that BDNF promotes the maturation of murine IVM oocytes by affecting key morphological characteristics, namely by improving the configuration of meiotic spindles and the localization and distribution of cortical granules at MI (Zhang *et al.* 2010a). BDNF also enhanced the rate at which parthenogenetically activated bovine embryos derived from large, more mature preovulatory oocytes reached the blastocyst stage (Martins da Silva *et al.* 2005). Unlike what has been reported in rodents, in the cow, BDNF did not appear to affect nuclear maturation (Martins da Silva *et al.* 2005). However, in a recent study, it was shown that BDNF does significantly increase the percentage of bovine MII oocytes, while no statistically significant changes were recorded for its effect on development to the blastocyst stage (Hong *et al.* 2009). As a potential candidate for improving *in vitro* culture and embryo

production of porcine oocytes, inclusion of BDNF in the IVM medium significantly increased first polar body extrusion, as well as enhancing oocyte developmental competence to reach the blastocyst stage following IVF and somatic cell nuclear transfer (Lee *et al.* 2007). In COCs derived from women undergoing laparoscopy who were then subjected to IVM, BDNF did not affect MII yields (Anderson *et al.* 2010). Indeed, paradoxically, inclusion of two different blocking antibodies against BDNF in the IVM medium improved the percentage of MII oocytes. In activated human oocytes, treatment with these same blocking antibodies resulted in the highest rate of abnormal cleavage, while failure to cleave was highest when BDNF was included in the culture media (Anderson *et al.* 2010). However, BDNF has also been reported to enhance the total number of human immature, cultured oocytes that reach MII (Zhao *et al.* 2011). From these combined findings, it is clear that BDNF signalling does play a role during the final stages of follicular development by affecting oocyte maturation but that its effects may be species dependent, or dependent on the particular paradigm applied for IVM (Table 2).

NGF was first shown to play a role during *Xenopus laevis* oocyte maturation, as the presence of its microinjected receptor RNA potentiated the effects of progesterone (Sehgal *et al.* 1988). Furthermore, *Xenopus* oocytes injected with *NTRK1* RNA and subsequently treated with NGF underwent breakdown of the germinal vesicle and activation of maturation-promoting factor, two markers of meiotic maturation (Nebreda *et al.* 1991). This finding was also observed in murine oocytes that were injected with NGF, which subsequently displayed an increased ability to form parthenogenetic pronuclei in both oocytes within COCs and denuded oocytes (Fedorushchenko *et al.* 1999). The same group also showed that addition of NGF to IVM media induced the resumption of meiosis in murine oocytes cultured without cumulus cells following gonadotrophin stimulation (Fedorushchenko *et al.* 1996). However, the developmental competence of one-cell stage bovine embryos produced by IVM or IVF was not enhanced after culture in the presence of NGF (Flood *et al.* 1993). During ovine IVM, NGF significantly enhanced cumulus cell expansion, also inducing the resumption of meiosis in oocytes to levels comparable to those achieved with gonadotrophin stimulation (Barboni *et al.* 2002). The finding that NGF levels increased in follicular fluid from sheep following the LH surge (Barboni *et al.* 2002) further supported that this neurotrophin plays a role during final oocyte maturation. However, in the pig, NGF failed to enhance the percentage of oocytes that reached the MII stage following IVM and did not affect the developmental competence of early embryos (Papp *et al.* 2005), which could, similar to the cow, be due to species differences. A summary of the effects of NGF on oocyte maturation is outlined in Table 3.

Putative mechanisms underlying neurotrophin expression and action in the postnatal ovary

One means by which GDNF may influence oocyte maturation is by affecting the expression of the germ cell-specific regulator deleted in azoospermia-like (DAZL). This relationship was investigated in our laboratory using porcine oocytes as a model, revealing that maximum levels of *DAZL* mRNA and DAZL protein are associated with MII oocytes (Liu *et al.* 2009). This finding suggests that DAZL, which is known to regulate translation of the synaptonemal complex component SYCP3, an essential player during meiosis (Reynolds *et al.* 2007), may also influence the meiotic maturation of oocytes. It has previously been shown that the presence of human *DAZL* mRNA is positively associated with blastocyst quality (Cauffman *et al.* 2005), and DAZL may therefore be required for mature oocytes to acquire full developmental competence to sustain preimplantation embryo development. Interestingly, inclusion of GDNF in the IVM media significantly increased *DAZL* mRNA and DAZL protein levels during porcine oocyte maturation, while exclusion of FSH or epidermal growth factor (EGF) from the IVM media decreased its mRNA level (Liu *et al.* 2009). These findings suggest that GDNF, FSH and EGF signalling cascades are likely converging to up-regulate DAZL expression, which in turn affects the expression of other key proteins, thereby enhancing oocyte maturation (Fig. 1).

A microarray analysis conducted by Dole *et al.* (2008) in the neonatal rat ovary examined changes in gene expression in response to GDNF. GDNF altered the expression of 28 genes, most notably those encoding known growth factors and secreted cytokines. Among these, the transcript for growth differentiation factor 9 (*Gdf9*), an oocyte-secreted factor essential for normal follicular development during the primary follicle stage

sustained until ovulation (Dong *et al.* 1996, Elvin *et al.* 1999), was up-regulated by GDNF (Dole *et al.* 2008). Other notable transcripts that increased in GDNF-treated ovaries that may play a role during the final stages of folliculogenesis and oocyte maturation included *Fgf8* and connective tissue growth factor (*Ctgf*; Dole *et al.* 2008). In the ovaries of adult rodents, the detection of *Fgf8* mRNA was restricted to oocytes derived from antral follicles, while no signal was detected in fetal ovaries or in primordial follicles of the adult ovary. This expression profile suggests that FGF8 plays a paracrine role, signalling between the oocyte and follicular somatic cells during the final stages of follicular maturation (Valve *et al.* 1997). However, it has been reported that *FGF8* is expressed in oocytes, granulosa cells and theca cells within bovine antral follicles (Buratini *et al.* 2005), suggesting an autocrine/paracrine mode of signalling. CTGF is a matrix-associated heparin binding protein that is regulated by gonadotrophins and is expressed in antral follicles and corpus lutea in the rat (Harlow *et al.* 2007). Its function in the ovary remains to be determined.

It has been established that NGF increases *Fshr* mRNA levels in the neonatal rat ovary in a cAMP-independent manner, as *Fshr* expression was significantly lower in NGF-null mutant mice (Romero *et al.* 2002). These findings suggest that by modulating FSHR levels, NGF mediates differentiation during the embryonic stages of folliculogenesis, facilitating the mechanism by which preantral follicles become gonadotrophin dependent. It has since been shown that NGF also plays a role in the adult ovary, affecting the function of human granulosa cells by increasing oestradiol secretion while reducing progesterone levels (Salas *et al.* 2006). Treatment of human granulosa cells with NGF increased *FSHR* mRNA levels, which was abrogated by the Trk tyrosine kinase blocker K252a. Also, NGF pretreatment of human granulosa cells resulted in higher secretion of oestradiol after exposure to FSH compared with cells that were not pretreated (Salas *et al.* 2006). These combined results demonstrate that one of the mechanisms by which NGF may influence antral follicles is by preventing early luteinization through inhibition of progesterone secretion, mediated by increased oestradiol production and increased expression of FSHRs, thereby making the maturing COCs more responsive to gonadotrophins. Based on these findings, a proposed model of putative mechanisms of neurotrophin action in the ovary is presented in Fig. 1.

Figure 1 Putative mechanisms of neurotrophin action in the ovary. In conjunction with FSH and EGF, GDNF enhances DAZL expression, an oocyte marker associated with oocyte developmental competence and blastocyst quality. NGF enhances follicular gonadotrophin responsiveness by increasing FSHR expression. In addition, FSH also increases the expression of GDNF, BDNF and NGF in antral follicles.

Potential mechanisms regulating the ovarian expression of neurotrophins and their respective receptors

Several studies offer insights into how ovarian neurotrophin expression is regulated. In the brain, oestrogen affects *BDNF* mRNA levels during neural development

and ageing (Toran-Allerand 1996a, 1996b). Whether local oestradiol produced by granulosa cells within the ovary also alters extraneuronal neurotrophin expression remains to be determined. However, it has been shown that circulating levels of oestradiol are positively associated with BDNF concentrations in women undergoing fertility treatment (Monteleone *et al.* 2007), and plasma BDNF levels fluctuate during a menstrual cycle (Cubeddu *et al.* 2011), declining in post-menopausal women (Lommatzsch *et al.* 2005, Begliuomini *et al.* 2007, Pluchino *et al.* 2009), but can be increased through hormone replacement (Begliuomini *et al.* 2007). Steroid hormones also influence NGF. The effects of oestrogen and progesterone on uterine NGF and NTRK1 expression have been evaluated in ovariectomized adult female golden hamsters, revealing that treatment with either hormone stimulates NGF and NTRK1 levels, although no additive effects were observed (Shi *et al.* 2006).

One possible pathway activated by BDNF through NTRK2-mediated signalling in murine cumulus cells and oocytes during IVM is via protein kinase B (PKB), as it was found that the pan-specific Trk inhibitor, K252a, together with BDNF, inhibited PKB phosphorylation. While BDNF also increased phosphorylation of MAPK in oocytes, K252a coupled with BDNF did not affect MAPK activation (Zhang *et al.* 2010b). During human oocyte maturation, cAMP has been identified as a signalling molecule that dose dependently increases the production and secretion of BDNF from cumulus cells (Seifer *et al.* 2002a). This result was confirmed using forskolin, which activates intracellular adenylate cyclase and in turn leads to increases in intracellular cAMP levels. Inclusion of forskolin in cumulus cell cultures resulted in a significant increase in BDNF production (Seifer *et al.* 2002a). Based on these findings, it was hypothesized that binding of gonadotrophins to cumulus cells results in activation of cAMP, thereby increasing the production and secretion of BDNF, which in turn elicits a paracrine effect by binding to NTRK2 present on oocytes (Seifer *et al.* 2002a).

The notion that gonadotrophins influence the expression of neurotrophins is also supported by the finding that NGF follicular fluid concentrations increased dramatically in ovine large, antral follicles in response to gonadotrophin stimulation (Barboni *et al.* 2002) and in golden hamsters, the LH surge induced NGF, NTRK1 and NGFR protein expression in ovarian interstitial cells (Weng *et al.* 2009). Furthermore, BDNF secretion by cumulus and granulosa cells was increased in women undergoing IVF in response to treatment with LH, human chorionic gonadotrophin (hCG) or menopausal urinary gonadotrophin (Feng *et al.* 2003), and a recent study conducted by Zhao *et al.* (2011) demonstrated that both *GDNF* and *BDNF* mRNA levels were slightly increased following FSH treatment of unstimulated human granulosa cells derived from women undergoing IVM (Zhao *et al.* 2011). This was similar to the changes in expression reported in mice following the

preovulatory LH/hCG surge (Kawamura *et al.* 2005, 2008). Furthermore, the expression of each transcript was significantly increased in response to treatment with either hCG or a combination of FSH and hCG (Zhao *et al.* 2011). In women undergoing IVM or IVF, *GDNF* mRNA levels were significantly increased after 36 h of hCG treatment (Zhao *et al.* 2011). These results are consistent with a study by Kawamura *et al.* (2008), which examined the regulation of *Gdnf*, *1Gfra1* and *Ret* mRNA levels in murine follicles after priming with pregnant mare serum gonadotrophin for 48 h and again after hCG treatment (Kawamura *et al.* 2008). *Gfra1* mRNA levels increased in cumulus cells and mural granulosa cells in response to hCG but did not change in oocytes. In addition, *Ret* receptor mRNA levels increased in both cumulus and mural granulosa cells but remained unchanged in oocytes (Kawamura *et al.* 2008). In further support that gonadotrophins influence the expression of ovarian neurotrophins, Perlman *et al.* (2006) used oligonucleotide gene chips to evaluate changes in transcript levels in human granulosa cells treated with FSH (Perlman *et al.* 2006). *BDNF* was among the differentially expressed genes, with its mRNA levels increasing significantly in response to FSH stimulation (Perlman *et al.* 2006).

In an ovarian follicle, activin increases the biosynthesis and enhances the actions of FSH, which is directly opposed by the action of inhibin at later states of folliculogenesis. In cultured human fetal ovaries treated with recombinant human activin A, the levels of *BDNF* mRNA increased significantly compared with untreated controls (Childs *et al.* 2010). However, there appear to be species differences in the activin A-mediated regulation of *BDNF* expression in fetal ovarian somatic cells, as no changes in *Bdnf* mRNA levels were reported in cultured cells derived from murine neonatal ovaries (Childs *et al.* 2010). It was speculated that activin A promotes signals that influence germ cell survival, down-regulating those pathways that ultimately lead to oocyte maturation (Childs *et al.* 2010). Whether BDNF levels are altered by activin in the adult ovary remains to be determined.

Potential clinical significance

It is clear that *GDNF*, *BDNF* and NGF are important ovarian factors that exert a significant influence on both developing and mature follicles. In particular, their roles during oocyte maturation and early development of the embryo make them attractive candidates for inclusion in IVM and IVF treatment regimens that could be of clinical significance. In women undergoing IVF and ICSI, it has been shown that treatment of immature oocytes and granulosa cells with gonadotrophins enhanced the expression of *GDNF* and *BDNF* in granulosa cells (Zhao *et al.* 2011). Furthermore, culturing immature oocytes collected from these patients in the presence

of GDNF or BDNF revealed that these ovarian neurotrophins promote oocyte maturation in a clinically relevant manner (Zhao *et al.* 2011). In women with endometriosis receiving assisted reproductive treatment, the follicular fluid concentration of BDNF was lower than in the control group composed of women with male factor infertility, while no differences in NGF concentrations were observed (Buyuk & Seifer 2008). Interestingly, the levels of NGF in follicular fluid derived from women with polycystic ovary syndrome (PCOS) were lower compared with the control group, but BDNF concentrations did not vary (Buyuk & Seifer 2008), suggesting an effect potentially attributable to the different aetiologies of infertility. This notion is supported by a recent study demonstrating that follicular fluid BDNF concentrations were only higher in women diagnosed with unexplained infertility following ovulation induction, while no relationship could be established between levels of BDNF or NGF in women with a history of either endometriosis or PCOS compared to controls (Sadeu *et al.* 2012). It should be noted that this particular study included only a small number of participants, the stage of disease was not factored into the measurements and there may be differences in ovulation induction procedures that vary between facilities that could explain the differences reported across different studies.

As gonadotrophins increase the expression of GDNF, BDNF and NGF in cumulus and granulosa cells (Seifer *et al.* 2002a, Zhao *et al.* 2011), and the concentrations of neurotrophins may be lower in the follicular fluid of women with infertility or diagnosed with reproductive disorders after ovarian stimulation, it is possible that the severity of different disease states may alter ovarian function and abrogate the gonadotrophin response of follicular cells through mechanisms that remain to be determined. Therefore, inclusion of these particular neurotrophic factors during IVM/IVF may provide maturing oocytes with sufficient concentrations of neurotrophins that are not available *in vivo* due to the aetiology of a given reproductive disorder.

From another clinical perspective, while neurotrophins mediate aspects of follicular maturation, it has also become evident that they may play a role in ovarian cancer, mediated through aberrant FSHR expression in ovarian surface epithelium and deregulated secretion of FSH by the pituitary gland (reviewed in Bose (2005)). GDNF may play a role in the aetiology of ovarian cancer, as its expression was initially low in *Fshr* knockout mice, but in ageing females, which displayed an increase in the incidence of ovarian tumours, GDNF levels were up-regulated (Aravindakshan *et al.* 2006). By contrast, BDNF is similarly expressed in normal and cancer cell lines and tissues (Au *et al.* 2009). However, NTRK2 was reported to be overexpressed in ovarian cancers compared to either benign tumours or normal ovarian epithelium (Yu *et al.* 2008, Au *et al.* 2009), and its overexpression has been linked with poor prognosis

in ovarian cancer patients (Au *et al.* 2009). It is therefore possible that BDNF/NTRK2-mediated signalling, coupled with crosstalk from other pathways, may be associated with tumour metastasis and chemotherapeutic resistance in ovarian cancer, making NTRK2 a putative therapeutic target (reviewed in Siu *et al.* (2009)). It has been shown that NGF and NTRK1 are expressed at very low levels in normal ovarian surface epithelium but are significantly more abundant in epithelial ovarian cancer cells (Campos *et al.* 2007, Tapia *et al.* 2011). Furthermore, NGF has been shown to dose dependently up-regulate vascular endothelial growth factor (VEGF) isoform expression in cancer explants (Campos *et al.* 2007), also increasing the proliferation, migration and differentiation of cultured ovarian cancer cells (Tapia *et al.* 2011), effects that were inhibited by either a NGF antibody or a K252a. These findings were corroborated by Julio-Pieper *et al.* (2009), who reported that NGF enhances the synthesis and secretion of VEGF from human granulosa cells in an NTRK1-dependent manner (Julio-Pieper *et al.* 2009).

Future research directions

Using models from various species, substantial evidence supports the beneficial functional role of GDNF, BDNF and NGF during oocyte maturation and early embryo development. However, substantial knowledge gaps exist, particularly with respect to the mechanisms that drive their effects in COCs upon meiotic resumption, as well as how the expression of these particular neurotrophic factors and their respective receptors is controlled in the postnatal ovary. Identifying the signalling networks regulating the expression of specific neurotrophins will be invaluable, as this could provide a means to intrinsically manipulate their levels in maturing COCs *in vitro*. Studies aimed at identifying the pathways and genes that are activated in response to neurotrophin stimulation in oocytes, cumulus cells and granulosa cells will provide insights into understanding their regulatory effects at the molecular level. None of these factors act alone, and in order to design a robust IVM regimen, it will be important to systematically dissect how GDNF, BDNF and NGF, in combination with other key ovarian growth factors and hormones, affect ovarian signalling during the final stage of folliculogenesis. In addition, it will be of interest to investigate the role of other neurotrophic factors on oocyte maturation and developmental competence. In particular, NTF3 and NTF4/5 are also expressed in the ovary, may play a role in primordial and primary follicles (Nilsson *et al.* 2009) and have been implicated in oocyte maturation in women (Seifer *et al.* 2002b). Future studies using animal models as a means to monitor the health of offspring generated by IVM/IVF carried out in media containing specific neurotrophins will help to validate their inclusion in assisted reproductive technologies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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