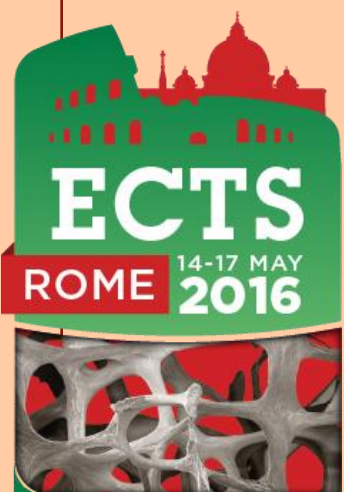


# OSTEOCALCIN INTERACTS WITH BRAIN-DERIVED NEUROTROPHIC FACTOR, NERVE GROWTH FACTOR BUT NOT OXYTOCIN IN THE REGULATION OF BONE, ENERGY, BRAIN AND REPRODUCTIVE FUNCTIONS



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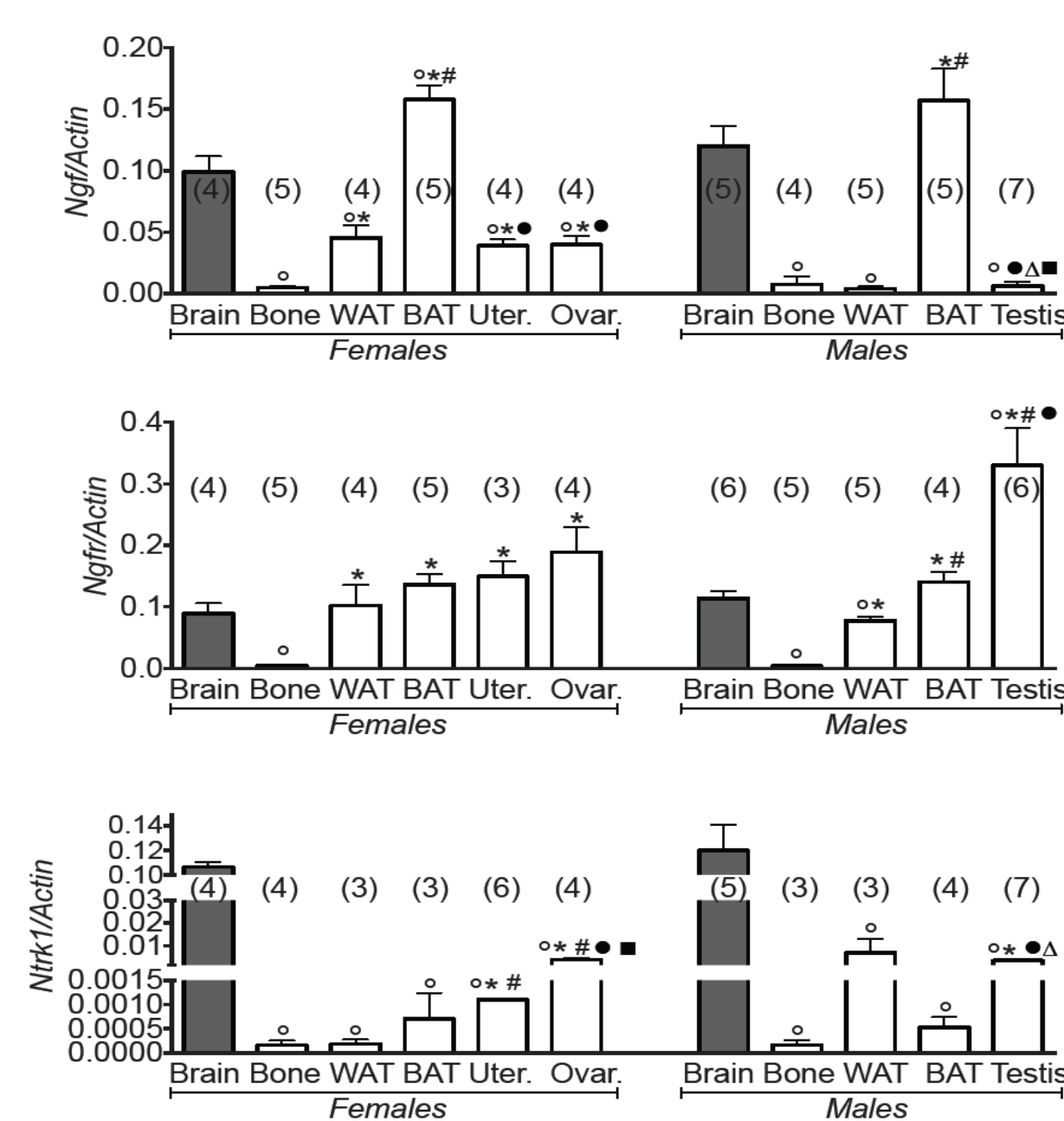
## Rational

The regulation of bone remodeling by adipocyte derived hormones implies that bone may exert a feedback control of energy homeostasis. Furthermore, the regulation of bone mass accrual by the gonads suggests that bone in turn in its endocrine capacity may affect the reproductive functions in one or both genders.

- **Osteocalcin.** Besides its role in bone metabolism, in its uncarboxylated form, osteocalcin acts as a hormone that links bone to other regulators of glucose homeostasis including leptin and insulin and reproduction (1, 2). The *Gprc6a* gene encoding for the osteocalcin receptor is expressed in testis but not in ovary, indicating that the action of osteocalcin on reproductive maturation is gender dependent. Nevertheless, (*Ocn*)<sup>-/-</sup> mice have low circulating testosterone levels despite an increase in circulating luteinizing hormone (LH), the major regulator of testosterone production (2). An emerging role of the osteocalcin has been recently also proposed in CNS (3).
- **The neurotrophins Nerve Growth Factor (NGF)/Brain-derived Neurotrophic Factor (BDNF).** NGF plays a role in reproduction being the “Ovulation Inducing Factor” (OIF) eliciting the surge of LH in the female (4). NGF may be implicated in the high LH levels of the *Ocn*<sup>-/-</sup> mice. Moreover, NGF genes are expressed in various osteoblastic cell lines, implicating a direct effect of NGF on bone (5). Last, NGF is produced by adipose tissue and stimulates leptin production (6). **BDNF** is implicated in energy and bone metabolism (7) while Brain-targeted BDNF conditional knockout mice (*Bdnf*(2lox/2lox)/93) show high bone mass and are a metabolic phenocopy of the leptin deficient *ob/ob* mice, but independent of adrenaline and serotonin (7).
- **Oxytocin (Oxt).** Oxt is involved in several functions including food intake and bone turnover (8, 9).
- **The aim of this study** is to better understand the role of NGF in bone, fat and reproductive organs and the possible gene interactions between NGF/BDNF, Osteocalcin and Oxt.

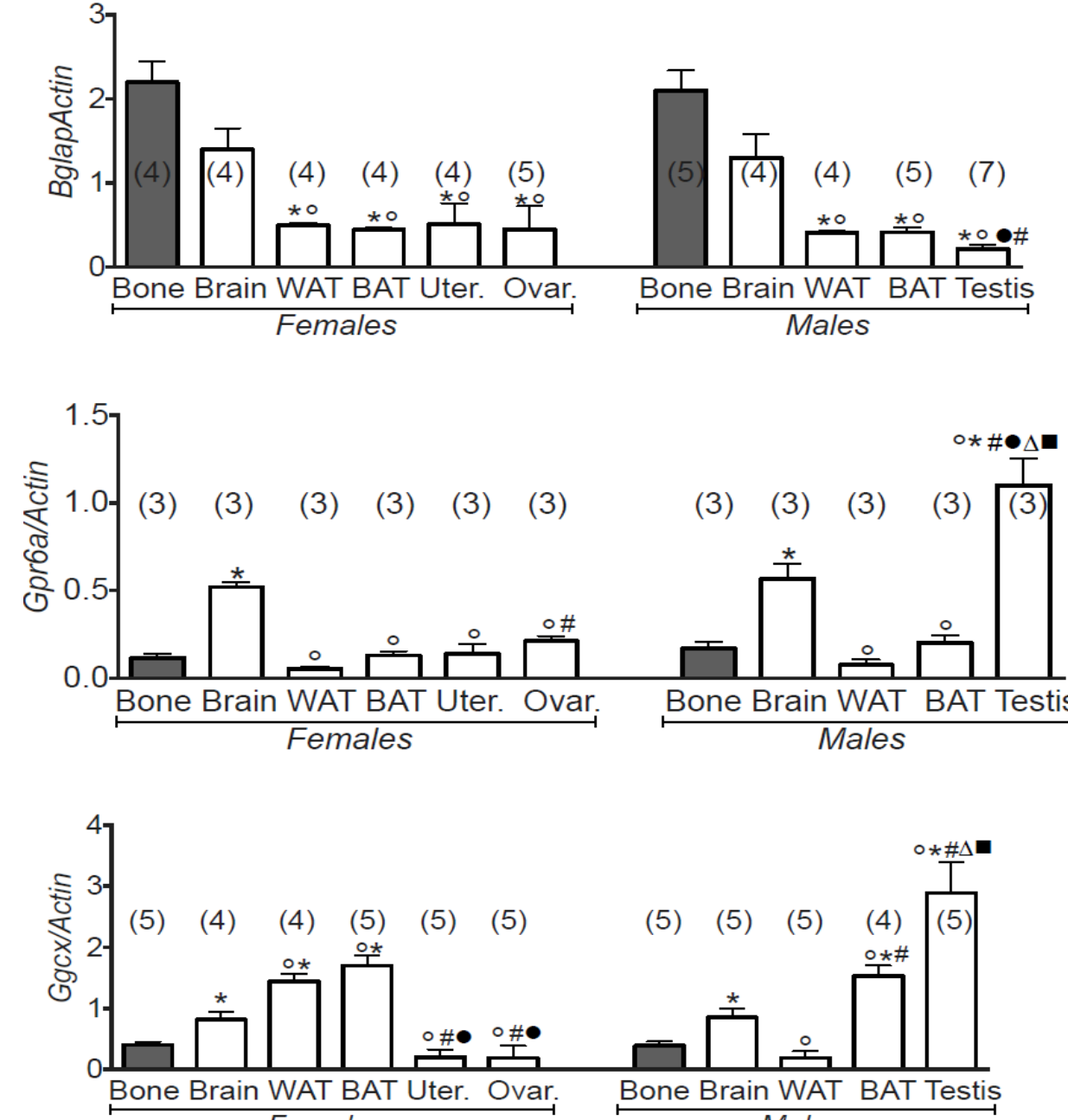
## Results

### EXPRESSION PROFILES OF THE NGF PATHWAYS



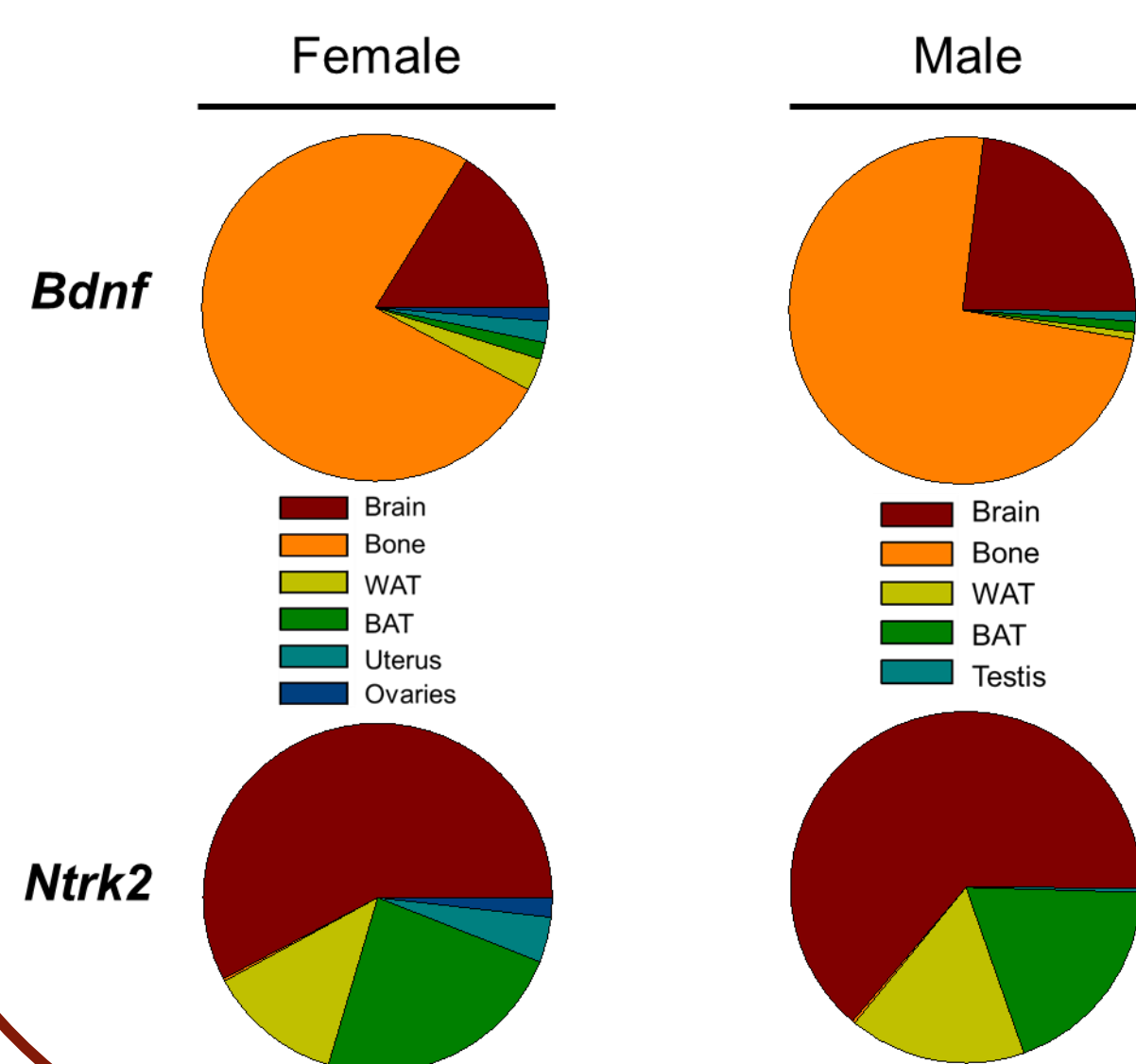
Significant differences (P<0.05) were found using unpaired Student's test versus brain (\*), bone (#), WAT (+), BAT (Δ), ovaries (□), and uterus (Δ); brain was used as a positive control.

### EXPRESSION PROFILES OF THE OSTEOCALCIN PATHWAY

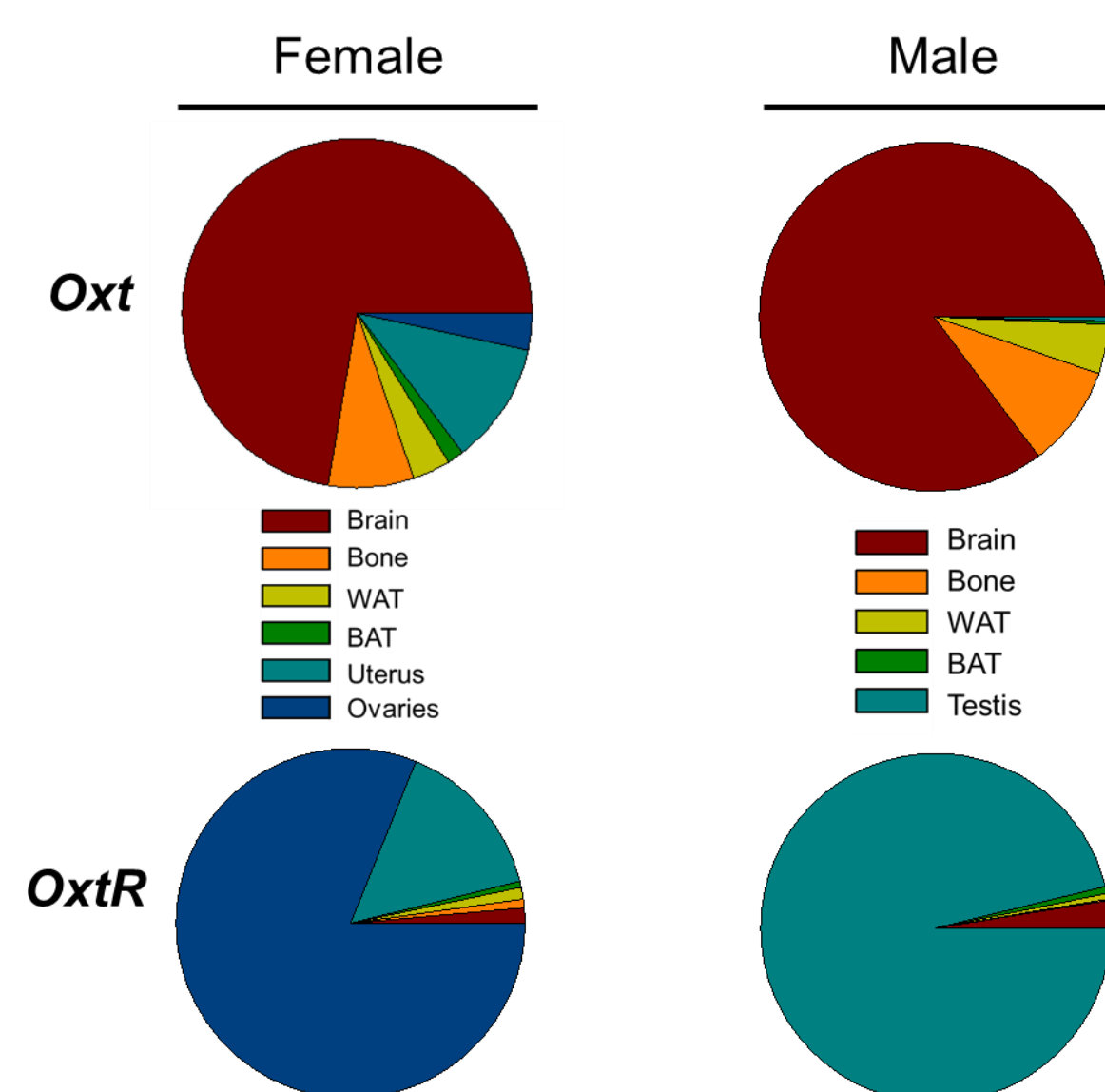


Significant differences (P<0.05) were found using unpaired Student's test versus brain (\*), bone (#), WAT (+), BAT (Δ), ovaries (□), and uterus (Δ); bone was used as a positive control.

### EXPRESSION PROFILES OF THE BDNF PATHWAY



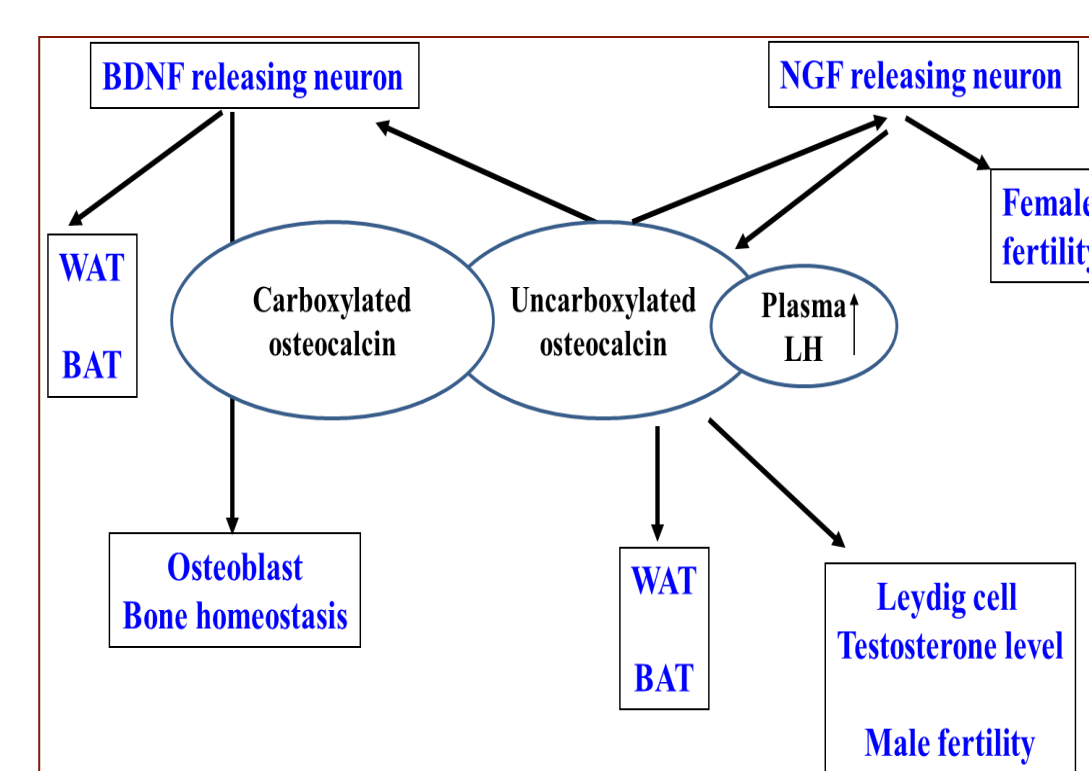
### EXPRESSION PROFILES OF THE OXT PATHWAY



- **Nerve growth factor (Ngf)** gene is expressed 50% higher in BAT vs brain of both genders, and it's significantly expressed in ovaries and uterus; the **nerve growth factor receptor (Ngfr)** gene is expressed 200% higher in testis vs Brain, and it's significantly expressed in ovaries and uterus; the **neurotrophic tyrosine kinase receptor type 1 (Ntrk1)** gene is poorly expressed in all tissues, vs Brain.
- **Osteocalcin (Bglap)** gene is highly expressed in Bone vs all other tissues; the **osteocalcin receptor (Gpr6a)** gene is expressed 150% higher in Brain of both genders vs bone and 400% higher in testis vs Bone; the **gamma-glutamyl carboxylase (Ggcx)** gene is expressed 200% higher in BAT of both genders and 500% in testis, vs Brain.
- **Brain derived neurotrophic factor (Bdnf)** gene is expressed 200% and 300% higher in Bone vs Brain of both genders; the **neurotrophic tyrosine kinase receptor type 2 (Ntrk2)** gene is also significantly expressed in WAT and BAT in both genders.
- **Oxytocin (Oxt)** gene is mostly expressed in Brain compared to all other tissues; the **oxytocin receptor (OxtR)** gene is mostly expressed in uterus and testis other than ovaries.

## Conclusions

- **NGF as a regulator of energy.** NGF and its receptors NGFR and NTRK1 genes are highly expressed in brown and white fat, at levels higher than brain used as positive control.
- **NGF may regulate the uncarboxylated form of osteocalcin.** The up-regulation of NGFR gene that we found in testis matches the *Gprc6a* gene expression in the same organ and can be responsible of the increased LH levels in the absence of osteocalcin observed in the (*Ocn*)<sup>-/-</sup> mice.
- **BDNF may regulate the carboxylated form of Osteocalcin** in its exclusive effects on bone. The effects on energy are independent of the leptin/serotonin pathway (7).
- **Osteocalcin may regulate neurotrophins expression in brain** as previously seen for estrogens (10).
- **A significant correlation was found between the expression levels of the osteocalcin gene and neurotrophin genes in different tissues** of both genders as determined by multiple correlation analysis. This suggests that changes in the expression levels of *Osteocalcin*, *Ngf* or *Bdnf* genes may affect the expression levels of the others. The calculated coefficient of correlation (*r*) was of 0.8904 (*b*<sub>0</sub> = 0.808, *b*<sub>1</sub> = -1.8611, *b*<sub>2</sub> = 0.6837) and of 0.9881 (*b*<sub>0</sub> = 0.37, *b*<sub>1</sub> = 0.059, *b*<sub>2</sub> = 0.228) between *Osteocalcin*, *Ngf* and *Bdnf* gene expression levels in different tissues of female and male mice, respectively.
- We failed to show correlation between oxytocin, neurotrophins and osteocalcin genes.



### Gene-relationship between osteocalcin and neurotrophins

*Osteocalcin*, *Ngf*, *Oxt* and *Bdnf* genes are highly expressed in brain of both genders while their receptors were instead expressed in the reproductive organs showing a gender specific profile. The signal-hormone is generated in the brain to act on peripheral target-organs.

**Material and Methods :** We analyzed by RT-PCR, in the same plate of reaction, the mRNA levels of *Ngf*, *Bdnf*, *Bglap* (osteocalcin) and associated receptors and of the *Ggcx* (gamma-glutamyl carboxylase) in brain, bone, fat and reproductive organs of 3 months old mice of both genders (seven male and five female mice). **Real-time PCR experiment:** The RNA extraction protocol was chosen based on the amount and type of tissue. In particular, brain, femur, uterus and white adipose tissues were extracted with Trizol (Invitrogen); the testis were extracted with RNeasy Tissue Mini Kit (Qiagen), while the ovaries were extracted with Tissue RNeasy Micro Kit (Qiagen). Synthesis of cDNA was performed by using random hexamers and Superscript II reverse transcriptase (Invitrogen). All genes were pre-amplified, by TaqMan PreAmp Master Mix (Invitrogen) before the real-time experiments. Each reaction was carried in triplicate on a single plex reaction. Each reaction contained 8 ng of cDNA, 0.5 μl of Probe TaqMan Gene Expression Assay 5 μl of TaqMan Universal PCR Master Mix, and nuclease free water for a final volume of 10 μl. The results were compared with a relative standard curve obtained by 6 points of 1:4 serial dilutions. The mRNA expression of the genes was normalized to the best housekeeping genes β-actin selected from β-actin *HPRT1*; 2β-microglobulin; *GAPDH* and *EEF2*. TaqMan Hydrolysis primer and probe gene expression assays were ordered from Applied Biosystems with the following IDs: hypoxanthine guanine phosphoribosyl transferase (*HPRT1*) IDs: Mm 00446968\_m1; beta-2 microglobulin (*2BM*) IDs: Mm 00437762\_m1; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) IDs: Mm 9999915\_g1; eukaryotic translation elongation factor 2 (*EEF2*) IDs: Mm 01171434\_g1; *BDNF* IDs: Mm 04230607\_s1; *NTRK1* IDs: Mm 01219406\_m1; *NTRK2* IDs: Mm 00435422\_m1; *NGFR* IDs: Mm 01309638\_m1 *NGF* IDs: Mm 00443039\_m1. **Statistics:** All experimental data were expressed as mean±standard error (SEM). Statistical analysis was performed using unpaired Student's t-test (P <0.05 or less) by GraphPad Prism version 6.

### References

1. Lee et al., *Cell* 2007; 2. Oury et al. *Cell*. 2011; 3. Oury et al. *Cell*. 2013; 4. Ratto et al., *PNAS* 2012; 5. Asaumi et al., *Bone* 2000; 6. Hristova et al., *Med Hypothesis* 2013; 7. Camerino et al., *Endocrinology* 2012; 8. Camerino C. *Obesity* 2009; 9. Camerino C. *Bone Key* 2009; 10. Sohrabji et al., *J Neurosci* 1994.