

# NTRK1 and NTRK2 receptors facilitate follicle assembly and early follicular development in the mouse ovary

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## Abstract

Recent studies have demonstrated that neurotrophins (NTs) and their NTRK tyrosine kinase receptors, thought to be exclusively required for the development of the nervous system, are also involved in controlling ovarian development. Here, we show that primordial follicle formation is decreased in the absence of nerve growth factor (NGF) or its receptor NTRK1, and in the absence of NTRK2, the receptor for neurotrophin-4 (NTF4) and brain-derived neurotrophic factor (BDNF). This deficiency is not due to premature oocyte loss, because the ovaries of *Ntrk1*<sup>-/-</sup> and *Ntrk2*<sup>-/-</sup> mice do not show an increased rate of oocyte death antedating the initiation of folliculogenesis. Moreover, exposure of NGF-deficient ovaries to NGF rescues the defect in follicular assembly, if NTRK1 receptors are present, suggesting that the absence of NTs causes a delay, and not an irretrievable loss, of follicle formation. Both the number of secondary follicles and FSH receptor (FSHR) expression are diminished in *Ntrk1*- and *Ntrk2*-null ovaries, but not in ovaries lacking the common NT receptor NGFR. Transient exposure of wild-type ovaries to NTF4 increases *Fshr* gene expression and enhances the ability of the ovary to respond to FSH with formation of cyclin D2, a cell cycle protein mediating the proliferative actions of FSH in the ovary. These results indicate that both NTRK1 and NTRK2 receptors are necessary for the timely assembly of primordial follicles and for sustaining early follicular development. They also suggest that a mechanism by which NTRK2 receptors facilitate subsequent follicle development is by inducing the formation of functional FSHR.

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## Introduction

Much is known about the hormonal mechanisms controlling ovarian development. More recently, a major focus of attention in the field has been the identification of regulatory pathways that, operating within the ovarian microenvironment, contribute to the acquisition of ovarian reproductive competence. Within this framework, our laboratory has developed the concept that neurotrophins (NTs) and their NTRK tyrosine kinase receptors, long thought to be exclusively required for the development of the nervous system, are also involved in the control of ovarian function (reviewed in (Dissen *et al.* 2004)).

A role for NTs in the control of ovarian maturation was initially suggested by the finding that the developing ovary not only contains four of the known NTs (nerve growth factor, NGF; brain derived neurotrophic factor, BDNF; neurotrophin 3, NTF3; and neurotrophin 4/5, NTF-4/5 (Ernfors *et al.* 1990, Lara *et al.* 1990, Berkemeier *et al.* 1991, Hallböök *et al.* 1991, Dissen *et al.* 1995,

1996), but also expresses the receptors for each of them (NGFR and the tyrosine kinase high-affinity receptors NTRK1, NTRK2, and NTRK3; Klein *et al.* 1989, Dissen *et al.* 1991, 1995, 1996, Lamballe *et al.* 1991, Paredes *et al.* 2004, Abir *et al.* 2005). More recent studies have made clear that the NTs and their respective receptors are expressed in fetoneonatal rodent ovaries and fetal human ovaries before the initiation of follicular assembly (Dissen *et al.* 1995, Anesetti *et al.* 2001, Anderson *et al.* 2002, Spears *et al.* 2003, Abir *et al.* 2005).

The importance of NGF in early follicular development was made evident by the reduction in the number of primary and secondary follicles, and the decreased expression of FSH receptor (FSHR) found at the end of the first postnatal week of life in *Ngf*-null mice (Dissen *et al.* 2001). These findings suggested that NGF not only promotes the early stages of follicle development, but also induces the initial biochemical differentiation of secondary follicles into gonadotropin-responsive structures.

Thus far, a role for NTF3 and its high-affinity receptor NTRK3 in follicle formation or follicle development has not been revealed (Spears *et al.* 2003). However, using *Ntrk2*-null mice, we (Paredes *et al.* 2004) and others (Spears *et al.* 2003) have recently demonstrated that NTRK2 signaling is required for oocyte survival and preantral follicular development. Spears *et al.* (2003) employed mutant mice lacking the intracellular domain of the receptor and found that these animals have a significantly lower number of oocytes and primordial follicles than wild-type (WT) controls, suggesting that NTRK2 signaling is required for germ cell survival before initiation of follicular assembly. Employing mutant mice lacking all NTRK2 isoforms, we found that the ovaries of these mice (or those lacking both NTF-4/5 and BDNF) suffer a stage-selective deficiency in early follicular development that compromises the ability of follicles to grow beyond the primary stage. Proliferation of granulosa cells – required for this transition – and expression of FSHRs, which reflects the degree of biochemical differentiation of growing follicles, are reduced in these ‘complete’ *Ntrk2*-null mice. To determine the importance of NTRK2 receptors for subsequent follicular development, and because *Ntrk2*-null mice die within the first 2 weeks of postnatal life, we grafted the ovaries from 4- to 5-day-old KO animals under the kidney capsule of WT adult female mice and examined the ovaries 2 weeks later. To our surprise, the *Ntrk2*-null ovaries failed to sustain follicular development and showed a striking loss of follicular organization, preceded by massive oocyte death. These results indicate that NTRK2 receptors facilitate the early development of ovarian follicles, and that the receptors become critical for oocyte survival after follicular assembly.

While the importance of NTRK2 receptors in early ovarian development appears now unquestionable, the general consistency – but also the specific differences – emerging from the aforementioned results (Spears *et al.* 2003, Paredes *et al.* 2004) raise an entirely new set of questions. For instance, the size of the primordial follicle population is relatively normal by the end of the first postnatal week of life in both *Ntrk1*- and *Ntrk2*-null mice (Dissen *et al.* 2001, Paredes *et al.* 2004), but it is significantly reduced in perinatal NTRK2-deficient ovaries (Spears *et al.* 2003), suggesting that NTRK2 and/or NTRK1 receptors may be required for the timely initiation of folliculogenesis. If this is the case, are NTRK2 and/or NTRK1 receptors required for oocyte survival before follicular formation? Are NTRK1 receptors, in addition to NTRK2 receptors, required for the acquisition of functional FSHRs? The present study addresses these questions and provides evidence that both NTRK1 and NTRK2 receptors contribute not only to the assembly of primordial follicles, but also to the subsequent development of the newly formed follicles, and to the acquisition of FSHRs by the growing follicles. In addition, these studies show that oocyte death does

not increase before the initiation of folliculogenesis in the absence of either NTRK1 or NTRK2 receptors, and that ligand-mediated activation of NTRK2 receptors enhances the capacity of the infantile ovary to respond to FSH with synthesis of cyclin D2, a cell cycle protein underlying the proliferative effects of FSH on granulosa cells (Sicinski *et al.* 1996).

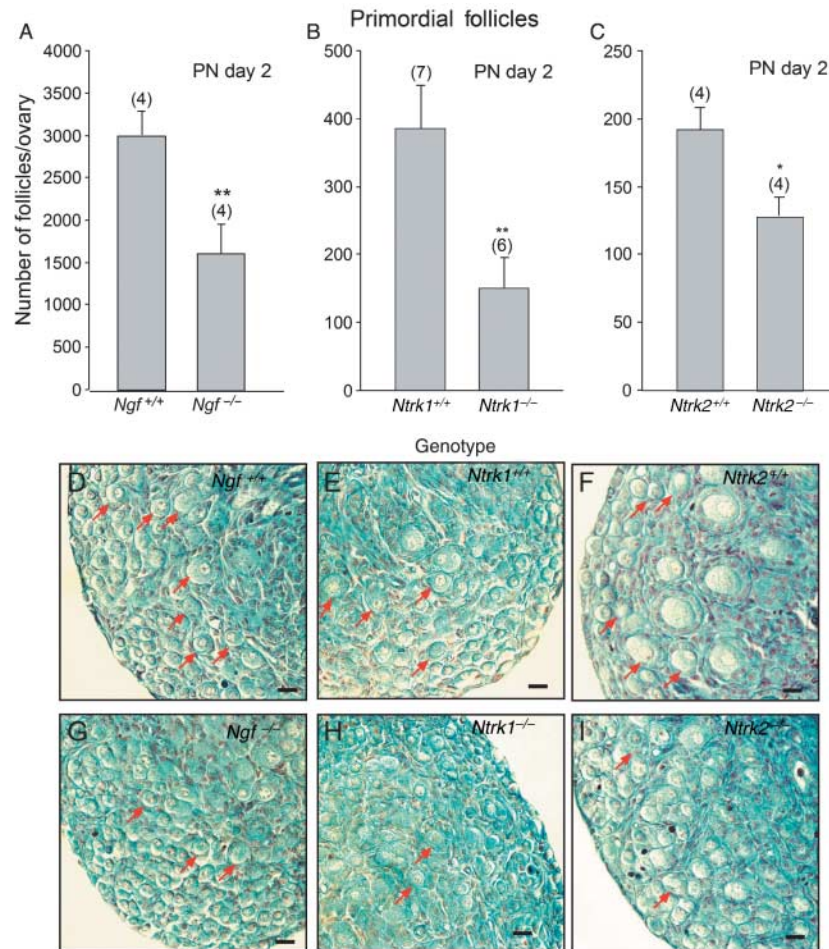
## Results

### **Absence of NT signaling delays follicle assembly**

It was previously shown that 7-day-old ovaries from *Ngf*<sup>-/-</sup> mice and from mice lacking all isoforms of the NTRK2 receptor have a deficiency of early follicle development, but a relatively normal complement of primordial follicles (Romero *et al.* 2002, Paredes *et al.* 2004). To determine whether this apparent normalcy represents a true lack of an NT role in facilitating follicular assembly or, instead, reflects a delay of follicular formation, we examined the ovaries of 2-day-old mice carrying null mutations of the genes encoding NGF and its high-affinity NTRK1 receptor, in addition to mice lacking all NTRK2 receptor isoforms. In all cases, and despite of the strain-related differences in the absolute number of follicles present at this early age, the number of primordial follicles was significantly lower in the mutant ovaries than that in the ovaries of their respective WT littermates (Fig. 1A–C). In each case, a clear immaturity of the ovary was apparent, with differences in the size of the primordial follicle population more clearly manifested in ovaries lacking NGF or its NTRK1 receptor (Fig. 1D, G and E, H respectively) than in *Ntrk2*-deficient ovaries (Fig. 1F and I). The KO ovaries not only have a reduced number of primordial follicles (examples denoted by red arrows), but also appear to exhibit a greater number of non-encapsulated oocytes than WT ovaries (Fig. 1G, H and I versus D, E and F).

### **The neonatal reduction in primordial follicles of *Ntrk1*- and *Ntrk2*-null ovaries is not due to perinatal death of oocytes**

Because the absence of NTRK1 and NTRK2-mediated signaling may result in oocyte death similar to that reported in mutant mice lacking the intracellular domain of the NTRK2 receptor (Spears *et al.* 2003), we used a TUNEL assay to examine the ovaries of *Ntrk1*<sup>-/-</sup> and *Ntrk2*<sup>-/-</sup> mice on the day of birth, i.e. preceding the initiation of folliculogenesis, for signs of apoptosis. Very few, if any, apoptotic cells (green color) were detected (Fig. 2A–D, arrows), including apoptotic oocytes, which were rarely observed (Fig. 2A–D; higher magnification images in E–G). Neither *Ntrk1*<sup>-/-</sup> nor *Ntrk2*<sup>-/-</sup> ovaries had a higher incidence of apoptotic oocytes than WT controls (Fig. 2A–D). This scarcity of somatic and germ



**Figure 1** Formation of primordial follicles is reduced in the ovaries of mice lacking the *Ngf*, *Ntrk1*, or the *Ntrk2* genes, as assessed by morphometric analysis of 2-day-old ovaries. (A) *Ngf*<sup>+/+</sup> and *Ngf*<sup>-/-</sup> mice. (B) *Ntrk1*<sup>+/+</sup> and *Ntrk1*<sup>-/-</sup> mice. (C) *Ntrk2*<sup>+/+</sup> and *Ntrk2*<sup>-/-</sup> mice. (D) Histological aspect of an *Ngf*<sup>+/+</sup> ovary showing an abundance of primordial follicles (examples denoted by red arrows), and the presence of primary follicles. (E) Similar aspect of an *Ntrk1*<sup>+/+</sup> ovary. (F) An *Ntrk2*<sup>+/+</sup> ovary showing that follicular development is more advanced in this strain at this time of postnatal life, with several primary follicles being apparent. (G) An *Ngf*<sup>-/-</sup> ovary showing a paucity of primordial follicles and many 'naked' oocytes. (H) An *Ntrk1*<sup>-/-</sup> ovary also showing a deficiency of primordial follicles, and an accumulation of naked oocytes. (I) Similar aspect of an *Ntrk2*<sup>-/-</sup> ovary. The numbers in parentheses above bars are number of animals per group, and vertical lines are s.e.m. \**P*<0.05 and \*\**P*<0.02 versus WT control groups. Scale bars = 20  $\mu$ m.

cell death is in contrast to 28-day-old ovaries (used as a positive control) in which granulosa cell apoptosis of an atretic follicle is widespread (Fig. 2H). As expected, all cells were TUNEL positive in sections treated with DNase I before performing the TUNEL reaction (Fig. 2I).

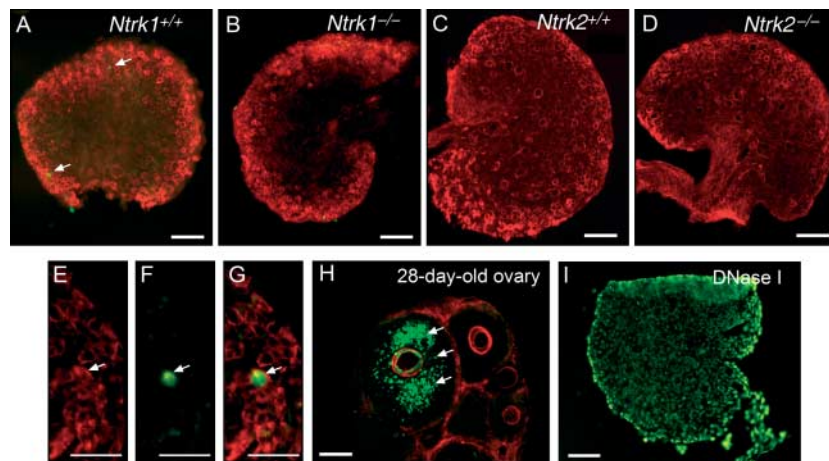
#### **NGF acting via NTRK1 receptors restores formation of primordial follicles in *Ngf*<sup>-/-</sup> mice**

To determine whether the reduced number of primordial follicles observed in *Ngf*<sup>-/-</sup> ovaries is specifically related to the absence of NGF-initiated signaling, we cultured ovaries from 1-day-old *Ngf*<sup>-/-</sup> mice for 4 days in the presence of NGF and determined the number of primordial follicles at the end of this period. Exposure to the NT distinctly (*P*<0.01) increased the number of primordial follicles in comparison with untreated

*Ngf*<sup>-/-</sup> ovaries (Fig. 3A). By contrast, NGF was ineffective in *Ntrk1*<sup>-/-</sup> ovaries (Fig. 3B), indicating that the facilitatory effect of NGF on follicular assembly is mediated by NTRK1 receptors.

#### **Early follicle development is impaired in the absence of NTRK1 and NTRK2 receptors**

To determine whether the lack of NTRK1 and NTRK2 receptors begins to impact follicle development as the follicles enter the proliferative pool, we examined the ovaries of 4-day-old mutant mice and compared the number of primordial, primary, and secondary follicles with those of WT littermates. In both *Ntrk1*- and *Ntrk2*-null ovaries, the size of the primordial follicle population was still significantly reduced (*P*<0.02), but no significant differences in the number of primary



**Figure 2** Representative sections from 0-day-old ovaries (day of birth) showing that the decreased number of primordial follicles observed in *Ntrk1*- and *Ntrk2*-null ovaries is not due to an earlier, postnatal increase in apoptotic oocyte death. Apoptosis was detected using a TUNEL assay (green color) and oocytes were identified with a MAB against CD9, a membrane protein abundant in oocytes (red color). (A) *Ntrk1*<sup>+/+</sup> ovary. (B) *Ntrk1*<sup>-/-</sup> ovary. (C) An *Ntrk2*<sup>+/+</sup> ovary. (D) An *Ntrk2*<sup>-/-</sup> ovary. Arrows point to TUNEL-positive cells. (E) Higher magnification image showing a region of a WT ovary containing many naked oocytes. (F) Image showing that one of the oocytes shown in (E) is apoptotic (arrow). (G) Merged image. (H) Prepubertal 28-day-old ovary showing an abundance of apoptotic cell nuclei (green color, arrows) in granulosa cells of an atretic follicle, and the lack of apoptotic cells in healthy, adjacent follicles. (I) Section from a day-0 WT ovary treated with DNase I. Scale bars=100  $\mu$ m.

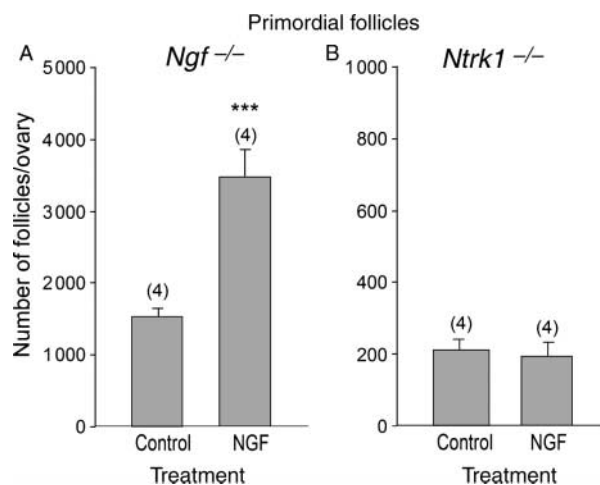
follicles were detected (Fig. 4A, B, D and E). By contrast, a marked decrease ( $P < 0.01$ ) in the number of secondary follicles was observed in both mutants (Fig. 4C and F), suggesting that recruitment of primary follicles into the proliferative pool is compromised in the absence of either NTRK1 or NTRK2 signaling.

#### Both NTRK1 and NTRK2 receptors contribute to the acquisition of FSHRs

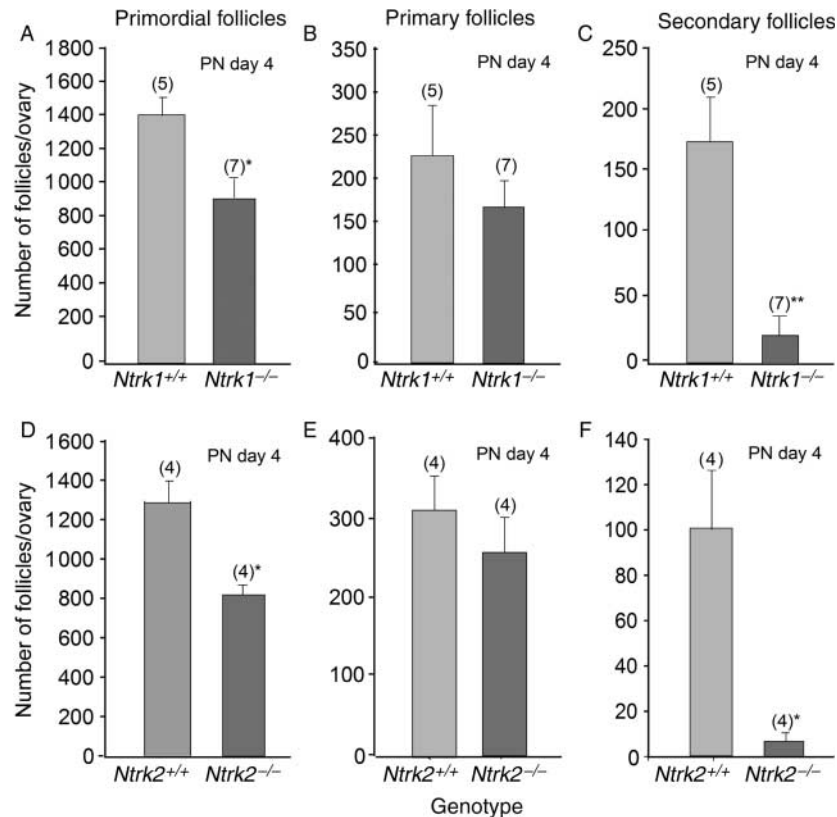
We previously showed that *Fshr* mRNA is decreased in the ovaries of *Ngf*<sup>-/-</sup> and *Ntrk2*<sup>-/-</sup> mice (Romero *et al.* 2002, Paredes *et al.* 2004), and that a short-term (8 h) exposure to NGF suffices to induce *Fshr* gene expression (Paredes *et al.* 2004). To determine whether the supportive effect of NGF on *Fshr* gene expression is mediated by high-affinity NTRK1 receptors or by the common NT receptor NGFR, we compared the abundance of *Fshr* mRNA in the ovaries of 7-day-old *Ntrk1*<sup>-/-</sup> and *Ngfr*<sup>-/-</sup> mice to that of WT littermates. As shown in Fig. 5, the absence of NTRK1 (A), but not that of NGFR (B) resulted in significantly ( $P < 0.05$ ) lower *Fshr* mRNA levels than in WT ovaries, suggesting that NGF maintains FSHR expression (Romero *et al.* 2002) via activation of NTRK1 receptors.

By the end of the first week of life, the ovaries from *Ntrk2*<sup>-/-</sup> mice show a marked reduction in granulosa cell proliferation, and a sustained reduction in the number of secondary follicles (Paredes *et al.* 2004). It was, therefore, important to determine: a) whether a transient exposure to the NTRK2 ligand NTF4 can increase *Fshr* gene expression, as previously shown for NGF (Romero *et al.* 2002), and b) if this treatment also results in the formation of FSHRs able to initiate a

proliferative signal, as measured by the ability of FSH to increase the synthesis of cyclin D2, a cell cycle protein known to mediate the stimulatory effect of FSH on granulosa cell proliferation (Sicinski *et al.* 1996). A short (8-h) *in vitro* exposure of 4-day-old WT ovaries to NTF4 (100 ng/ml) significantly ( $P < 0.01$ ) increased *Fshr* mRNA levels (Fig. 6A). Ovaries treated with NTF4 for 8 h and then with FSH for 24 h, in the absence of the NT, responded to FSH with an increase ( $P < 0.05$ ) in cyclin D2 levels (Fig. 6B). This increase was not seen in ovaries treated with NTF4 for 8 h and then cultured in medium



**Figure 3** NGF acting via NTRK1 receptors rescues the number of primordial follicles that fail to form in *Ngf*<sup>-/-</sup> mice. (A) The number of primordial follicles is increased in *Ngf*<sup>-/-</sup> ovaries explanted on the day of birth (day 0) after a 4-day exposure to NGF (100 ng/ml). (B) The same treatment is ineffective in the absence of NTRK1 receptors. Vertical lines represent the S.E.M. and the numbers in parentheses are number of animals per group. \*\*\* $P < 0.01$  versus untreated control group.



**Figure 4** A decreased number of primordial follicles persist 4 days after birth in both *Ntrk1*<sup>-/-</sup> and *Ntrk2*<sup>-/-</sup> ovaries, which in addition show a reduced number of secondary follicles. (A–C) *Ntrk1*<sup>+/+</sup> and *Ntrk1*<sup>-/-</sup> ovaries. (D–F) *Ntrk2*<sup>+/+</sup> and *Ntrk2*<sup>-/-</sup> ovaries. Vertical lines represent S.E.M. and the numbers in parentheses on top of bars are number of animals per group. \**P*<0.05 and \*\**P*<0.02 versus respective WT groups.

alone for 24 h or in ovaries cultured in medium alone for 8 h and then with FSH for 24 h (Fig. 6B). These results suggest that the supportive effect of NTRK2-mediated signaling on follicle development is mediated, at least in part, by facilitating the formation of biologically active FSHRs capable of increasing the expression of a key cell cycle protein underlying the proliferative effect of FSH on the developing ovary.

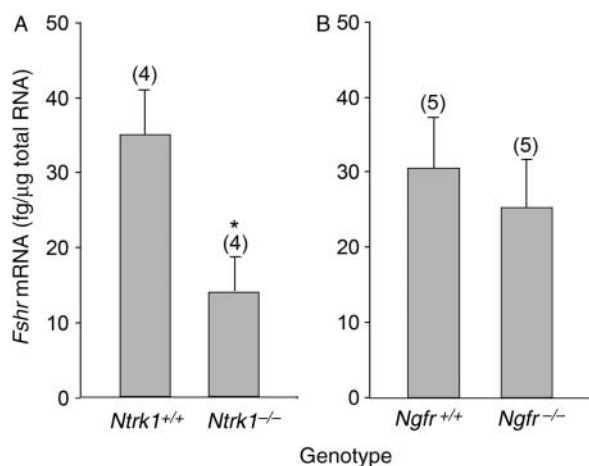
## Discussion

The present results demonstrate that mouse ovaries lacking either NTRK1 or NTRK2 receptors exhibit a reduced number of primordial follicles and a deficiency in early follicular development. The results also indicate that the defect in primordial follicle assembly is not due to premature oocyte death. Instead, it appears to result from an NT deficiency-dependent inability of somatic and germ cells to engage into the bidirectional communication process required for follicular organization. The subsequent delay of follicle development is likely related to the absence of an NT-dependent proliferative signal followed by reduced formation of FSHR.

The observation that folliculogenesis is impaired in mice lacking either NGF or its NTRK1 receptor complements and expands the results of a previous

study examining follicular development in *Ngf*<sup>-/-</sup> mice (Dissen *et al.* 2001). In that study, we found that the number of primordial follicles of *Ngf* KO ovaries collected on postnatal day 7 was similar to that of WT littermates, implying that follicular assembly does not require NGF. However, an increased number of 'naked' (i.e. non-encapsulated) oocytes were also noted, suggesting that, instead of preventing follicle assembly, the absence of NGF-mediated signaling may have delayed primordial follicle formation. The present results, obtained using ovaries collected at the time of initiation of folliculogenesis (PN day 2) and when follicle assembly is progressing in earnest (PN day 4), support this notion. Our findings also indicate that the supportive effect of NGF on follicular formation is mediated by NTRK1 receptors, because the absence of these receptors results in an ovarian phenotype indistinguishable from that of *Ngf*<sup>-/-</sup> mice.

Confirming the findings of Spears *et al.* (2003), we have now observed that the ovaries of 2- and 4-day-old *Ntrk2*<sup>-/-</sup> mice contain fewer primordial follicles than WT controls. When the ovaries were examined at a later age (PN day 7), only a trend toward a lower number of primordial follicles was observed (Paredes *et al.* 2004). Together, these earlier findings and the present observations indicate that – as is the case of NGF- and



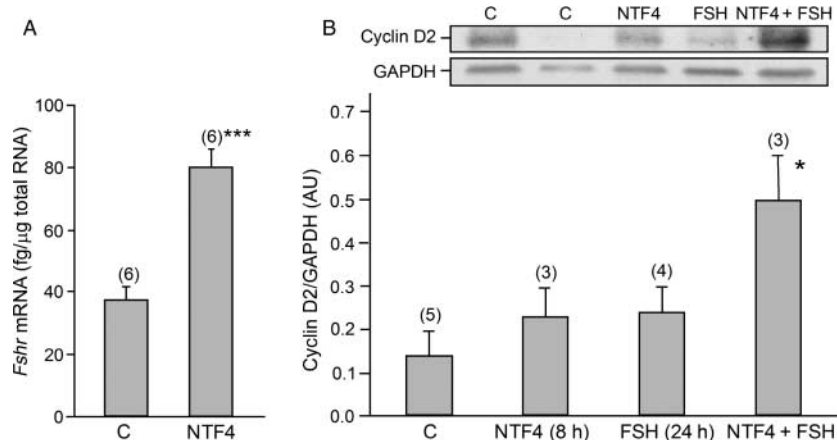
**Figure 5** *Fshr* mRNA abundance is sustained by NTRK1-mediated signaling, but does not require NGFR. (A) *Fshr* mRNA levels in 7-day-old *Ntrk1*<sup>-/-</sup> ovaries and their age-matched *Ntrk1*<sup>+/+</sup> controls. (B) *Fshr* mRNA in *Ngnr*<sup>-/-</sup> ovaries and their age-matched WT controls. Vertical lines represent s.e.m. and the numbers in parentheses on top of bars are number of animals per group. \**P* < 0.05 versus *Ntrk1*<sup>+/+</sup> group.

NTRK1-deficient mice – the absence of NTRK2-mediated signaling delays, but does not prevent, follicle assembly.

Mice lacking the intracellular domain of the NTRK2 receptor show a reduced number of oocytes 4–6 days after birth (Spears *et al.* 2003), suggesting that the lower number of primordial follicles observed in these animals may be a consequence of prior oocyte death, which would reduce the pool of oocytes able to organize somatic cells into primordial follicles. We have now examined the ovaries of *Ntrk1*<sup>-/-</sup> and *Ntrk2*<sup>-/-</sup> mice lacking all isoforms of the NTRK2 receptor on the day of birth, i.e. 48 h before the initiation of folliculogenesis,

and found no evidence for an increased incidence of oocyte death. In fact, we detected very few apoptotic oocytes in WT ovaries, and this low number remained unchanged in both *Ntrk1*<sup>-/-</sup> and *Ntrk2*<sup>-/-</sup> ovaries. Moreover, using *Ngf*<sup>-/-</sup> mice as a model to define the effects of NTs on primordial follicle formation, we observed that ovaries treated with NGF from the day of birth respond to the NT with increased formation of primordial follicles, but only when NTRK1 receptors are present. These findings indicate that the loss of primordial follicles resulting from the absence of NT signaling can be prevented by restoring NTs to the deficient ovary, as long as the corresponding high-affinity NTRK receptors are also present. This conclusion does not exclude the possibility of a prenatal increase in oocyte death caused by the absence of NTRK receptors. Such a loss, if it occurs, may exacerbate the natural loss of oocytes that occurs before birth (McClellan *et al.* 2003). Because in the present study we did not count the total number of ‘non-encapsulated’ follicles present on the day of birth, we cannot formally rule out this possibility. Nevertheless, our findings are consistent with the notion that the loss of primordial follicles seen in NT/NTRK receptor-deficient ovaries is not an irretrievable event, because it can be reversed by exposing the ovary to an NT (NGF), and it is no longer evident by the end of the first postnatal week of life (Disson *et al.* 2001, Paredes *et al.* 2004). The cellular mechanisms underlying the supportive effect of NTs on primordial follicle formation remain to be identified.

Both *Ntrk1*- and *Ntrk2*-null ovaries, in addition to the ovaries from *Ngf*<sup>-/-</sup> mice, exhibit a reduced number of secondary follicles. Although 7-day-old *Ngf*<sup>-/-</sup> ovaries also have a reduced number of primary follicles (Disson *et al.* 2001), this was not the case of the younger ovaries examined in the present study. The most parsimonious



**Figure 6** NTF4 increases *Fshr* mRNA levels and induces the formation of biologically active FSHR (as measured by the ability of FSH to increase the synthesis of cyclin D2, an FSH-responsive gene) in explanted ovaries from 4-day-old mice. (A) *Fshr* mRNA abundance, determined by real-time PCR, increases after an 8-h exposure to NTF4 (100 ng/ml). (B) A short-term (8-h) exposure to NTF4 (100 ng/ml) enhances the ability of the ovary to respond to FSH (500 ng/ml) with an increase in cyclin D2 formation, as determined by western blot analysis. A representative western blot is shown on top of the bar graph depicting the quantitative analysis of this experiment. Vertical lines are s.e.m. and the numbers in parentheses above bars are number of animals per group. \**P* < 0.05; \*\*\**P* < 0.01 versus untreated control groups (C).

explanation for this difference is that the size of the primary follicle population reflects an equilibrium between the number of primordial follicles that differentiate into follicles containing one-cell layer of cuboidal granulosa cells and those that enter the proliferative pool. If a defect in primordial-to-primary follicle differentiation is more pronounced than a defect in primary follicle recruitment to the proliferative pool (primary to secondary follicle transition), as it happens in 7-day-old NGF-deficient mice (Dissen *et al.* 2001), the number of primary follicles is reduced. However, if the recruitment defect is more prevalent, then the number of primary follicles may not change.

The cellular mechanisms underlying the supportive effect of NTs on follicle development have not been elucidated. However, in both NGF- and NTRK2-deficient ovaries, somatic cells proliferation is reduced (Romero *et al.* 2002, Paredes *et al.* 2004), suggesting that follicle development is stunted because follicular cells fail to proliferate. This functional relationship appears more obvious in the case of the NTRK2 receptor, because the proliferative defect observed in NTRK2-deficient ovaries takes place in the granulosa cell compartment (Paredes *et al.* 2004). The present results show that a transient (8 h) exposure of 4-day-old ovaries to NTF4 enhances the ability of the ovary to respond to FSH with formation of cyclin D2, a cell cycle protein known to mediate the stimulatory effect of FSH on granulosa cell proliferation (Sicinski *et al.* 1996). Such an effect of NTF4, coupled to the proliferative deficit observed in *Ntrk2*-null ovaries, strongly suggest that one mechanism by which activation of NTRK2 receptors supports follicular development is by endowing the developing follicles with the capacity to respond to FSH with proliferation. It is also clear, however, that the defect in follicle development becomes evident before the follicles reach the secondary stage and, consequently, acquire responsiveness to FSH (Dierich *et al.* 1998). This early deficiency strongly suggests that NTs exert proliferative actions on their own, in the absence of FSH. The present report does not directly address this likely possibility, which needs to be examined in detail by future studies.

In contrast to *Ntrk2*<sup>-/-</sup> ovaries, the ovaries of *Ngf*<sup>-/-</sup> mice show a reduced proliferation of mesenchymal cells (Romero *et al.* 2002), implying the existence of a directional communication pathway that, initiated in these cells and acting on granulosa cell/oocytes, supports follicle development. Such a pathway was previously postulated based on morphological evidence (Rajah *et al.* 1992), but the molecules involved remain to be identified.

In mice lacking FSHR, follicular development proceeds normally until follicles reach the secondary stage (Dierich *et al.* 1998), indicating that they become gonadotropin dependent only at this time. Our results suggest that NTs acting via NTRK receptors are

intraovarian factors that promote this biochemical differentiation process because FSHR expression is low in the absence of NGF (Romero *et al.* 2002) or its NTRK1 receptor (present results), and also in ovaries lacking NTRK2 receptors (Paredes *et al.* 2004). Conversely, *Fshr* mRNA levels increase within 8 h of exposing ovaries to either NGF, the NTRK1 ligand (Romero *et al.* 2002) or NTF4 (present results), one of the NTRK2 ligands. The common NT receptor NGFR does not appear to play a role in this process, because *Ngfr*-null mice have normal *Fshr* mRNA levels. This result was not entirely unexpected because in the rodent ovary NGFR is expressed only in mesenchymal cells long before the initiation of follicle assembly (Dissen *et al.* 1995), and remains highly expressed in thecal cells throughout the natural history of follicle development (Dissen *et al.* 1991). Because the loss of NGFR expression is only partial in existing *Ngfr* KOs (von Schack *et al.* 2001, Paul *et al.* 2004), it may be necessary to use conditional KO mice in which the cell-specific loss of the receptor is complete to fully understand the contribution that this receptor may have to ovarian development.

The cell-to-cell signaling pathways underlying the action of NTs on follicle organization and subsequent development are not known. Because in the perinatal mouse ovary NTRK receptors are located in both somatic and germ cells (Dissen *et al.* 2001, Anderson *et al.* 2002, Paredes *et al.* 2004, Abir *et al.* 2005), it appears plausible that part of the mechanism used by NTs to facilitate folliculogenesis and the progression of follicular development to the secondary stage involves the activation of reciprocal mesenchyma–granulosa cell and granulosa cell–oocyte communication pathways. The nature of the cell–cell signaling molecules involved remains to be established, but in recent studies examining the changes in gene expression that accompany the absence of NTRK1 and NTRK2 receptors, we have observed that a cell–cell communication pathway affected in both mutant ovaries involves the Jagged1 ligand and its Notch receptor (Kerr *et al.* 2006).

Altogether, the present results provided substantial support to the concept that NTs are intraovarian factors that promote ovarian development before the ovary becomes subjected to gonadotropin control.

## Materials and Methods

### *Ntrk1*<sup>-</sup>, *Ntrk2*<sup>-</sup>, *Ngfr*<sup>-</sup>, and *Ngf*-null mice

*Ntrk1*<sup>+/-</sup> (129Sv-C57BL/6) mice (Liebl *et al.* 2000) were a generous gift of Dr Lino Tessarollo (National Cancer Institute, Frederick, MD, USA). *Ngf*<sup>+/-</sup> (C57BL/6-AB1) mice (Crowley *et al.* 1994) were kindly provided by Dr Heike Phillips (University of California at San Francisco, CA, USA) and *Ngfr*<sup>+/-</sup> (BALB/c) mice (Lee *et al.* 1992) were generously provided by Dr Kuo-Fen Lee (The Salk Institute, La Jolla,

CA, USA). *Ntrk2*<sup>+/-</sup> mice (C57BL6-DBA) were generated as previously described (Paredes *et al.* 2004). The mutant mice were bred to WT animals of the same genetic background, and the null mutant and WT mice used in this study were obtained by crossing F1 heterozygous individuals. The animals were housed under controlled conditions of temperature (23–25 °C) and light (12 h light:12 h darkness; lights on from 0700 to 1900 h), and were given *ad libitum* access to food (LabDiet 5001, PMI Nutrition International Brentwood, St Louis, MO, USA) and tap water. The use of mice was duly approved by the ONPRC Animal Care and Use Committee, in accordance to the guidelines provided by the NIH Guide for the Care and Use of Laboratory Animals.

### Collection of ovarian tissue and genotyping

Ovaries from entire litters were collected at 0 (day of birth), 2 and 4 days after birth, and assigned to different procedures (organ culture, RNA extraction or morphometric analysis) before establishing each genotype. Once this was known, the ovaries were assigned to either a WT or a KO group. No heterozygotes were studied. Genotyping was performed via PCR analysis of tail DNA, employing specific oligodeoxynucleotide primers that amplify a DNA segment comprising both the targeting vector and the gene sequence specific to each mutant animal (Lee *et al.* 1992, Crowley *et al.* 1994, Liebl *et al.* 2000, Paredes *et al.* 2004).

### Culture of ovaries

To determine the effect of NGF on primordial follicle formation, ovaries from 0-day-old *Ngf*<sup>-/-</sup> and *Ntrk1*<sup>-/-</sup> mice were dissected under a stereomicroscope using aseptic conditions, placed on sterile lens paper and cultured on metal grids in a 24-well plate at the interface of air/culture medium, under an atmosphere of 60% O<sub>2</sub>-35% N<sub>2</sub>-5% CO<sub>2</sub>, as described (Romero *et al.* 2002, Paredes *et al.* 2004). One ovary from each animal was cultured in presence of NGF (100 ng/ml) for 4 days; the contralateral ovary served as an untreated control. At the end of this period, the ovaries were collected, fixed in Kahle's fixative (Hirshfield & DeSanti 1995), embedded in paraffin, serially sectioned at 6 µm and stained for morphometric analysis, as reported earlier (Dissen *et al.* 2001).

To determine the effect of NTRK2 activation on *Fshr* mRNA levels, and on cyclin D2 abundance, ovaries from 4-day-old WT mice were dissected and cultured as outlined above. When used for measurement of *Fshr* mRNA, the ovaries were cultured for 8 h in presence or absence of NTF4 (100 ng/ml), then frozen in dry ice, and stored at -85 °C until RNA extraction. Cultures employed for western blot analysis of cyclin D2 protein levels were subjected to the same experimental protocol we used earlier to document an effect of NGF on the formation of functional FSHR (Romero *et al.* 2002). In brief, the ovaries were treated with NTF4 for 8 h or left untreated. At the end of this period, the medium was replaced with fresh medium alone or medium containing FSH (500 ng/ml), in the absence of NTF4. Twenty-four h later, the ovaries were collected, frozen on dry ice, and stored at -85 °C until protein extraction.

### RNA extraction and real-time PCR

Total RNA was isolated from cultured ovaries treated with NTF4 using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) as previously described (Paredes *et al.* 2004). *Fshr* mRNA was detected by real-time PCR using a procedure previously described (Romero *et al.* 2002) with some modifications. After reverse transcribing 200 ng of total RNA, aliquots of each reaction (10 ng cDNA/µl) were diluted 1:10 before using 2 µl for real-time PCR. Each sample was run in triplicate along with a relative and an absolute standard curve. Relative standard curves, generated by serially diluting one sample 1:10 to 1:10 000 times, served to estimate the content of 18S rRNA of each sample. The primers used to detect 18S rRNA were purchased as a kit (TaqMan Ribosomal RNA Control Reagents Kit, Perkin Elmer Applied Biosystems, Foster City, CA, USA). Absolute standard curves were constructed by using serial dilutions (1:10) of sense *Fshr* RNA (2 ag-2 ng, see below). The threshold cycle number (C<sub>t</sub>) from each sample was referred to this curve to estimate the corresponding RNA content, and each RNA value was then normalized for procedural losses using the 18S rRNA values estimated from the relative standard curve. C<sub>t</sub> was the fractional cycle number at which the fluorescence accumulated to a level 10 times > 1 s.d. from basal values. The *Fshr* and 18S rRNA primers employed were those already reported (Romero *et al.* 2002). These primers and the fluorescent probes for real-time PCR were selected with the assistance of the program, Primer Express (Perkin Elmer Applied Biosystems). Real-time PCRs were performed in a total volume of 10 µl, each reaction containing 2 µl of the diluted reverse transcribed sample or 2 µl of sense *Fshr* mRNA standard, 5 µl TaqMan Universal PCR Master Mix (Perkin Elmer Applied Biosystems), 250 nM of each gene specific- and ribosomal fluorescent probes, 300 nM of each gene specific primer, and 10 nM of each ribosomal primer.

To construct the sense *Fshr* RNA standard curve used to quantify *Fshr* mRNA abundance in the ovary samples, we used a 346 bp cDNA generated by RT-PCR of total RNA derived from adult mouse ovaries. The primers used were a 5' forward primer (5'-GCCCTGGCCTTTGTGGTCATCTGT-3') corresponding to nucleotides 1675–1698 in mouse *Fshr* mRNA (NM\_013523) and a 3' reverse primer (5'-AAATCTGGGCTTGACCTCA-TAAC-3') complementary to nucleotides 1997–2020. To amplify this fragment, we used a PCR amplification protocol consisting of 33 cycles of denaturing at 94 °C (30 s), annealing at 57 °C (60 s), and extension at 72 °C (60 s). The resulting PCR product was cloned into the pGEM-T vector (Promega Corp.), and sequenced from both ends to verify its identity.

### Morphometric analysis

Ovaries from 2- and 4-day-old mice, and 0-day-old ovaries maintained for 4 days in organ culture were fixed in Kahle's fixative, embedded in paraffin, serially sectioned at 6 µm, stained with Weigert's iron hematoxylin, and counterstained with picric acid-methyl blue, as reported (Dissen *et al.* 2001, Romero *et al.* 2002, Paredes *et al.* 2004). Every other section was imaged as described (Paredes *et al.* 2004) and the degree of follicle development was morphometrically analyzed counting only follicles in which the nucleus of the oocyte was visible



(Paredes *et al.* 2004). The total number of follicles per ovary was then calculated by first dividing the total number of follicles counted per ovary by the number of sections examined and then multiplying this number by the total number of sections made from each ovary. Because each ovary yielded about 140 sections, we counted about 50 sections per ovary. All counts were performed without previous knowledge of the animal's genotype. The follicles were classified in different developmental stages according to well-established criteria (Peters 1969) that we have previously used (Disson *et al.* 2001, Romero *et al.* 2002, Paredes *et al.* 2004). Briefly, primordial follicles are the initial result of follicular assembly; they contain an oocyte surrounded by a single layer of flattened pregranulosa cells (Peters 1969). Primordial follicles become primary follicles (type 3a; Peters 1969) by a process that results in the differentiation of the flattened granulosa cells into a cuboidal morphology (Peters 1969, Hirshfield 1991). Granulosa cell proliferation and oocyte growth begin at this point resulting in the formation of larger (type 3b) primary follicles first, and secondary follicles with two (type 4) or more layers of granulosa cells (type 5 and larger) subsequently.

### Assessment of apoptosis

To determine whether the decrease in the number of primordial follicles observed in *Ntrk1*<sup>-/-</sup> and *Ntrk2*<sup>-/-</sup> mice on postnatal days 2 and 4 is due to death of oocytes before they become organized into follicular structures, we used ovaries from 0-day-old mice. The ovaries were immersed in Zamboni's fixative overnight at 4 °C and processed as described (Paredes *et al.* 2004), before preparing 14 µm cryostat sections. Six–eight randomly selected sections derived from three mice per group were then subjected to combined immunohisto-fluorescence-TUNEL. Oocytes were identified using a rat MAB (Mab KMC8, 5 µg/ml, BD Pharmingen, San Diego, CA, USA) that recognizes mouse CD9, a member of the tetraspanin family of membrane proteins expressed in the plasma membrane of oocytes (Zhu *et al.* 2002). CD9 is also present in several cell types of the immune system (BD Pharmingen, product specifications). After an overnight incubation at 4 °C with the CD9 antibody, the immunoreaction was developed by incubating the sections for 1 h at room temperature with Alexa 594 donkey anti-rat gamma globulin (1:500). Following extensive rinsing, the sections were subjected to the TUNEL reaction, which was performed following the manufacturer's instructions. To detect apoptotic ovarian cells by TUNEL, we used an *in situ* cell death detection kit coupled with fluorescein detection (Roche Diagnostics Co). Positive controls for the TUNEL reaction included sections from the ovary of a 28-day-old mouse (to detect apoptotic granulosa cells) and sections from day-0 ovaries treated with DNase I (Ambion, Austin, TX, USA; 10 U/ml for 20 min at room temperature).

### Western blots

Proteins and RNA derived from the experiments described in the subsection 'Culture of ovaries' were extracted simultaneously, as recommended (Morse *et al.* 2006, Tolosa *et al.* 2007) using a nucleic acid column extraction kit

(RNeasy minikit, Qiagen Inc). Briefly, pools of four ovaries (i.e. from two animals) were homogenized using an ultra-Turrax homogenizer in 300 µl of a guanidine hydrochloride-containing buffer (RLT buffer), provided with the kit supplemented with 10% β-mercaptoethanol. The RNA was then extracted using the RNeasy minicolumn, following the manufacturer's instructions. The flow-through containing the proteins was collected at every step and pooled. The proteins were precipitated overnight at -20 °C, the precipitates were collected by centrifugation at 10 000 g (15 min at 4 °C), and the pellets were washed three times with 100% cold ethanol (each wash for 30 min at -20 °C), before collecting the proteins by centrifugation at 10 000 g for 30 min at 4 °C. Thereafter, the pellets were air-dried and each sample was resuspended in 40–50 µl Laemmli sample buffer (187 mM Tris-base, 9% SDS, 15% glycerol, and 15% β-mercaptoethanol, pH 6.8), boiled 5 min, and fractionated in a 4–20% precast SDS-PAGE gel (Invitrogen). Due to the small amount of tissue per sample, the protein concentration of each sample was not determined before gel loading. After electrophoresis at 130 V for 2 h, the proteins were transferred for 1.5 h at 4 °C onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk for 1 h. A rat MAB raised against recombinant cyclin D2 (34B1-3; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a 1:1000 dilution (overnight at 4 °C) followed by a goat anti-rat HRP antibody (1 h at room temperature, 1:25 000 sc-2006, Santa Cruz Biotechnology). The signal was developed by ECL using the Super Signal West Dura Extended Duration Substrate (Pierce Biotechnology Inc., Thermo Fisher Scientific Inc., Rockford, IL, USA). For quantitation purposes, the membrane was washed several times in Tris-buffered saline 0.5% Tween-20 before an overnight exposure (at 4 °C) to a mouse MAB against GAPDH (Abcam Inc., Cambridge, MA, USA; 1:10 000 dilution), followed by an anti-mouse HRP antibody (Invitrogen; 1 h at room temperature, 1:50 000 dilution). To develop the signal, an ECL substrate (Western Lighting, Perkin Elmer) was used.

### Statistical analysis

Before specific analyses were performed, the data were subjected to a normality test. Data passing this test were then analyzed using the two-tailed Student's *t*-test to assess the differences between two groups of animals or independent observations, or a one-way ANOVA followed by the Student–Neuman–Keuls multiple comparison test for unequal replications, when comparing several groups. When the data failed the normality test, they were analyzed using an ANOVA test on ranks followed by the Kruskal–Wallis one-way ANOVA on ranks test (SigmaStat, Systat Software Inc., v3.11, San Jose, CA, USA). A *P* value <0.05 was considered statistically significant.

### Declaration of interest

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

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