https://doi.org/10.1016/j.joca.2021.02.005

Title: The cholinergic system in joint health and osteoarthritis: a narrative-review

Running title: Cholinergic system and OA

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Key words: Acetylcholine, osteoarthritis, articular cartilage, endochondral ossification, inflammation

Acknowledgement: This work was supported by Research Grants Council of Hong Kong Early Career Scheme (PolyU 251008/18M) and General Research Fund (PolyU151061/20M), PROCORE-France/Hong Kong Joint Research Scheme (F-PolyU504/18), and also Health and Medical Research Fund Scheme (01150087#, 15161391#, 16172691#). The study funders/study sponsors had no involvement in the study design, data collection, analysis or interpretation of the study, or in the writing of, or decision to submit the manuscript.

Conflict of interests: none

Abstract (250 words)

Osteoarthritis (OA) poses a major health and economic burden worldwide due to an increasing number of patients and the unavailability of disease-modifying drugs. In this review, the latest understanding of the involvement of the cholinergic system in joint homeostasis and OA will be outlined.

First of all, the current evidence on the presence of the cholinergic system in the normal and OA joint will be described. Cholinergic innervation as well as the non-neuronal cholinergic system are detected. In a variety of inflammatory diseases, the classic cholinergic anti-inflammatory pathway lately received a lot of attention as via this pathway cholinergic agonists can reduce inflammation. The role of this cholinergic anti-inflammatory pathway in the context of OA will be discussed. Activation of this pathway improved the progression of the disease. Secondly, chondrocyte hypertrophy plays a pivotal role in osteophyte formation and OA development; the impact of the cholinergic system on hypertrophic chondroblasts and endochondral ossification will be evaluated. Cholinergic stimulation increased chondrocyte proliferation, delayed chondrocyte differentiation and caused early mineralisation. Moreover, acetylcholinesterase and butyrylcholinesterase affect the endochondral ossification via an acetylcholine-independent pathway.

Thirdly, subchondral bone is critical for cartilage homeostasis and metabolism; the cholinergic system in subchondral bone homeostasis and disorders will be explored. An increase in osteoblast proliferation and osteoclast apoptosis is observed. Lastly, current therapeutic strategies for OA are limited to symptom relief; here the impact of smoking on disease progression and the potential of acetylcholinesterase inhibitors as candidate disease-modifying drug for OA will be discussed.

Introduction

Osteoarthritis (OA), a degenerative joint disease characterized by pain, stiffness, and disability, represents an increasing health challenge and socioeconomic burden worldwide. OA is a serious disease that confers higher risk of cardiovascular accident and all-cause mortality than those without OA. With population ageing and the obesity pandemic, the prevalence of OA surges (1,2). Unluckily, there is no cure for OA once it is established.

The pathological changes observed in OA joints are associated with a metabolic shift from cells in a resting state to a highly metabolic state associated with an increased production of inflammatory and catabolic factors. This shift is observed in subchondral bone, cartilage and synovium (3). The hallmark of OA is the loss of articular cartilage that cushions the joint during movement. Chondrocytes are no longer in a resting state but actively proliferate and undergo hypertrophy. It is assumed that the hypertrophic chondrocytes undergo apoptosis which is associated with extracellular matrix degradation and calcification. This will stimulate angiogenesis which attracts osteoblast and osteoclasts and results in osteophyte formation. This activation closely looks like the endochondral ossification pathway as both are defined by the presence of hypertrophic and apoptotic chondrocytes (4–7). Micro-architectural deterioration of subchondral bone is another typical characteristic of OA. In early OA, an increased bone turnover and structural deterioration is observed and although the mechanism is not fully understood, it is linked to several factors such as microdamage repair, increased vascularisation, and enhanced bone-cartilage crosstalk. In late stage OA, the subchondral bone will become sclerotic presenting an increased density and low mineralisation. Histopathological changes in bone associated with the progression of OA include micro-damages, bone marrow oedema and subchondral cyst (7–9). OA is also characterized by synovitis with low-grade inflammation (10). This is associated with the invasion of mononuclear cells, thickening of synovial membrane and the imbalance between anti-inflammatory (for example Il10 and Il4) and inflammatory (for example TNF and Il-1b) cytokines and growth factors. Mounting evidence suggest that synovitis is associated with pain and structural progression of OA (11,12). Currently, most OA research focusses on the understanding of the mechanisms behind the activation of the inflammatory, pro-catabolic and pro-resorptive pathways. Less interest goes to endogenous regulation. Here, we evaluate the role of the cholinergic system in the OA joint as an endogenous regulator that can be activated to protect joint damaging and OA.

The cholinergic system is defined by the presence of acetylcholine (Ach) accompanied by its synthesizing enzymes, transporters, receptors, and degrading enzymes. It is mostly known for its function as neurotransmitter in the nervous synapses i.e. neuronal cholinergic system. Ach as neurotransmitter plays an important role in the autonomic nervous system, which is the nervous system responsible for unconsciously controlling the organs. It consists of two antagonistic-operating systems: the parasympathetic and sympathetic system. The parasympathetic system is mostly represented by the vagus nerve which is a mixed nerve containing 80% afferent and 20% efferent fibers. Next, evidence suggests that the cholinergic system

is also found outside the nervous system in non-neuronal cells. In these cells, Ach is responsible for the interaction with the extracellular environment, hormones, growth factors and the nervous system. The presence and function of Ach outside the nervous system is called the non-neuronal cholinergic system (NNCS) (13,14). The investigation of the cholinergic system can be challenging. Quantification of acetylcholine (Ach) in *in vivo* systems is difficult due to the fast-enzymatic hydrolysis of the small molecule by acetylcholinesterase (AchE). Moreover, Ach can bind on two acetylcholine receptors (AchR): the muscarinic (coupled G protein receptor) and the nicotinic receptors (pentameric ligand-gated ion channels). Five subtypes of muscarinic receptors and approximatively 17 combinations of nicotinic receptors are known. The knowledge about the cellular distribution of these subtypes is advantageous as a thorough understanding of the function and location is needed to develop novel drugs and to better comprehend the cholinergic system. However, most antibodies to detect these subunits are inaccurate and non-specific. Therefore, it remains difficult to establish where and under which circumstances a certain receptor subtype is upregulated which impacts the downstream effects (15).

In this narrative review, the goal is to delineate the presence and the involvement of the cholinergic system in the different substructures of the synovial joint during OA development. Our literature review revealed that cholinergic nerves can be present in subchondral bone and that most of the joints tissues and especially cartilage, known as avascular and devoid of nerve terminations, expressed a non-neuronal cholinergic system. The influence of acetylcholine in synovitis, bone remodeling and inflammatory activation of chondrocytes is described, whereas its role in chondrocyte hypertrophy in OA is still largely unknown. Therefore, as data are available on the endochondral ossification process, it will be employed as model to evaluate the role of the cholinergic system in chondrocyte hypertrophy. Finally, the cholinergic system as emerging target in the treatment of OA will be addressed.

Cholinergic system in the synovial joint

An overview of the cholinergic innervation and the non-neuronal cholinergic system in the joint is shown in **figure 1.**

Cholinergic innervation of synovial joints

Courties et al. recently observed in a first small-scale study that cholinergic peripheral nerves (ChAT+) are present in subchondral bone in OA patients (16). These fibers did not connect with the vagus nerve. Two hypotheses can be formulated about the origin of these fibers: they can be considered true-parasympathetic or false-parasympathetic fibers as they can arise from sympathetic fibers that have modified their phenotype. The latter is in accordance with previous reports of cholinergic fibers producing Ach in periosteum (17,18). On the other hand, in rat metaphysis bone (19), the cholinergic fibers (VAchT+) were probably parasympathetic since the pseudorabies virus injected in the metaphysis projected to the sacral spinal cord segment which is parasympathetic. In human, such experiments are not possible and therefore the origin of the cholinergic fibers in subchondral bone remains unknown today.

In synovium, sympathetic innervation is described, but less is known about the parasympathetic innervation. It is suspected that also here cholinergic fibers are present that arise from sympathetic rather than parasympathetic centres (18,20). As expected no cholinergic fibers could be detected in cartilage (16). More studies are needed to demonstrate the local effect of Ach produced by these nerves on subchondral bone and synovium. Thus, based on initial data, cholinergic nerve fibers might be present in joint tissue which, when confirmed, could lead to a direct local effect of neuronal acetylcholine on joint homeostasis.

Cholinergic system and its role in inflammation

The stimulation of the vagus nerve has been studied in inflammatory models of arthritis (i.e rheumatoid arthritis) showing systemic immunomodulatory and anti-inflammatory properties that decrease the synovial inflammation and pain (21,22). These properties are attributed to a homeostatic control mechanism, balancing pro- and anti-inflammatory pathways, activated via the vagus nerve. This mechanism is an important part of the defense mechanism of the body against harmful stimuli such as pathogens, damaged cells, and irritants as it prevents excessive or uncontrolled inflammation which causes tissue damage and disease. It was first discovered by Tracey et al. This group proved that direct electrical activation of the peripheral vagus nerve and thus the vagal efferent fibers, inhibited TNF production and prevented shock when lethal endotoxemia was induced in rats (23). Activation released Ach from the efferent vagal nerve which inhibits the release of pro-inflammatory cytokines (for example TNF) from macrophages. This process is mediated via the nicotinic acetylcholine receptor α7 (α7-nAchR) of macrophages and is called the cholinergic anti-inflammatory reflex (24).

Regulation of the joint inflammation via the anti-inflammatory pathway can be achieved via the vagus nerve, a systemic indirect effect.

The non-neuronal cholinergic system in the joint

The different components of the NNCS are found in the various constituents of the synovial joint. In the synovial membrane the two enzymes responsible for synthesis of Ach, choline acetyltransferase (ChAT) and carnitine acetyltransferase (CarAT), were observed (25). ChAT was detected in fibroblast-like cells and mononuclear-like cells. Synovial choline transport and release is mediated via the organic cation transporter (OCT) and choline transporter-like family (CTL). The CTL transporters were found in macrophages-like and fibroblasts-like cells. Moreover, the two degrading enzymes, butyrylcholinesterase (BuChE) and acetylcholine esterase (AchE), were detected as also several receptor subunits. The presence and activation of the receptors is determined in human synovium samples via RT-PCR. The subunits α3, α5, α6, α7, α9, α10 of the nicotinic receptor and muscarinic receptors M1, M3, M4 and M5 were present. Special attention goes to the α7nAchR which is expressed in the synovial layer as this receptor plays an important role in the cholinergic anti-inflammatory pathway (25–27).

Next, the expression of the cholinergic system in human OA chondrocytes and murine wild-type chondrocytes from wild-type C57BL/6 mice was evaluated. The entire cholinergic system including CarAT (synthesis), VAchT (transport), AchE (degradation) and a functional α7nAChR was observed. Moreover, also several other subunits of the nicotine receptor were expressed on the cell surface. Subunits α5, α6, α7 and β4 were detected in human and murine cells, while β2 was only found in human and α4 only in murine cells. ChAT and BuChE expression was not detected in chondrocytes (28).

The presence of the NNCS in subchondral bone is not yet evaluated. However, the components of the NNCS are found in bone more specifically in osteoblasts and osteoclasts. Osteoblasts can produce Ach via the non-traditional carnitine acetylcholine transferase (29). Uptake of choline and release of Ach in osteoblasts is mediated through OCT or VAChT (29,30). Both nicotinic and muscarinic acetylcholine receptors are reported to be expressed in osteoblasts and osteoclasts (30–32) and also AChE has been identified (33,34).

The presence of the non-neuronal cholinergic system in all joint tissues supports a role of ACh as local actor on the receptors within the joint.

The role of cholinergic system in the arthritic joint

Chondrocyte hypertrophy

The formation of hypertrophic chondrocytes is considered an important pathway in the progression of OA (5,6). According to our knowledge, no literature is available on the effects of acetylcholine on chondrocyte and chondrocyte hypertrophy in OA. The pathological development of chondrocytes to hypertrophic chondrocytes in OA is quietly closed from the biological endochondral ossification pathway (35,36). Thus, to estimate the effect of the cholinergic system on hypertrophic chondrocytes in OA, the effect on the endochondral ossification pathway is evaluated

Endochondral ossification, the embryonic development of long bones via a cartilage intermediate, begins with mesenchymal stem cells aggregating into a condensate. In these condensates the mesenchymal cells differentiate into chondrocytes which subsequently will start to proliferate to expand the cartilage. Next, the chondrocytes in the middle of the cartilage template start to enlarge to form hypertrophic chondrocytes. The ultimate destination of the hypertrophic chondrocytes depends on its location, the hypertrophic chondrocytes closest to perichondral osteogenic cells differentiate into osteoblast while other chondrocytes, further away, undergo apoptosis (37–39).

The effects of the cholinergic system on chondrocyte proliferation and differentiation in the endochondral ossification can be divided into Ach-dependent and Ach-independent effects.

The Ach-dependent activation results in stimulation of chondrocyte proliferation, premature mineralisation and incomplete chondrogenic differentiation. Although more chondrocytes are present, they need more time to differentiate which will lead to a delay in growth rate and thus smaller animals at birth with a disturbed cartilage and bone structure. This was confirmed in several *in vivo* and *in vitro* models (38,40–43). Spieker at al. implanted Ach- and ChAT-soaked beads in mice which stimulated the proliferation of chondroblast in the growth plate. The degree of effect depended on the local concentration (40). Moreover, the same group evaluated the knockout of BuChE and AchE alone or together. An increased chondroblast proliferation in combination with an incomplete chondrogenic differentiation was observed. It is postulated that these degrading enzymes influences proliferation via establishing an Ach gradient between epiphysis and diaphysis as the protein itself is not present at proliferation locations. Moreover, an acceleration of the onset of mineralisation and prematurely completion of cartilage remodelling/mineralisation was detected in BuChE knockout and combination of BuChE and AchE knockout (38). Moreover, *in vitro* results showed that addition of nicotine reduced hypertrophic chondrocyte formation/chondrocyte differentiation and matrix production in growth plate via α7nAchR (41). After pre-natal nicotine exposure in rats, early differentiation of chondrocytes was suppressed leading to an accumulation of hypertrophic chondrocytes and a delayed formation of the ossification centre. Blocking the α9α10 nicotinic receptor rescued these effects (42).

Next to these Ach-dependent effects, Ach-independent effects are also observed. The involvement of an Ach-independent mechanism was suspected after a delay in mineralisation was identified in AchE knockout mice. Under normal circumstances, AchE knockout would increase the Ach concentration which would

accelerate rather than delay mineralisation. Thus, these results need to be related to another mechanism. AchE is detected at locations of differentiation and mineralisation and thus the delay in mineralisation could be attributed to AchE itself. Previously, the AchE amidase side activity and its non-enzymatic adhesion function were already involved in other development processes (44,45). This was also confirmed after administration of BW284c51, an acetylcholinesterase inhibitor (AchEI) which not only inhibit the catalytic site but also the peripheral anionic site, the site responsible for the non-cholinergic activity. It is postulated that the Ach-independent activity is related to its adhesion function. Interaction between AchE and extra-cellular matrix components such as laminin, ALP and collagen is already established before (34,43,45). Knockout of BuChE also identified Ach-independent effects for BuChE as an accelerated secondary ossification is observed. It is hypothesized that BuChE forms complexes with several proteins balancing the speed of ossification (43).

Finally, the role of the cholinergic system in terminal differentiation of hypertrophic chondrocytes is investigated. Two pathways are assumed: differentiation into osteoblasts or apoptosis. It is already established that AchE plays an important role in apoptosis. AchE expression is observed during apoptosis in different primary cultures and cell lines naturally occurring or after induction. Deficiency or low levels of AchE make cells less sensitive to apoptosis. In contrast, overexpression stimulates apoptosis although it is not considered an initiator (46). The role of AchE in chondrocyte differentiation into osteoblast is not yet evaluated. However, Spieker et al. estimate that AchE promotes entry into the last cycle of hypertrophic cells. Thus after administration of an AchEI an increase in osteoblast formation and reduction in mineralisation should be observed (43).
The effects of the cholinergic system on the endochondral ossification pathway are summarised in **figure 2**.

The influence of Ach-dependent and independent pathways on chondrogenic differentiation during endochondral ossification is demonstrated. Therefore, it could be hypothesized that AchEI can affect the development of OA, even though more data need to be provided to substantiate this statement.

Subchondral bone cyst and sclerosis

Alterations in the subchondral bone structure are observed during the progression of OA. In early stage OA is characterised by bone loss and a low bone density while in late stage OA bone sclerosis is observed. Bone cysts, bone marrow oedema and bone sclerosis will arise as a result of these changes. The subchondral bone modifications are closely linked to the RANK/RANKL/OPG system in osteoblast and thus the activation of osteoclast formation (47,48). This causes an imbalance in osteoblast and osteoclast, similar to osteoporosis.

The influence of the cholinergic system on subchondral bone is not yet investigated. However, several studies examined the role of Ach and AchE in bone homeostasis in the context of osteoporosis. Acetylcholine or exogenous activation *via* nicotine or muscarine, stimulates osteoblast proliferation (30,31,33) and osteoclast apoptosis (49). Binding of Ach to the nicotinic receptor leads to an upregulation of cyclin D which will promote cell proliferation in osteoblasts (30). Activation of the muscarinic receptor showed to increase the intracellular calcium level and promote cell proliferation in osteoblast (31,50). Oppositely, osteoclast apoptosis is stimulated by activation of nicotinic receptors (19) and a downregulation of the nAChRα2 and mAChR M3 receptor is observed in the process of osteoclast formation (49). Moreover, next to its enzymatic hydrolytic function AchE exerts a non-enzymatic function as bone matrix protein. It is demonstrated that AchE mediates osteoblast function and has an influence on bone development via cell-matrix interaction (33,34,40)

Thus, AchE can influence bone homeostasis via an enzymatic and non-enzymatic pathway. Therefore, inhibition of AchE would be beneficially.

Low-grade synovitis

Acetylcholine and the cholinergic anti-inflammatory pathway lately received a lot of attention, especially for their possible role in the treatment of inflammatory diseases. As inflammation is considered an important part of OA, the influence of the cholinergic anti-inflammatory pathway in OA joints needs to be assessed. However, the role of the cholinergic system in OA synovitis has not been thoroughly investigated. Recently, Courties et al observed no difference in synovitis score between wild type and α7-nAchR knockout mice (28). Therefore, it is hypothesised that the cholinergic system can influence OA synovitis via the systemic anti-inflammatory pathway. This is possible since cholinergic fibers are present in the different joint structures. More research is needed to substantiate this hypothesis

Cartilage degradation

Here the focus will be on the non-neuronal stimulation of the α7-nAchR in chondrocytes. This is possible as all the components necessary for activation of this pathway are present in the joint (see above).

A role for the α7nAChR in the inflammation response of chondrocytes was confirmed by Courties et al.(28). In this study, the activation of the nicotinic receptor in human and murine chondrocytes, stimulated with IL-1β to induce inflammation, decreased the inflammatory and catabolic response. Moreover, no effect on inflammation was observed after administration of nicotine to Chrna7-/- chondrocytes. This substantiates a role for the α7nAChR in the anti-inflammatory response after cholinergic stimulation. Depending on the differentiation state of the chondrocytes, activation of the α7nAChR triggers response via influx of calcium or via a direct effect on the associated pathways (28). Similar results were obtained after activation of rat chondrocytes with IL-1β. After administration of nicotine the inflammatory response decreased. More specifically, a decrease in phosphorylation of p38, Erk1/2, JNK MAPKs and NF-κB p65 is observed. This reduces the action of signalling pathways such as the mitogen activated protein kinase and the nuclear factor-kappa B pathway which play a role in chondrocyte activation (52). Moreover, in mice, nicotine showed a reduced cartilage degradation after monosodium iodoacetate (MIA) induced OA. This was attributed to the suppression of matrix metalloproteinase-9 (MMP-9) production by macrophages after activation of the α7nAChR. Activation of this receptor increases phosphorylation of PI3K and Akt and a decreases the transcription of NF-κB (53). In contrast with the above mentioned studies, Bock et al. could not find a positive effect of nicotine on the development of MIA-induced OA (54). This could be attributed to the small scale of the study in combination with the use of conservative statistical models. However, a positive tendency was observed.

An overview of the observed effects after cholinergic activation are given in **figure 3** and an overview of the literature can be found in **table 1.**

The cholinergic system as emerging target for OA prevention and treatment

Nicotine substitution as model for cholinergic activation in OA

Nicotine, an exogenous stimulator of the cholinergic system, is an important component of cigarette smoke. Therefore, evaluation of the effects of smoking on OA can be used as a model to evaluate cholinergic stimulation via nicotine. Several studies investigated the role of smoking on the development of OA. In 2011, a meta-analysis of 48 observational studies including knee, hip, spine and hand OA observed less OA in smokers. However, such a protective effect was only significant in case-control studies and not in cohort studies. In these case-control studies the effect was most pronounced for knee OA in hospital setting (55). These results were confirmed in several other studies (56–58). A systematic review and meta-analysis including 46 cohort studies analysed the risk and protective factors for the onset of knee OA, no statistically significant risk or protective effect of smoking on knee OA was observed (56). Another systematic review including cohort and case-control studies found a protective effect of smoking on OA. However this evidence was not sustained when only looking at cohort studies (57). A new meta-analysis conducted in 2017 investigated the relationship with knee OA in cohort, case-control and cross sectional studies and detected an inverse relationship between knee OA and smoking (58). In contrast, several meta-analysis studies did not detect any effects of smoking on incidence and progression of OA when analysing observational studies (59,60). Although the positive effects of smoking on OA are mainly associated with case-control studies, which are more prone to selection bias and considered less reliable, a protective effect cannot be excluded. However, the presence of several other toxic components in cigarettes, the co-occurrence of smoking with other OA risk factors such as a sedative lifestyle will most likely reverse the protective effect and lead to deleterious effects of smoking on health and joint homeostasis.

Pharmacological possibilities to target the cholinergic system in OA

The involvement of the cholinergic system in OA as described above supports its role as a novel potential target for treatment and prevention of OA. The development of improved treatment options is necessary as current treatment is mainly symptomatic and non-pharmacological.

Previous research evaluated the role of GTS21, an α7nAchR agonist, in human endotoxemia. Although GTS21 was associated with a lower cytokine level no significant differences in inflammatory mediators could be observed (61). Moreover, in humans a partial duplication of the CHRNA7 gene, encoding for the α7nAchR, exist known as CHRFAM7A.The latter is a dominant negative regulator of α7nAchR resulting in a decrease of the ion channel function and thus reduced the anti-inflammatory effect of Ach (62,63).

Therefore, it could be useful to directly target Ach for example by using acetylcholinesterase inhibitors (AchEI). Several AchEI inhibitors were already tested *in vitro* for their role in attenuating inflammation in arthritis. Donepezil, a selective and potent AchEI, was added to human chondrocytes. Donepezil suppressed the TNF-induced activation of MMP-13 via inhibition of the STAT1/IRF1 and prevents collagen II degradation (64). Addition of the AchEI, BW284c5, to a micro-mass culture of differentiated chondroblasts and osteoblasts subjected to TNF, lead to a decrease in the TNF-induced inflammation (65). Moreover, the

effects of AchEI were also assessed *in vivo* using models of rheumatoid arthritis. Galantamine, an AchEI and allosteric binder to the α7nAChR, reduced all biomarkers for inflammation (for example TNF) in adjuvant-induced arthritis in rats. Moreover, blockade of the α7nAChR blunted the anti-inflammatory effect of galantamine (66,67). Administration of neostigmine, a peripheral AchE inhibitor, to mice with antigen-induced arthritis reduced neutrophil recruitment and hyperalgesia (68).

Finally, besides cholinergic stimulation using α7 agonists or AchE inhibitors, the cholinergic anti-inflammatory pathway can also be stimulated via the vagal nerve. Recent evidence showed that transcutaneous vagal stimulation decreases inflammation and pain in erosive hand OA(69). A randomised clinical study (ClinicalTrials.gov.Identifier: NCT04520516) will start soon to further substantiate this result.

Conclusion

In this review, the cholinergic system in joint physiology and OA was discussed. Cholinergic innervation was observed in subchondral bone and synovium and the non-neuronal cholinergic system was detected in synovial cells, bone cells and chondrocytes. This presence potentially affects all structures of the synovial joint. Here, the role of the cholinergic system in bone, endochondral ossification and OA related inflammation was explored as to-date no information is available on the effect of the cholinergic system on subchondral bone, hypertrophic chondrocytes, or synovitis in OA joints. The knowledge of the cholinergic system in OA is limited to the systemic activation of the nAchR by vagus nerve stimulation or by chemical cholinergic agonists which may protect cartilage against OA lesions probably via the a7nAChR since knockout mice exhibit more severe cartilage lesions. Therefore, the specific and local role of the cholinergic system in OA should be further evaluated as well as the treatment with AchEI which could prolongate the function of endogenous Ach. **Figure 4** shows a summary of this review.

Research Agenda

- Determine the presence of the NNCS in subchondral bone
- Evaluate the function of the subchondral cholinergic fibers in OA
- Determine the role of terminal differentiation of hypertrophic chondrocytes in OA
- Evaluate the enzymatic and non-enzymatic function of AchE in OA chondrocytes
- Evaluate the enzymatic and non-enzymatic function of AchE in the terminal differentiation of hypertrophic chondrocytes to osteoblast
- Evaluate the role of AchEI on chondrocyte hypertrophy in vitro and in vivo
- Evaluation of effect of AchEI in murine model of OA
- Proof of concept study in human OA

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