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Substance P and Norepinephrine Modulate Murine Chondrocyte Proliferation and Apoptosis

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Objective. **Substance P (SP) and norepinephrine (NE) containing sensory and sympathetic nerve fibers innervate bone and fracture callus. They are involved in controlling vascularization and matrix differentiation during skeletal growth. Both types of nerve fibers are known to modulate growth and metabolic activity of osteoblasts and osteoclasts. The aim of this study was to understand the roles of SP and NE in chondrocyte metabolism and their impact on chondrocyte proliferation, apoptosis, and cell adhesion.**

Methods. **Primary costal chondrocytes were isolated from newborn mice. Micromass and monolayer cell culture regimens were used to analyze the effects of SP and NE on matrix formation, as determined by quantitative polymerase chain reaction and immunohistochemistry. The effects of SP and NE on proliferation, adhesion, and apoptosis of chondrocytes were determined by enzyme-linked immunosorbent assay, bromodeoxyuridine, TUNEL, and morphometric analyses.**

Results. **SP, neurokinin type 1 (NK-1) receptor, α-adrenergic receptor (α-AR), and β-AR were abundantly expressed in primary costal chondrocytes. Stimulation with SP or NE did not affect extracellular matrix formation with respect to types I, II, and IX collagen and aggrecan in micromass pellets. SP dose-dependently increased the rate of proliferation of chondrocytes via the NK-1 receptor, whereas NE decreased the apoptosis rate of chondrocytes by stimulating β-AR. Both neurotransmitters induced the formation of focal adhesion contacts.**

Conclusion. **Transmitters of sympathetic and sensory nerve fibers modulate the metabolic activity of chondrocytes. Endogenous SP, NK-1 receptor, and adrenoceptor expression in chondrocytes implicates as-yet-unknown, presumably trophic, functions of neurotransmitter for skeletal growth and might be of interest for use in cartilage regenerative medicine.**

The importance of the nervous system in maintaining body homeostasis has previously been described, and it has been suggested that organogenesis and tissue repair are under neuronal control (1). Recent published studies suggest that the peripheral nervous system is involved in bone metabolism, osteogenesis, bone formation, and bone remodeling (2–4). Nerve fibers of sympathetic and sensory origin innervate bone and fracture callus (5–7) and are involved in controlling vascularization and matrix differentiation during endochondral ossification in embryonic development (8).

In contrast to permanent cartilage (i.e., articular cartilage), temporary cartilage is destined to be replaced by bone, for example, during skeletal development in a process called endochondral ossification. Skeletal development is initiated by the formation of the cartilage templates for the future bones. These bone anlagen derive from mesenchymal progenitor cells (MPCs) recruited into areas where bones are to be built. In these locations, MPCs condense, differentiate into chondrocytes, and prefigure the future bone. Within the bone anlagen, the differentiated cartilage cells then transit through a temporospatial cascade of late differentiation events that sequentially include proliferation and several steps of maturation, culminating in chondrocyte hypertrophy. After invasion of blood vessels, the majority of hypertrophic cells undergo apoptosis, and the cartilage template is remodeled into trabecular bone. This pro-

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cess of bone formation, including the chondrogenic differentiation of MPCs, chondrocyte proliferation, chondrocyte differentiation, and mineralization, is referred to as endochondral ossification (9).

Substance P (SP) has a widespread distribution in the central and peripheral nervous systems (10) and belongs to a class of neuropeptides known as tachykinins. In addition to SP, the best-known members of the tachykinin family are neurokinin A (NKA) and NKB. In mammalian species, the actions of tachykinins are mediated by 3 G protein–coupled receptors, NK-1, NK-2, and NK-3 receptors; among these, the NK-1 receptor has the highest affinity for SP (11). SP stimulates osteogenesis (12), and it affects osteoblastic bone formation by acting directly on osteoblasts through the NK-1 receptor (13). It induces mitosis in several cell types (14) and stimulates bone marrow–derived stem cell proliferation and is thus involved in the regulation of local bone turnover (15).

Norepinephrine (NE) belongs to the catecholamine family of tyrosine-derived neurotransmitters of the sympathetic nervous system. The subtype of sympathetic nerve fibers, characterized by the expression of tyrosine hydroxylase (TH), has been identified in bone marrow, in the periosteum, and in bone-adherent ligaments (16,17), indicating that the growth and metabolic activity of bone tissue are regulated by sympathetic neurotransmitters. Catecholaminergic effects are mediated by the adrenergic receptor (AR) family; functional α_1-, α_2-, and β_2-AR are present on osteoblasts and osteoclasts (18,19). NE is able to induce apoptosis in several cell types (20,21) and to regulate the proliferation of osteoblasts, osteoblast-like cells, and mesenchymal stem cell lines through β-AR and α_1-AR (22–24).

However, the influence of catecholamines and SP on chondrocyte metabolism is largely unknown. SP is expressed in adult articular chondrocytes, where it is involved in mechanotransduction via the NK-1 receptor (25). SP-containing sensory nerve fibers have been found in the rat knee epiphysis at birth, where they are located at the interface between growth cartilage and bone, indicating an unknown function in endochondral ossification (26). Activation of the β_2-AR subtype on murine growth plate chondrocytes inhibited the expression of type X collagen and Indian hedgehog, hinting at a contribution of sympathetic regulation in growth cartilage differentiation during endochondral bone development (27).

This study addresses the roles of SP and NE in chondrocyte metabolism and their impact on chondrocyte proliferation, apoptosis, and cell adhesion, demonstrating a novel role of peripheral neurotransmitters in chondrocyte physiology. Notably, we chose to examine chondrocytes from newborn mice because, from a pathologic point of view, in diseases affecting the musculoskeletal system, such as osteoarthritis (OA), and in musculoskeletal trauma, such as bone fracture, chondrocytes again adopt a phenotype resembling that in the newborn state. It is commonly believed that in OA and during fracture healing, embryonic skeletal development processes are recapitulated and, in that way, are improperly activated. Therefore, with regard to pathophysiology of the musculoskeletal system, the use of chondrocytes from newborn mice provides a valid model that is also representative of adult humans.

MATERIALS AND METHODS

Isolation of chondrocytes. There are no specific cell surface markers to unambiguously identify chondrocytes and discriminate them from other mesenchymal cells (i.e., fibroblasts). Markers described in the literature might be used to separate chondrocytes within different zones in the growth plate but are not specific for chondrocytes and can be used only after isolating the growth plate in total by microdissection (28). We therefore isolated chondrocytes by optimized sequential enzymatic digestion. The anterior ribcage and sternum of newborn C57BL/6 mice (Charles River) were harvested, rinsed with phosphate buffered saline (PBS), placed in 2 mg/ml of Pronase (Roche Applied Science), and incubated for 60 minutes at 37°C, with continuous shaking to loosen connective tissue, followed by incubation with collagenase D (2 mg/ml; Roche Applied Science) in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen) for 90 minutes at 37°C. Afterward, the nondigested cartilaginous parts of the sterna and ribs were rinsed with PBS and then digested overnight at 37°C with 2 mg/ml of collagenase D. Released chondrocytes were passed through a 70-μm nylon mesh (Falcon) to remove residual bone fragments, centrifuged at 200*g* for 5 minutes, and resuspended in DMEM/F-12. For all assays using monolayer cultures, cells were kept in DMEM/F-12 supplemented with 10% fetal calf serum (FCS), 50 μg/ml ascorbate phosphate, 1 m*M* cysteine, and 1 m*M* pyruvate.

Micromass pellet culture. For cultivation in a serum-free 3-dimensional micromass pellet system using chondrogenic medium, which selects for cells that confer chondrogenic differentiation potential, 200-μl aliquots containing 2×10^4 cells were placed into 96-well plates with a conical bottom (Nunc/Fisher Scientific) and pelleted at 2,000*g* for 10 minutes at room temperature. All pellets were maintained up to 21 days in chondrogenic medium without FCS (29), and for some experiments (analysis of type X collagen gene and protein expression), 50 ng/ml of L-thyroxine (Sigma) was added. During the first 7 days, cells were incubated daily with vehicle (PBS), SP (10^{-9}–10^{-11} moles/liter), or NE (10^{-6}–10^{-8} moles/liter) (all from Sigma).

RNA isolation, end point, and quantitative reverse transcription–polymerase chain reaction (RT-PCR). RNA was isolated with Qiagen RNeasy Mini kits. Chondrocyte pellets were first digested for 4 hours in a 3:1 mixture of DMEM/F-12 and CTH (3 mg/ml of type II collagenase, 25 mg/ml of trypsin, and 10 mg/ml of hyaluronidase). Gene expression was determined using One-Step RT-PCR (Invitrogen), where each reaction consisted of 250 ng of total RNA. Gene expression of murine GAPDH was used as a loading control. Primers were designed from the National Institutes of Health GenBank database (data available upon request from the author). The identity of the amplified PCR products was confirmed by sequencing.

Relative quantitative RT-PCRs for Col1a1, Col9a1, Col10a1, and matrix metalloproteinase 13 (MMP-13) were performed with SYBR Green Dye according to established protocols (29). Experiments were performed in triplicate for each sample. Murine primers were designed with Primer3Plus software (data available upon request from the author). Relative quantification was determined according to the $\triangle\triangle C_t$ method using GAPDH as endogenous control and day 0 unstimulated cells as calibrator. The mean relative quantification (RQ) values were plotted semilogarithmically using RQ study application version 1.1 software (ABI Prism 7000 SDS software version 1.1).

Enzyme immunoassay (EIA) for SP. The production and release of SP were analyzed with an ACE competitive EIA for SP (Cayman Chemical). Micromass pellets and monolayer were lysed (150 m*M* NaCl, 50 m*M* Tris HCl, pH 8.0, 0.5% Nonidet P-40, and Protease Inhibitor Complete Mini [Roche]) and subjected to EIA. Supernatants (chondrogenic medium) were harvested after 24 hours, and 50-μl aliquots were subjected to EIA.

Proliferation and apoptosis in micromass pellet culture. In micromass pellets, the number of apoptotic cells was monitored by TUNEL on paraffin-embedded sections. Apoptotic cells were identified by TUNEL according to the instructions for the fluorescent In Situ Cell Death Detection kit (Roche Diagnostics). Photographs of random fields were taken, and the numbers of dead and live cells were counted. A minimum of 1,000 cells were counted for each treatment and plotted as the percentage of cell death. Proliferation was monitored by detection of the proliferating cell nuclear antigen (PCNA). The deparaffinized and rehydrated sections were incubated with 0.3% H_2O_2 for 10 minutes at room temperature to block endogenous peroxidase activity. For the staining of sections with the mouse anti-PCNA antibody, a biotinylation kit was used (Dako). The sections were counterstained with Weigert's hematoxylin. Photographs of random fields were taken, and the number of PCNA-positive cells was counted. A minimum of 1,000 cells were counted for each treatment and plotted as the percentage of proliferation.

Adhesion assay in monolayer culture. Chondrocytes were seeded into 96-well plates at a density of 10,000 cells per well. Cells were allowed to adhere for 6 hours in the presence of SP (10^{-8}–10^{-12}*M*), NE (10^{-5}–10^{-9}*M*), or vehicle (PBS). Adherent cells were fixed for 15 minutes at room temperature with 1% (volume/volume) glutaraldehyde solution (Merck) for 30 minutes and stained with 0.02% (weight/volume) crystal violet solution (Sigma). Bound crystal violet was removed by incubation for 3 hours with 70% (v/v) ethanol (Roth). Crystal violet adsorption at 600 nm was measured with a microplate reader (Tecan). Each experiment was performed in triplicate.

Immunofluorescence staining for focal adhesion contacts. Chondrocytes were cultured overnight on glass slides at a density of 20,000 cells/ml and in the presence of SP (10^{-8}–10^{-12}*M*), NE (10^{-5}–10^{-9}*M*), or vehicle (PBS). Medium was removed, and cells were fixed for 5 minutes in 4% (w/v) paraformaldehyde, and autofluorescence was blocked for 10 minutes with 0.1*M* NH_4Cl (both from AppliChem). Blocking was performed overnight at 4°C in PBS containing 1% normal goat serum (Dako). Primary rabbit antibody against human vinculin (Sigma) and Alexa Fluor 488–conjugated secondary antibody (Invitrogen), both diluted 1:500 in blocking buffer, were added sequentially for 1 hour at 37°C. After staining of cell nuclei with DAPI (Invitrogen), slides were evaluated using laser scanning microscopy (C1 confocal microscope; Nikon).

Proliferation assay in monolayer culture. Proliferation of chondrocytes was determined using a bromodeoxyuridine (BrdU) cell proliferation kit (Roche). Cells were incubated with BrdU labeling medium in 96-well chambers for 2 days in the presence of SP (10^{-8}–10^{-12}*M*), NE (10^{-5}–10^{-9}*M*), or vehicle (PBS). BrdU incorporation was quantified using a microplate reader (Tecan). To test for specificity of receptor-mediated effects of SP, the NK-1 receptor antagonist L733,060 (10^{-5}*M* and 10^{-6}*M*; BioTrend), with or without SP (10^{-9}*M*), was included in the incubation medium in some experiments.

Caspase activity assay in monolayer culture. Apoptosis of chondrocytes was determined using an Apo-ONE caspase 3/7 assay (Promega). Cells were placed in 96-well chambers and were treated for 2 days with SP (10^{-8}–10^{-12}*M*), NE (10^{-5}–10^{-9}*M*), or vehicle (PBS), followed by incubation for 3 hours with the assay reagents. The fluorescence was measured using a microplate reader (Tecan). To test for specificity of the receptor-mediated effects of NE, the α_1-antagonist doxazosin, the α_2-antagonist yohimbine, or the β_{1-3}-antagonist nadolol (10^{-5}*M*; Sigma) were included in the incubation medium as indicated. To demonstrate receptor subtype specificity, cells were incubated with the β_1-antagonist CGP 20712A, the β_2-antagonist ICI 118,551 hydrochloride, or the β_3-antagonist SR 59230A (10^{-5}*M*; Tocris), with or without 10^{-7}*M* NE.

Localization of SP, NK-1 receptor, and TH in monolayer and micromass pellet cultures. Both chondrocytes grown for 24 hours on poly-L-lysine–coated chamber slides (BD Biosciences) and deparaffinized micromass pellet sections were blocked for 1 hour with 5% bovine serum albumin (Biomol), followed by overnight incubation in primary antibody at 4°C. Primary antibodies were diluted in blocking solution at 1:100 for anti-SP, 1:250 for anti–NK-1 receptor, and 1:250 for anti-TH (all from Chemicon). After incubation with the primary antibody, sections were incubated for 60 minutes at 37°C with secondary antibodies (Alexa Fluor 488–labeled donkey anti-rabbit IgG for SP and TH; Alexa 488–labeled donkey anti-goat IgG for NK-1 receptor; Invitrogen).

Localization of types I, IX, and X collagen in micromass pellet cultures. The following antibodies were used for the localization of specific collagen types: mouse anti–type I collagen (1:1,000 dilution; Sigma), rabbit anti–type IX collagen

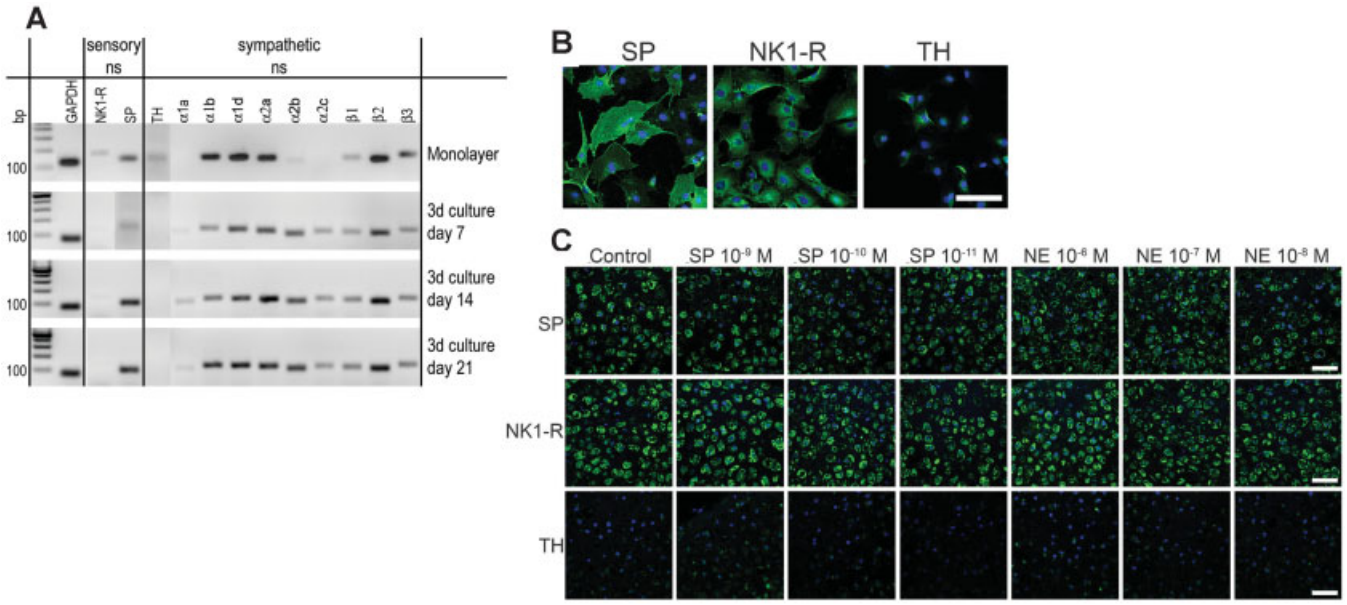

Figure 1. Gene and protein expression of substance P (SP), tyrosine hydroxylase (TH), neurokinin type 1 (NK-1) receptor, and adrenoceptor subtypes. **A,** Expression of mRNA for SP, NK-1 receptor, TH, and α- and β-adrenergic receptor subtypes in chondrocyte monolayers and 3-dimensional (3d) micromass pellet cultures, as determined by reverse transcription–polymerase chain reaction analysis. GAPDH expression was used as a loading control. ns = nervous system. **B,** Detection of cells positive for SP, NK-1 receptor, and TH in monolayer cultures of murine costal chondrocytes. Bar = 100 μm. **C,** Detection of cells positive for SP, NK-1 receptor, and TH in unstimulated micromass pellets or in micromass pellets stimulated with the indicated concentrations of SP or norepinephrine (NE) on day 21 of culture. Cell nuclei were counterstained with DAPI. Sections incubated without primary antibody served as negative controls and showed no staining (results not shown). Bars = 100 μm. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

(1:500 dilution; ref. 30), and mouse anti–type X collagen (1:25 dilution; Quartett).

The deparaffinized and rehydrated sections were treated as described previously (31), followed by overnight incubation at 4°C with primary antibodies in blocking buffer and incubation with secondary antibodies (biotinylated anti-rabbit immunoglobulins for 60 minutes at 37°C; Dako). For detection of types I and X collagen, the primary antibodies were incubated directly on the sections in order to biotinylate them with the animal research kit ARK (Dako). Subsequently, the sections were incubated for 60 minutes at 37°C with streptavidin–peroxidase and horseradish peroxidase substrate solution and were counterstained with Weigert's hematoxylin.

Statistical analysis. Data are expressed as the median with 95% confidence intervals. Differences between the groups were assessed using the Mann-Whitney U test using SPSS statistical software. *P* values less than 0.05 were considered significant.

RESULTS

Sensory and sympathetic neurotransmitter/receptor profile. Murine costal chondrocytes expressed messenger RNA (mRNA) for SP, NK-1 receptor, TH, and α- and β-adrenergic receptor subtype, as studied by end-point RT-PCR (Figure 1A). Chondrocytes in monolayer constitutively expressed SP and NK-1 receptor mRNA and contained mRNA transcripts of adrenergic receptors, except for the α_{1a} and α_{2c} subtypes. They also expressed TH, the rate-limiting enzyme for the biosynthesis of catecholamines. Micromass pellet culture resulted in the expression of all adrenergic receptor subtypes, while the level of mRNA for NK-1 receptor and TH markedly decreased during the culture period (Figure 1A).

Endogenous production of SP. By competitive EIA, we demonstrated that chondrocytes maintained in micromass pellets contain an average of 35 pg of SP in lysates of 2×10^4 cells and 528 pg of SP in the supernatant calculated for the same cell number. Chondrocytes in monolayer culture were more productive; lysates of 2×10^4 cells contained 96 pg of SP and supernatants contained 1,292 pg of SP.

Immunocytochemical localization of SP, NK-1 receptor, and TH. Protein expression of SP, NK-1 receptor, and TH was detected in chondrocyte monolayers (Figure 1B) and in micromass pellet cultures for up to 21 days (Figure 1C). Nearly all chondrocytes were positive for SP and its receptor, while expression of TH

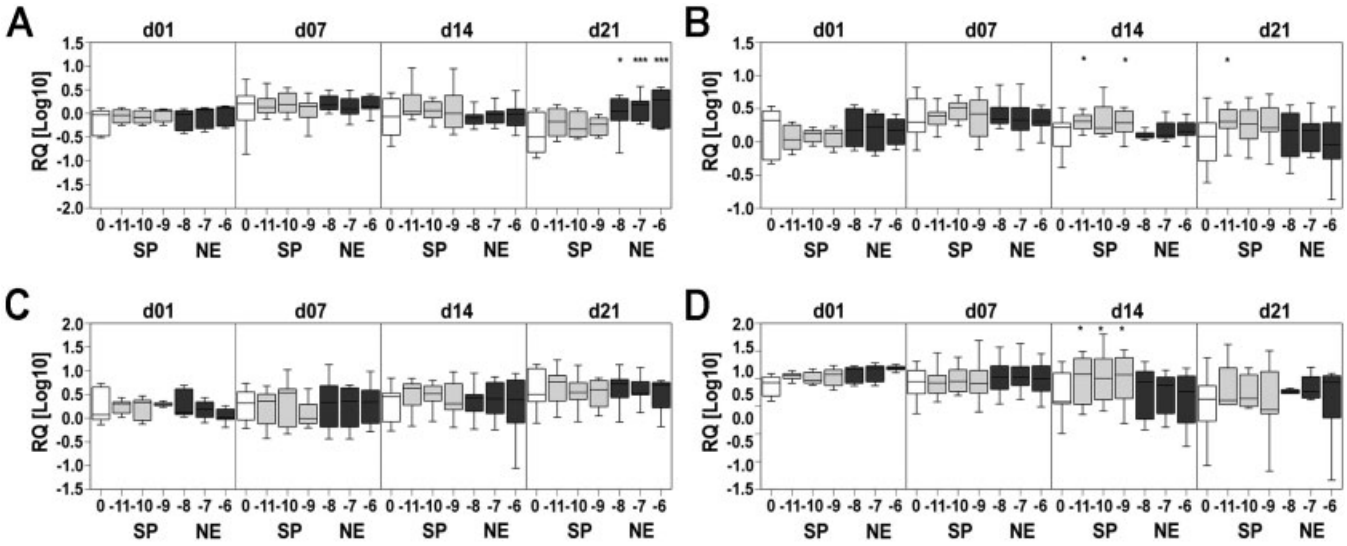

Figure 2. Gene expression of extracellular matrix formation markers and matrix metalloproteinase 13 (MMP-13). Expression of mRNA for **A,** Col1a1, **B,** Col9a1, **C,** Col10a1, and **D,** MMP-13 in unstimulated micromass pellets (open boxes) or in micromass pellets stimulated with substance P (SP) (lightly shaded boxes) or norepinephrine (NE) (darkly shaded boxes) was determined by real-time polymerase chain reaction analysis on days 1, 7, 14, and 21 of culture. The relative expression levels of each gene were determined according to the $\Delta\Delta C_t$ method. GAPDH expression was used as a loading control. Values for day 0 unstimulated micromass pellets (calibrator) were set at 0. All data were log-transformed to base 10 and are expressed as the mean relative quantification (RQ) values. Numbers along the x-axis are the log-molar concentrations of SP and NE. Data are shown as box plots. Each box represents the 95% confidence intervals. Lines inside the boxes represent the median. Lines outside the boxes represent the SD; n = 8 independent experiments. $* = P < 0.05$; $*** = P < 0.001$ versus unstimulated controls.

was restricted to a few cells. To determine whether there might be negative or positive feedback mechanisms that affect endogenous production of these neurotransmitters or their key enzymes, we added external SP or NE. We also investigated whether stimulation with external SP affects endogenous expression of its NK-1 receptor. Administration of SP and NE to micromass pellets appeared not to modulate protein expression of endogenous SP/NK-1 receptor and TH when analyzed with immunohistologic staining.

Effect of exogenously administered SP and NE on gene expression levels of chondrocyte differentiation markers. To maintain a stable chondrocytic phenotype, chondrocytes were cultured as micromass pellets for up to 21 days. The gene expression levels of Col1a1, Col9a1, Col10a1, and MMP-13 were partly affected by SP and NE (Figure 2).

Gene expression of Col1a1, an indicator of chondrocyte dedifferentiation, was significantly up-regulated when stimulated with NE on day 21 (Figure 2A). Up-regulation was most profound at $10^{-6}/10^{-7}M$, indicating signal transduction through the β-adrenoceptors. Stimulation with SP resulted in significantly up-regulated gene expression of Col9a1 (maturation marker) on day 14 and day 21 (Figure 2B).

Col10a1 gene expression, a marker of terminally differentiated hypertrophic chondrocytes, was not affected by SP or NE (Figure 2C). We analyzed the gene expression of MMP-13, representative of collagen-degrading MMPs, which was induced significantly on day 14 by SP (Figure 2D).

Extracellular matrix formation of SP/NE-stimulated micromass pellet cultures. After 7 days, a homogeneous cell pellet was formed, producing an Alcian blue–positive cartilage matrix, indicative of sulfated glycosaminoglycans (GAGs). GAG production, and thus aggrecan deposition, was not affected by SP or NE stimulation (Figure 3B). In addition, type I collagen (inset in Figure 3B), type IX collagen (Figure 3A), and type X collagen (inset in Figure 3A) showed no changes of accumulation in the extracellular matrix following SP or NE stimulation. Notably, type X collagen staining was slightly induced on day 21, indicating a tendency toward hypertrophic differentiation (due to administration of L-thyroxine) without being affected by the application of SP or NE.

Effect of SP and NE on proliferation and apoptosis in micromass pellet cultures. In comparison to the unstimulated controls, the highest SP concentrations ($10^{-9}/10^{-10}M$) stimulated proliferation until day 7 (Fig-

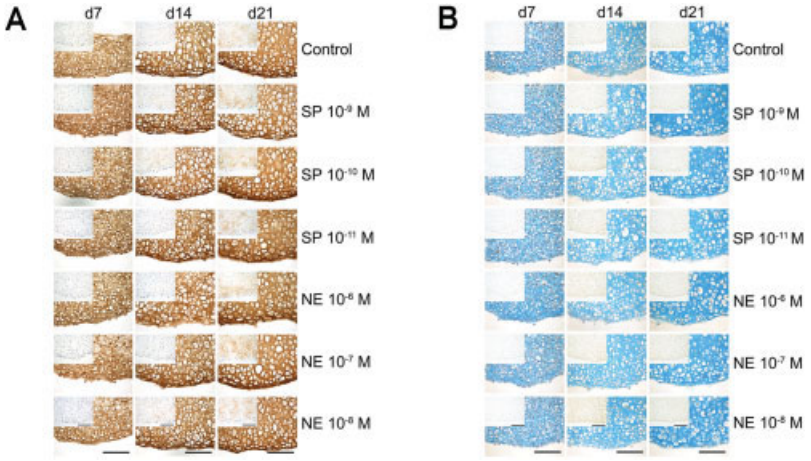

Figure 3. Glycosaminoglycan and collagen production during micromass culture. **A,** Expression of type IX collagen and type X collagen (**insets**) was unaffected by stimulation with the indicated concentrations of substance P (SP) or norepinephrine (NE), as determined on days 7, 14, and 21 of culture. **B,** Alcian blue–stained sections of micromass pellets revealed a cartilage-like extracellular matrix development that was unaffected by stimulation with the indicated concentrations of SP or NE, as determined on days 7, 14, and 21 of culture. Immunohistochemical detection of type I collagen in corresponding sections (**insets**) did not reveal an effect of SP or NE. Sections incubated without primary antibody served as negative controls and showed no staining (results not shown). Bars = 100 μm.

ure 4A). Thereafter, proliferation decreased significantly on day 14 and recovered to control levels on day 21. In contrast, chondrocyte proliferation remained unaffected by NE (data available upon request from the author).

In comparison to the unstimulated controls, all NE concentrations tested reduced apoptosis up to 14 days (Figure 4B). The maximum effect was observed when NE was present at a concentration of $10^{-6}M$ and $10^{-7}M$ on day 14, while on day 21, the control level was

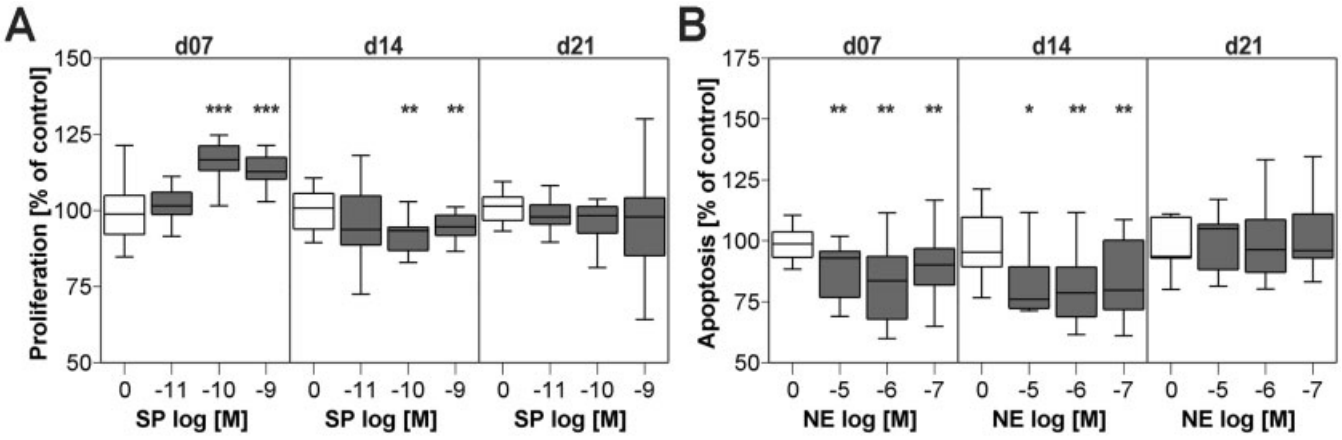

Figure 4. Influence of substance P (SP) and norepinephrine (NE) on chondrocyte proliferation and apoptosis in micromass pellet culture. **A,** Percentages of proliferating cell nuclear antigen–positive chondrocytes as an indicator of cell proliferation were determined in unstimulated micromass pellets (open boxes) or in micromass pellets stimulated with SP (shaded boxes) on days 7, 14, and 21 of culture. **B,** Percentages of TUNEL-positive chondrocytes as an indicator of apoptosis were determined in unstimulated micromass pellets (open boxes) or in micromass pellets stimulated with NE (shaded boxes) on days 7, 14, and 21 of culture. Sections incubated without primary antibody served as negative controls and showed no staining (data not shown). Data are shown as box plots. Each box represents the 95% confidence intervals. Lines inside the boxes represent the median. Lines outside the boxes represent the SD; n = 4 independent experiments. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ versus unstimulated controls.

regained. In contrast, the chondrocyte apoptosis rate remained unaffected by SP (data available upon request from the author).

NK-1 receptor antagonist L733,060 blockade of SP-induced stimulation of proliferation. Chondrocyte monolayer cultures were used to investigate the role of the NK-1 receptor in regulating the mitotic effect of SP. Culturing chondrocytes for 2 days in the presence of exogenous SP induced a significant dose-dependent increase in proliferation as compared to the control culture. The maximum effect was observed at a concentration of 10^{-10}–$10^{-8}M$ (Figure 5A).

Addition of the NK-1 receptor antagonist L733,060 resulted in a dose-dependent inhibition of proliferation (Figure 5B). This observation suggests that endogenously synthesized and secreted SP also modulates the proliferation of chondrocytes in vivo. In the presence of $10^{-9}M$ SP, addition of L733,060 reduced chondrocyte proliferation to a highly significant degree (Figure 5B).

NE-induced antiapoptotic effects mediated via β_2/β_3-adrenoceptors. Chondrocyte monolayer cultures (2 days) were used to investigate the role of adrenergic receptors in regulating the antiapoptotic effect of NE. In the presence of NE, a significant dose-dependent decrease in apoptosis was observed (Figure 5C).

In order to determine the adrenergic receptor subtype involved, chondrocytes were incubated in the presence of the α_1-adrenoceptor antagonist doxazosin, the α_2-adrenoceptor antagonist yohimbine, or the β_{1-3}-adrenoceptor antagonist nadolol. Without addition of NE, none of the antagonists affected apoptosis (Figure 5D). Since the apoptosis-effective concentrations mainly promote signaling through β-adrenoceptors, we assumed that the modulating effects are mediated via β-adrenoceptors. To specify the β-adrenoceptor subtype, we administered the β_1-receptor antagonist CGP 20712A, the β_2-receptor antagonist ICI 118,551, and the β_3-receptor antagonist SR 59230A with and without NE (Figure 5E). Both the β_2-receptor antagonist ICI 118,551 and the β_3-receptor antagonist SR 59230A significantly induced apoptosis. The apoptosis rate was unchanged upon application of the β_1-receptor antagonist CGP 20712A.

Effect of SP and NE on chondrocyte adhesion. Both SP and NE increased the adhesion of chondrocytes at all concentrations tested (Figures 6A and C). Addition of the NK-1 receptor antagonist L733,060 alone but not in the presence of SP resulted in a dose-dependent inhibition of adhesion (Figure 6B), indicating an effect of endogenously produced SP.

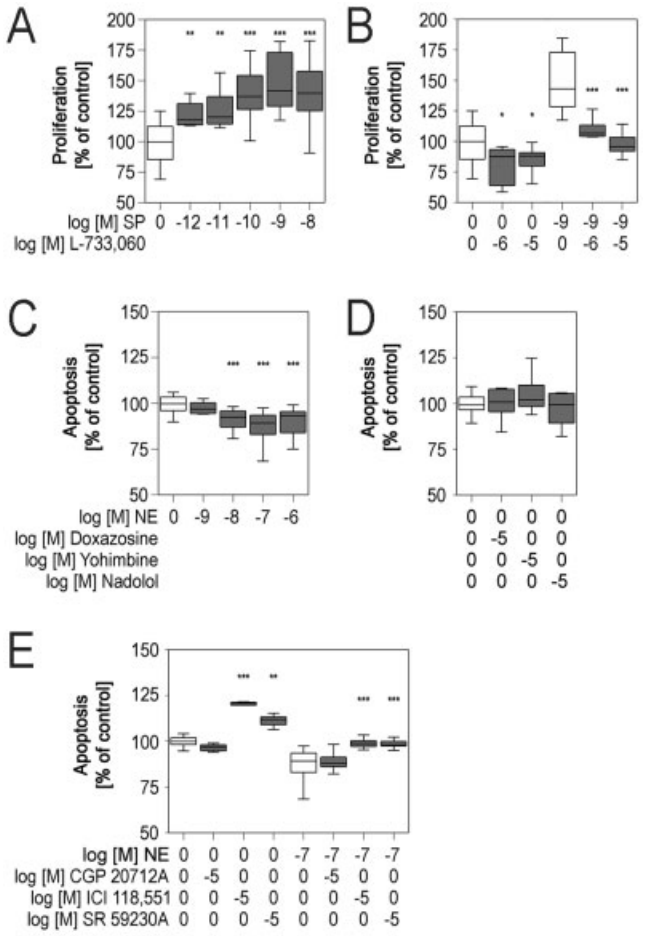

Figure 5. Analysis of neurokinin type 1 (NK-1) receptor involvement in proliferation and identification of adrenergic receptor subtypes involved in apoptosis. **A,** Proliferation of chondrocytes in unstimulated monolayer culture (open box) or in monolayer cultures stimulated with increasing concentrations of substance P (SP) (shaded boxes). **B,** Proliferation of chondrocytes in unstimulated cultures, in unstimulated cultures blocked with 2 different concentrations of the NK-1 receptor antagonist L733,060, or in cultures stimulated with $10^{-9}M$ SP and blocked with 2 different concentrations of L733,060, as indicated. **C,** Apoptosis of chondrocytes in unstimulated monolayer culture (open box) or in monolayer cultures stimulated with increasing concentrations of norepinephrine (NE) (shaded boxes). **D,** Apoptosis of chondrocytes in unstimulated monolayer cultures or in cultures treated as indicated with doxazosin (α_1-receptor), yohimbine (α_2-receptor), or nadolol (β_{1-3}-receptors) to block the indicated adrenergic receptor subtypes. **E,** Apoptosis of chondrocytes in unstimulated monolayer cultures or in cultures treated as indicated with the β_1-receptor antagonist CGP 20712A, the β_2-receptor antagonist ICI 118,551, the β_3-receptor antagonist SR 59230A, or with $10^{-7}M$ NE together with the β_1-, β_2-, or β_3-receptor antagonists. Data are shown as box plots. Each box represents the 95% confidence intervals. Lines inside the boxes represent the median. Lines outside the boxes represent the SD; n = 8 independent experiments. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ versus unstimulated controls.

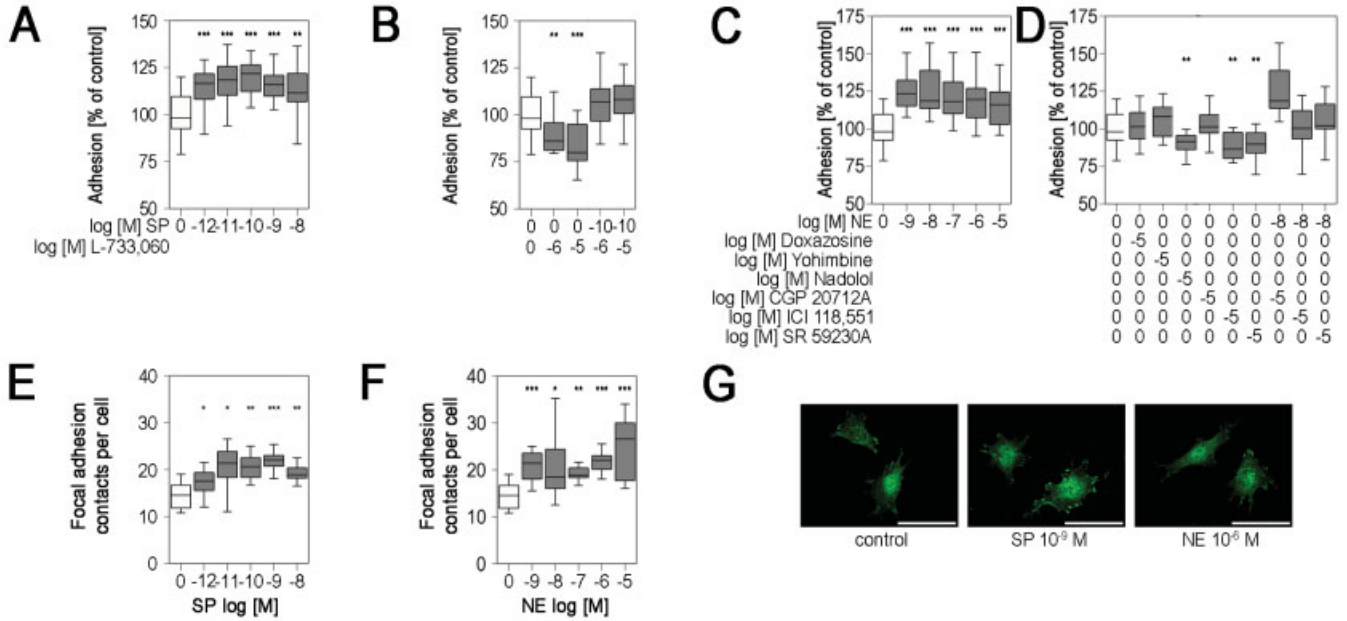

Figure 6. Effects of substance P (SP) and norepinephrine (NE) on chondrocyte adhesion and the formation of focal adhesion contacts (FACs). **A,** Adhesion of chondrocytes seeded in 96-well plates and allowed to adhere for 6 hours in the absence of stimulation (open box) or in the presence of the indicated concentrations of SP (shaded boxes). **B,** Adhesion of chondrocytes in unstimulated cultures, in unstimulated cultures blocked with 2 different concentrations of the neurokinin type 1 (NK-1) receptor antagonist L733,060, or in cultures stimulated with $10^{-10}M$ SP and blocked with 2 different concentrations of L733,060, as indicated. **C,** Adhesion of chondrocytes seeded and allowed to adhere as in **A**, in the absence of stimulation (open box) or in cultures stimulated with increasing concentrations of NE (shaded boxes). **D,** Adhesion of chondrocytes in unstimulated monolayer cultures or in cultures treated as indicated with the α_1-antagonist doxazosin, the α_2-antagonist yohimbine, the β_{1-3}-antagonist nadolol, the β_1-antagonist CGP 20712A, the β_2-antagonist ICI 118,551 hydrochloride, or the β_3-antagonist SR 59230A, or with $10^{-8}M$ NE with the β_1-, β_2-, or β_3-receptor antagonists. **E** and **F,** Adhesion of chondrocytes seeded in glass chamber slides and allowed to adhere for 6 hours in the presence of the indicated concentrations of SP (**E**) or NE (**F**). The numbers of FACs per cell were counted. Data are shown as box plots. Each box represents the 95% confidence intervals. Lines inside the boxes represent the median. Lines outside the boxes represent the SD; n = 8 independent experiments in **A–D** and n = 5 independent experiments in **E** and **F**. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ versus unstimulated controls. **G,** Representative images of vinculin-stained murine costal chondrocytes for visualization of FACs. Bars = 50 μm. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

In order to determine the adrenergic receptor subtype involved, the α_1-, α_2- and β-adrenoceptor antagonists (see above) were added. Again, only the β-adrenoceptor antagonist nadolol significantly reduced adhesion, indicating effects of endogenously produced catecholamines (Figure 6D). To specify the β-adrenoceptor type, we administered β_1-, β_2-, and β_3-adrenoceptor antagonists (see above) (Figure 6D). Application of CGP 20712A (β_1) left the adhesion rate unchanged. When $10^{-8}M$ NE was administered together with $10^{-5}M$ ICI 118,551 (β_2) or SR 59230A (β_3), the effect on chondrocyte adhesion was abolished (Figure 6D).

To determine whether the effect of SP or NE on adhesion is translated into an increased formation of focal adhesion contacts (FACs), FACs were counted. Both SP and NE stimulated in a dose-dependent manner the formation of FACs on chondrocytes (Figures 6E and F).

DISCUSSION

Neurotransmitters of the sensory and the sympathetic nervous system are involved in bone biology, including osteogenesis, osteoclastogenesis, and bone resorption, presumably acting as trophic factors in this context (13,15,18,32,33). However, studies demonstrating the effects of peripheral neurotransmitters on chondrocyte biology are scarce.

We observed a dose-dependent increase in cell proliferation in both monolayer and micromass pellet cultures when chondrocytes were stimulated with exogenous SP and L733,060, indicating that they bear functional and specific NK-1 receptor. A proliferation-

stimulating effect of SP has previously been shown for fibroblasts during tendon repair (34,35), for colonic epithelial cells during the tissue healing phase in chronic colitis (36), for neural progenitor cells after ischemic trauma (37), and for bone marrow stromal cells (15). Notably, Im et al (38) reported no effect of SP on the proliferation of normal human adult articular chondrocytes. These data indicate a specific mitotic effect of SP on mesodermal cells after injury or trauma during the regenerating/healing phase and on precursor cells during skeletal growth, which is consistent with our results. Presumably, in adults, once tissue and organ formation is finished, this effect is abrogated.

We observed only a small influence of SP and NE on the expression of matrix-forming genes, such as Col1a1 and Col9a1, or protease genes, such as MMP-13. Up-regulation of these genes was statistically significant but did not reach physiologic significance (>2-fold), as corroborated by our observations for type I and type IX collagen protein expression in micromass pellet cultures. Im et al (38) reported that SP, via the NK-1 receptor pathway, accelerates matrix degradation by stimulating the production of cartilage-degrading enzymes in adult articular chondrocytes and by reducing the deposition of proteoglycans. This discrepancy is most probably due to the use of different culture conditions (i.e., monolayer cultures), only 24 hours of stimulation, and use of markedly higher SP concentrations. Reports from studies of rabbit intervertebral disc chondrocytes or bovine articular chondrocytes, where only small effects of SP on matrix production and differentiation have been observed, corroborate our results (39,40).

Inhibition of Col10a1 gene expression upon stimulation of murine costal chondrocytes with β_2-adrenergic receptor agonists but no effect on proteoglycan deposition has been described (27), while we found no effect of NE on the level of mRNA for Col10a1 during 3 weeks of culture. Again, this difference is presumably due to different chondrocyte culture conditions, which in our study, were maintained as 3-dimensional micromass pellets. Micromass pellets stabilize a chondrogenic phenotype, while in the study by Lai and Mitchell (27), the chondrocytes were cultured in monolayer, which destabilizes the chondrogenic phenotype and promotes the expression of dedifferentiation and hypertrophic markers.

Our experimental setup did not reveal an influence of SP on the apoptosis of chondrocytes, while stimulation of chondrocytes with NE decreased apoptosis in both monolayer and micromass pellet cultures, but had no effect on proliferation. Other investigators also did not observe an influence of catecholamines on the proliferation of growth plate chondrocytes (41). The application of the subtype-specific adrenoceptor antagonists ICI 118,551 and SR 59230A indicated that antiapoptotic effects in chondrocytes are mediated via β_2- and β_3-adrenoceptors. Stimulation of β-AR with agonists regulates apoptosis in other cell types, as in cardiomyocytes, where stimulation of β_1-AR induces apoptosis while stimulation of β_2-AR exerts an antiapoptotic effect (42). Using an ex vivo tibiotarsal model, NE induced longitudinal bone growth in embryonic chicken without altering the proliferation rate of condylar cells (41). The authors suggest that the increase in bone growth is induced by stimulation of chondrocyte maturation through NE, which up-regulates type X collagen gene expression. This observation appears to be in contrast to our data, which might be explained by differences in the species studied (mammalian versus avian) (41). However, they did not analyze chondrocyte apoptosis, which can be considered an important means of controlling skeletal growth in addition to cellular proliferation rates. Since NE concentrations of $10^{-4}M$ and $10^{-5}M$ were used, which stimulate β-adrenergic receptors, it is possible that antiapoptotic effects add to the increase in longitudinal bone growth.

The interaction of extracellular components, such as fibronectin, collagens, and laminins, with chondrocyte surface adhesion molecules plays a critical role in skeletal growth and focal cartilage defect repair in cellular implants. Reduction of adhesion molecule expression may be responsible for delayed or impaired engraftment of chondrocytes subsequent to implantation. Consequently, modulating the expression of adhesion molecules on chondrocytes could help to overcome difficulties in the integration of chondrocyte implants. Our experiments demonstrated a dose-dependent increase in chondrocyte adhesion in the presence of SP or NE, which was mediated through increased FAC formation. FACs mediate cell–matrix interactions by mechanically anchoring the actin cytoskeleton to the extracellular matrix (43).

Previous studies revealed that a disruption of cell contact to the surrounding extracellular matrix could induce anoikis, an apoptosis-like phenomenon. Data from studies of ovarian cancer cells suggest that stress hormones such as NE may protect cells from anoikis by activating and modulating the focal adhesion kinase (44). This unfavorable effect of catecholamines with respect to tumor growth might be favorable in other clinical settings, such as the pathophysiology of the joint. Here, increased cell adhesion in combination with re-

duced apoptosis mediated through β-adrenergic receptors might lead to an earlier increase in local cartilage-like matrix production, which is imperative for the successful integration of cellular constructs into focal cartilage lesions.

In this study, we demonstrated that SP and NE selectively affect the apoptosis, proliferation, and adherence of chondrocytes. SP induced proliferation, and NE reduced apoptosis. Both neurotransmitters stimulated the formation of FACs in chondrocytes. These effects are mediated specifically through the NK-1 receptor and the β_2/β_3-adrenoceptors. In chondrocyte physiology, endogenous SP production by chondrocytes seems to act as a trophic factor and not as a classic neuropeptide. We suggest that SP and NE play a distinct role in modulating skeletal growth and joint regeneration processes.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Grässel had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Opolka, Straub, Grifka, Grässel.
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