Musculoskeletal disorders represent the 3rd greatest burden on health in the developed world. Osteoarthritis is the single greatest cause of chronic pain, has no cure, and affects 8.5 and 27 million in the UK and US respectively. Osteoarthritis commonly occurs after joint injury, particularly affecting younger patients. Painful joints are often treated with injections of steroid or hyaluronic acid (HA), but treatments to prevent subsequent joint degeneration remain elusive. In animals, joint injury increases glutamate release into the joint, acting on nerves to cause pain, and joint tissues to cause inflammation and degeneration. This study investigated synovial fluid glutamate concentrations and glutamate receptor (GluR) expression in injured human joints and compared efficacy of GluR antagonists with current treatments in a mouse model of injury-induced osteoarthritis (ACL rupture). GluRs were expressed in ligament and meniscus after knee injury and synovial fluid glutamate concentrations ranged from 19–129 µM. Intra-articular injection of NBQX (GluR antagonist), administered at the time of injury, substantially reduced swelling and degeneration in the mouse ACL rupture model. HA had no effect and depo-medrone reduced swelling for 1 day, but increased degeneration by 50%. Intra-articular administration of NBQX was both symptom and disease modifying to a greater extent than current treatments. There is an opportunity for repurposing related drugs, developed for CNS disorders, with proven safety in man, to […]

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Title:
AMPA/Kainate glutamate receptor antagonists prevent post-traumatic osteoarthritis

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Abstract

Musculoskeletal disorders represent the 3rd greatest burden on health in the developed world. Osteoarthritis is the single greatest cause of chronic pain, has no cure, and affects 8.5 and 27 million in the UK and US respectively. Osteoarthritis commonly occurs after joint injury, particularly affecting younger patients. Painful joints are often treated with injections of steroid or hyaluronic acid (HA), but treatments to prevent subsequent joint degeneration remain elusive. In animals, joint injury increases glutamate release into the joint, acting on nerves to cause pain, and joint tissues to cause inflammation and degeneration. This study investigated synovial fluid glutamate concentrations and glutamate receptor (GluR) expression in injured human joints and compared efficacy of GluR antagonists with current treatments in a mouse model of injury-induced osteoarthritis (ACL rupture).

GluRs were expressed in ligament and meniscus after knee injury and synovial fluid glutamate concentrations ranged from 19-129µM. Intra-articular injection of NBQX (GluR antagonist), administered at the time of injury, substantially reduced swelling and degeneration in the mouse ACL rupture model. HA had no effect and depot-medrone reduced swelling for 1 day, but increased degeneration by 50%.

Intra-articular administration of NBQX was both symptom and disease modifying to a greater extent than current treatments. There is an opportunity for repurposing related drugs, developed for CNS disorders, with proven safety in man, to prevent injury-induced osteoarthritis. This could quickly reduce the substantial burden associated with osteoarthritis.
**Introduction.**

Musculoskeletal disorders represent the 3rd greatest burden on the health of the world’s population in developed countries causing 21.3% of the total years lived with disability globally (1). Osteoarthritis (OA) affects 8.5 million in the UK (2, 3) and 27 million in the US (4), causing substantial physical, psychological, and socioeconomic burden (4-7). At least 12% of the current OA population was caused by prior joint injury (8, 9), so called post-traumatic OA (PTOA). For example, anterior cruciate ligament rupture (ACLr) causes knee OA in 50-90% of patients 5-15 years later (10-12). This example, along with other joint injuries (13-16), potentially inflicts OA at a young age. Since there are no disease modifying treatments for OA, which usually manifests after substantial joint damage has already occurred, effective interventions at the time of injury offer a new opportunity for prevention of PTOA.

Current treatments for unresolved joint pain, early OA and joint injuries include intra-articular (i.a.) steroid (e.g. depo-medrone) or hyaluronic acid (HA), but few preclinical studies test whether these, or other agents, given i.a. at the time of joint injury influence OA progression. Intra-articular amnion/chorion membrane 24 hours post-meniscal transection surgery in rats (17), dexamethasone immediately following surgical drill injury in rabbits (18) and interleukin 1 receptor antagonist (IL-1RA), given immediately post-articular fracture in mice (19, 20), all reduced joint degeneration to some extent. Of these, IL-1RA has progressed to Phase 2 trials for prevention of PTOA (ClinicalTrials.gov: NCT02930122), whereas dexamethasone (ClinicalTrials.gov: NCT02318433) is undergoing Phase 1 trials for this indication.
Glutamate signalling contributes to pain, inflammation, bone remodelling and degradation in arthritis (21, 22). Synovial fluid (SF) glutamate concentrations increase ~54 fold in arthritic patients compared to cadaveric non-OA samples (23), and double after inflammatory arthritis induction (24), and ACL transection induced OA (25), in rats in vivo. Increased glutamate released during arthritis acts on nerves to drive peripheral pain, with locally delivered glutamate receptor (GluR) antagonists inhibiting pain behaviour in carrageenan induced arthritis (26), monosodium-iodoacetate induced arthritis (27), antigen induced arthritis (AIA) (22), and inflammatory pain in arthritic mice (28). Glutamate also regulates inflammation. Rheumatoid arthritis (RA) patient SF glutamate concentrations correlate with chemokine levels and GluR agonists induce tumour necrosis factor α (TNFα) release in RA synovial cells (29) and increase TNFα and RANTES release by human synovial cells (30). Human RA synoviocytes express functional AMPA/kainate GluRs, which regulate IL-6 release (31), an essential mediator of arthritic joint degradation (32). Glutamate also influences pathological processes. We showed that a single i.a. injection of NBQX (AMPA/kainate antagonist) in rat AIA significantly reduced knee swelling by 33%, histological synovial inflammation by 34% and joint degeneration scores by 27%, exceeding efficacy of etanercept, infliximab and methotrexate (22). Others have shown that memantine (NMDA GluR antagonist, intra-peritoneal every 12-24hrs) reduces synovitis and bone erosions in collagen induced arthritis (33).

Previously, we showed that subchondral bone remodelling, an early driver of OA pathology (34-36), was substantially reduced by NBQX treatment in rat AIA (22). Spontaneous OA animal models show that subchondral bone remodelling precedes cartilage damage (36-40) and correlates with cartilage lesions (41). In humans, ACLr leads to subchondral bone
remodelling that precedes cartilage destruction (34), with bone bruises occurring in 80% of patients within 2 weeks of ACLr that later associate with cartilage damage (42). Thus, using AMPA/kainate GluR antagonists following trauma to inhibit these early pathologic changes in bone, may reduce subsequent cartilage degradation.

We hypothesised that glutamate drives OA initiation following acute injury and that GluR antagonists could prevent OA symptoms and joint degeneration. Glutamate signals through ionotropic (iGluRs- AMPA, kainate, NMDA) and metabotropic (mGluR1-8) GluRs expressed by all joint tissues (21, 22). Drugs targeting these receptors have been thoroughly investigated for disorders of the nervous system such as epilepsy, motor neurone disease, stroke and migraine, and offer a rich opportunity for quick translation to new indications.

Although AMPA/kainate GluR antagonists are disease modifying in AIA (22) and SF glutamate concentrations increase in human and animal arthritis (23-25), it is not known whether glutamate concentrations vary at the time of traumatic injury in humans or animals, or whether GluR antagonists given at the time of injury are protective. Here, we measure SF glutamate concentrations after human ACL injury and demonstrate that i.a. NBQX treatment given at the time of injury was disease modifying in the mouse ACLr model, where controlled loading of the joint ruptures the ligament in the absence of surgery (43). NBQX efficacy exceeded that of depo-medrone and HA, which when given in the same way at doses used clinically, were either ineffective (HA) or increased joint degeneration (depo-medrone). AMPA/kainate receptor antagonists originally developed for epilepsy, migraine and pain, and already safety tested in man, represent a promising translational opportunity to repurpose for prevention of injury-induced osteoarthritis.
Results.

Glutamate concentration is similar in OA and post-injury and GluRs are expressed in knee tissues from injured patients.

Mean glutamate concentrations were highest in RA SF (62.3µM±8.5), followed by ACLr (55.2µM±4.98), meniscal tear (43.1µM±14.42) and OA (39.2µM±9.44) (Figure 1A, n=5/group, except ACLr where n=27 and RA where n=3). Glutamate concentrations varied significantly with time post ACL injury (ANOVA p=0.03), being greater at 0-20 (58.3µM±5.84, p<0.05, n=12) and 21-100 (70.64µM±10.32, p<0.01, n=8) weeks post-injury, compared with 100-500 (34.9µM±7.79, n=7) weeks post-injury (Tukeys, Figure 1B). Across the whole patient cohort, SF glutamate concentrations showed a trend towards reduced concentrations with increasing age (Pearson’s correlation coefficient -0.330, p=0.053, Figure 1C). There were no differences in male and female SF glutamate concentrations (Figure 1D).

AMPA2 and kainate-1 (KA1) were expressed in ACL fibroblasts and meniscal chondrocytes from patients with ACLr or meniscal injury (Figure 1E-H).

NBQX reduces inflammation in ACLr PTOA and is more effective than HA or steroid.

ACLr significantly increased knee swelling in sH2O vehicle treated mice (p<0.001, GLM) and this was significantly reduced by NBQX treatments (p<0.001, GLM) (Figure 2A). On days 1, 2, 3 and 7 post ACLr, knee swelling in vehicle treated mice (0.98-0.75mm) was significantly greater than day 0 swelling (all p<0.001, tukey), whereas NBQX treated mice (0.552-0.419mm) only showed significant increases on day 1 (p=0.007, tukey) and day 2 (p=0.023, tukey) compared to day 0 (Figure 2A). NBQX treatment significantly reduced knee swelling after ACLr on days 1 and 2 compared to vehicle treated mice (by 44% and 45%, p=0.007, p=0.02 respectively, tukey) (Figure
Steroid (depo-medrone) treatment significantly reduced knee swelling on day 1 compared to vehicle \( (p=0.001, \text{tukey}) \) and HA \( (p=0.017, \text{tukey}) \) (Figure 2B). Knee swelling in steroid (all \( p<0.01, \text{tukey} \)), HA (all \( p<0.001, \text{tukey} \)) and vehicle (all \( p<0.001, \text{tukey} \)) treated mice remained significantly higher than day 0 (pre-surgery) measurements until 14 days post-rupture (Figure 2B).

At day 21, synovial hyperplasia and infiltrate induced by ACLr was similar for NBQX, HA and their respective vehicle controls (Kruskal Wallace with Mann Whitney post hoc test) (Figure 2C-E). Steroid treatment increased mean inflammation score, although not significant (Figure 2D&E).

**NBQX does not influence pain related behaviour in ACLr PTOA.**

Lameness score reduced over time for both NBQX and vehicle treated ACLr mice \( (p<0.001, \text{GLM}) \), becoming significantly lower than day 1 scores by day 7, with NBQX treatment having no significant effect on lameness compared to vehicle (Figure 2F). A significant interaction between time and treatment \( (p<0.001, \text{GLM}) \) reveals that NBQX treated ACLr mice were more lame on days 1-3 but were not lame on days 14 and 21 compared to vehicle treated ACLr mice. Irrespective of time, steroid and HA treatment reduced lameness score compared to saline (treatment \( p<0.001 \text{ GLM} \), \( p<0.001 \), steroid vs saline (Tukey); \( p<0.001 \), HA vs saline (Tukey)), although no significant differences were found on individual days and mice remained lame until day 14 (Figure 2G).

**NBQX reduces joint degradation in ACLr PTOA whereas steroid increases damage.**

After ACLr, NBQX treatment reduced cartilage and bone pathology by 29%, from a severity score of 31.3±1.23 to 22.2±2.72 \( (p<0.001, \text{Two-Sample t-Test}) \), although not to naïve values (7.6±0.67).
Individual parameters of the scoring system reveal that ACLr caused substantial loss of cartilage (13.65±0.6), proteoglycan loss (11.4±0.5) and subchondral bone remodelling (6.41±0.39), whereas NBQX reduced these by 26% (10.05±1.12, p=0.001), 25% (8.5±0.96, p=0.004) and 43% (3.65±0.74, p<0.001) respectively (Two-Sample t-Tests, Figure 3C). Significant reductions in joint severity score caused by NBQX treatment were seen in both the medial (29%, from 23.56±1.07 to 16.63±2.65, p<0.05, Mann-Whitney test) and lateral (28%, from 7.77±0.4 to 5.58±0.32, p<0.001, Two-Sample t-Test) sides of the joint (Figure 3D).

Steroid (depo-medrone) (43.79±2.5) treatment significantly increased joint degradation by 52% and 53% compared to HA (28.8±3.03) or saline vehicle treatment (28.71±3.49) respectively (p<0.05, one-way ANOVA) (Figure 3B).

Representative images reveal that the subchondral bone thickening, developing osteophytes and cartilage loss, markedly on the medial side of the joint following ACL rupture, were substantially reduced by NBQX treatment (Figure 3E). Joints treated with HA appeared similar to vehicle controls, however steroid treatment caused severe bone loss down through the growth plate on both medial and lateral sides and also the formation of large chondrophytes (developing osteophytes) and ectopic bone (Figure 3E).

**Double dose of NBQX is most effective at preventing joint degradation in the ACLr model.**

Both the number of injections (1 v 2 v 3, p=0.024) and the type of treatment (NBQX v vehicle, p=0.02) significantly affect joint degeneration after ACLr (Two-factor GLM, Supplementary Figure 1A). Two injections significantly reduce joint severity scores compared to a single injection following ACLr (p<0.02, GLM) (Supplementary Figure 1A). Two i.a. doses of NBQX (at time of
rupture and 24 hours later) reduce mean knee severity score to 10.35±2.6 compared to a single dose (19.4±3.5), three doses (24.1±4.0) and also two doses of vehicle control (21.7±4.2) (Supplementary Figure 1A). Knee severity score following two doses of NBQX was restored to that of control, uninjured knees, whereas all other treatments remained significantly higher (Supplementary Figure 1A). When broken down into parameters, 2 doses of NBQX significantly reduced scores compared to 2 doses of vehicle (p=0.002, GLM) (Supplementary Figure 1B). Two doses of NBQX reduced OA changes by 51%, bone changes by 78% and proteoglycan loss by 42% (Supplementary Figure 1B). Knee swelling and lameness over time, and day 21 histological inflammation, were not affected by dosing regimen (Supplementary Figure 1C-E).

**NBQX treatment alters GluR expression in ACLr PTOA.**

To determine whether AMPA and kainate GluRs are expressed in PTOA, reveal potential target tissues, and determine whether expression patterns changed with NBQX treatment, we immunolocalised GluRs in each treatment group at disease end stage (21 days) (Figure 4). Expression patterns are summarised in Table 1. It is important to note that due to the progression of OA after ACLr, surface chondrocytes are often not present, and it is not possible to distinguish which cartilage zones remain.

AMPAR2 stained the synovial lining strongly in both NBQX treated (Figure 4a2) and vehicle treated (Figure 4c2) ACLr mice but was less abundant in intact ACL (Figure 4e2). AMPAR2 staining of chondrocytes was present throughout the cartilage in all 3 groups (Figures 4 A, a1, C, c1, E, e1). Osteocytes stained for AMPAR2 in NBQX treated (~50%) (Figure 4a3) and vehicle treated (~75%) (Figure 4c3) ACLr mice, whereas only ~20% of osteocytes were positive in intact ACL (Figure 4e3). Bone lining cells stained strongly for AMPAR2 in vehicle treated ACLr mice (Figure
AMPAR2 and KA1 staining was abundant in ruptured ACL from vehicle (Figure 4K-N) and NBQX (Figure 4G-J) treated ACLr mice, but KA1 staining was almost absent in intact ACL (Figure 4O-R, Supplementary Figures 1F-I). Significantly less positive KA1 staining was seen in intact ACL compared to ruptured ACL following 2 doses of either vehicle or NBQX (Supplementary Figure 1I).
**Discussion.**

We report glutamate concentrations in human injured knee joint fluids for the first time. Patients with ACLr (55µM) or meniscal tear (43µM) had similar mean SF glutamate concentrations to those with OA (39µM), within reported ranges of patients with OA (266µM, range 0-530µM) and RA (326µM, range 4-608µM) and exceeding ranges in post-mortem non-arthritic humans (6.25µM, range 0.82-22µM) and living non-arthritic rabbits (4.23µM) and rats (2.72µM-5.91µM) (23). Glutamate concentrations varied over time post ACL injury, being greatest before 100 weeks post-injury. Mean SF concentrations ranged from 58µM at 0-20 weeks and 71µM 21-100 weeks post-ACL rupture, to 35µM at >100 weeks post injury. This temporal elevation in SF glutamate concentrations, along with our observation that AMPA and kainate GluRs are expressed after knee injury, and in arthritic joints (22), provides evidence that GluR antagonists may be an appropriate acute intervention in human PTOA. Both temporal and patient-specific variability in SF glutamate concentrations may reveal an opportunity for therapeutic targeting.

Our previous work indicated that AMPA and kainate GluRs on human synoviocytes regulated IL-6 release, an essential mediator of joint degeneration in inflammatory arthritis (31) and that NBQX intervention in inflammatory arthritis reduced pain, inflammation and degeneration (22). We have recently described a mouse model of joint injury, where a single controlled load ruptures the ACL non-invasively, inducing very early inflammatory effects including increased IL-6 expression (43). We therefore assessed the protective effect of 20mM NBQX administered i.a. at the time of acute injury in the mouse ACLr model, as a mimic of acute joint injury (43, 44).

A single i.a. NBQX treatment after ACLr reduced knee swelling by up to ~45% throughout the 7-days following injury and reduced joint severity score, representing cartilage and bone pathology,
by 29%. NBQX significantly reduced all components of the joint severity score but was most effective in reducing the bone score by 43%, in keeping with our previous studies showing its control of bone changes in rat AIA (22). Intra-articular injection ensures bioavailability of the drug in the joint, and controls dosing, but small molecules, like NBQX, rapidly diffuse (0.23h half-life within humans (45)). Two doses of NBQX (at time of rupture and 24 hours later) reduced joint severity score by 48%, restoring values to those of uninjured knees. The two-dose efficacy may reflect the intervention window in the ACLr model, as 3 doses (days 0, 1 and 7 post-rupture) were less effective. However, repeated i.a. injections may increase inflammation and mask protective effects of longer-term treatments.

There are surprisingly few reports of other agents given i.a. acutely at the time of injury. In surgical models, i.a. delivery of dehydrated amnion/chorion membrane given 24 hours postsurgery (17) appeared protective, but was not conventionally scored, whereas four repeated i.a interleukin-1 receptor antagonist (IL-1RA, 6mg per 40µl injection) injections reduced cartilage histopathology after ACL transection in rats (46). Corticosteroid data are contradictory and have been reported to damage joint structures (47, 48); one i.a injection of dexamethasone (0.5mg/kg) immediately following surgical drill injury in rabbits reduced Mankin score by approximately 40% (18), whereas 0.5mg i.a injection of dexamethasone given 7 days post-surgery increased joint degradation in rabbit PTOA (49). Studies reporting the effect of i.a. drugs at the time of injury in non-surgical PTOA are limited to those on IL-1RA (20). IL-1RA, which has progressed to human trials, given immediately post-injury in a mouse articular fracture model of PTOA, improved mean degeneration scores by 30%, restoring them to control values (19, 20). The disease modifying effect of one i.a. injection of NBQX matched that of IL-1RA, and 2 doses exceeded its efficacy.
No studies have tested i.a. injections of corticosteroids or HA at the time of injury in non-surgical rodent models of osteoarthritis, even though these drugs are often used in painful, injured joints in humans. Therefore, we compared efficacy of NBQX with i.a. steroid (depo-medrone) or HA used at equivalent i.a doses in mice as recommended in man. Following ACLr, NBQX reduced knee swelling by ~45% over days 1-7 whereas HA had no effect and depo-medrone reduced knee swelling by 50% on day 1 only. NBQX increased lameness score by ~25% over days 1-3, but prevented lameness after day 7, whereas depo-medrone and HA treatment reduced lameness scores by ~17% compared with vehicle, but mice remained lame until day 14. Finally, NBQX reduced joint degeneration scores by 29% (1 dose) to 48% (2 doses), whereas a single dose of HA had no effect on degeneration and depo-medrone significantly increased joint severity score by 50% compared to saline control. The degenerative effects of depo-medrone are consistent with reports of its dose-dependent deleterious effects on cartilage morphology, histology, and cell viability both in vitro and in vivo (48), and the limited evidence of beneficial effects of i.a injections of steroids in man (50). Therefore, NBQX was more effective at reducing swelling and degeneration than steroid and HA after ACLr in mice.

There is a plethora of drugs developed to antagonise GluRs, some of which have passed Phase 1 safety trials when used systemically (51). AMPA and kainate GluR antagonists have predominantly been tested for central nervous system (CNS) diseases such as epilepsy, with perampanel approved by EMA and FDA for epilepsy treatment. Although some of these drugs, taken orally as a daily medication, have been associated with side effects, most are safe and tolerated. Intra-articular injection into the joint acutely at the time of injury will reduce sustained bioavailability of the drug in the brain, which is likely to reduce side effects.
There are a number of limitations to this study. The SF glutamate concentrations after ACL rupture were only measured in 27 patients, reducing the power of detecting associations with time since injury, and although significant differences were observed with time, we did not measure glutamate concentrations for healthy controls. Whilst mouse ACLr models are widely accepted PTOA models (52), limitations include thin articular cartilage, no zonal organisation of cartilage, ossified meniscal cartilage, altered joint mechanics and no closure of growth plates. We utilised day 21 post-ACL rupture for data collection on endpoint OA disease and matched this to longitudinal measurements of \textit{in vivo} changes in swelling and lameness. Further studies including earlier timepoints would allow for the timeline of OA progression to be compared between treatment groups. Although our previous work suggests glutamate may be acting on the arthritic joint via IL-6 signalling (22, 31, 43), our current study lacks a mechanistic correlation with IL-6 and further \textit{in vitro/ex vivo} studies are required to address this. Finally, the NBQX concentrations used in these experiments (20mM) may elicit non-specific effects on other GluRs within the joint (51), and further studies are required to establish the minimum effective dose and optimum dose regime.

In conclusion, SF glutamate concentrations vary following knee injury in humans and i.a. administration of the AMPA and kainate GluR antagonist NBQX administered at the time of injury, was more effective at reducing swelling and degeneration than current treatments for joint pain and early OA. Repurposing clinic ready AMPA/kainate GluR antagonists with proven safety in man, would substantially reduce the burden of injury-induced OA, and represents a major advance that could quickly address this unmet need.
Materials and Methods.

Patients.

Matched SF, ACL and menisci were obtained (informed consent, Ref: 10/MRE0928) from randomly selected patients undergoing total knee replacement (TKR) for OA (2 men, 1 woman), ACL reconstruction (2 men, 1 woman) or meniscal arthroscopy (2 women) respectively. Additional SF was obtained from 2 ACL reconstruction (2 men), 3 meniscal arthroscopy (2 men, 1 woman), 2 OA TKR (1 man, 1 woman) and 2 RA TKR (1 man, 1 woman) patients. To examine SF glutamate levels at different times post-ACL injury, 24 additional patients (18 men, 6 women) were identified with known injury date and sample extraction date. Age, sex, BMI and time since injury were recorded (Supplementary Table 1).

Animals.

Procedures were performed according to Home Office and ARRIVE guidelines (53) on male C57/BL/6J mice (12 weeks old) housed in groups of 5 (Envigo, UK; 12 hour light/dark cycles, ad libitum food and water). Animals were randomly assigned to the various experimental groups and treatments randomly distributed amongst cages. The data were collected and processed randomly. All animal studies were blinded. Investigators did not know which animals had received which treatments during data acquisition and subsequent scoring of samples and were unaware of the allocated treatment at the time of induction of PTOA. Animals were monitored for welfare (weight, limping, posture) daily in the first week and weekly thereafter.

ACLR model.

Custom built cups (54) were used to hold the right knee of anaesthetised mice in flexion with a pre-load of 0.5N prior to the application of a 12N load at a velocity of 1.4mm/s (ElectroForce®)
3200, BOSE, USA). This force and velocity was chosen based upon prior experiments showing that a 12N threshold force ruptured the ACL immediately on application of load and 1.4mm/s resulted in a mid-substance tear of the ligament (43). ACLr was identified through the waveform as a continued increase in displacement following release of the applied compressive force with an audible ‘popping’ sound. Histology and x-ray analysis confirmed rupture (data not shown). Contralateral knees served as unloaded controls. All mice received Temgesic (0.05mg/kg) subcutaneously at the start of the experiment and moved freely after loading. All measurements were taken at days 0 (baseline), 1, 2, 3, 7, 14 and 21, unless stated. Day 21 was the predefined endpoint representing advanced/end stage arthritis for assessment of degeneration. Animals were excluded before this time only due to culling for welfare reasons or if there was no audible “pop” and change in displacement on loading (n=2 for NBQX).

Following power calculations, pilot studies used n=5 (detects differences of 50-80% in degeneration in ACL-rupture versus uninjured controls) and the main study used n=10 (detects differences in degeneration between ACLr+NBQX v ACLr+veh of 23-35% with 80% power).

**Drug treatments.**

1. **NBQX.**

A single i.a. injection of NBQX (20mM in 10µl sterile water) was administered to 10 animals immediately following ACLr, whilst another 15 received an i.a. injection of vehicle (sterile water) immediately following ACLr. 20mM NBQX was chosen as it had been reported to be safe in mice (45). Contralateral knees (intact ACL, n=15) were used as controls. To test dosing regimen, following ACLr, mice received either a single i.a. injection (day 0) of 20mM NBQX (or sterile water control), two injections (NBQX or vehicle, day 0, day 1) or 3 injections (NBQX or vehicle,
day 0, day 1, day 7) (n=5 for each group). Contralateral knees (intact ACL with no load, n=5) and mice who received i.a. 20mM NBQX without load (intact ACL, to assess effects of NBQX in normal joint) were used as controls.

2. Steroid and HA.

Steroid (depo-medrone (Pfizer), 10mg/kg, n=7), HA (Durolane™ (Bioventus), 8mg/kg, n=8) or vehicle control (saline, n=7) were administered as a single i.a. injection (10µl) immediately following ACLr. Depo-medrone concentrations were of the same order of magnitude, taking into consideration SF volume (55-58), as those used clinically in the joints of dogs, horses, and humans (59, 60).

**Sample processing.**

Human ACL, meniscus and day 21 whole mouse knees were fixed (2 days, 10% neutral buffered formalin, Sigma), decalcified (4°C, 10% EDTA, Fisher Scientific), paraffin embedded and coronally sectioned (6µm).

**Histology.**

Consecutive sections from all animal experiments (day 21, numbers as above), were stained with haematoxylin and eosin (synovial inflammation), toluidine blue/safranin-O (cartilage and bone) or retained for immunohistochemistry.

Two independent observers blinded to treatment, used published scoring systems to assess mouse synovial inflammation (61) and knee degeneration (62) (Supplementary Tables 2-3) in two sections ~200µm apart. Average scores from both observers and both sections are presented.
Immunohistochemistry.

GluRs were immunolocalised in sequential sections from human ACL, meniscus and mouse knees (numbers as above) using antibodies to kainate receptor-1 (KA1) and AMPA receptor-2 (AMPAR2) (anti-KA1 (ab67402), anti-iGluR2 (ab52176); Abcam, Supplementary Methods). Sections underwent antigen retrieval (1mg/ml trypsin, Sigma), peroxidase blocking, overnight incubation (4°C) with primary antibody, and detection (Vectastain Elite ABC kit, nickel enhanced diaminobenzidine, Vector Laboratories). Tris buffered saline and IgG controls were included in every run.

To quantify positively staining cells in the ACL, 2-3 regions of interest were chosen (top, bottom and middle of ACL if possible) and the total cells and positive staining cells counted. The number of positive staining cells were then presented as a percentage of total cells counted in the ACL.

Knee swelling and lameness.

Mouse knee diameters were measured (mean of three readings per knee, Mitutoyo digital calliper, RS Components Ltd, observer blinded to treatment) and the difference between left and right knees presented. Lameness was scored on a scale of 0-10 where 0 represents full weight-bearing and no evidence of gait abnormality and 10 is non-weight-bearing (adapted from (63)). Scores were averaged from 2 assessors blinded to treatment.

Glutamate ELISA.

Glutamate concentrations were quantified in human SF (numbers as above) according to manufacturer’s instructions (Glutamate ELISA kit KA1909, Novus Biologicals).
**Statistics.**

Vehicle and drug treatments were compared using Minitab 18 software. Data were tested for normality and equal variances prior to two-sample t-Tests, one-way ANOVA or general linear model (GLM) 2-way or 3-way ANOVAs with Tukey *post hoc* tests. Non-parametric data used Kruskal-Wallis or Sheirer-Ray-Hare with Mann-Whitney *post hoc* tests. P<0.05 was considered significant. Means ± standard deviation (SD) or boxplots representing interquartile range, median and all data points (including mean, indicated by crossed circle) are presented.

**Study approval.**

Written informed consent was obtained from participants prior to inclusion in the study as per the Research Protocol which has been reviewed and approved by the Health and Care Research Wales ethics committee, Wales REC 3, reference 10/MRE09/28, IRAS project number 51853. Animal procedures were performed in compliance with the Animals (Scientific Procedures) Act 1986 (Home Office licences 30/2959 and P287E87DF) according to Home Office and ARRIVE guidelines.

**Author Contribution**

CSB designed research studies, conducted experiments, acquired data, analysed data and wrote the manuscript. SJG conducted experiments, acquired data, analysed data and edited the manuscript. EJB conducted experiments, acquired data, and edited the manuscript. ASW designed research studies, analysed data and edited the manuscript. DJM conceived and designed research studies, analysed data, and wrote the manuscript. The authors have declared
that no conflict of interest exists. Dr Bonnet and Dr Mason have granted patents US 9,918,986, EP3016636 and ZL201480047577.6 in respect of this technology.

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References.


Figures and figure legends.

Figure 1. Glutamate concentrations and receptors in patients. (A) Glutamate was detectable in synovial fluid samples from 27 ACL reconstruction, 5 osteoarthritic total knee replacement (TKR), 5 meniscal arthroscopy and 3 rheumatoid arthritis (RA) patients. (B) Glutamate concentrations were significantly greater at 0-20 (n=12) and 21-100 (n=8) weeks post ACL injury, compared with 100-500 (n=7) weeks post-injury (ANOVA p=0.03, Tukey post-hoc: *p<0.05, **p<0.01). (C) Synovial fluid glutamate concentrations appear to reduce with increasing age, although not significant (Pearson’s correlation coefficient -0.330, p=0.053). (D) There are no differences in male (n=25) and female (n=11) synovial fluid glutamate concentrations. (E-H) Matched tissue samples from patients in (A) were used for AMPAR2 and Kainate-1 (KA1) immunohistochemistry. AMPAR2 and KA1 receptors were expressed in ACL fibroblasts (E&F) and meniscal chondrocytes (G&H) from ACL and meniscus injury patients respectively. Dashed boxes indicate location of x40 objective image. Arrows in x40 objective images indicate positive staining. Scale bars: E-H, 100μm; x40 images, 50μm. Data presented as boxplots representing interquartile range, median and all data points (including mean, indicated by crossed circle) in A, B & D.
Figure 2. Knee swelling, histological inflammation and pain behaviour in ACLr mice treated with 20mM NBQX (n=10) versus vehicle (water, n=15), and HA (n=8) or depo-medrone (n=7) versus vehicle (saline, n=7). (A) On days 1, 2, 3 and 7 post ACLr, knee swelling in vehicle treated mice was significantly greater than day 0 swelling (**p<0.001, Tukey), whereas NBQX treated mice showed no significant increase compared to day 0. NBQX treatment significantly reduced knee swelling on days 1 and 2 compared to vehicle treated mice (++p<0.01, +p<0.05 respectively, Tukey). (B) Steroid (depo-medrone) treatment significantly reduced knee swelling on day 1 compared to vehicle (***p<0.001) and HA (*p<0.05, Tukey). Knee swelling in steroid (○○○p<0.001, ○○p<0.01, Tukey), HA (ΔΔΔp<0.001, Tukey) and vehicle (###p<0.001, Tukey) treated mice remained significantly higher than day 0 (pre-surgery) measurements until 14 days post-rupture. At day 21, synovial inflammation score was similar for NBQX (C), HA (D) and respective vehicle treated mice, whereas steroid treatment increased mean inflammation score, although not significantly (D). Contralateral knees with intact ACL displayed no signs of inflammation. (E) Intact ACL mice displayed normal synovial lining, 2–4 cells thick (black arrow), with underlying adipose tissue (black asterisk). ACLr with vehicle treatment induced synovial hyperplasia (red arrow) and infiltrate (red asterisk) that were also present following NBQX, HA and steroid treatment. FC, femoral condyle; TP, tibial plateaux; M, meniscus. Scale bars: 100µm. (F) Lameness score reduced over time for both NBQX treated and vehicle control ACLr mice, becoming significantly lower than day 1 measurements from day 7 (+++p<0.001 NBQX, ***p<0.001, **p<0.01 vehicle, Tukey). (G) Steroid and HA treatment reduced lameness score (independent of time) compared to saline (GLM for treatment p<0.001, Tukey post-hoc test: ◊◊◊p<0.001, steroid vs saline; ###p<0.001, HA vs saline), although no significant differences were found on individual days. By day 14, lameness score was significantly lower for saline, HA and steroid compared to day 1 scores (***p<0.001 saline, +++p<0.001 HA, ○○○p<0.001 steroid, Tukey). Data presented as mean ±SD in A, B, F, G and
boxplots representing interquartile range, median and all data points (including mean, indicated by crossed circle) in C & D.
Figure 3. Histological knee joint severity in ACLr mice treated with 20mM NBQX (n=10) versus vehicle (water, n=15), and HA (n=8) or depo-medrone (n=7) versus vehicle (saline, n=7). (A) At day 21, NBQX treatment significantly reduced joint severity score compared to vehicle (**p<0.001, Two-Sample t-Test). (B) Steroid (depo-medrone) treatment significantly increased joint degradation by ~50% compared to HA or saline vehicle treatment (*p<0.05, one-way ANOVA with Tukey post-hoc). (C) When broken down into parameters, NBQX significantly reduced cartilage loss (OA damage, ***p<0.001), proteoglycan loss (**p<0.01) and bone changes (**p<0.001, Two-Sample t-Tests). (D) Significant reductions in joint severity score caused by NBQX treatment were seen in both the medial (*p<0.05, Mann-Whitney test) and lateral (**p<0.001, Two-Sample t-Test) sides of the joint. (E) Healthy bone (b), covered by a smooth articular cartilage (c), is clearly present in knees with an intact ACL, however, following ACLr (with vehicle), severe cartilage loss is evident (red arrows), in conjunction with bone thickening and remodelling (black arrows) and chondrophyte formation (asterisk). Joint damage was sometimes so severe, cartilage and bone loss occurred down to the growth plate (black arrowhead). A single i.a. injection of NBQX reduced cartilage (red arrow) and bone changes (black arrows) and joints retained more structural integrity. Two injections of NBQX maintained joint structure similar to that seen in intact ACL samples. HA had similar effects to vehicle, however, intra-articular depo-medrone was highly damaging to the joint, causing bone loss down through the growth plate (black arrowheads) in both the medial and lateral compartments and the formation of large chondrophytes (asterisk). FC, femoral condyle; TP, tibial plateaux; ACL, anterior cruciate ligament; m, meniscus. Scale bars: 50µm. Data presented as boxplots representing interquartile range, median and all data points (including mean, indicated by crossed circle).
Figure 4. GluR expression in bone, cartilage and ligament in the ACL rupture model. Strong AMPAR2 staining of synovial lining after NBQX (a2) and vehicle (c2) treatment was less abundant in intact ACL (e2). AMPAR2 stained chondrocytes throughout cartilage under all conditions (A, a1, C, c1, E, e1). AMPAR2 stained osteocytes after NBQX (a3) and vehicle (c3) treatment, but fewer osteocytes were positive in intact ACL (e3). AMPAR2 stained bone lining cells after vehicle treatment (c3), but was less abundant after NBQX treatment (a3) and in intact ACL (e3). KA1 stained synovial lining after vehicle treatment (d2) but was not detected after NBQX treatment (b2) or in intact ACL (f2). KA1 stained chondrocytes after NBQX treatment (B & b1) and in intact ACL (F & f1), but not after vehicle treatment (D & d1). KA1 was not expressed in osteocytes (b3, d3, f3). KA1 weakly stained bone lining cells after NBQX (b3) and vehicle (d3) treatment but was less abundant in intact ACL (f3). AMPAR2 and KA1 staining was abundant in ACL fibroblasts from vehicle treated ACL ruptured mice (K-N), but was less abundant in NBQX treated mice (G-J) and almost absent in intact ACL (O-R). Positive staining indicated by black arrows. FC, femoral condyle; TP, tibial plateau; M, meniscus. Scale bars: 50µm in 1, 2, 3 images and x40 images, 100µm in all others.
Tables

Table 1. AMPAR2 and KA1 protein expression in healthy mouse stifle and after ACL rupture with vehicle or NBQX treatment.

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**KEY:** - = absent; +/- = weak; + = present; ++ = abundant