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ORIGINAL ARTICLE

Serotonin mediates PGE_2 overexpression through 5-HT_{2A} and 5-HT_3 receptor subtypes in serum-free tissue culture of macrophage-like synovial cells

Matthias F. Seidel · Bernd L. Fiebich · Gudrun Ulrich-Merzenich · Eduardo Candelario-Jalil · Franz-Walter Koch · Hans Vetter

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Abstract Serotonin antagonists show impressive analgesic efficacy in rheumatoid arthritis, osteoarthritis (OA) or fibromyalgia; however, this effect is not well understood. We examined the mechanism of serotonin-induced inflammation and its antagonists in OA. Serotonin receptor subtypes and COX-2 were analysed by RT-PCR from synovial tissue. Serum-free cultures were stimulated with 10 μM serotonin and/or the antagonists ketanserin (5-HT_{2A}), tropisetron (5-HT_3) and parecoxib (COX-2). Prostaglandin E_2 (PGE_2), tumour necrosis factor alpha (TNF-α), interleukin 1β (IL-1β) and leukotriene B4 (LTB4) were measured by an immunoassay in the supernatants. RT-PCR results showed mRNA for 5-HT_{2A} and 5-HT_3 receptors, and COX-2. PGE_2 in the supernatants increased by 261.2% ± 56.7 (mean ± SEM; $P = 0.007$) in response to serotonin. TNF-α, IL-1β and LTB4 levels did not change. Ketanserin, tropisetron and parecoxib suppressed PGE_2. The serotonin-induced PGE_2 overexpression appeared thus to be mediated by 5-HT_{2A} and 5-HT_3 receptors. This activation might involve COX-2. The findings may explain the potent benefit of 5-HT_3 antagonists.

M. F. Seidel (✉) · G. Ulrich-Merzenich · H. Vetter
Rheumatology, Medizinische Universitäts-Poliklink,
Wilhelmstr. 35-37, 53111 Bonn, Germany
e-mail: Matthias.Seidel@ukb.uni-bonn.de

B. L. Fiebich · E. Candelario-Jalil
Psychiatrie und Psychotherapie, Universitätsklinikum Freiburg,
Hauptstr. 5, 79104 Freiburg, Germany

F.-W. Koch
Orthopädie, St. Josefs-Hospital, Hospitalstr. 45,
53840 Troisdorf, Germany

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Background

Rheumatic diseases are characterised by a complex network of mediators that trigger cartilage erosion and osteodestruction. The nervous system is a powerful modulator of the immune system and down-regulates arthritis activity after loss of physiologic neuronal function [1]. Neuron-derived peptides and transmitters are of exceptional interest because these molecules appear to activate autoimmune events. Substance P is a neurotransmitter of nociceptor-activated neurons and may initiate local inflammation in case of chronic pain or many rheumatic conditions [2, 3]. Similarly, serotonin (5-hydroxytryptamine, 5-HT) modulates the immune response in a variety of autoimmune conditions [4] and is found in synovial inflammation [5]. It may be released from platelets but not mast cells in human disease [6]. However, the precise mechanism of serotonin overexpression is not known. In animal experiments, serotonin is upregulated in antigen-induced arthritis and stimulates pain and inflammation after intra-articular injection [7]. Pharmacological depletion of serotonin results in a significant reduction of disease severity [8]. In rheumatoid arthritis (RA), high levels of serotonin in serum are associated with progression of erosion, suggesting that it mediates multiple autoimmune mechanisms [9].

The serotonin-induced inflammation appears to be activated by several receptor subtypes. In a rat paw formalin model, peripheral nociception associated with acute inflammation was significantly reduced by the co-administration of selective antagonists for 5-HT_1 and 5-HT_3

receptor subtypes [10]. 5-HT_{2A} receptors on small dorsal root ganglia neurons expressing calcitonin gene-related peptide (CGRP) in rats were involved in the potentiation of inflammatory pain in the periphery [11].

The role of serotonin in human autoimmune joint diseases is not well understood. Using a serum-free synovial tissue culture model we have recently shown that similar to interleukin-1β (IL-1β), 10 μM serotonin stimulates the production of prostaglandin E_2 (PGE_2) [12]. These cultures are characterised by a mixed cellular phenotype that express CD68 and HLA DR, thus sharing many characteristics with mature tissue macrophages [13]. The lack of CD14 expression matches findings in undissociated synovial tissue and suggests the absence of blood monocytes. This serum-free technique permits the study of individual inflammation mediators without interference or pre-activation of foetal calf serum factors.

Several studies suggest an intimate role of the 5-HT_3 receptor sub type in pain and inflammation. Local injections of tropisetron, a selective 5-HT_3 receptor antagonist, demonstrated potent analgesic and antiphlogistic effects in arthritis [14] and tendinopathies [15]. Results from our culture model suggest that tropisetron completely blocks the serotonin-induced overexpression of PGE_2 [12]. In another ex vivo model of isolated peripheral blood monocytes, mediators such as tumor necrosis factor-alpha (TNF-α) and IL-1β were downregulated by tropisetron [16].

Macrophages are key elements in arthritis, and up to date the mechanism of serotonin-induced activation of these cells is not well understood. Both, autoimmune joint diseases such as RA and osteoarthritis (OA) share autoimmune inflammatory characteristics. For example, OA patients show elevated serum C-reactive protein levels when determined by a plasma high-sensitivity assay [17]. Furthermore, digital OA is rarely a consequence of degeneration or malalignment suggesting that this disease shares an autoimmune background. Thus, OA tissue is similar to autoimmune synovitis and specimens are reliably available as a rich source for synovial macrophages from routine surgery.

The objective of our study was to more closely examine the effect of serotonin in OA synovitis. We analysed the expression patterns of serotonin receptors by RT-PCR in synovial tissue and compared the results to our macrophage-like tissue culture model. We then analysed 5-HT_{2A} and 5-HT_3 receptors in more detail as two candidates that may mediate inflammation using selective receptor antagonists. We further investigated whether serotonin stimulates the release of TNF-α, IL-1β and leukotriene B4 (LTB4). In inflammatory synovitis the COX-2 isoenzyme is upregulated and we examined the involvement of serotonin in this pathway using RT-PCR analyses and selective inhibitors.

Methods

Tissue culture of macrophage-like synovial cells

All reagents were purchased from Sigma (Taufkirchen, Germany) unless otherwise specified. The project was approved by the local ethics committee and subjects' written consents were obtained according to the declaration of Helsinki. Macroscopically inflamed synovial tissue was obtained from patients undergoing routine endoprosthetic replacement due to knee OA ($n = 5$; mean age ± SD: 68.7 ± 8.4 years). Patients were evaluated for clinical history and disease duration, co-morbidity and medication. Tissue culture experiments were performed as previously described [13]. Briefly, synovial tissue was dissociated in collagenase type I for 90 min at 37°C. The cell suspension was washed, filtered through a metal mesh and plated at a density of 50,000/cm^2 in poly-D-lysine coated 48-well tissue culture plates. The cultures were kept in Iscove's Modified Dulbecco's Medium supplemented with 25 μg/ml insulin and 100 μg penicillin/streptomycin for 10 days in a standard tissue culture incubator. Cultured cells were photographed under an inverted phase contrast microscope (Krüss, Germany) using a Nikon Coolpix 950 digital camera.

RT-PCR for 5-HT receptor subtypes and COX-2

Synovial tissue from OA patients ($n = 4$) and from patients with traumatic knee lesions ($n = 6$) was frozen in liquid nitrogen and stored at −70°C. Total RNA from these specimens and from subsequently cultured OA cells ($n = 4$) was extracted using a standard Tri-reagent. For RT-PCR, 1 μg total RNA was reverse transcribed using MuMLV-reverse transcriptase (Gibco, Eggenstein, Germany) and random hexamers. Primers for human 5-HT receptors (Table 1) were designed using PrimerSelect Software from DNA Star Inc. (Madison, WI, USA). PCR was then carried out adding Taq polymerase (Promega, Germany) and a pair of primers for human 5-$HT_{1A,}$ 5-HT_{1B}, 5-HT_{2A}, 5-$HT_{3,}$ 5-$HT_{4,}$ 5-HT_7 receptors [18] and the COX-2 isoenzyme [19] ($n = 4$). Positive controls consisted of human monocytes and human astrocytoma U373 MG cells. Internal positive controls consisted of β-actin. Negative controls consisted of RNA samples prior to RT-synthesis. PCR products were separated electrophoretically on a 2% agarose gel.

Table 1 PCR primers used for serotonin receptor subtype RT-PCR analyses

Product	Sense	Anti-sense	Annealing T (°C)	Cycles	size
5-HT 1A	5′-GCC GCG TGC GCT CAT CTC G-3′	5′-GCG GCG CCA TCG TCA CCT T-3′	64	40	411 bp
5-HT 1B	5′-CAG CGC CAA GGA CTA CAT TTA CCA-3′	5′-GAA GAA GGG CGG CAG CGA GAT AGA-3′	62	40	460 bp
5-HT 2A	5′-ACT CGC CGA TGA TAA CTT TGT CCT-3′	5′-TGA CGG CCA TGA TGT TTG TGA T-3′	64	40	359 bp
5-HT 3	5′-CCG GCG GCC CCT CTT CTA T-3′ 352 bp	5′-GCA AAG TAG CCA GGC GAT TCT CT-3′	64	40	448/
5-HT 4	5′-GGC CTT CTA CAT CCC ATT TCT CCT-3′	5′-CTT CGG TAG CGC TCA TCA TCA CA-3′	60	40	411 bp
5-HT 7	5′-GCG CTG GCC GAC CTC TC-3′	5′-TCT TCC TGG CAG CCT TGT AAA TCT-3′	64	40	436 bp
$\tilde{\beta}$ actin	5′-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′	5′-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3′	60	25	838 bp

Stimulation of macrophage-like synovial cells

After 10 days, cultures were stimulated for 48 h. Negative controls consisted of culture medium or an equal concentration of didimethylsulfoxide (DMSO) alone. Stimulation was carried out with 10 μM serotonin. Positive controls consisted of stimulations with 50 IU/ml IL-1β. Selective antagonists were used either alone or together with 10 μM serotonin and included 10 μg/ml ketanserin in DMSO, 25 μg/ml tropisetron or 200 ng (1 μg/ml) parecoxib [20], in aqueous solution. Experiments were conducted in triplicate wells for each condition. Cultures were checked for cellular integrity by phase contrast optics. After stimulation, the tissue culture supernatants were removed and stored at −70°C until further analysis.

Determination of PGE_2, IL-1β, TNF-α and LTB4

Protein extracts from cultured cells were obtained by incubation for 10 min on ice using a typical lysis buffer (20 mM Tris, 50 mM NaCl, 50 mM NaF, 10 mM EDTA, 20 mM sodium pyrophosphate) supplemented with 1% Triton X100 and 10 μg/ml proteinase inhibitor cocktail (Sigma). Protein concentrations were measured by the Bradford method (BioRAD, Germany). PGE_2, IL-1β, TNF–α and LTB4 concentrations in the culture supernatants were determined using commercial immunoassay kits (R&D Systems, Germany) according to the manufacturer's instructions. Cytokine concentrations were expressed in relation to the total protein content of the cultured cells as an index of cell mass. The findings had to be expressed as percent of the untreated controls because total responses varied in each patient and thus could not be calculated as absolute numbers. For statistics, SigmaStat version 3.5 (Erkrath, Germany) was used. Prior to the Mann-Whitney Rank Sum Test or the *t* test normalities and equal variances were analysed.

Results

RT-PCR shows mRNA of serotonin receptor subtypes and COX-2

In OA synovial tissue and OA culture, RT-PCR analyses demonstrated low or no expression of 5-$HT_{1A,}$ 5-HT_{1B}, 5-HT_4 and 5-HT_7 receptors. Strong expression was found for 5-HT_{2A}, 5-HT_3 (Fig. 1a) and the COX-2 isoenzyme (Fig. 1b). In synovial tissues from traumatic knee lesions, RT-PCR showed positive results in all patients for the 5-HT_{2A} receptor. Four patients were positive for the 5-HT_3 receptor (not shown). Positive controls showed appropriate amplification of the β-actin fragment. Human monocytes and human astrocytoma U373 MG cells showed PCR fragments of 5-HT receptor subtypes (not shown).

Serotonin-induced PGE_2 overexpression is blocked by ketanserin, tropisetron and parecoxib

Macrophage-like synovial cells showed multiple cellular phenotypes (Fig. 2) as previously described [13]. Similar to our previous study, serum-free cultures were constantly quiescent without significant proliferation over time as judged by phase contrast optics. The overall magnitude of serotonin-induced responses varied in patients and did not depend on clinical history, pre-medication or radiological criteria. Incubation with serotonin or the antagonists did not change the cellular morphology or total protein content (data not shown). Stimulation with 50 IU/ml IL-1β induced a 207.1% ± 38.4 increase of PGE_2 (mean ± SEM; $P = 0.03$). Stimulation with 10 μM serotonin increased PGE_2 concentrations by 261.2% ± 56.6 ($P = 0.007$). When compared to unstimulated controls, pre-incubation with tropisetron completely blocked the serotonin-induced PGE_2 increase (107.4% ± 26.5; $P = 0.4$). Similarly,

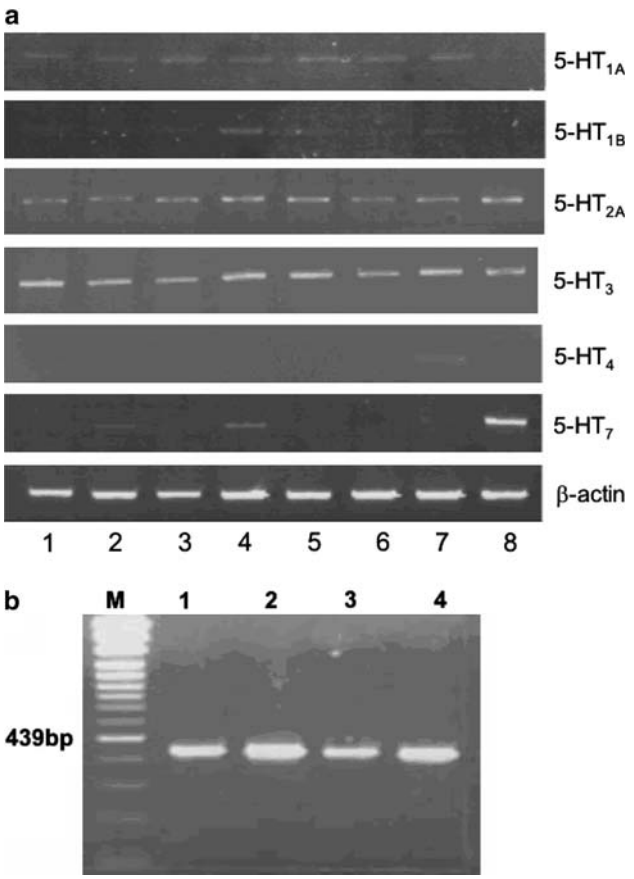

Fig. 1 PCR for human 5-HT receptors and COX-2. **a** RT-PCR for human 5-HT receptors. RNA from OA synovial membranes (*lanes 1*, *3*, *5* and *7*) and subsequently serum-free cultured macrophage-like synovial cells (*lanes 2*, *4*, *6* and *8*) were reverse transcribed and assayed using primer pairs as outlined. Low or no expression of 5-HT_{1A} (411 bp), 5-HT_{1B} (460 bp), 5-$HT_{4,}$ (411 bp), and 5-HT_7 (436 bp) receptors. Strong expression of 5-HT_{2A} (359 bp) and 5-HT_3 (448 bp) receptor mRNA. **b** COX-2 expression in serum-free cultured macrophage-like synovial cells using PCR primer pairs as outlined [18]

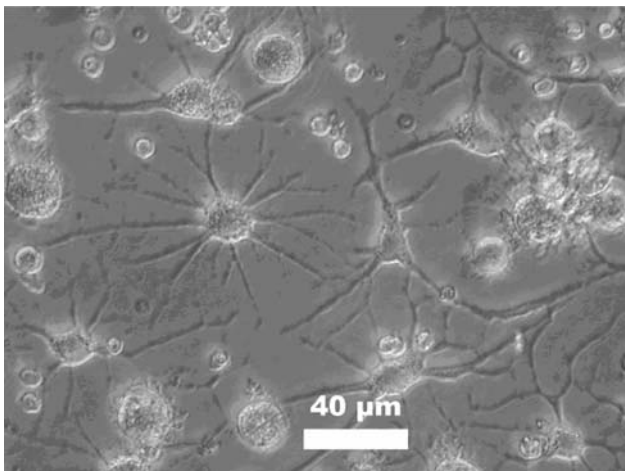

Fig. 2 Phase contrast micrograph of cultured macrophage-like synovial cells. Synovial tissue was dissociated and cultured using serum-free, insulin-supplemented Iscove's Modified Dulbecco's Medium for 10 days as described. Cultures show multiple cellular phenotypes suggesting a mixed cell population

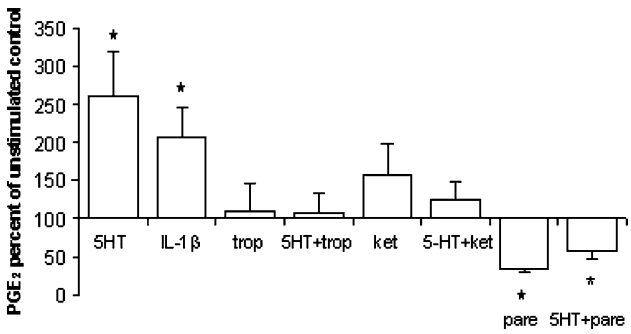

Fig. 3 Serotonin-induced PGE_2 release in cultured synovial macrophage-like cells. Dissociated osteoarthritis tissue was cultured for 10 days in serum-free medium and stimulated with 10 µM serotonin (5HT), 50 IU/ml interleukin-1β (IL-1β) and/or 25 µg/ml tropisetron (trop), 10 µg/ml ketanserin (ket) and 1 µg/ml parecoxib (par). After 48 h, PGE_2 concentrations in the supernatants were determined by a commercially available EIA and expressed as percent of unstimulated controls. 10 µM serotonin induced the release of PGE_2 that is blocked in the presence of 25 µg/ml tropisetron, 10 µg/ml ketanserin and 200 ng parecoxib. *Asterisks* indicate *P*-values equal to or smaller than 0.05 for comparisons between stimulation with serotonin and unstimulated controls

ketanserin reduced serotonin-induced PGE_2 levels to 125.1% ± 23.2 ($P = 0.9$). 200 ng parecoxib significantly decreased serotonin-induced PGE_2 levels below unstimulated controls (56.6% ± 11.2, $P = 0.01$). Tropisetron and ketanserin alone did not alter baseline PGE_2 levels significantly (110.3% ± 36.7, $P = 0.4$ and 155.8% ± 42.2, $P = 0.9$, respectively). Parecoxib alone, on the other hand, significantly reduced baseline PGE_2 expression to 34.5% ± 6.5 ($P = <0.001$). Comparisons of antagonists alone with the antagonists and serotonin did not show significant differences (tropisetron/5-HT: $P = 0.7$; ketanserin/5-HT: $P = 0.9$; parecoxib/5-HT: $P = 0.3$). Serotonin did not stimulate TNF–α, IL-1β or LTB4 expression (data not shown). The effects of serotonin on PGE_2 release are summarised in Fig. 3.

Discussion

The nervous system regulates rheumatic disease activity. We examined the potential role of serotonin as an activating mediator in a tissue culture model of human autoimmune synovitis. The serum-free technique excluded receptor pre-activation by serotonin prior to stimulation experiments, thus making our tissue culture model a valuable tool for these in vitro analyses. Conventional foetal calf serum-containing culture conditions include an undefined concentration of serotonin and other factors that

might interfere with mediators and antagonist studied here. Furthermore, human synovial cells never make contact with foetal calf serum that in addition to its mitogenic effects may induce a variety of immune responses in vitro. The serum-free cultures, on the other hand, lack these serum-induced effects. Our model is characterised by a mixed cell population with phenotypes expressing CD68 and HLA DR resembling multiple macrophage subtypes found in vivo [21]. Stimulation of these cells with 50 IU/ml IL-1β as an inductor of inflammation demonstrated an overexpression of PGE_2. Confirming findings from our previous study [12], we show a serotonin-induced increase of PGE_2. The in vitro results suggest that PGE_2 overexpression is one potential mechanism of synovial inflammation induced by serotonin in vivo. The selected serotonin concentration for stimulation in our experiments was of a similar order of magnitude as under pathological conditions in vivo [5]. The observations are in part in contrast to the results from human, LPS-stimulated peripheral blood polymorphnuclear leucocytes [22]. These cells responded to serotonin with an increase of IL-1β, whereas our cells neither upregulated IL-1β nor TNF-α. Differences in the extracellular environment, the cellular phenotype and finally the degree of pre-activation with LPS might explain this contrast. Furthermore, previous multiplex RT-PCR analyses from our OA cell cultures confirmed a subordinate expression of these cytokines [13]. Other co-factors that were absent in our chemically defined culture environment might be necessary to further increase the serotonin-induced activation of inflammatory mediators studied here. Cultures from more active autoimmune conditions such as RA may show additional cytokine responses.

In OA synovial tissue and cultured synovial cells, results from RT-PCR experiments suggested the expression of 5-HT_{2A} and 5-HT_3 receptor subtypes albeit a quantitative analysis was not possible by the technique used here. Control tissue from acute traumatic knee lesions demonstrated profiles similar to the chronic condition of long-standing OA synovitis. Here, we show for the first time that 5-HT_{2A} and 5-HT_3 receptors are also expressed in cultured macrophage-like synovial cells, thus reflecting the expression pattern from the originating synovial tissue. These results are similar to our previous study that demonstrated a comparable expression of CD68 and HLA DR in both, synovial tissue and subsequent cultures. Thus, our serum-free culture model appeared as an appropriate tool to examine the effects of serotonin in more detail.

Several aspects of serotonin receptor-specific characteristics have to be considered when analysing the data obtained here: We examined cultures with selective 5-HT_{2A} and 5-HT_3 antagonists because these receptors showed strong expression by RT-PCR. The data suggest that both, tropisetron and ketanserin had a powerful PGE_2-suppressive effect. Unlike ketanserin, tropisetron lacked intrinsic stimulation of PGE_2. Cellular viability did not appear to be effected by tropisetron or ketanserin because the cellular phenotype, the total protein content and baseline PGE_2 concentrations were the same as in unstimulated controls. Suppression of the same biological effect by two different inhibitors suggests receptor cross-talk or shared receptor subunits that have been described for several 5-HT receptor subtypes [23, 24]. This might also explain the intrinsic stimulation by ketanserin, which may in part activate shared subunits. Furthermore, we exposed our cultures to serotonin for 48 h although a more rapid desensitization has been described for 5-HT_3 receptors [25]. However, this effect was observed in basolateral amygdala neurons that do not compare to the non-neuronal in vitro system used here. In addition, the distinct experimental and cellular synovial cell characteristics may also exhibit an IC50 different from <30 nM that is usually found for antagonising 5-HT_3 receptors. The putative excess concentration of tropisetron used here did not interfere with cellular viability and induced a reproducible PGE_2-suppressive effect. Separate dose-response experiments would further clarify the precise characteristics of the 5-HT_3 receptors expressed on our non-neuronal cells.

In vivo, the 5-HT_3 receptor appears to be the predominant mediator of inflammation because intra-articular tropisetron or granisetron, another 5-HT_3 antagonist, have shown impressive clinical benefits in OA, RA and scleroderma [26–28]. In addition, tropisetron has been effective in the treatment of cardiac autonomic dysfunctions in patients with fibromyalgia syndrome [29]. In agreement with these clinical studies, our results thus suggest that a substantial part of the serotonin-induced pathology is mediated by 5-HT_3 receptors. In contrast, clinical trials with ketanserin have not shown a significant therapeutical effect (unpublished communication Wolfgang Müller, Basel, Switzerland).

The results from RT-PCR analyses suggested the expression of the COX-2 isoenzyme in vivo and in vitro. COX-1 is expressed in a variety of tissues while COX-2 is upregulated in conditions with pain and inflammation similar to our positive controls from traumatic knees or OA. Suppression of PGE_2 concentrations below baseline levels suggests the presence of a robust intrinsic OA inflammation that can be blocked by parecoxib. Serotonin thus appeared to be one of several COX-2 stimulators.

In conclusion, our results suggest an involvement of serotonin in OA synovitis. This inflammation might be triggered by stimulation of 5-HT_{2a} and 5-HT_3 receptor subtypes with consecutive overexpression of PGE_2.

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