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Human specific duplicate CHRFAM7A gene is associated with more severe osteoarthritis and amplifies pain behaviors

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Abstract

Objectives: CHRFAM7A is a uniquely human fusion gene that functions as a dominant negative regulator of alpha 7 acetylcholine nicotinic receptor (α7nAChR) *in vitro*. This study determined the impact of CHRFAM7A on α7nAChR agonist responses, osteoarthritis (OA) severity and pain behaviors and investigated mechanisms.

Methods: Transgenic CHRFAM7A (TgCHRFAM7A) mice were used to determine the impact of CHRFAM7A on knee OA histology, pain severity in OA and other pain models, response to nAchR agonist and IL-1β. Mouse and human cells were used for mechanistic studies.

Results: Transgenic (Tg) TgCHRFAM7A mice developed more severe structural damage and increased mechanical allodynia than WT mice in the destabilization of medial meniscus model of OA. This was associated with a decreased suppression of inflammation by α7nAchR agonist. TgCHRFAM7A mice displayed a higher basal sensitivity to pain stimuli and increased pain behavior in the monoiodoacetate and formalin models. Dorsal root ganglia (DRG) of TgCHRFAM7A mice showed increased macrophage infiltration and expression of the chemokine fractalkine and also had a compromised antinociceptive response to the α7nAchR agonist nicotine. Both native CHRNA7 and CHRFAM7A subunits were expressed in human joint tissues and the CHRFAM7A/CHRNA7 ratio was increased in OA cartilage. Human chondrocytes with two copies of CHRFAM7A had reduced anti-inflammatory responses to nicotine.

Conclusion: CHRFAM7A is an aggravating factor for OA-associated inflammation and tissue damage and a novel genetic risk factor and therapeutic target for pain.

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Keywords

osteoarthritis; pain; nicotinic receptors; cholinergic system; CHRFAM7A

INTRODUCTION

Osteoarthritis (OA) is the most prevalent joint disease associated with progressive structural damage to the joint tissues and pain and function limitations affecting patients' quality of life [1]. Pharmacological approaches to structure modification are not available and pain management is challenging due to reduced efficacy during chronic use and adverse events caused by the pain medications [2]. Thus, there is a major need for better understanding mechanisms of OA pathogenesis and pain.

The nicotinic acetylcholine receptors (nAChRs) are ionotropic channels, assembled as pentamers of hetero or homologous subunits [3]. Among them, the Cholinergic Receptor Nicotinic Alpha 7 Subunit (CHRNA7) is widely expressed by neuronal and non-neuronal cells and mostly assembles as α7nAChR homopentamer [4]. The α7nAChR activation displays protective effects in a range of pathological processes such as central nervous system diseases (CNS), pain and inflammation [5–8].

Protective functions of α7nAChR in OA have been recently shown. Mice lacking the CHRNA7 subunit had more severe cartilage lesions after meniscectomy while systemic activation of α7nAChR by nicotine decreased monoiodoacetate (MIA)-induced OA lesion severity and pain [9–11]. One possible explanation of the anti-catabolic effect of CHRNA7 could be local action in the joint since cartilage expressed all modulators of non-neuronal ACh metabolism and subchondral bone is innervated by cholinergic fibers [9,12–14]. Furthermore, α7nAChR activation limits chondrocyte cytokine and metalloproteinase production and apoptosis induced by inflammatory stress [9,15]. Systemic or local CHRNA7 activation might therefore constitute a possible therapeutic target in human OA.

During evolution a human specific partial duplication of exons 5–10 of the CHRNA7 has emerged and rearranged with another duplicated exon, called FAM7A to form the hybrid gene CHRFAM7A [16]. This results in production of the dupα7 protein which assembles with α7 subunits and acts as a dominant negative inhibitor of receptor function because it lacks the binding site for agonist [17,18]. Indeed, CHRFAM7A could be associated with an inflammatory phenotype [19] and decreased agonist efficacy in human CNS diseases, explaining discrepancies between mice and humans in studies on Alzheimer's disease and schizophrenia [20,21]. Most of the human population (78%) expresses two copies of CHRFAM7A while 20% have one and around 1% have no copy [22,23]. Transgenic (Tg) mice expressing CHRFAM7A (TgCHRFAM7A) were recently shown to manifest increased inflammation and a higher hematopoietic stem cell reservoir toward monocyte/macrophage lineage, leading to increased myeloid migration in a lung injury model [24].

Since CHRNA7 is involved in OA pathophysiology in animal models, it is essential to understand the involvement of the specific human duplicate CHRFAM7A in OA pathophysiology and pain. The objectives of this study were to determine the functional

consequences of the human specific duplicate CHRFAM7A in human joint tissue cells *in vitro* and using TgCHRFAM7A mice to assess function and OA-associated tissue damage and pain *in vivo*.

RESULTS

CHRFAM7A increases the severity of structural damage in experimental OA and impairs anti-inflammatory nicotinic receptor activation

We confirmed the expression of CHRFAM7A mRNA in blood, joint tissues, spinal cord, and dorsal root ganglia (DRG) of TgCHRFAM7A mice (Supp. Fig. S1).

TgCHRFAM7A mice had more severe OA-associated tissue damage 8 weeks after destabilization of medial meniscus (DMM) than their littermate controls with an OARSI score of mean±SEM 21.2±2.3 *versus* 12.2±1.9 (on a 0–48 scale, $p<0.05$, Figure 1A). This increased cartilage damage was associated with more severe synovitis (Figure 1B) including increased macrophage invasion in synovium (Figure 1C). In the anterior compartment of the synovium between the tibia and meniscus we observed 1326±164.2 macrophages/mm^2 in TgCHRFAM7A synovium *versus* 875±124.9 macrophages/mm^2 in WT, $p=0.04$ (Figure 1C). No differences in OARSI and synovitis scores were observed after SHAM surgery between the TgCHRFAM7A and WT mice. However, the macrophage invasion was higher after SHAM in TgCHRFAM7A than in WT, $p<0.001$. In the MIA model, TgCHRFAM7A mice had also higher OARSI scores than WT with a mean OARSI score of 4.0±0.6 *versus* 1.9±0.5, $p=0.02$ (Figure 1D) but there was no significant difference in synovitis scores (Figure 1E).

There were no differences in body weights between TgCHRFAM7A and WT mice throughout the entire duration in both OA models (Supp. Fig. S2).

We hypothesized that the increased severity of experimental OA in TgCHRFAM7A was due to a blunted effect of local endogenous agonist on α7nAChR. To confirm the decrease of local agonist response, we evaluated the effect of intraarticular (IA) injection of nicotine on IL-1β-induced expression of cytokines and proteases. WT and TgCHRFAM7A received IA injection of IL-1β (30 ng) or nicotine (10 μM) or a combination of both. After 2 hours, knee joint tissues were collected for RNA extraction and PCR analysis. In WT mice, nicotine 10μM (n=5) induced a significant decrease of *Il6* and *Mmp3* in meniscus (−26% for *Il6* and −28% for *Mmp3*, Figure 2A) and synovium (−46% for *Il6* and −41% for *Mmp3*, Figure 2B). For cartilage we also observed a trend to decreased *Mmp3* (−29%, $p=0.09$) with no effect on *Il6* expression (Figure 2C). The anti-inflammatory effects of nicotine were not observed in TgCHRFAM7A knee joint tissues, neither in meniscus (Figure 2D), synovium (Figure 2E) or cartilage (Figure 2F) suggesting a complete loss of α7nAChR response in those tissues.

To further examine the impact of CHRFAM7A on the joint cell responses, we confirmed as previously shown [9] that in immature murine chondrocytes (iMACs) from WT mice, activation of nAChR by nicotine prevented IL-1β induced *Il6* (−58%, $p<0.05$), *Mmp3* (−43%, $p<0.05$) and *Mmp13* (−52%, $p<0.05$) expressions (Figure 2G). Nicotine also significantly prevented TNFα-induced *Il6* and *Mmp3* expressions in iMACs from WT mice

(Supp. Fig. S3). However, iMACs from TgCHRFAM7A mice, showed a loss of agonistic effect since nicotine pre-treatment had no effect on gene expression induced by IL-1β or TNFα (Figure 2H–Supp. Fig. S3).

CHRFAM7A enhances pain-related hypersensitivity and increases OA pain behaviors in mice

As α7nAChR activation is known to decrease pain [11,25], we evaluated the impact of TgCHRFAM7A on mechanical allodynia in DMM and MIA models [26].

Using von Frey filaments (VF), we found that TgCHRFAM7A mice had a significantly more severe allodynia compared to WT after DMM in ipsilateral paw (genotype effect $F(1,22)=14.28$, $p=0.001$) with a significant difference at D+14, D+28 and D+56 in post-hoc analysis (Figure 3A). Notably, TgCHRFAM7A had also more allodynia after SHAM surgery compared to WT mice (genotype effect $F(1,28)=13.55$,$p=0.001$) with significant differences at D+14, D+42 and D+56 in post-hoc analysis (Figure 3B). In the MIA model, we used two different methods to evaluate allodynia: VF and pressure application measure (PAM). We found an overall effect of the genotype on pain for both analysis methods with a significantly higher allodynia in TgCHRFAM7A compared to WT mice using VF (genotype effect, $F(1,17)=11.44$, $p=0.0035$) but with no difference in post-hoc analysis at different time points (Figure 3C) and PAM (genotype effect, $F(1,17)=16.29$, $p<0.001$) with significant differences at D+3 and D+7 (Figure 3D).

We performed more detailed pain analyses since we observed not only increased pain behaviors of the TgCHRFAM7A mice during induced OA, but also at baseline prior to any injury (D-7: Figure 3A and 3B, Baseline: Figure 3C and 3D). Twelve-weeks old naïve TgCHRFAM7A mice had a lower threshold in the VF testing (0.65±0.04 *versus* 1.01±0.07, $p<0.0001$) (Figure 3E) and in PAM (433.9±15.98 *versus* 486.9±10.06, $p=0.01$) (Figure 3F) compared to WT mice. We observed no difference in spontaneous nocifensive behavior such as licking, biting, or paw-lifting. We confirmed these results in 20 weeks old TgCHFRFAM7A (Supp. Fig. S4). To extend these findings in another pain model, we applied the formalin pain test [27], and observed an increased reactivity after formalin application in TgCHRFAM7A mice especially during the second inflammatory phase (177±23 sec in WT *versus* 276±19 sec in TgCHRFAM7A mice, $p<0.001$; Figure 3E).

TgCHRFAM7A mice display altered patterns of macrophage infiltration and chemokine expression in DRG

To address mechanisms for the differences in pain behaviors, we first evaluated the innervation of knee joints (synovium and vascular meniscus) using pan neuronal marker PGP 9.5 in naïve WT and TgCHRFAM7A mice and found no difference in nerve fiber density between the two strains (Supp. Fig. S5A). As well, we found no difference in the expression of spinal cord c-Fos as a marker of central neuronal activation (Supp. Fig. S5B).

We hypothesized that TgCHRFAM7A mice might have more pain due to increased macrophage invasion in DRG because TgCHRFAM7A mice have elevated numbers of hematopoietic stem cells in bone marrow toward macrophage lineages [24] and macrophage invasion in DRG has been linked to pain [28,29]. In the L3-L5 DRG, naïve 12-weeks old

mice, TgCHRFAM7A had an increased number of F4/80+ macrophages/mm^2 prior to any injury (Figure 4A and 4B). After DMM, right ipsilateral L3-L5 DRG showed increased numbers of F4/80+ macrophages, with significantly more in TgCHRFAM7A compared to WT (p=0.05) (Figure 4C and 4D). Interestingly, the mean number of macrophages in right L3-L5 DRG negatively correlated with the VF threshold (Figure 4E).

Because macrophage recruitment in DRG is mediated by cytokines and chemokines, we examined mRNA expression in whole L3-L5 DRG of naïve WT and TgCHRFAM7A mice. We found a significant increase in *Cx3cl1/Fractalkine* (Cx3cl1/Fkn) expression in TgCHRFAM7A mice DRG compared to WT while there was no difference for its receptor *Cx3cr1* (Figure 4F) nor for *Ccl2/Mcp-1* and its *Ccr2 receptor*, *Il6*, *Il1β* or *Tnf* expression (nor for any neuronal markers) (Supp. Fig. S6A and S6B). The increase of CX3CL1/FKN expression was confirmed by immunofluorescence (Figure 4G and 4H) in naïve DRG TgCHRFAM7A compared to WT. While the protein expression of CX3CL1/FKN in naïve WT DRG was almost undetectable, it was significantly increased in TgCHRFAM7A DRG (Figure 4G and 4H). The overall CX3CL1/FKN protein expression correlated with numbers of macrophages in each DRG (r=0.6, p=0.01).

To investigate differences in DRG more comprehensively, we performed RNA-sequencing on L3-L5 DRG from 12 weeks-old naïve mice, and mice with SHAM or DMM surgery.

In DRG of naïve TgCHRFAM7A mice, we found 755 upregulated genes and 550 downregulated genes compared to WT using p-value <0.05 (Supp. Table 1). DRG of SHAM TgCHRFAM7A mice had 891 upregulated genes and 732 downregulated genes while DRG of DMM TgCHRFAM7A mice had 302 upregulated genes and 387 downregulated genes compared to WT SHAM and DMM DRG, respectively (Supp. Table 2 and 3). As shown on the Venn diagram, only 10 genes were differentially expressed (DE) in the 3 conditions (i.e, Naïve, SHAM and DMM) between TgCHRFAM7A and WT (Figure 5A and 5B). Among these 10 genes, the expression was increased or decreased in TgCHRFAM7A DRG with a similar pattern in naïve and DMM DRG compared to SHAM. *Cacnb3* (Calcium Voltage-Gated Channel Auxiliary Subunit Beta 3) was the only gene known to be involved in pain [30]. It encodes the beta subunit of High voltage-activated calcium channels (HVACCs). *Cacnb3* expression is increased in DRG during spinal nerve ligation (SNL) while its inhibition by siRNA is decreased the pain-induced by SNL [30].

The genes highly DE (logFC≥2 or logFC<−2 and p <0.05) were used for gene ontology (GO) pathway analysis for biological processes (BP) (Supp. Table 1–3). In naïve DRG, the 317 genes upregulated with log FC≥2 gave 24 significantly enriched GO BP (Supp. Fig S7). The most enriched GO BP were related to immune and inflammatory responses (Figure 5C).

Even in SHAM DRG, the 291 genes upregulated in TgCHRFAM7A mice with log FC≥2 gave 11 significantly enriched GO BP and the most enriched were similar as those seen in the DMM knees (Figure 5D). Finally, for DMM DRG only 28 genes were upregulated with log FC≥2 which gave 6 significantly enriched GO BP which were all related to leukocyte recruitment or activation (Figure 5E). Four genes (Pro-Platelet Basic Protein [*Ppbp*] encoding the chemokine Chemokine (C-X-C motif) ligand 7 (*Cxcl7*), C-X-C Motif

Chemokine Receptor 2 [*Cxcr2*], *Cd177*, and Proteinase 3 [*Prtn3*]) were involved in the enrichment for all these pathways.

This confirms the enhancement of immune and inflammatory processes within the DRG in TgCHRFAM7A mice. The 101, 221 and 29 downregulated genes with a logFC<−2 in DRG of naïve, SHAM and DMM of TgCHRFAM7A compared to WT, respectively did not yield any significant GO terms.

CHRFAM7A decreases agonist binding to α7nAChR and nicotine anti-nociceptive effect in DRG

We hypothesized that the differences in pain behaviors associated with increased F4/80+ macrophage recruitment were due a loss of the α7nAChR anti-nociceptive functional response. *Chrna7* mRNA levels in DRG were similar between WT and TgCHRFAM7A mice while, as expected, *Chrfam7a* mRNA was only detectable in TgCHRFAM7A mice (Figure 6A). Despite no difference in *Chrna7* mRNA, DRG in TgCHRFAM7A mice had a significant loss of ligand binding capacity as shown using the specific biotinylated α7nAChR antagonist α-bungarotoxin; 5 μg/L (Figure 6B and 6C). We thus evaluated the effect of CHRFAM7A on the anti-nociceptive response to exogenous agonist using nicotine in the DMM model. As shown above (Figure 3A), 8 weeks after DMM, WT and TgCHRFAM7A mice had developed allodynia. In WT mice, IP injection of nicotine 0.5 and 1 mg/kg decreased knee allodynia in a dose dependent manner with a maximum effect at 5 minutes ($p<0.05$ between PBS and nicotine 1 mg/kg). In contrast, TgCHRFAM7A mice showed no detectable effect on allodynia at any dose of nicotine ($p=0.006$ and $p=0.01$ between nicotine 1mg/kg in TgCHRFAM7A and WT at 5 and 15 minutes, respectively) (Figure 6D). Furthermore, in the MIA model, we observed a significant difference in the decreased allodynia between the two strains ($p=0.05$ between nicotine 1 mg/kg WT and TgCHRFAM7A) (Figure 6E).

CHRFAM7A is expressed in human joint tissues and is associated with decreased human chondrocyte responses to nicotinic agonist

To extend and validate our observations in the mouse models, we analyzed human tissues and cells (see Suppl. Table 4 for donor information). CHRNA7 and CHRFAM7A mRNA were both expressed in joint (cartilage, meniscus, synovium) and neuronal tissues (DRG, spinal cord), except that 2 out of 5 DRG did not express CHRFAM7A. The overall mRNA expression of CHRFAM7A and CHRNA7 in the joint tissues was in a similar range as the expression in the neuronal tissues. While CHRFAM7A expression did not differ between the tissues, the CHRNA7 was significantly higher in synovium, spinal cord and DRG compared to meniscus and cartilage (Figure 7A and 7B). The CHRFAM7A/CHRNA7 ratio was >1 in vascular, avascular meniscus and cartilage, indicating that CHRFAM7A is predominant in these tissues while it was always <1 in spinal cord, DRG and, in most donors, in synovium (Figure 7C). In the joint tissues, the ratio of CHRFAM7A/CHRNA7 was increased in OA cartilage (n=10) compared to normal cartilage (n=7) and not in other tissues (Figure 7D). These results suggest that the high expression of CHRFAM7A in cartilage, especially during OA, might decrease the response to endogenous and/or exogenous α7nAChR agonists and might dampen articular protective effect mediated by α7nAChR.

Indeed, with increasing copy number of CHRFAM7A the ratio of CHRFAM7A/CHRNA7 also increased (Figure 7E). In human OA chondrocytes from patients with one copy number, nicotine pre-treatment still decreased IL-1β-induced IL6 in a dose dependent manner with a significant decrease at 100 μM (−34% compared to IL-1β alone, p=0.03) (Figure 7F). This effect of nicotine on IL-1β-induced IL6 expression was blunted and non-significant in OA chondrocytes from patients with two copies of CHRFAM7A (Figure 7G).

DISCUSSION

This study demonstrates the impact of the human specific duplicate CHRFAM7A on OA-associated tissue damage and OA-associated pain. Unexpectedly, we observed that the sole presence of the gene duplication imparts an increased basal sensitivity in normal mice and an enhanced response in several different pain models. The expression of CHRFAM7A was associated with decreased α7nAChR responses to agonists and with the loss of anti-inflammatory effects α7nAChR on joint tissues and cells from mice and humans. The decrease of functional α7nAChR appears to be responsible for an increased CX3C1/FKN release associated with an activation of immune and inflammation pathways and macrophage recruitment in DRG of TgCHRFAM7A mice. These two features are known to be associated with increased pain phenotypes [28,31–33]. We showed that human CHRFAM7A is expressed in all joint tissues and that the copy number has a dose-dependent effect on the response to agonist in non-neuronal cells.

The impact of CHRFAM7A on OA histopathological severity in Tg mice could be due to a decreased anti-inflammatory and anti-catabolic effect of endogenous local non-neuronal and/or neuronal acetylcholine on the native α7nAChR. We have found earlier that chondrocytes expressed all components responsible for Ach metabolism and that cholinergic nerves are present in human subchondral bone, which has been recently confirmed in mice [9,13,14]. Furthermore, nicotinic receptor activation by intra-articular nicotine injection decreased joint tissue responses to inflammatory stress confirming a local effect on joint tissues in mice. CHRFAM7A is associated with a decreased response of joint tissues *in vivo*, and chondrocyte response to nicotinic receptor agonist ligand *in vitro* both in murine and human cells. This is consistent with the more severe post-traumatic OA in α7nAChR KO mice [9]. Thus, the activation of the local non-neuronal cholinergic system is a novel and interesting target for disease-modifying OA drugs. While the high local expression of CHRFAM7A in cartilage and meniscus might be challenging for the application of agonists, drugs that interfere with the expression, stability, or competing function of the mutant protein are potentially better approaches.

This study for the first time implies that CHRFAM7A is involved in determining overall pain sensitivity. In mice, the systemic α7nAChR activation has previously been found to have anti-nociceptive effects in several pain models [25,34] and in OA [35]. As well, CHRNA7 mRNA expression in DRG decreased after DMM [31], but the causal involvement of this CHRNA7 decrease in pain was not understood until a recent study showed that the local injection of *Chrna7* siRNA in DRG aggravated allodynia [32]. Here, we found, that CHRFAM7A expression decreases the function of α7nAChR in DRG and that this was associated with increased sensitivity to pain stimuli as measured by von Frey testing and

PAM in post-traumatic OA induced by DMM, in the MIA model, and even in mice with SHAM surgery or without any OA induction. The difference between the two genotypes seems to be mostly associated with the baseline hypersensitivity that persists in all pain models. However, as expected, the profile of pain varied between the two models of OA [36]. Mice had higher mechanical allodynia after MIA than DMM but the overall effect of genotype on allodynia still remained and was higher in TgCHRFAM7A than in WT.

We confirmed that nicotine displays acute anti-nociceptive effects in WT mice after DMM and MIA, but CHRFAM7A almost totally abolished this effect. We can therefore propose that CHRFAM7A expression is responsible for a loss of α7nAChR anti-nociceptive response to ACh in DRG. However, we cannot rule out a specific and Chrna7-independent effect of CHFRAM7A or a central effect, especially because the effect of nicotine analgesia was short. The decrease of functional α7nAChR was associated with an increase in local CX3CL1/FKN release and with increased immune processes that allow macrophage recruitment in DRG [37]. Since TgCHRFAM7A mice have higher numbers of hematopoietic stem cells toward monocyte/macrophages lineages [24], we can hypothesize that the impact of CX3CL1/FKN on macrophage recruitment to DRG is of importance in TgCHRFAM7A mice. While we analyzed several additional inflammatory mediators including CCL2/MCP1, CCR2, IbA1, TNF or IL6 they were not significantly different in DRGs of transgenic mice compared to WT. In this regard, RNA-sequencing confirmed the enrichment in the activation of several immune pathways in DRG of naïve TgCHRFAM7A mice and after, SHAM and DMM surgery. The close association between CHRFAM7A, immunity and inflammation raised questions about potential benefits of this gene in humans. We can speculate that it has emerged to enhance the response of humans to different types of stress by regulating the activity of the native a7nAchR. Furthermore, since the ratio of CHRNA7 and CHRFAM7A is very different in neuronal and non-neuronal tissues, it might have emerged for a better local regulation of cholinergic immune regulation in specific tissues, and that is why tissue-specific expression of the transgene in mice is needed in future studies.

This study has some limitations. First, we evaluated the post-traumatic pain during 8 weeks after DMM while the OA-related chronic pain develops at later timepoints following DMM [38,39]. Even if we observed a significant difference between SHAM and DMM level of pain from the week 42 for WT and 56 for TgCHRFAM7A, longer evaluation could have been more efficient to separate pain triggered by surgery versus OA. Furthermore, we conclude that the specific effect of TgCHRFAM7A is due to the higher inflammatory state but since we did not modulate inflammation in TgCHRFAM7A, we do not have direct proof for this link. However, inflammation in DRG and in joint are known to promote both pain and OA-associated tissue damage. We cannot rule out the association with a systemic effect of the CHRFAM7A/CHRNA7 axis since TgCHRFAM7A mice express CHRFAM7A in all tissues. Tissue specific transgenic mice could allow to a better understanding of the transgene's local effect. However, humans express CHRFAM7A ubiquitously and the effect could be also both, local and systemic in humans. Finally, we did not evaluate the impact of the transgene on the neuronal electrophysiological response to agonist which might be reduced in DRG since CHRFAM7A is a dominant negative regulator of the native a7nAchR in vitro [17,21,40]. However, we evaluated an indirect marker of neuronal activation using

c-fos expression and we did not see significant differences between WT and transgenic mice.

Most of the human population has one or two copies of the CHRFAM7A, but a polymorphism with a 2bp deletion has appeared in the exon 6 of CHRFAM7A only. This deletion polymorphism modulates the effect of CHRFAM7A as a null allele for some or as a more potent negative α7nAChR regulator for others in xenopus oocytes [17,21]. Evaluation of the impact of this polymorphism on anti-inflammatory and anti-nociceptive effect of α7nAChR is of interest to a better understanding of its impact in human as it could better define functional CHRFAM7A carriers (75%) and non-carriers (25%). Comparison of copy number and this deletion between patients with and without OA, after controlling for confounding factors, must be evaluated in large cohorts with respect to sensitivity to pain stimuli.

To conclude, we observed that CHRFAM7A is associated with a more severe structural damage and pain in OA. This is due to a decrease of agonist binding with negative effects on protective signaling through the native Ach/α7nAChR axis. Evaluation of agonist α7nAChR as a therapy in humans should be done according to the genotype to better understand its relevance in humans. Finally, CHRFAM7A appears especially important in setting the threshold of sensitivity to pain which can be explained by increased FKN which induces macrophage recruitment and pain sensitization. These findings reveal CHRFAM7A as a new and promising target for OA disease modification and for pain management not only in OA but also in other pain conditions.

MATERIALS AND METHODS

Experimental procedures are provided in online supplemental materials and methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability:

All data relevant to the study are included in the article or uploaded as online supplemental information. The GEO accession numbers for all the datasets utilized in the present study will be made available upon acceptance of the manuscript and included in the final version.

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Key messages:

What is already known about this subject?

Alpha 7 nicotinic acetylcholine receptor (α7nAChR) activation decreases pain and inflammation in several conditions such as osteoarthritis (OA) while its deletion aggravates post-traumatic knee OA in mice. CHRFAM7A is a specific human gene duplicate of the native gene Chrna7 encoding for the α7 subunit. CHRFAM7A functions as a dominant negative regulator of α7nAChR *in vitro* but its role in OA-associated joint tissue damage and pain is unknown.

What does this study add?

Transgenic CHRFAM7A expression in mice increased OA histological severity and OA pain due a decrease in agonist response of the native α7nAChR, compromising its attenuation of inflammation and pain. Furthermore, CHRFAM7A modulates pain behavior and decreases baseline threshold sensitivity to pain due to an increased recruitment of macrophages in dorsal root ganglia (DRG) associated with a local CX3CL1/fractalkine overexpression.

How might this impact on clinical practice or future developments?

In humans, genotyping for CHRFAM7A is necessary to determine patients' ability to respond to treatment with α7nAChR agonists. As CHRFAM7A is a determinant for pain threshold in humans, it represents a novel target for pain control.

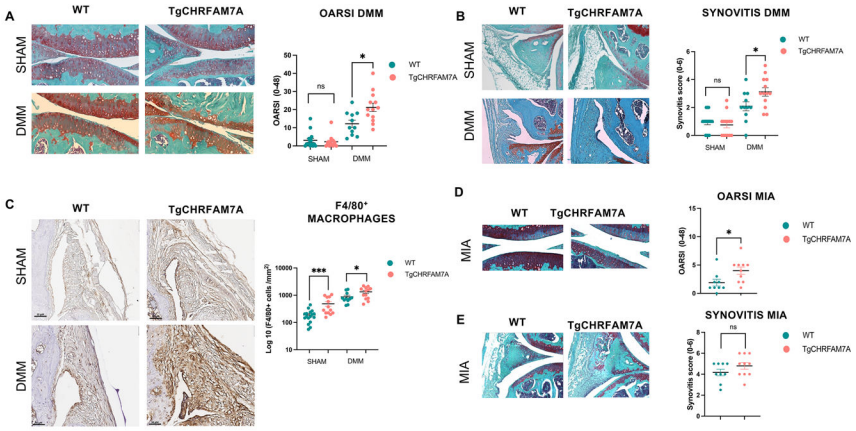

Figure 1: CHRFAM7A is associated with more severe OA.

Cartilage OARSI (0–48) (**A**) and (**B**) synovitis histological scoring (using semi-quantitative methods (0–6)) of knees from WT and TgCHRFAM7A mice 8 weeks after DMM (n=11 WT, n=13 Tg CHRFAM7A) or SHAM surgery, (n=16 WT, n= 14 TgCHRFAM7A); (**C**) Representative and quantitative analysis of F4/80+ macrophage invasion in synovium of WT and TgCHRFAM7A mice after SHAM and DMM surgeries. Cartilage OARSI (0–48) (**D**) and synovitis (**E**) histological scoring (using semi-quantitative methods (0–6)) of knees from WT and TgCHRFAM7A mice 3 weeks after MIA (n=9 WT, n=10 TgCHRFAM7A). Two group comparisons (WT and TgCHRFAM7A) were done using unpaired non-parametric t-tests, *p<0.05, ***p<0.001.

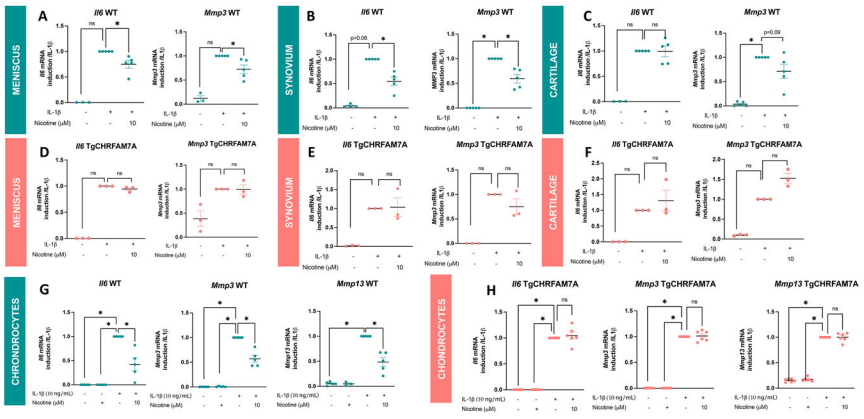

Figure 2: CHRFAM7A blunts anti-inflammatory effects of nicotinic receptor activation in murine joint tissues and chondrocytes.

Mouse knees were injected with PBS, IL-1β (30 ng) alone or with nicotine (10 μM) and tissues were collected 2h later for RNA isolation and RTqPCR. Expression of *Il6* and *Mmp3* mRNA in meniscus **(A)**, synovium **(B)** and cartilage **(C)** of 11 to 13 weeks old WT mice or meniscus (**D**), synovium (**E**) and cartilage (**F**) of TgCHRFAM7A mice. Chondrocytes from WT (n=3 independent littermates, 1 to 2 replicates) (**G**) and TgCHRFAM7A mice (n=3 independent littermates, 2 replicates) (**H**) were pretreated with 10 μM nicotine for 15 minutes and stimulated with 10 ng/ml IL-1β for 24 hours and RTqPCR was performed for *Il6, Mmp3* and *Mmp13* mRNA. Results are normalized on IL-1β condition, non-parametric paired t-test *p<0.05, ns: not significant.

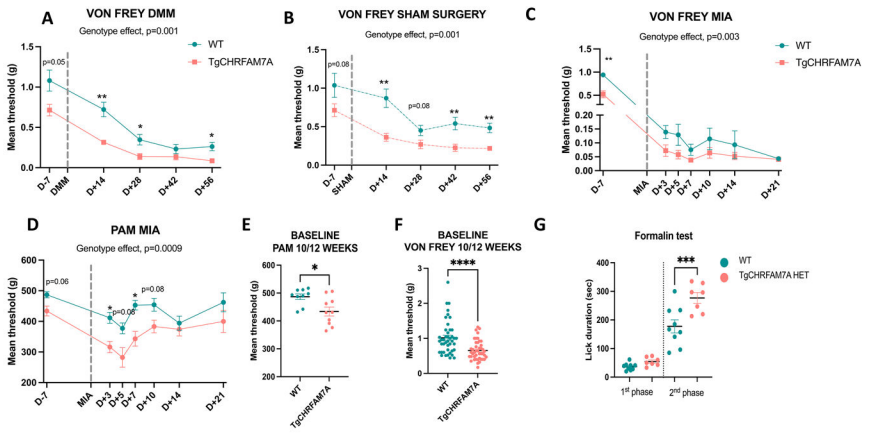

Figure 3: CHRFAM7A regulates pain-related hypersensitivity and increases pain behaviors in mice.

Mechanical allodynia was determined by mean threshold of withdrawal in von Frey filament testing on the paws of the limb with DMM (**A**) or SHAM surgery (**B**) of WT (green) and TgCHRFAM7A mice (red) at the indicated time points (7 days before surgery [D-7], 14, 28, 42 and 56 days after surgery [D+14, D+28, D+42, D+56]). Mechanical allodynia testing of the paw from the MIA injected limb using von Frey (**C**) or pressure application measure (PAM) device (**D**) of WT (green) and TgCHRFAM7A (red) mice at different time points (2 days before surgery as baseline, 3, 5, 7,10, 14 and 21 days after surgery. Data are expressed as mean ±SEM, two-way ANOVA with Holm-Šidák multiple comparisons post-tests between WT and TgCHRFAM7A mice over time. Comparison of mean withdrawal threshold between 12 weeks-old WT and TgCHRFAM7A mice using von Frey Filament testing applied on the right hind paw **(E)** and PAM on the right knee **(F).** Time intervals (seconds) of paw licking were measured in WT (green) and TgCHRFAM7A (pink) mice after formalin injection (**G**) during the first phase (0–5 minutes) and the second phase response (20–40 minutes). Unpaired non-parametric t-test or one-way ANOVA for two-groups and multiple group comparison, respectively, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

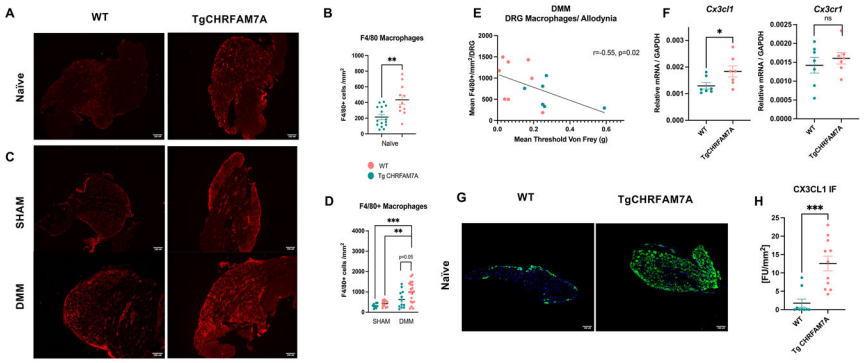

Figure 4: Macrophage infiltration and chemokine expression in DRG.

Representative images (**A**) and quantification (**B**) of macrophages (F4/80+ cells per mm^2) in L3-L5 DRG of naïve 12 weeks-old mice and (**C**) (**D**) SHAM/DMM WT and TgCHRFAM7A mice as determined by immunofluorescence using anti-rat F4/80+ (1:300) and analyzed by using Fiji. Each dot represents one lumbar L3-L5 DRG. (**E**) Spearman correlation between macrophages (F4/80+ cells per mm^2) in L3-L5 DRG and allodynia (von Frey mean threshold) in WT (green) and TgCHRFAM7A mice (red). Each dot is the mean number of macrophages in one mouse (L3-L5 DRG). (**F**) RTqPCR analysis of *Cx3cl1/Fkn* and its receptor *Cx3cr1* in DRG of naïve WT and TgCHRFAM7A mice. Representative images (**G**) and quantification (**H**) of CX3CL1/FKN immunofluorescence in DRG of naïve WT and TgCHRFAM7A mice. Each dot represents one lumbar L3-L5 DRG. Data are expressed as mean ±SEM. Two-group comparisons were performed using non-parametric t-test and multiple groups comparison was performed using one-way ANOVA with Tukey multiple comparisons post-tests. *p<0.05, **p<0.01, ***p<0.001.

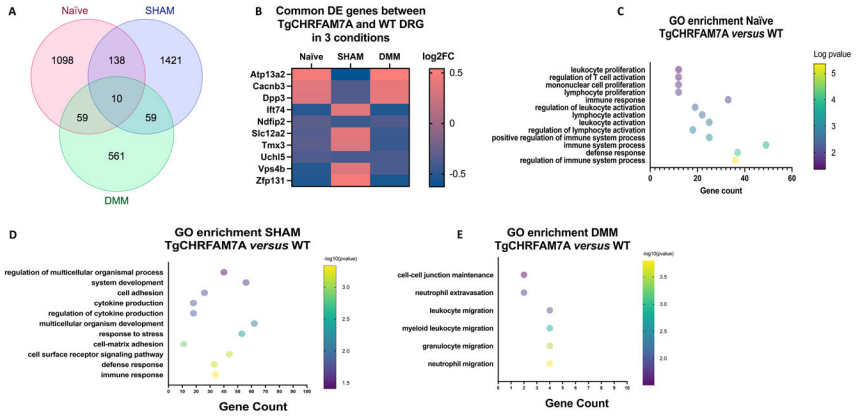

Figure 5: RNA-sequencing of naïve, and 8 weeks after SHAM and DMM in right L3-L5 DRG of WT and TgCHRFAM7A mice.

Venn diagram (**A**) of the DE genes between WT and TgCHRFAM7A in each condition. (**B**) The 10 common genes differentially expressed in right L3-L5 DRG TgCHRFAM7A (Naïve, SHAM and DMM, 8 weeks after surgery) compared to WT. Gene ontology (GO) biological pathways significantly enriched (p-adjusted< 0.05, represented as −log10 on a color scale on the right) in Naïve (with most relevant pathways in term of function) (**C**), SHAM (**D**) and DMM (**E**) DRG TgCHRFAM7A mice compared to DRG WT using DE genes upregulated with a logFC≥2 and a p<0.05.

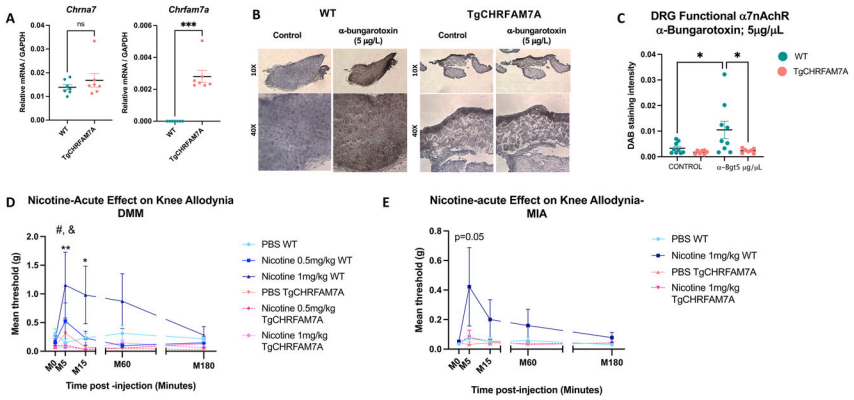

Figure 6: TgCHRFAM7A mice have a decreased agonist binding in DRG and a decreased anti-nociceptive response.

Quantitative expression of CHRNA7, CHRFAM7A mRNA in DRG of WT and TgCHRFAM7A mice **(A)**. Representative images **(B)** DAB quantification **(C)** of the α-bungarotoxin (5 µg/L) staining on DRG of 12-weeks old naïve WT and TgCHRFAM7A mice. Nicotine anti-nociceptive effect on mechanical allodynia induced by DMM (8 weeks after surgery) (**D**) and MIA (3 weeks after injection) (**E**) in WT and TgCHRFAM7A mice: mean threshold of DMM or MIA right paw withdrawal at the indicated von Frey filaments in WT and TgCHRFAM7A mice before and 5, 15 minutes, 1 and 3 hours after intra-peritoneal injection of PBS, Nicotine 0.5mg/kg (only for DMM) or nicotine 1 mg/kg. #$p<0.05$ between PBS WT and nicotine 1mg/kg WT, &$p<0.05$ between nicotine 0.5 mg/kg WT and nicotine 0.5mg/kg TgCHrFAM7A, ** $p<0.01$ and*$p<0.05$ between nicotine 1mg/kg in WT and nicotine 1mg/kg in TgCHRFAM7A mice. Data are expressed as mean ±SEM. For A to E, comparisons were performed using non-parametric t-test. Results from behavioral testing were analyzed by two-way ANOVA with Holm-Šidák multiple comparisons post-tests between WT and TgCHRFAM7A mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

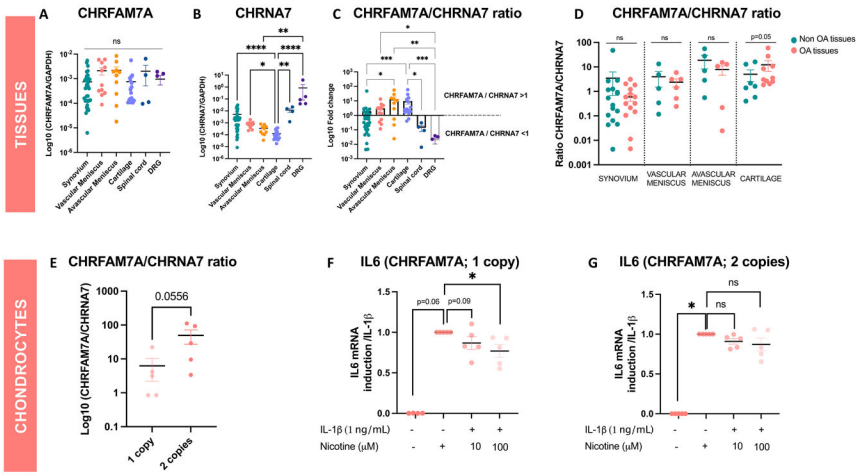

Figure 7: CHRFAM7A is expressed in all joint tissues with an increased CHRFAM7A/CHRNA7 ratio in OA cartilage and its expression is associated with a decreased chondrocyte response to agonist.

Relative mRNA expression of CHRFAM7A **(A)**, CHRNA7 **(B)** and of the CHRFAM7A/CHRNA7 ratio **(C)** in human synovium (n=39), vascular meniscus (n=11), avascular meniscus (n=10) cartilage (n=17), spinal cord (n=4) and lumbar DRG (n=5). Comparison of the CHRFAM7A/CHRNA7 ratio between human normal and OA synovium, meniscus, and cartilage (**D**) and in human OA chondrocytes according to the number of copies of CHRFAM7A (one or two copies, n=5 per group) (**E**). Relative quantitative expression of IL-6 mRNA in human OA chondrocytes pretreated with 1, 10, or 100 μ*M* nicotine for 15 minutes and stimulated with 1 ng/ml IL-1β for 24 hours among patients with one copy (**F**) and two copies (**G**) of CHRFAM7A. Data are expressed as mean ±SEM. Comparison were performed using non-parametric t-tests or one-way ANOVA with Tukey multiple comparisons post-tests if gaussian distribution was obtained. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant.