Imbalance between excitatory and inhibitory amino acids at spinal level is associated with maintenance of persistent pain-related behaviors

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\textbf{A B S T R A C T}

Although the postsynaptic events responsible for development of pathological pain have been intensively studied, the relative contribution of presynaptic neurotransmitters to the whole process remains less elucidated. In the present investigation, we sought to measure temporal changes in spinal release of both excitatory amino acids (EAAs, glutamate and aspartate) and inhibitory amino acids (IAAs, glycine, \(\gamma\)-aminobutyric acid and taurine) in response to peripheral inflammatory pain state. The results showed that following peripheral chemical insult induced by subcutaneous bee venom (BV) injection, there was an initial, parallel increase in spinal release of both EAAs and IAAs, however, the balance between them was gradually disrupted when pain persisted longer, with EAAs remaining at higher level but IAAs at a level below the baseline. Moreover, the EAAs–IAAs imbalance at the spinal level was dependent upon the ongoing activity from the peripheral injury site. Intrathecal blockade of ionotropic (NMDA and non-NMDA) and metabotropic (mGluRI, II, III) glutamate receptors, respectively, resulted in a differential inhibition of BV-induced different types of pain (persistent nociception vs. hyperalgesia, or thermal vs. mechanical hyperalgesia), implicating that spinal antagonism of any specific glutamate receptor subtype fails to block all types of pain-related behaviors. This result provides a new line of evidence emphasizing an importance of restoration of EAAs–IAAs balance at the spinal level to prevent persistence or chronicity of pain.

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1. Introduction

There is substantial evidence indicating that the excitatory amino acids (EAAs), namely glutamate (Glu) and aspartate (Asp), are critically important for normal transmission of nociceptive information from the periphery to the spinal cord \[1,2\]. They are major neurotransmitters released from the central and peripheral terminals of nociceptive primary afferent fibers \[3,4\]. Numerous studies have been associated with estimating spinal release of EAAs in response to acute mechanical, chemical and electrical nociceptive stimuli \[5\]. Moreover, intradermal injection of formalin \[6–8\] or capsaicin \[9,10\] could also produce certain amounts of Asp and Glu release in the spinal cord, although these changes may specifically vary in amplitude and duration with different pain models \[11\]. Inhibitory amino acids (IAAs), including glycine (Gly), \(\gamma\)-aminobutyric acid (GABA) and taurine (Tau), are believed to work as counterparts of EAAs at the spinal level and play very important roles in keeping tonic inhibition of nociceptive input under physiological state. However, whether EAAs–IAAs balance is changed or not in response to peripheral persistent nociception remains less clear. Thus, a parallel measurement of spinal release of both EAAs and IAAs is essential for a better understanding of the roles of EAAs and IAAs in development of pain persistence or chronicity as well.

In the past several decades, pharmacological blockade of ionotropic glutamate receptors (iGluRs) activation at the spinal level has been demonstrated to be effective in inhibition of both thermal and mechanical hyperalgesia \[12–17\] as well as the capsaicin- or formalin-induced spontaneous nociception \[18,19\]. Moreover, the antagonism against metabotropic glutamate receptors (mGluRs) at the spinal level was also demonstrated to be effective in anti-nociception \[1,20–24\]. However, discrepancies were frequently observed across uses of different animal models.
and nociceptive stimulus modalities (electrical, thermal, mechanical and chemical) [12]. Therefore, effects of spinal antagonism at specific glutamate receptor subtypes (iGluRs vs. mGluRs) are also required to be re-evaluated by the use of an animal model displaying multiple “phenotypes” of pain. The bee venom (BV) test, a well-established experimental animal model mimicking honeybee sting-induced natural tissue injury, is produced by subcutaneous (s.c.) injection of a given dose of honeybee venom into one hind paw of rats [25–28]. This model of clinically relevant pathological pain is behaviorally characterized by persistent spontaneous nociception (PSN) as well as prolonged thermal and mechanical hyperalgesia (primary or secondary) related to peripheral inflammation [12,25–27,29,30]. Previous electrophysiological studies have shown that BV-elicited PSN and hypersensitivity are mediated by a long-lasting increase in spontaneous discharges and subsequent enhanced heat/mechanical responsiveness of wide dynamic range neurons in the spinal dorsal horn [31–35]. All of these BV-induced behavioral and electrophysiological changes have been proved to be peripheral-dependent, since either peripheral sciatic nerve blockade or local administration of N-methyl-D-aspartate (NMDA) receptor antagonists or destruction of capsaicin-sensitive primary afferent fibers could effectively eliminate them [27,31,32,35,36]. In addition, a wealth of evidence has been accumulated supporting the viewpoint that BV-evoked different “phenotypes” of pain might be mediated by different spinal signaling pathways [29,30,37–40]. Thus, the BV test is the most appropriate model for examining the roles of spinal amino acids and their receptors in mediating different “phenotypes” of pain. The present study was designed to assess the time course and extent of spinal EAA (Glu and Asp) release after s.c. BV injection as well as its peripheral-dependence. Changes in IAs (Gly, GABA and Tau) and other amino acids or metabolites, including threonine (Thr), arginine (Arg), alanine (Ala) and glutamine (Gln), at spinal level, were also quantified. Besides, we further examined putative roles of central iGluRs (NMDA and non-NMDA receptors) and mGluRs in induction and/or maintenance of BV-induced PSN, primary thermal and mechanical hyperalgesia.

2. Materials and methods

2.1. Animals

The experiments were performed on male Sprague–Dawley albino rats (provided by Experimental Animal Center of the Capital Medical University, CCMU) weighing from 200 to 250 g. All experiments were carried out with the approval of the Institutional Animal Care and Use Committee at the CCMU. The animals were maintained and cared for in accordance with the guidelines set forth by the International Association for the Study of Pain [41]. All rats were housed and maintained in plastic boxes on a 12 h light/dark cycle at 24–26 °C with food and water available ad libitum. The rats were acclimatized to the laboratory and habituated to the test boxes for at least 30 min each day for 5 days before testing. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Experimental groups

To examine whether BV-induced release of spinal amino acids and PSN were dependent upon the ongoing primary afferent inputs from the periphery, rats were randomly divided into four groups: (1) Saline-treated group: rats with s.c. injection of 50 μl 0.9% sterile, isotonic saline solution into the hind paw; (2) BV-inflamed group: rats with s.c. injection of 50 μl solution of whole BV (200 μg BV dissolved in 0.9% sterile saline, Apis mellifera, Sigma); (3) Bup_contrib + BV group: rats with s.c. injection of 200 μl bupivacaine (0.75%, 10 min before BV treatment) into the same hind paw (identical to the BV injection site); (4) Bup_contrib + BV group: rats with s.c. injection of 200 μl bupivacaine (0.75%, 10 min before BV treatment) into the corresponding site of the contralateral hind paw (symmetrical to the BV injection site). Notably, the Bup_contrib + BV group of rats was only used in behavioral assays of PSN in the present study.

In experiments regarding pharmacological examination of roles of Glu receptor types in BV-induced nociception and hyperalgesia, two major groups of animals were assigned: (1) pre-treatment group: CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, a competitive non-NMDA receptor antagonist, dissolved in dimethyl sulfoxide (DMSO) at 3.5 nmol/10 μl, 35 nmol/10 μl, or 100 nmol/10 μl, Sigma) or MK-801 (a non-competitive NMDA receptor channel blocker, dissolved in 0.9% sterile saline at 1.2 nmol/10 μl or 24 nmol/10 μl, Sigma) or vehicle (DMSO or saline) was intrathecally administered 5 min prior to s.c. BV injection for studying the induction of PSN; (2) post-treatment group: intrathecal (i.t.) administration of 10 μl CNQX (100 nmol) or 10 μl MK-801 (24 nmol) or vehicle (DMSO or saline) was performed at either 5 min or 2 h after s.c. BV injection for testing maintenance of PSN or primary hyperalgesia, respectively. In a separate set of experiments, the same volume of AIDA (1-aminoindan–1,5-dicarboxylic acid, a group I mGluR antagonist, dissolved in DMSO at 10 nmol/10 μl, Sigma), EGLU ((S)-α-ethylglutamic acid, a group II mGluR antagonist, dissolved in DMSO at 20 nmol/10 μl, Sigma) or MSOP ((RS)-α-methylserine-O-phosphate, a group III mGluR antagonist, dissolved in DMSO at 10 nmol/10 μl, Sigma) was intrathecally applied at 2 h after s.c. BV injection to explore their possible roles in mediating thermal and mechanical hypersensitivity. The doses of all drugs used in the present study were determined according to our preliminary data. It should be noted that only the post-treatment paradigm was adopted in behavioral assays of primary hyperalgesia in the current study. It is also important to mention here that our preliminary experiments found no significant effects of each drug on basal thermal latency or mechanical threshold under normal conditions (without BV-initiated inflammation), so the single drug group was not incorporated in the present experimental design.

2.3. Surgery

For the in vivo i.t. microdialysis of spinal amino acid release in response to various experimental manipulations, a spinal triple lumen catheter with a single loop (Marsil Scientific, San Diego, USA) was chronically implanted into the subarachnoid space of each rat as described previously [7,42]. In brief, rats were first anesthetized with 7% trichloroacetaldehyde (1.2–1.5 g/kg, i.p.), and then placed properly in a stereotaxic apparatus. After shaving off the air on the top of the head, a 1-cm long midline incision was made across the occipital bone edge, and cervical muscles were split by using a blunt forceps, so the atlanto-occipital membrane was fully exposed. Subsequently, a triple dialysis catheter was carefully implanted into the subarachnoid space through an incision at the atlanto-occipital membrane, with the loop membrane of the catheter eventually sutured to the rostral margin of the lumbar enlargement. After closing the incision, sterile normal saline was flushed through the catheter, and then the three free ends of the microdialysis catheter were externalized through the skin at the top of the head and squeezed tightly. At last, 5 ml of normal saline was subcutaneously injected into the abdominal area of each rat to replenish the lost body fluid. Following recovery from anesthesia, the rat was returned to its cage and monitored for 5 days before further experiments were performed. All those animals showing motor weakness or signs of paralysis or sensory impairment during the observation period were abandoned.

For i.t. administration of drugs in pharmacological experiments, chronic i.t. catheterization was performed according to our modi-
ified methodology [37,38,40]. Briefly, a 2-cm-long skin incision was made and the muscles were separated from the C7 to T4 vertebrae under ketamine anesthesia (50 mg/kg, i.p.). A laminectomy was then performed at T2 or T3 level and the dura was opened. To prevent the inset tubing from moving, a polyethylene (PE)-8 tube (0.2 mm i.d., 0.5 mm o.d.) was passed through a 1-cm-long muscle tunnel and then advanced caudally (3–5 cm distance between the entry and the target level) through the subarachnoid space to the rostral side of the lumbar enlargement (namely, the caudal tip of the catheter ended between spinal levels L3 and L4). The outer end of the PE-8 tubing was firmly fixed to the paravertebral muscles. The wound was washed with sterile saline, treated with antibiotics and the muscles and skin were sutured by layers. The whole operation was performed in strictly sterile conditions. Rats showing any neurological deficits postoperatively were sacrificed. After testing, placement of the inner end of the tube was verified, and animals with the tube in the wrong place or with local pathological changes were also excluded from the final analysis.

2.4. Microdialysis

2.4.1. Perfusion of catheters and sample collection

Spinal microdialysis was carried out in freely moving rats following 5 days of recovery from surgery [42]. First, the rat was put into a round Lucite cubicle (CMA/120, CMA/Microdialysis, Sweden) designed to limit the mobility of the animal) for 15 min before the probes were connected. To initiate dialysis, one of the externalized PE connections was attached to a 30 cm length of PE tubing (inflow) and the other arm to a 25 cm length of PE tubing (outflow). Then the dialysis tubing, connected to a microdialysis pump (CMA 100, CMA/Microdialysis, Sweden) with a 2.5 ml plastic syringe, was perfused with modified Ringer’s solution (NaCl 147 mM, KCl 4 mM, and CaCl2 2.3 mM) at a flow rate of 5 μl/min. After a 2 h wash-out period to establish a diffusion equilibrium, 3 baseline dialysates were collected sequentially by a microfraction collector (CMA/142, CMA/Microdialysis, Sweden) at 20-min intervals and then the following experiments were performed separately:

1. To investigate possible changes in spinal amino acid release after inflammatory pain, rats were subjected to s.c. injection of 50 μl BV solution after establishing the baseline. Then, samples were collected every 20 min for 2 h.
2. As a control group, rats were treated with s.c. injection of equal volume of normal saline in the hind paw after baseline testing. Then samples were collected as described above.
3. To examine whether amino acid release was dependent upon the ongoing primary afferent input from the site of peripheral injury, rats were injected with 0.75% bupivacaine 10 min prior to s.c. BV treatment in the same hind paw. Dialysates were then collected at the same time points as mentioned above.

2.4.2. Histology

After completion of the microdialysis experiment, all rats were euthanized with an overdose of trichloroacetaldehyde and anatomical observation was performed by an observer unaware of the rats’ treatments to check the location of the microdialysis membrane. The majority of the dialysis catheter sites were in the dorsal horn of L5–L6 spinal segments (lumbosacral enlargement). If it was not in the proper location, the rat was excluded from the further analysis.

2.5. Amino acids analysis of dialysates

Dialysate samples were collected on ice and immediately analyzed for amino acid concentrations by using the high performance liquid chromatography (HPLC) method [43,44].

2.5.1. Reagents and equipments

Standard amino acid reagents (including Asp, Glu, Gly, Tau, Ala, Gln, Thr, and Arg) and phthalaldehyde (derivatization reagent) were purchased from Sigma Chemical Co. (USA). Methanol (chromatographic grades) and tetrahydrofuran (THF, chromatographic grades) were purchased from Fisher Scientific Co. (USA). Sodium acetate anhydrous (NaAc) was obtained from Beijing Chemical Industry (Beijing, P.R. China).

HPLC was performed by using an Agilent 1050 series chromatographic system which consisted of a 1050 series Quaternary pump, a 1050 series programmable fluorescence detector, a 1050 series auto sample injector, a 1100 series online degasser, an analysis column temperature controlling chamber and an Agilent Phoenix DOS chromatographic workstation (Agilent, USA).

2.5.2. Mobile phase

Mobile phase “A”: Mobile phase “A” consisted of 410 ml 0.5 M NaAc, 85 ml methanol, and 5 ml THF. This solution was adjusted to pH 6.8 with acetic acid (10 N) and filtered through 0.4-μm pore filters.

Mobile phase “B”: Mobile phase “B” consisted of 110 ml 0.5 M NaAc, 385 ml methanol, and 5 ml THF. This solution was also adjusted to pH 6.8 and filtered as above.

2.5.3. Gradient

First, a mixture of 95% A: 5% B was used at a flow rate of 0.8 ml/min. Then the gradient was gradually changed to 100% B for about 3 min. At 35 min the initial conditions were returned and maintained for at least 15 min to stabilize the column for the next run.

A Waters C18 column (3.9 mm × 150 mm, 5 μm) was set at a constant temperature of 40 °C. A 20-μl portion of sample (dialysate or standard) was taken up and 20 μl phthalaldehyde was then added. Pre-column derivatization was completed by the auto sample injector. Excitation and emission wavelengths were selected at 232 and 440 nm, respectively. The order of elution of the amino acids was Asp, Glu, Gln, Gly, Thr, Arg, Tau, and Ala in a typical HPLC trace obtained in these experiments.

2.6. Assessment of spontaneous pain-related behaviors

As previously described [26,27], a 30 cm × 30 cm × 30 cm transparent plexiglas test box with a transparent glass floor was placed on a supporting frame of 30 cm high above the experimental table to allow the experimenters to observe the paws of the animals without obstruction. The rat was placed in the test box for at least 30 min before administration of any chemical agents. After the acclimation period, s.c. injection of BV or saline was made into the center of the plantar surface of one hind paw with slight restraint. The rat was then returned to the test box, and pain-related spontaneous behavioral responses were determined by counting the number of paw flinches occurring during 5 min intervals for 1 h (pharmacological experiments) or 2 h (peripheral blockade studies) following intraplantar injection.

2.7. Behavioral assays of pain sensitivity

2.7.1. Quantitative measurement of thermal pain sensitivity

Thermal pain sensitivity of rats was determined by testing paw withdrawal thermal latency (PWTL, s) in response to heat stimuli applied onto the injection site of the inflamed hindpaw. As described previously [26,27], the rat was placed on the surface of a 2 mm thick glass plate covered with a plastic chamber (20 cm × 20 cm × 25 cm) to measure its sensitivity to heat stimuli with a TC-1 radiant heat stimulator (new generation of RY-3 made in Xi’an Bobang Technologies of Chemical Industry Co. Ltd.,
P.R. China). The radiant heat source was a high intensity halogen lamp bulb (150 W) positioned under the glass floor directly beneath the target area on the hind paw. The rate of heating is 1 °C per 1 s from pre-set baseline temperature (about 35 °C, human body temperature). The distance between the projector lamp bulb and lower surface of the glass floor was adjusted to produce a light spot on the floor surface with 5 mm diameter. The heat stimulus was applied to the same site for 5 times and the latter three values were averaged as the mean PWLT. The inter-stimulus interval was between 10 and 15 min. The thermal latency was determined as the duration from the onset of heat stimulus to the occurrence of hind paw withdrawal reflex. The stimulus was stopped if the latency exceeded 30 s so as to avoid excessive tissue injury and the region was considered to be completely unresponsive.

2.7.2. Quantitative measurement of mechanical pain sensitivity

Mechanical pain sensitivity of rats was determined by testing paw withdrawal mechanical threshold (PWMT, mN) in response to mechanical stimuli applied in ipsilateral hindpaw produced by ascending graded individual von Frey monofilaments with bending forces of 21.07, 27.44, 34.30, 44.10, 53.90, 76.44, 107.80, 147.00, 196.00, 245.00, 294.00, 392.00, 490.00, and 588.00 mN. The rat was placed on a metal mesh floor covered with the same plastic chamber and von Frey filaments were applied from underneath the metal mesh floor to the testing site of the target hind paw. A von Frey filament was applied 10 times (several seconds for each stimulus) to each testing area. The bending force of the von Frey filament being able to evoke an approximate 50% occurrence of paw withdrawal reflex was expressed as the PWMT. The stimulus was stopped if the threshold exceeded 588.00 mN (cutoff value). Noticeably, it was not really the case that all of the 14 forces were applied to the same testing site in the experiment. Contrarily, we tended to select a range of von Frey filament forces (245.00–490.00 mN) as the priority over other forces according to our previous experiences in performing such behavioral testing. If the first filament used could elicit an appropriate 50% occurrence of paw withdrawal reflex across ten times of stimulation, then its force was considered as the PWMT value at that time point. If the selected filament force could not, the next higher force was tested. Similarly, if the filament elicited a more than 50% occurrence of paw withdrawal, the next lower force was used. The whole process continued until the optimal intensity of the filament was found. What we did in the testing avoided excessive poking of the hind-paw.

2.8. Experimental protocol

For evaluating the effects of MK-801 and CNQX on initiation and maintenance of PSN, the two drugs (for doses, see above) were intrathecally administered 5 min prior to or 5 min after s.c. BV injection and the testing of PSN began immediately after BV or drug administration in a pre- and post-administration paradigm, respectively. For examining ionotropic and metabotropic receptor antagonists on established thermal and mechanical hypersensitivity, all those compounds were injected at 2 h after s.c. BV injection and testing of thermal or mechanical hypersensitivity was performed between 2 and 4 h after s.c. BV injection. In addition, for each group of animals, the baseline values of PWTL and PWMT were measured prior to any treatment. The mechanical and thermal testing were done in the same group of animals in the present study. In general, the experimenting orders of these two measurements were randomly arranged to rule out the effects of expectation of rats. It is noteworthy that all behavioral testing procedures were performed blind to the treatment of the animals.

2.9. Statistics

2.9.1. Microdialysis data

Concentrations of amino acids were calculated based on the normalized peak areas with the external standards. Otherwise stated, all sample values were expressed as a percentage of baseline concentration (mean ± S.E.M.). Normalized data in one group across time and at each time point in different groups were analyzed by one way analysis of variance (ANOVA) followed by individual post hoc multiple comparisons (Fishers PLSD test). A statistical difference was accepted as significant at $P < 0.05$.

2.9.2. Behavioral data

All data were expressed as mean ± S.E.M. Two-way repeated measure ANOVA was used to analyze group differences in mean time courses of pain. One-way ANOVA (post hoc Fisher’s PLSD test) was applied to compare differences in averaged mean number of flinching reflex per 5 min as well as the thermal or mechanical hyperalgesia measurements. A level of $P < 0.05$ was accepted as significant.

3. Results

3.1. Peripheral-dependence of BV-induced spontaneous nociceptive behaviors

Consistent with our previous reports [26,27], s.c. injection of whole BV, but not normal saline solution, produced marked spontaneous pain-related behaviors characterized by increased paw flinch responses. The number of flinches peaked at 10 min and returned back to baseline levels before the 1 h time point (Fig. 1, BV-inflamed). Local pre-administration of an anesthetic agent (0.75% bupivacaine, 10 min prior to BV injection) resulted in an almost complete blockade of PSN (Fig. 1, Bupipsil + BV). To exclude a possible systemic effect, bupivacaine was also subcutaneously injected into a region on the contralateral hindpaw symmetrical to the BV injection site. It was found that contralateral bupivacaine application did not significantly affect the development of BV-evoked PSN (Fig. 1, Bupipsil + BV).

![Fig. 1](image-url)
3.2. Activity-dependent release of spinal amino acids following BV-evoked persistent nociception

3.2.1. Changes in extracellular concentrations of Glu, Asp and Gln

After collecting the baseline for 60 min, s.c. injection of BV into the rat hind paw elicited an apparent increase in spinal release of Glu (Fig. 2A) and Asp (Fig. 2B), whereas no appreciable changes were observed in the saline control group. It appeared clear that both Glu and Asp concentrations began to rise immediately after BV injection, with a peak attained at about 20 min (174% and 204% of baseline, n = 6 for each group, P < 0.05 and P < 0.001 vs. saline control for Glu and Asp respectively), and then declined gradually at 40 min. Increased responses recovered to the baseline at 60 min after s.c. BV treatment (P > 0.05 vs. saline control for all). Peripheral bupivacaine pre-treatment (0.75%, 10 min prior to BV injection) completely inhibited BV-evoked increase in release of Glu and Asp, even the levels of these two EAAs being much lower than those of baseline (Fig. 2A and B).

The extracellular amount of Gln, a metabolic product of Glu [45–47], also peaked at 20 min after BV injection (208% of baseline, n = 8, P < 0.05 vs. saline control), and returned to baseline at 40 min. Interestingly, its level continually decreased and became even lower than baseline at 60 min (48% of baseline, P < 0.05 vs. saline control). This decrease of Gln release continued until 120 min after BV injection. The temporal profile of Gln release in Bupipsi + BV group was almost the same as that of Glu and Asp (Fig. 2C).

3.2.2. Changes in extracellular concentrations of Gly, Tau and GABA

BV-induced persistent pain elicited a significant increase in the amount of extracellular Gly (Fig. 3A) and Tau (Fig. 3B), with a maximum level reaching at 20 min (136% and 270% of baseline, n = 7 and 8, P < 0.05 and P < 0.01 vs. saline control for Gly and Tau respectively). Then increased concentrations of the two IAs went back to the baseline at 40 min after BV injection (Fig. 3A and B). Subsequently, the concentration of Tau remained constant (P > 0.05 vs. saline control, Fig. 3B), while the level of Gly further decreased and became lower than baseline at 60 min (62% of baseline, P < 0.05 vs. saline control). Peripheral bupivacaine pre-treatment fully abolished BV-evoked release of Gly and Tau (Fig. 3A and B). While the baseline level of Gly release was also reduced by bupivacaine-induced local anesthesia, that of Tau remained less influenced.

Here, it is noteworthy that γ-aminobutyric acid (GABA), another principal inhibitory neurotransmitter involved in nociceptive modulation [48], was below detection limits under baseline conditions. However, from 20 min towards 60 min after s.c. BV injection, a certain amount of extracellular GABA became detectable in some cases. Because of the so small amount of GABA release, we quantified it by measuring the peak area under the HPLC curve instead of converting it to percentage of baseline. At 20 min after BV injection, the extracellular GABA content raised from undetectable level to 0.9829 ± 0.4272 Norm. Then, the GABA release decreased, with the peak area being 0.6100 ± 0.2672 Norm and 0.5100 ± 0.2261 Norm at 40 min and 60 min, respectively. After that, the GABA level became undetectable again until the end of the detection period (2 h after BV injection).

3.2.3. Changes in extracellular concentrations of Ala, Arg and Thr

The extracellular concentrations of Ala (Fig. 4A), Arg (Fig. 4B) and Thr (Fig. 4C) increased to their maximum at 20 min after administration of BV (153%, 127% and 157% of baseline, n = 7–8, P < 0.05 vs. saline control for Ala, Arg and Thr respectively). Their concentra-

![Fig. 2](image_url) Temporal changes in extracellular concentrations of aspartate, glutamate and glutamine at the spinal level. The rats were treated with subcutaneous injection of saline (n = 6 for each), bee venom (n = 6–8 for each) and Bupipsi + BV (local administration of 0.75% bupivacaine 10 min prior to BV injection in the ipsilateral paw, n = 4 for each). Big reverse arrows indicate the time of saline or BV injection, while small reverse arrows denote the time of bupivacaine administration. Vertical bars: ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 vs. BV-inflamed group; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. saline-treated group.

![Fig. 3](image_url) Temporal changes in extracellular concentrations of glycine and taurine at the spinal level. The rats were treated with subcutaneous injection of saline (n = 6 for each), bee venom (n = 7–8 for each) and Bupipsi + BV (local administration of 0.75% bupivacaine 10 min prior to BV injection in the ipsilateral paw, n = 4 for each). Big reverse arrows indicate the time of saline or BV injection, while small reverse arrows denote the time of bupivacaine administration. Vertical bars: ± S.E.M. P < 0.05 vs. BV-inflamed group; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. saline-treated group.
reverse arrows denote the time of bupivacaine administration. Vertical bars:

3.3. Effects of i.t. CNQX and MK801 on BV-induced spontaneous nociception and hyperalgesia

3.3.1. Prevention and reversal of PSN by spinal blockade of NMDA and non-NMDA receptors activation

In comparison with vehicle control, i.t pre-treatment with CNQX (3.5 nmol, 35 nmol and 100 nmol) and MK-801 (1.2 nmol and 24 nmol) resulted in a dose-dependent suppression of BV-induced persistent paw flinching reflex over the 1 h time course of observation (Fig. 5A and B). The averaged mean number of flinching reflex per 5 min was shown in the left panel of Fig. 5D and E for i.t. pre-treatment with CNQX and MK-801, respectively. The three doses of CNQX produced 19.42% (3.5 nmol, n = 8, P < 0.05), 37.27% (35 nmol, n = 8, P < 0.05) and 57.32% (100 nmol, n = 8, P < 0.001) inhibition of PSN, while the values of MK-801 were 32.42% (1.2 nmol, n = 8, P < 0.05) and 57.97% (24 nmol, n = 8, P < 0.001), respectively.

Intrathecal post-treatment with CNQX and MK-801 at 5 min after s.c. BV could also produce 31.98% (100 nmol, n = 8, P < 0.001) and 44.81% (24 nmol, n = 8, P < 0.001) inhibition of the BV-induced PSN in the subsequent 55 min period (Fig. 5C and the right panel of Fig. 5D and E). Taken together, the above results suggest that activation of spinal NMDA and non-NMDA receptors plays crucial roles in both inducing and maintaining processes of BV-evoked PSN.

3.3.2. Spinal inhibition of NMDA and non-NMDA receptors fails to reverse primary thermal and mechanical hyperalgesia in the BV model

Post-treatment with i.t. administration of the highest dose of CNQX (100 nmol) or MK-801 (24 nmol) used in the current study produced no significant effect on basal pain sensitivity to either thermal or mechanical stimuli (data not shown). There were still no obvious changes in either PWTL or PWMT when the same doses of CNQX and MK-801 were intrathecally applied in BV-inflamed rats (Fig. 6), indicating no involvement of GluRs in the maintenance of BV-induced primary heat and mechanical hyperalgesia.

3.4. Effects of i.t. mGluR antagonism on BV-evoked thermal and mechanical hyperalgesia

To further elucidate the Glu receptor subtypes mediating its putative influence on thermal and mechanical hyperalgesia, we investigated effects of i.t. post-treatment with three kinds of mGluR antagonists: AIDA for group I [49,50], EGLU for group II [21,51] and MSOP for group III mGluRs [51,52]. As illustrated in Fig. 6, i.t. administration of AIDA (10 nmol) produced a partial reversal of BV-evoked mechanical (but not thermal) hyperalgesia, whereas post-injection of EGLU (20 nmol) and MSOP (10 nmol) elicited an almost full reversal of BV-induced thermal (but not mechanical) hyperalgesia.

4. Discussion

The present study demonstrated a rapid and significant release of EAAs (Asp and Glu), IAAs (Gly, Tau and GABA) and other amino acids (Ala, Arg and Thr) in the spinal cord following BV-induced persistent nociception. Peripheral afferent inputs from the injection site were found to be required for both behavioral PSN and spinal amino acid release. Furthermore, our pharmacological experiments showed that i.t. administration of CNQX or MK-801 could significantly prevent the induction and reverse the maintenance of BV-induced persistent spontaneous nociception, whereas i.t. mGluRs antagonism resulted in an apparent reversal of BV-evoked thermal or mechanical hypersensitivity.

One gain of this work lies in the finding that extracellular concentrations of spinal Glu and Asp profoundly increased in the BV model of inflammatory pain. These results are in large accordance with previous observations from other animal pain models [6–11,53–55], although the specific release profile of EAAs varies a lot among each other (see [11] for a brief summary), possibly reflecting inter-model differences in EAAs responses (magnitude or duration). The divergence might also be explained by subtle differences in experimental conditions (such as the exact location of the dialysis fiber, the accuracy or sensitivity of the detection method, the animal species etc.). In the present study, the sample collection interval was set at 20 min. Although one cannot gain any certain knowledge of when the EAAs level began to rise substantially after BV injection (maybe earlier than 20 min), the general release profile of spinal EAAs paralleled well with the time course of BV-induced PSN, suggesting a strong correlation between them.

The present results also revealed a smaller and shorter increase in spinal IAAs release following BV-induced persistent pain.
Enhanced release of IAAs under the pain state has also been reported by a multiplicity of previous papers [79,53]. This transient increase in concentrations of spinal IAAs may be interpreted as a reflection of activation of an endogenous spinal analgesia system or descending inhibitory response, based on the previous suggestion that the large increase in EAAs was often accompanied by a corresponding rise in IAAs level in an attempt to reinstate the homeostasis [11,53]. A distinct feature of the present spinal IAAs release profile was that the level of Gly continued to decrease after returning to the baseline, becoming much lower at the end of the

Fig. 5. Effects of intrathecal pre- and post-treatment with CNQX and MK801 on the induction and maintenance of persistent spontaneous flinching reflex induced by subcutaneous BV injection. Curve graph (A, B, C) shows the time courses of CNQX and MK-801 effects on mean number of paw flinches recorded at each 5 min time block. (A) Pre-treatment with CNQX at three doses (3.5 nmol, 35 nmol and 100 nmol); (B) pre-treatment with MK-801 at two doses (1.2 and 24 nmol); (C) post-treatment with CNQX (24 nmol) and MK-801 (100 nmol). Column graph (D, E) illustrates the mean number of paw flinches per 5 min averaged from the observation period. (D) Effects of pre- and post-treatment with CNQX; (E) effects of pre- and post-treatment with MK-801. Values are mean ± S.E.M. (n = 8 for each group).* P<0.05, *** P<0.001 vs. vehicle group.

Fig. 6. Effects of intrathecal post-treatment with CNQX (100 nmol), MK801 (24 nmol), AIDA (10 nmol), EGLU (20 nmol), and MSOP (10 nmol) on maintenance of BV-induced primary thermal (A) and mechanical (B) hyperalgesia. Vertical bars: ± S.E.M. (n = 8 for each). Baseline, averaged values of PWTL or PWMT obtained from the other groups of animals prior to any treatment. PWTL, paw withdrawal thermal latency; PWMT, paw withdrawal mechanical threshold. * P<0.05 vs. BV + vehicle group.
dependence of BV-induced behavioral and electrophysiological contributions to the behavioral expression of thermal and mechanical under BV-produced inflammatory pain state, which, at least in part, contributes to the behavioral expression of thermal and mechanical hypersensitivity [57–63].

Consistent with our previous demonstrations of the peripheral-dependence of BV-induced behavioral and electrophysiological responses [27,31,32,35], in this study, local pre-administration of bupivacaine could cause a complete abolition of PSN and spinal amino acid release. The possibility that this inhibitory effect might be due to a systemic effect of the locally injected anesthetic agent seems unlikely because peripheral injection of the same dose of bupivacaine into the non-injured hindpaw caused no significantly pharmacological influence on BV-evoked persistent spontaneous paw flinching reflex (Fig. 1). Of primary interest is the finding that, in some cases, local anesthesia of the hindpaw resulted in not only an entire abolition of BV-induced EAA and IAAs release, but also an even lower level than the baseline (Figs. 2 and 3). This result is in general agreement with one electrophysiological study, in which blockade of the sciatic nerve with 4% lidocaine suppressed not only formalin-induced spike discharges but also background activities of spinal nociceptive neurons [64]. Based upon the above results, we propose that EAAs–IAAs imbalance at the spinal level, requiring the intact primary afferent inputs from the periphery, serves as another major mechanism underlying occurrence/maintenance of central sensitization and chronicity of pain state. And this concern will be important to explain why pain treatment (such as local nerve blockade) should be given as early as possible before the balance between nociception and anti-nociception is disrupted.

A growing number of earlier reports have provided compelling evidence for key roles of NMDA and non-NMDA receptors in numerous behavioral manifestations of pathological pain by using various pain models [65–67]. According to previous studies, NMDA but not non-NMDA receptor antagonists could block the persistent nociceptive responses induced by s.c. injection of formalin [18], while both NMDA and non-NMDA receptor antagonists were effective in inhibiting the nociceptive behaviors produced by intraplantar injection of capsaicin into the mouse hindpaw [19]. There are also a large number of previous reports indicating complicated roles of iGluRs in multiple kinds of inflammatory or neuropathic pain [13–16,68–71]. Taken together with those findings, it might be logically supposed that specialized roles of NMDA and non-NMDA receptors in development and maintenance of pain and hyperalgesia may be different from case to case and need to be further studied by using a pathological pain model being able to reflect multiple “phenotypes” of pain-related behaviors.

The unique behavioral “phenotypes” of nociception and hyperalgesia identified in the rodent BV test are believed to reflect a complex pathological state of inflammatory pain and might be appropriate to the study of phenotype-based mechanisms of pain and hyperalgesia [26,28,30,31,37,39]. Using this unique model of pain following chemical tissue injury, we performed series of pharmacological experiments to investigate potential roles of peripheral and central NMDA or non-NMDA receptors in BV-induced multifarious pain-related behaviors and electrophysiological alterations in spinal nociceptive neurons [12,17,32,35,72]. In the present study, we further contributed the new findings that activation of both NMDA and non-NMDA receptors in the spinal cord is involved in the induction and maintenance of BV-induced PSN. However, they were not likely to contribute to the maintenance of primary hyperalgesia produced by intraplantar BV injection. The failure of NMDA and non-NMDA receptor antagonisms to reverse established thermal and mechanical hyperalgesia by i.t. route was not likely due to the dose insufficiency, because the same dose of both drugs could effectively repress BV-initiated PSN. Moreover, our previous report demonstrated that systemic administration of MK801 (0.01 mg/kg, i.p.) was not effective either [12]. Collectively, these results, on one hand, suggest that sufficient amounts of EAAs released from spinal cord (as mentioned above) are likely acting on iGluRs to mediate the development and persistence of BV-evoked PSN, while on the other hand, further confirm our previous hypothesis that different neurochemical components (or intracellular messenger-mediated signal transduction pathways) are likely to be involved in mediating different “phenotypes” of nociception and hypersensitivity [29,30,37–40,73].

In another series of experiments, we found that i.t. antagonism of different subgroups of mGluRs could effectively block established thermal and mechanical hyperalgesia in BV-inflamed rats. Previous behavioral and electrophysiological evidence has been accumulating showing involvement of mGluRs in spinal nociceptive processing [1,20–24,74,75]. An important finding added by the present study is that blockade of spinal group I mGluRs activation by AIDA suppressed BV-induced primary mechanical hyperalgesia, while inhibition of group II and III mGluRs activity by EGLU and MSOP markedly reduced the extent of primary thermal hypersensitivity. This result suggests that the BV-evoked primary hyperalgesia is maintained by a central sensitized state caused by not only spinal disinhibition but also sustained activation of mGluR subtypes and the sequential signaling cascades. The exact sources of Glu that is recruited and responsible for such prolonged activation of mGluRs are still not clear but likely ascribed to evoked release by peripheral heat or mechanical stimuli when testing being performed. In addition, the present pharmacological blockade observations also suggest that activation of iGluRs or mGluRs is state- or stimulus modality-dependent, and spinal antagonism of any specific glutamate receptor subtype fails to block all behavioral “phenotypes” of pathological pain, implicating possible involvement of inhibitory influences during the whole story.

In summary, it is likely that there is an EAAs–IAAs balance at the spinal level under normal state, however, the balance would be disrupted by the arrival of ongoing activity from peripheral persistent nociception (prolonged pathological state), leading to abnormal changes in spinal synaptic transmission and modulation (central sensitization) which in turn contribute to persistence or chronicity of pain. Based upon this hypothesis, the new therapeutic strategy of analgesia should focus not only on the EAAs receptor-mediated signaling targets but also on the restoration of EAAs–IAAs balance at the spinal level to prevent persistence or chronicity of pain at the early stage of pathological processing.

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