INTRODUCTION
Fracture repair constitutes the sequence of cell biological events following bone injury and recapitulates the steps of endochondral ossification observed during embryonic skeletal development and growth. Because of the different phases of fracture healing (inflammation, cartilage formation and remodeling) the fracture callus provides an excellent tool for analysis of cartilage and bone formation in adults (1,4). There is evidence that the peripheral nervous system is involved in fracture healing and bone remodelling (3). Nerve fibres of sympathetic and sensory origin containing neurotransmitters and neuropeptides are known to innervate bone and fracture callus, and the absence of sensory innervation alters callus size and bone formation and may result in non-united fractures (2). In addition, neurotransmitters are known to control vascularization and matrix differentiation during endochondral ossification in embryonic development (5). However, little is known about their role in fracture healing and their influence on callus maturation and bone formation. The current research intends to understand the role of the peripheral nervous system for organization and differentiation of a cartilaginous callus and its impact on endochondral ossification.

METHODS
Tibial fractures were set in 8-10 week old mice. Bone calli were dissected on day 1, 5, 9, 13, 16 and 20 after setting the fractures and parafomaldehyde-fixed paraffin-sections were stained with antibodies against specific cartilage components or neural factors.

For the pellet culture system costal chondrocytes were isolated from rib cages of newborn (P1-3) C57Bl/6 mice. The rib cages were carefully dissected, pre-digested in pronase and collagenase (2mg/ml), and after washing with PBS placed in a Petri dish for over night digestion in fresh collagenase (2mg/ml). Chondrocyte pellets were formed by centrifugation at 500x g for 10 min. and cultured in DMEM plus 50 µg ml^-1 ascorbate, 1 mM cysteine, 1 mM pyruvate, and 1% penicillin/streptomycin. Chondrocyte pellets were stimulated with substance P (SP; 10^-9 M, 10^-10 M, and 10^-11 M) and nor epinephrine (NE; 10^-6 M, 10^-7 M, and 10^-8 M) for 7 days and the medium was changed every day throughout the culture period. 1, 4, and 7 days after stimulation pellets were collected for RNA isolation and paraffin embedding.

RESULTS
In our animal fracture model we studied the time-dependent in-growth of sympathetic and sensory nerve fibres into the fracture callus. Both, substance P (SP)- and tyrosine hydroxylase (TH)-positive nerves penetrate the callus in early stages of the healing process (day 1), mainly innervating blood vessels in the early granulation tissue. At later time points, when a cartilaginous matrix has been developed SP- and TH-positive nerves retract towards the callus periphery. At day 5 and onward they innervate the perichondrium, and the periosteum. TH-positive fibres additional innervate the blood vessels which penetrate the cartilage templates in the course of endochondral bone formation. After day 9 of fracture healing process both fibre types were only sparsely detected.

Both, SP- and TH-positive nerve fibres seem not to penetrate the cartilage tissue as well as the newly formed woven bone whereas chondrocytes originated from callus tissue express SP and the neurokinin 1 receptor (NK1-R). Earliest expression is detected at day 5 and prolongs until day 13.

In order to analyze the function of substance P expressed by chondrocytes we established a pellet culture system using costal chondrocytes from newborn mice. Both, primary costal chondrocytes f and costal chondrocyte cultured as micromass pellets express substance P and NK1-R at mRNA and protein level. Preliminary results indicate that stimulation of chondrocyte pellets with SP and NE alters chondrocyte metabolism and matrix formation differently. Whereas stimulation with SP leaves the proliferation rate of chondrocytes unchanged, NE stimulation affects the proliferation rate in a dose-dependent manner. In comparison with the un-stimulated control, the highest NE concentration (10^-8 M) leads to an increase of the proliferation rate. In contrast, stimulation with 10^-7 or 10^-8 M NE seems to have no effect on the proliferation rate. Preliminary results suggest further that the matrix formation is suppressed after SP stimulation. In comparison to the stimulation with NE and un-stimulated controls collagen II and alcin blue staining of SP-stimulated pellets indicate less cartilage matrix formation.

CONCLUSIONS
Our fracture model demonstrated characteristic stage-specific localisations of TH- and SP-positive fibres during the healing progress. Both, TH- and SP-positive nerves penetrate the callus in early stages of the healing process, while at later time points when a cartilaginous matrix has been formed they retract towards the callus periphery.

TH-positive nerve fibres innervate blood vessels within the fracture callus, implicating a role in blood-flow regulation during callus differentiation. Furthermore the release of neural factors from the cartilage periphery may directly affect chondrocyte differentiation and matrix formation during callus maturation.

Chondrocytes originated from callus tissue express SP and its receptor (NK1-R). This implicates yet unknown, possible trophic functions of neuropeptides during cartilage differentiation and endochondral ossification in adults. Preliminary results of our chondrocyte pellet culture system suggest opposite effects of SP and NE on chondrocyte proliferation and matrix formation. Possibly, SP limits formation of a cartilaginous matrix and promotes formation of a bony matrix during endochondral ossification of callus in fracture healing.

References