

Original Article

Excitatory amino acids display compartmental disparity between plasma and synovial fluid in clinical arthropathies

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Abstract: Background: Previous studies have demonstrated elevated levels of excitatory amino acids (EAA) glutamate (Glu) and aspartate (Asp) in the synovial fluid (SF) of patients with active arthritis. The source of SF EAA concentrations are thought in large part to be secondary to passive diffusion from the plasma across synovial membranes and less so, reflective of local synovial pathology. Objective: This descriptive report assesses the hypothesis that the SF EAA levels reflect inflammatory processes of the joint and are not dependent on plasma levels. Methods: Simultaneously drawn plasma and SF samples were obtained from 14 recently deceased cadavers and 10 patients with active arthritis. Plasma and SF EAA and other amino acid (AA) levels were determined by HPLC. SF: Plasma compartment concentration ratios were calculated to assess if SF EAA levels were similar to plasma levels. Results: In the cadavers with no *antemortem* arthritis, the mean SF: Plasma ratios for Glu and Asp were 4-5-fold lower than the mean ratios seen for 9 other AAs, showing specific discrepancies of EAA levels between plasma and synovial fluid. In 9 patients with active arthritis, the SF: Plasma concentration ratios were higher in samples derived from inflammatory arthropathies. Conclusions: Clinical samples demonstrated distinct, independent compartmental EAA concentrations between blood and joint compartments in support that local arthritic processes rather than plasma influence SF EAA concentrations. The SF EAA levels, whether from local cell production, local neurogenic sources, and/or transport-gradient mechanisms, parallel local pathology in the joint compartment and thus serve as surrogate biomarkers of local inflammatory processes.

Keywords: Glutamate, aspartate, synovial fluid, arthritis, biomarker, neurotransmitter

Introduction

Previous studies have demonstrated elevated levels of excitatory amino acids (EAA) and other neurotransmitters in synovial fluid (SF) extracted from patients with active arthritic conditions [1-3] [McNearney TA, Goel N, Lisse JL, Speegle D, Cao S and Westlund KN. Neurotransmitter excitatory amino acids in synovial fluids demonstrate distinct temporal fluctuations in active arthritis, submitted]. Additional studies have reported elevated plasma amino acid (AA) levels in patients with rheumatoid arthritis compared to normal controls [4]. The source(s) of increased SF EAA levels is not known but possibilities include local cell production, neurogenic exudation, or passive diffusion from the

blood or plasma across synovial membranes. The concentration elevations of SF EAA in symptomatic arthropathies and their reported association with SF RANTES, and MIP1-alpha concentrations promotes the hypothesis that local inflammatory joint processes rather than passive diffusion from plasma determines SF EAA concentrations [5]. To assess this, simultaneously drawn plasma and synovial fluids from the knees of 14 recently deceased cadavers and 9 patients with active arthritis were obtained and measured for EAA and other AA levels to assess the compartmental SF: Plasma concentration ratios. Disparate ratios between these compartments would support the concept that EAA and other AA levels are primarily determined by local inflammatory arthritic pro-

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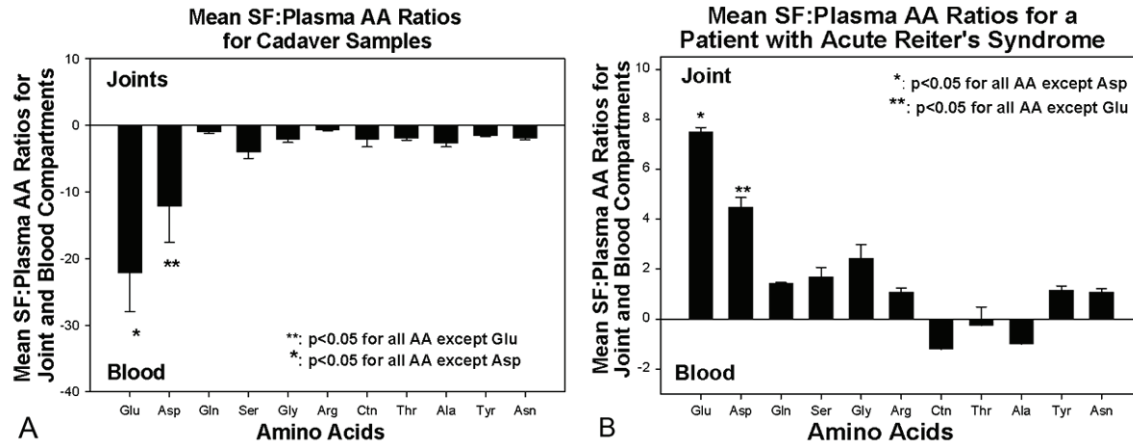


Figure 1. A: SF: Plasma ratios of 12 amino acid concentrations (μM) determined by HPLC derived from 14 cadavers. SF: Plasma ratios are shown as mean values \pm standard error. EAA: glutamate (Glu); aspartate (Asp) and glutamine (Gln). Other amino acids include serine (Ser); glycine (Gly); arginine (Arg); citrulline (Ctn); threonine (Thr); alanine (Ala); tyrosine (Tyr); taurine (Tau) and asparagine (Asn). *: $p < 0.01$ comparing the mean Glu SF: Plasma ratio to non EAA amino acids. **: $p < 0.01$ comparing the mean Asp SF: Plasma ratio to non EAA amino acids. These data support compartmental differences for EAA relative to other AA. B: SF: Plasma ratios of amino acid concentrations (μM) of plasma and knee SF determined by HPLC derived from a patient with active Reiter's syndrome. SF: Plasma ratios are shown as mean values \pm standard error. In a patient with active inflammatory synovitis, the mean SF: Plasma ratios for EAA were much higher compared to the ratios of other amino acids. *: $p < 0.01$ comparing the mean Glu SF: Plasma ratio to non EAA amino acids. **: $p < 0.01$ comparing the mean Asp SF: Plasma ratio to non EAA amino acids.

cesses and are independent of the plasma concentrations.

Materials and methods

Informed consent

All clinical samples collected and medical record reviews were under approved protocols by the University of Texas Medical Branch Institutional Review Board (IRB) with appropriately authorized informed consent.

Cadavers

Autopsy specimens were considered acceptable for study if samples were harvested within 24 hours after witnessed death. Written consent was obtained from the family or authorized persons for fluid harvest, medical record review, and physical examination before plasma and synovial fluids were harvested, per IRB protocol. Cadaveric samples (N=14) were available for which there was no clinical or *antemortem* history of active arthritis at the time of death. Blood was obtained by venipuncture or cardiac puncture, as routinely obtained for autopsy cases in tubes with EDTA. Synovial fluids were obtained by introduction of a 16-gauge needle below the patella and aspiration of the joint

fluid. In nonedematous or nonarthritic conditions, the synovial fluid harvest was usually between 0.5-2.0 ml of viscous, clear, or pale yellow fluid. In patients with peripheral (generalized) edema or *antemortem* arthritic conditions, it was possible to harvest larger volumes (5-8 ml) of fluid from the knee joints.

Patients

After written consent was obtained, SF from the active arthritic knee was obtained at the time of diagnostic or therapeutic arthrocentesis. Venipuncture was performed simultaneously. Sample processing has been previously described [3]. The arthritic conditions were diagnosed by faculty members of the University Rheumatology division based on diagnostic criteria [6]. These included: rheumatoid arthritis, acute gout, acute Reiter's syndrome, osteoarthritis, pseudogout and sympathetic effusion in a patient with catastrophic antiphospholipid antibody syndrome (APAS).

High pressure liquid chromatography (HPLC), experimental standards and quality control

The protocols, standards and internal controls for HPLC determination have been previously described [3].

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Quantities (in μM) from HPLC are presented as the mean SF: Plasma EAA concentration ratios or mean SF: Plasma AA concentration ratios. The neurotransmitter EAA's measured were glutamate (Glu) and aspartate (Asp). Other AAs measured included serine (Ser) and glycine (Gly), as neurotransmitter inhibitory amino acids (IAA) and AA used as metabolic controls: glutamine (Gln), arginine (Arg), threonine (Thr), alanine (Ala), taurine (Tau), tyrosine (Tyr) and asparagine (Asn).

Statistics

Each sample had three determinations. Data are presented as average values \pm SE. Analyses of concentrations were determined by Mann-Whitney U tests. A p value <0.05 was considered significant. Average SF: Plasma concentration ratios were also determined and plotted with positive values showing higher values in the joint and negative values showing higher values in the plasma (blood).

Results

Mean SF: plasma EAA concentration ratios demonstrate significant compartmental disparity

Figure 1A depicts the mean SF: Plasma concentration ratios of the EAA and other AA from 14 cadavers without evidence of active *antemortem* arthritis. The mean SF: Plasma concentration ratios for 14 cadaveric samples were <1 for all EAA and other AA. However, the mean SF: Plasma concentration ratio disparity for Glu was much greater compared to the other AA (Glu: -22.15 -fold, $p < 0.01$ compared to all other AA except Asp). The mean Asp SF: Plasma concentration ratios were much greater for other AA except for Tau, (Asp: -17.16 -fold $p < 0.01$ all non EAA except Tau; Asp vs. Tau $p = 0.059$). The mean SF: Plasma concentration ratios of other AA's were much less disparate and are as follows: Gln: -1.61 ; Ser: -3.96 ; Gly: -2.11 ; Arg: -0.68 ; Ctn: -2.13 ; Thr: -1.92 ; Ala: -2.69 ; Tau: -1.57 ; Tyr: -5.71 and Asn: -1.93 . Nonarthritic cadaver SF Glu and Asp levels were in close agreement with previously reported baseline levels [3]. For all cadavers with no *antemortem* arthritis, mean SF Glu concentrations ranged from $0.28 - 26.26 \mu\text{M}$ and mean Asp concentrations ranged from $0 - 11.93 \mu\text{M}$. From the plasma samples, mean plasma Glu

concentrations ranged from $9.48 - 80.50 \mu\text{M}$ and mean plasma Asp concentration ranged from $0 - 73.36 \mu\text{M}$ among the cadavers.

Figure 1B depicts the mean EAA and other AA SF: Plasma concentration ratios from samples derived from a patient with acute Reiter's syndrome. The mean SF: Plasma concentration ratio for Glu: 7.50 ± 0.18 -fold, $p < 0.01$ compared to all AA except Asp. The mean Asp SF: Plasma concentration ratios for Asp: 4.49 ± 0.39 -fold $p < 0.01$, compared to other AA except Glu. The samples from the patient with acute Reiter's syndrome with symptomatic synovitis demonstrates that higher SF EAA concentrations reflect the intense inflammatory pathology of the joint compartment relative to the plasma concentrations (blood compartment) in this systemic illness.

Figure 2 demonstrates mean SF: Plasma EAA concentration ratios of 9 patients with active arthritis or synovial effusion who underwent simultaneous blood draws and arthrocenteses. Patient 1 had Reiter's syndrome (RS); patients 2 and 3 had rheumatoid arthritis (RA); patients 4 and 5 had acute gout; patients 6 and 7 had osteoarthritis; patient 8 had pseudogout (PG) and patient 9 had a sympathetic synovial effusion from complications of antiphospholipid antibody syndrome (APAS). Patients 1-5 generally had mean SF: Plasma EAA concentration ratios >1 , reflecting higher SF EAA concentrations in the joint compartment. Patients 6-9 had arthritic conditions with mean SF: Plasma ratios <1 , reflecting lower inflammatory arthritic activity. relative to any systemic processes. Patients 8 and 9 had severe systemic illnesses (acute bacterial pneumonia and catastrophic APAS, respectively) which resulted in significantly higher concentrations of plasma EAA and other AA. Thus, these patients had lower mean SF: Plasma ratios (<1), as inordinately higher plasma EAA levels reflected severe systemic illnesses. To serve as templates, the far right of the graph depicts SF: Plasma concentration ratios from cadaveric samples from a RA patient who had active synovitis at the time death (+) and from a patient who had no *antemortem* history of active arthritis (-). **Table 1** is a summary of the pertinent clinical laboratory results of the nine patients from whom the samples were derived, including the SF WBC counts, which serve as inflammatory indices for synovial effusions. **Table 1** also demonstrates

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Table 1. Summary of patient diagnoses, age and cell counts

Patient #	1	2	3	4	5	6	7	8	9
Diagnosis	RS	RA	RA	Gout	Gout	OA	OA	PG	APAS
Age, yrs	37	73	29	63	67	54	41	79	27
PI WBC #	10	8.0	6.9	13.6	6.5	NA	8.6	8.1	6.3
SF WBC #	44.0	6.0	31.0	19.2	31.0	NA	280	0.33	0.123
SF Lym #	3.15	0.24	1.5	NA	0.3	NA	002	0.04	0.006
Intracell. Crystals	---	---	---	++ UA	++ UA	---	---	++ CPPD	---

Diagnosis: RS: Reiters syndrome, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus; OA: osteoarthritis, PG: pseudogout, APAS: antiphospholipid antibody syndrome; Age: in years; PI WBC #: plasma white blood cell count, /mm³x1000; SF WBC #: synovial fluid white blood cell count /mm³x1000; SF Lym. #: synovial fluid lymphocyte count/mm³x1000. Intracell. Crystals: intracellular crystals in SF, UA: uric acid crystals, CPPD: intracellular calcium pyrophosphate dihydrate; ND: not done.

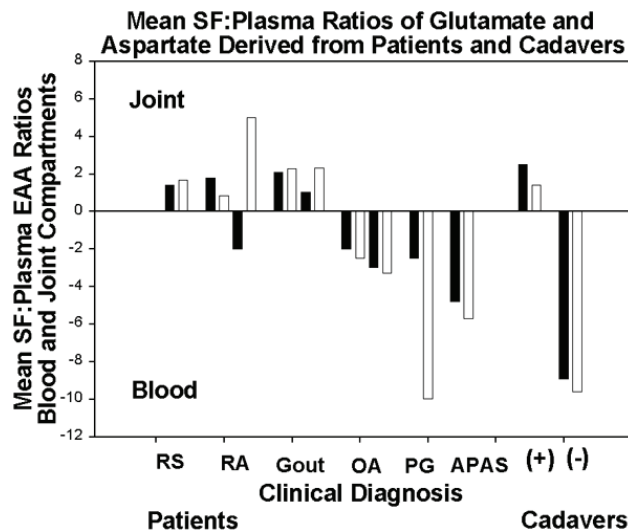


Figure 2. SF: Plasma ratios of amino acid concentrations (μM) of plasma and knee SF determined by HPLC derived from patients with active arthritis. SF: Plasma ratios are shown as mean values \pm standard error. The lanes depict patients with, 1: Reiter's syndrome (RS); 2 and 3: rheumatoid arthritis (RA); 4 and 5: acute gout; 6 and 7: osteoarthritis (OA); 8: pseudogout (PG) and 9: sympathetic effusion in a patient with antiphospholipid antibody syndrome (APAS). The values above the x-axis are conditions where the EAA concentrations are higher in the joint fluid compared to blood. The values below the x-axis represent conditions where the plasma EAA values are higher than the SF levels. Closed bars depict glutamate (Glu); open bars depict aspartate (Asp).

that blood WBC counts or SF white cell counts alone do not account for the SF or plasma EAA concentrations or the SF: Plasma EAA concentration ratios.

Discussion

This descriptive study is the first to assess the relative compartmental concentrations of amino acids between plasma and synovial fluid and indicates a clinical compartmental relevance to increased SF Glu and Asp concentra-

tions in patients with active arthritis. The sources of elevated SF Glu and Asp concentrations in active arthritis are unknown, but likely candidates include plasma, local production from synoviocytes or osteocytes in the joint capsule or local secretion from nerve fibers. One might expect that SF Glu and Asp would be in full equilibrium with the plasma, based on size, as small physiologic molecules are usually in full equilibrium between plasma and synovial fluid [7]. However, the samples from the cadavers with no *antemortem* arthritis had significantly decreased EAA SF: Plasma concentration ratios compared to nine other AA. The significantly greater compartmental ratio differences of SF Glu and Asp indicate that plasma is not the sole or even major source of SF EAA. Higher SF: Plasma concentration ratios in one cadaver with *antemortem* arthritis and several patients with active inflammatory arthritic processes also support the hypothesis that SF EAA concentrations reflect local physiologic processes in the joint. High AA concentrations obtained in several cadaver plasma samples might be explained by *antemortem* systemic inflammatory conditions or possibly reflect *post-mortem* hemolysis and cytolysis. However,

the mean plasma concentrations of cadavers with no active *antemortem* infectious or inflammatory conditions are in close agreement to the plasma levels obtained from 15 normal healthy controls (Data not shown). Moreover, SF samples with no *antemortem* arthritis are in close agreement to baseline levels obtained in healthy rats (Data not shown).

The SF: Plasma EAA ratio discrepancies might also reflect the necessity of energy dependent glutamate transporter proteins in peripheral

tissues, including bone, cartilage and synovium [8-10]. Sodium or potassium dependent Glu and Asp transport proteins may play role in the maintenance of SF EAA concentrations in the normal joint. In the CNS, high affinity glutamate/aspartate carrier proteins are capable of transporting the neurotransmitters against several thousand fold concentration gradients using the sodium, potassium electrochemical gradient as a driving force, under control conditions [11]. Persistent depolarization of nerves reverses the function of the Glu transporter pumps, however, resulting in a dumping of amino acids [12, 13]. Microdialysis uptake of inflammatory perturbations may also induce local synovium-based carrier proteins to inappropriately allow an increased influx of normally excluded substances from the plasma, including Glu and Asp. Alternatively, the Glu and Asp may require additional mechanisms to migrate through the SF, and its accumulation points to a failure of normal exclusion physiology during joint inflammation. In addition to EAA sources from the plasma, SF Glu and Asp concentrations are secondary to local production by resident cells in the joint capsule [14, 15].

A more likely source may be the stimulated release from the primary afferent nerve terminals supplying the joint, as is thought for substance P release into the joint [16]. Substance P has been demonstrated to have SF: Plasma ratios of 2-fold in an inflammatory arthropathy [17]. The SF EAA values derived from normal rat suggest that the low values might be physiologic in the absence of active arthritis and are elevated in inflamed joints [18, 19]. Previous studies have demonstrated increased Glu immunoreactivity in the median articular nerve supplying inflamed joints of monkeys [20]. Thus, it is reasonable to assume that glutamate might also be released into the joint by nerve fibers. In a k/c induced arthritis model in rats, the expected increase in SF Glu was abrogated with pretreatment with intra-articular lidocaine, which decreases neurotransmitter release from peripheral nerves [18]. Local glutamate and aspartate can bind and activate peripheral receptors on local osteocytes, chondrocytes and synoviocytes to enhance or perpetuate local inflammation and pathologies [15, 19, 21-25]. In a previous study, our group demonstrated a correlation between SF EAA levels and SF RANTES, MIP1-alpha and IL-8 lev-

els [5]. In the same study, SF WBC counts significantly correlated to SF IL-8 levels but not to EAA levels.

The disparity of EAA levels between body compartments in this study demonstrates a spectrum of host pathologic processes and supports a physiologic relevance of the EAA levels and their regulation in the joint. Taken together, SF EAA elevations reflect the dynamics of joint inflammation and pain. Further studies might identify SF EAA concentrations as novel direct or surrogate biomarkers that reflect local pathologic arthritic processes.

Conflict of interest

There are no financial disclosures or conflicts of interest with the authors.

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References

- [1] Appलगren A, Appलगren B, Eriksson S, Kopp S, Lundeberg T, Nylander M and Theodorsson E. Neuropeptides in temporomandibular joints with rheumatoid arthritis: a clinical study. *Scand J Dent Res* 1991; 99: 519-521.
- [2] Larsson J, Ekblom A, Henriksson K, Lundeberg T and Theodorsson E. Concentration of substance P, neurokinin A, calcitonin gene-related peptide, neuropeptide Y and vasoactive intestinal polypeptide in synovial fluid from knee joints in patients suffering from rheumatoid arthritis. *Scand J Rheumatol* 1991; 20: 326-335.

EAA compartmental differences reflect arthritis

- [3] McNearney T, Speegle D, Lawand N, Lisse J and Westlund KN. Excitatory amino acid profiles of synovial fluid from patients with arthritis. *J Rheumatol* 2000; 27: 739-745.
- [4] Trang LE, Furst P, Odeback AC and Lovgren O. Plasma amino acids in rheumatoid arthritis. *Scand J Rheumatol* 1985; 14: 393-402.
- [5] McNearney T, Baethge BA, Cao S, Alam R, Lisse JR and Westlund KN. Excitatory amino acids, TNF-alpha, and chemokine levels in synovial fluids of patients with active arthropathies. *Clin Exp Immunol* 2004; 137: 621-627.
- [6] Klippel JH. *Primer of the Rheumatic Diseases*. Atlanta, Arthritis Foundation, 2001.
- [7] McCarty D. *Arthritis and Allied Conditions*. Edited by Koopman WJ. Baltimore: Williams and Wilkins, 1997; pp: 81-102.
- [8] Huggett JF, Mustafa A, O'Neal L and Mason DJ. The glutamate transporter GLAST-1 (EAAT-1) is expressed in the plasma membrane of osteocytes and is responsive to extracellular glutamate concentration. *Biochem Soc Trans* 2002; 30: 890-893.
- [9] Mason DJ. The role of glutamate transporters in bone cell signalling. *J Musculoskelet Neuronal Interact* 2004; 4: 128-131.
- [10] Mason DJ, Suva LJ, Genever PG, Patton AJ, Steuckle S, Hillam RA and Skerry TM. Mechanically regulated expression of a neural glutamate transporter in bone: a role for excitatory amino acids as osteotropic agents? *Bone* 1997; 20: 199-205.
- [11] Nicholls D and Attwell D. The release and uptake of excitatory amino acids. *Trends Pharmacol Sci* 1990; 11: 462-468.
- [12] Rothstein JD, Jin L, Dykes-Hoberg M and Kuncl RW. Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. *Proc Natl Acad Sci U S A* 1993; 90: 6591-6595.
- [13] Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N and Kuncl RW. Localization of neuronal and glial glutamate transporters. *Neuron* 1994; 13: 713-725.
- [14] Hinoi E, Ohashi R, Miyata S, Kato Y, Iemata M, Hojo H, Takarada T and Yoneda Y. Excitatory amino acid transporters expressed by synovial fibroblasts in rats with collagen-induced arthritis. *Biochem Pharmacol* 2005; 70: 1744-1755.
- [15] Skerry TM and Genever PG. Glutamate signalling in non-neuronal tissues. *Trends Pharmacol Sci* 2001; 22: 174-181.
- [16] Yaksh TL, Bailey J, Roddy DR and Harty GJ. Peripheral release of substance P from primary afferents. *Proceedings from the Vth World Congress on Pain*. Edited by Dubner R, Gebhart GF, Bond MR. Amsterdam: Elsevier, 1988, pp: 51-54.
- [17] Marshall KW, Chiu B and Inman RD. Substance P and arthritis: analysis of plasma and synovial fluid levels. *Arthritis Rheum* 1990; 33: 87-90.
- [18] Lawand NB, McNearney T and Westlund KN. Amino acid release into the knee joint: key role in nociception and inflammation. *Pain* 2000; 86: 69-74.
- [19] Lawand NB, Willis WD and Westlund KN. Excitatory amino acid receptor involvement in peripheral nociceptive transmission in rats. *Eur J Pharmacol* 1997; 324: 169-177.
- [20] Westlund KN, Sun YC, Sluka KA, Dougherty PM, Sorkin LS and Willis WD. Neural changes in acute arthritis in monkeys. II. Increased glutamate immunoreactivity in the medial articular nerve. *Brain Res Brain Rese Rev* 1992; 17: 15-27.
- [21] Flood S, Parri R, Williams A, Duance V and Mason D. Modulation of interleukin-6 and matrix metalloproteinase 2 expression in human fibroblast-like synoviocytes by functional ionotropic glutamate receptors. *Arthritis Rheum* 2007; 56: 2523-2534.
- [22] Gu Y, Genever PG, Skerry TM and Publicover SJ. The NMDA type glutamate receptors expressed by primary rat osteoblasts have the same electrophysiological characteristics as neuronal receptors. *Calcif Tissue Intl* 2002; 70: 194-203.
- [23] Laketic-Ljubojevic I, Suva LJ, Maathuis FJ, Sanders D and Skerry TM. Functional characterization of N-methyl-D-aspartic acid-gated channels in bone cells. *Bone* 1999; 25: 631-637.
- [24] McNearney TA, Ma Y, Chen Y, Taglialatela G, Yin H, Zhang WR and Westlund KN. A peripheral neuroimmune link: glutamate agonists upregulate NMDA NR1 receptor mRNA and protein, vimentin, TNF-alpha, and RANTES in cultured human synoviocytes. *Am J Physiol Regul Integr Comp Physiol* 2010; 298: R584-598.
- [25] Ramage L, Martel MA, Hardingham GE and Salter DM. NMDA receptor expression and activity in osteoarthritic human articular chondrocytes. *Osteoarthritis Cartilage* 2008; 16: 1576-1584.