行政院國家科學委員會專題研究計畫 成果報告

肉毒桿菌毒素-A 對麻醉大白鼠骨盆-尿道反射增益現象的抑制效應及訊息路徑的調節經由骨盆臟器間對話的去敏感機轉(第 3 年)

研究成果報告(完整版)

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中文摘要： 近年來已證實 Eph 接受器（EphBR）為酪氨酸激酶，與其配體 ephrinB 參與調控脊髓上與疼痛有關的神經可塑性。利用此現象假設腰薦部脊髓上的 EphB 接受器活化，可能透過 Src 家族酪氨酸激酶的活化，使麩胺酸性 NMDA 接受器 NR2B 次單元蛋白質磷酸化，進而參與調控骨盆尿道反射增益現象。因此在大鼠上由椎管內給予 ephrinB2 後，分別記錄外尿道括約肌肌動作電位的反射活性和分析腰薦部（L6-S2）脊髓背角的神經上蛋白質的表現量。與給予 vehicle 比較，當由椎管內給予外源性的 ephrinB2（5 ug/rat）誘發反射增益現象，其伴隨著 ERK1/2、 Src 家族激酶、NR2B 次單元的 Y1336 和 Y1472 的酪氨酸接合位的磷酸化產生。此外，分別由椎管內給予 EphB1 和 EphB2 的免疫球蛋白聚合蛋白質（10 ug/rat）阻斷由 ephrinB2 引起的反射增益現象以及蛋白質磷酸化的現象。由椎管內前處理 Src 家族激酶的拮抗劑 PP2（50 uM, 10 ul），可逆轉反射增益現象以及 Src 家族激酶、NR2B 次單元蛋白質的磷酸化。總結上述結果可證實 ephrinB2 使 EphB 接受器活化之後，其藉由 Src 家族激酶使腰薦部的脊髓背角上的 NMDA 接受器 NR2B 次單元蛋白質磷酸化，其機轉對於使脊髓上反射增益現象轉變成腹腔疼痛/骨盆腔疼痛過敏的現象極為重要。

中文關鍵詞： 骨盆腔疼痛、尿道、 Src 家族激酶、NMDA

英文摘要： EphrinB2 induces pelvic-urethra reflex potentiation via Src kinase-dependent tyrosine phosphorylation of NR2B. Am J Physiol Renal Physiol 300: F403–F411, 2011. First published December 8, 2010； doi:10.1152/ajprenal.00520.2010.—Recently, the role of EphB receptor (EphBR) tyrosine kinase and their ephrinB ligands in painrelated neural plasticity at the spinal cord level have been identified. To test whether Src-family tyrosine kinase-dependent glutamatergic N-methyl-D-aspartate receptor NR2B subunit phosphorylation underlies lumbosacral spinal EphBR activation to mediate pelvic-urethra reflex potentiation, we recorded external urethra

sphincter electromyogram
reflex activity and analyzed protein expression in the
lumbosacral (L6-S2) dorsal horn in response to intrathecal ephrinB2
injections. When compared with vehicle solution, exogenous ephrinB2
(5 g/rat it)-induced reflex potentiation, in associated with
phosphorylation of EphB1/2, Src-family kinase, NR2B Y1336 and
Y1472 tyrosine residues. Both intrathecal EphB1 and EphB2 immunoglobulin
fusion protein (both 10 g/rat it) prevented ephrinB2-
dependent reflex potentiation, as well as protein phosphorylation.
Pretreatment with PP2 (50 M, 10 l it), an Src-family kinase
antagonist, reversed the reflex potentiation, as well as Src kinase and
NR2B phosphorylation. Together, these results suggest the ephrinB2-
dependent EphBR activation, which subsequently provokes Src kinase-
mediated N-methyl-D-aspartate receptor NR2B phosphorylation
in the lumbosacral dorsal horn, is crucial for the induction of spinal
reflex potentiation contributing to the development of visceral pain
and/or hyperalgesia in the pelvic area.

英文關鍵詞： pelvic pain； urethra； Src-family kinase； N-methyl-D-aspartate

前言、研究目的、文獻探討：

IN THE LUMBOSACRAL DORSAL horn, neurotransmission mediated by glutamatergic N-methyl-D-aspartate receptors (NMDARs) has been implicated in processing nociceptive afferent inputs coming from the lower urinary tract (Birder and De Groat, 1992, Haley et al., 1990). Spinal administration of NMDAR agonists has dose-dependently facilitated the visceromotor reflex, together with pressor responses to pelvic noxious stimulation (Kolhekar and Gebhart, 1994). Conversely, blockage of NMDAR using pharmacological antagonists has inhibited pain behavior caused by irritation of the pelvic viscera (Olivar and Laird, 1999). Among subunits of NMDAR, the role of the NR2B subunit in pain development has been intensively investigated, as electrophysiological studies have demonstrated that phosphorylation of specific NR2B tyrosine residues is an important determinant for NMDAR-mediated currents (Moon et al., 1994), which defines the role of NMDARs in pain-related neural plasticity (Berberich et al., 2005, Liu et al., 2004, Luque et al., 1994, Massey et al., 2004). The signaling of Src kinases, a family of protooncogenic tyrosine kinases, has been shown to modulate NMDAR-mediated synaptic transmission and plasticity (Ali and Salter, 2001). In inflammatory animal models, the intrathecal administration of Src-family kinase inhibitors prevented spinal NR2B phosphorylation and behavior hyperalgesia, implying that Src-dependent phosphorylation of NMDAR NR2B subunits plays a crucial role in the development of postinflammatory pain and/or hyperalgesia (Slack et al., 2008).

EphB receptors (EphBRs), transmembrane molecules, were initially identified as guidance cues during neural development (Wilkinson, 2001). In the last decade, studies have demonstrated that the interactions between EphBRs and their ephrinB ligands modulate neural plasticity induction in the mammalian central nervous system, mainly, but not exclusively, via exhibiting effects on NMDAR (Grunwald et al., 2001, Henderson et al., 2001). A recent study also demonstrated that, in association with thermal hyperalgesia, EphBR activation caused by immunoglobulin fusion protein of ephrinB2 (ephrinB2-Fc)-induced, Src kinase-dependent NR2B phosphorylation in the spinal cord (Battaglia et al., 2003), suggesting that ephrinB2-EphBR tyrosine kinase interactions could probably modulate pain signaling via spinal Src-dependent NMDAR phosphorylation (Battaglia et al., 2003).

Although further proof is still needed for the physiological and/or pathophysiological relevance, the induction of pelvicurethra reflex potentiation (Lin, 2003; 2004), a form of NR2B phosphorylation-dependent neural plasticity (Chang et al., 2010, Peng et al., 2011, Peng et al., 2009, Wu et al., 2010), has been linked to the development of visceral pain from pelvic organs (Peng et al., 2008a; 2008b; 2009; 2010a; 2010b). Studies have shown that the activation of nociceptive afferent fibers expressing transient receptor potential vanilloid/transient receptor potential ankyrin by the instillation of irritants into the uterus (Peng et al., 2008a; 2008b) and the descending colon (Peng et al., 2009) facilitated pelvic-urethra reflex activity in a crossorgan manner. Recently, we reported that acute colonic nociceptive stimulation sensitized pelvic-urethra reflex activity via the upregulation of endogenous ephrinB2 expression, which activates EphB1 and EphB2 receptors and leads to Src-family kinase-dependent NR2B phosphorylation in the lumbosacral spinal cord (Peng et al., 2010). Accordingly, we hypothesized that the interactions between ephrinB2 and EphBRs, as well as downstream Src-dependent NR2B phosphorylation, are involved in the induction of pelvic-urethra reflex potentiation. We tested this hypothesis with direct spinal application of ephrinB2 and simultaneous recordings, which evoked urethra reflex activity in intact animal preparations, and we analyzed the protein

expression/phosphorylation of the lumbosacral spinal cord. Our results dem onstrated that the activation of EphBR can initiate downstream phosphorylation of Src-dependent NMDAR NR2B Y1336 and Y1472 tyrosine residues in the lumbosacral dorsal horn to mediate the induction of pelvic-urethra reflex potentiation, which is presumed to underlie the development of pelvic visceral pain/hyperalgesia.

研究方法：

Animal Preparations

One hundred and ninety-eight female Sprague-Dawley rats (205–290 g) were used in the present experiment, which was reviewed and approved by the Institutional Review Board of Chung-Shan Medical University in Taiwan. Rats were anesthetized with urethane (1.2 g/kg ip). A PE-10 catheter was inserted through a slit made at the atlantooccipital membrane and passed caudally to the L6-S2 spinal cord, which is the spinal level regulating urogenital system, for the dispensing of test agents. The left pelvic nerve was dissected and mounted on a pair of wire electrodes for stimulation. Oligo-/single unit action potentials in the external urethra sphincter electromyogram (EUSE) activity were recorded by a pair of wire electrodes and were continuously recorded on a recording system (MP30, Biopac, Santa Barbara, CA). Single shocks at a fixed suprathreshold strength were repeated at 1 stimulation/30 s [test stimulation (TS)] and given through the stimulation electrodes.

Application of Drugs

Drugs administered included the following: N-methyl-D-aspartic acid (NMDA; 10 uM, 10 ul it, Sigma) (Peng et al., 2010), a selective glutamatergic NMDAR agonist; D-2-amino-5-phosphonovalerate (APV, 10 uM, 10 ul it, Sigma) (Peng et al., 2010), a glutamatergic NMDAR antagonist; ephrinB2-Fc chimera (5 ug/rat it, Sigma) (Battaglia et al., 2003), EphBR ligand; EphB1-Fc chimera (10 ug/rat it, Sigma) (Battaglia et al., 2003), EphB1 selective antagonist; EphB2-Fc chimera (10 ug/rat it, Sigma) (Gogas, 2006), EphB2 selective antagonist; and 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolol[3,4-d]pyrimidine (PP2, 50 uM, 10 ul it, Tocris) (Peng et al., 2010), a Src-family kinase inhibitor. In all cases, solvent solutions of identical volume to tested agents were dispensed to serve as the vehicle control.

Western Blotting

After the experimental procedures have been finished, animals were decapitated, and the dorsal half of the left spinal cord segments from L6-S2, the spinal level regulating urogenital system, was dissected because the left pelvic nerve was stimulation. Protein samples (20 ug) were separated on SDS-PAGE (8 and 12%) and transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat milk and probed with antibodies against ephrinB2 (1:2,000, Santa Cruz), phosphorylated EphB1/2 (pEphB1/2, 1:2,000, Milipore), phosphorlated Src (1:1,000, Milipore), or phosphorylated tyr1336/tyr1472-NR2B (1:1,000 Milipore). The blots were incubated with horseradish peroxidase-conjugated antibody (1:2,000) for 1 h at room temperature and visualized with ECL solution (5 min) followed by film exposure (2 min). Densitometric analysis of the WB membranes was done with Science Lab 2003 (Fuji, Japan).

Experimental Protocols

Protocols for assessing effects of various kinds of reagents on reflex activity were as follows.

Protocol 1: Baseline reflex activity. Single shocks of TS (1 stimulation/30 s) were given through stimulation electrodes for 30 min.

Protocol 2: Agonist-induced reflex potentiation After equilibration (usually 30 min), vehicle solution, NMDA, or ephrinB2 was injected via the intrathecal catheter at 2 min before TS onset.

Protocol 3: Pharmacological antagonization. EphB1, EphB2, EphB1 and EphB2, APV, or PP2 was injected intrathecally 5 min before, and then ephrinB2 or NMDA was done 2 min before, TS stimulation onset.

Protocol 4: Effects of NMDA. In some studies, NMDA was intrathecally injected after PP2 (5 min before) and ephrinB2 (2 min before) at 1 min before stimulation onset.

Data Analysis

The reflex excitability was assayed by recording the EUSE resulting from the pelvic afferent nerve stimulations. We, therefore, counted the spike number within 1 min following each shock applied. The responses at 5, 10, 15, and 30 min were offline analyzed and were plotted as a line chart, and the evoked activity at 30 min was used to create the bar charts. The data from each specific time point were averaged from three evoked events in each animal, and then the data from all of the animals in this experiment were averaged in the statistical charts. For the data from the Western blot analysis, the density of specific bands was measured with a computer-assisted imaging analysis system (LAS-3000; Fuji, Kanagawa, Japan). After normalized against corresponding loading control bands, the intensity of specific bands was expressed as percentage of loading control. Data were analyzed by using SigmaPlot 10.0 (Systat Software, San Jose, CA). All data in the text and Figs. 1–3 in this study are expressed as means ± SE. For serial measurements over time (i.e., spikes of EUSE and protein expression in response to capsaicin instillation at different time point), two-way repeated-measures ANOVA was used to assess changes in values before and after treatment. In other cases, two-way ANOVA was used to analyze data. In all cases, a post hoc Tukey test was used to compare means for groups when an adequate F ratio was achieved, and significance was assigned at a $P < 0.05$.

結果與討論：

Results

Involvement of EphB1 and EphB2 Receptors

The role of spinal EphB1 and EphB2 receptors in ephrinB2-dependent reflex potentiation was tested by administering selective Eph1B and Eph2B receptor antagonists, Eph1B-Fc and Eph2B-Fc, respectively. As described, intrathecal ephrinB2 (5 ug/rat, Fig. 1, A and E, TS + ephrinB2), but not vehicle solution (TS + VEH), induced reflex potentiation characterized by an elongated firing in animal preparations. Rather than vehicle solutions (data not shown), prior administration of EphB1-Fc (10 ug/rat it, 5 min before stimulation onset) and EphB2-Fc (10 ug/rat it, 5 min before stimulation onset) both prevented ephrinB2-dependent reflex potentiation by decreasing the mean spike count evoked by the TS (TS + EphB1 + ephrinB2 and TS + EphB2 + ephrinB2, respectively), whereas neither EphB1-Fc nor EphB2-Fc affected the TS-evoked baseline reflex activity (Fig. 1, A and D, TS + EphB1 and TS + EphB2, respectively). The participation of EphB1/2 in ephrinB2-dependent reflex potentiation was further investigated by immunoblotting analysis. Compared with vehicle solution (Fig. 2A, TS + VEH), intrathecal ephrinB2 (5 ug/rat, bar: ephrinB2, TS) significantly upregulated the expression of pEphB1/2 (pEphB2) in the lumbosacral dorsal horn without affecting the levels of total EphB1 and total EphB2 (EphB1 and EphB2, respectively). Prior EpB1-Fc and EphB2-Fc administrations prevented EphB1/2 phosphorylation caused by ephrinB2 by decreasing pEphB1/2 expression (TS + EphB1 and TS + EphB2, respectively).

Involvement of NMDAR

Then we investigated the role of the interactions between the EphB1/2 receptor and NMDAR in ephrinB2-dependent reflex potentiation. Compared with the animals that received the ephrinB2 injection (5 ug/rat, Fig. 1, TS + ephrinB2), the intrathecal APV (10 uM, 10 ul) injection prevented the ephrinB2-dependent reflex potentiation by decreasing the mean spike count evoked by the TS (Fig. 1, B and E, TS + APV + ephrinB2). Furthermore, after both EphB1 and EphB2 had been blocked by the coadministration of selective antagonists, Eph1B-Fc and Eph2B-Fc (TS + EphB1 + EphB2), intrathecal NMDA injection (10 uM, 10 ul) provoked reflex potentiation characterized by an elongated firing in these preparations (TS + EphB1 + EphB2 + NMDA). On the other hand, immunoblotting showed that prior APV administration failed to prevent ephrinB2-dependent EphB1/2 phosphorylation in the lumbosacral dorsal horn (Fig. 2A, TS + APV).

Involvement of Src-Family Kinases

The role of Src-family kinases in ephrinB2-dependent reflex potentiation was studied using a selective antagonist, PP2. Electrophysiological recordings showed that, while pretreatment with PP2 (50 uM, 10 ul it) exhibited no effects on the TS-evoked baseline reflex activity (TS + PP2), it prevented ephrinB2-dependent reflex potentiation by decreasing the mean spike count evoked by the TS (Fig. 1, B and F, TS + PP2 + ephrinB2). Moreover, after ephrinB2-dependent reflex potentiation had been abolished by PP2, intrathecal NMDA reversed PP2-induced antagonization by increasing the mean spike count (TS + PP2 + ephrinB2 + NMDA). The role of Src family kinases was further investigated using Western blotting analysis. Although ephrinB2-dependent EphB1/2 phosphorylation in the lumbosacral spinal cord was not antagonized by prior PP2 injection (Fig. 2A, TS + PP2), in the animals that received the TS, immunoblotting showed that the

expression level of phosphorylated Src (Fig. 2B) but not total Src had increased significantly due to the intrathecal ephrinB2 administration (5 ug/rat, bar: ephrinB2 TS) compared with those given vehicle solution (TS + VEH). Pretreatment with EpB1-Fc, EphB2-Fc, and PP2 (TS + EphB1, TS + EphB2, and TS + PP2, respectively) but not APV (TS + APV) prevented ephrinB2-dependent Src phosphorylation in the lumbosacral dorsal horn.

The Role of NMDAR NR2B Subunit

The involvement of Src-mediated phosphorylation of NR2B Y1336 and Y1472 tyrosine residues in ephrinB2-dependent reflex potentiation was explored using Western blotting analysis. Compared with intrathecal vehicle solution (Fig. 2C, TS + VEH), ephrinB2 enhanced the expression of phosphorylated Y1336 and phosphorylated Y1472 without affecting the level of total NR2B in the lumbosacral dorsal horn of animals that received the TS. All of the prior EpB1-Fc, EphB2-Fc, and PP2 treatments (TS + EphB1, TS + EphB2, and TS + PP2, respectively) prevented ephrinB2-dependent NR2B Y1336 and Y1472 phosphorylation in the lumbosacral spinal cord.

Discussion

In this study, we found ephrinB2 administration in the lumbosacral spinal cord induced pelvic-urethra reflex potentiation with corroboration by EphB1/2, Src-family kinases, as well as phosphorylation of NR2B Y1336 and Y1472 tyrosine residues. Pretreatment with EphBR selective antagonists prevented agonist-induced reflex potentiation and all protein phosphorylation. Intrathecal application of a Src-family kinase inhibitor blocked reflex potentiation, which occurred with a reversal in phosphorylation of Src kinase and NR2B tyrosine residues. Taken together, these results suggest that induction of pelvic-urethra reflex potentiation caused by spinal ephrinB2 administration may be, at least in part, due to the phosphorylation of specific tyrosine residues of the NMDAR NR2B subunit caused by a Src-family tyrosine kinase downstream of EphBR activation at the lumbosacral spinal cord level.

It has been shown that interactions between ephrinB2 and EphBRs in the spinal cord are required for the onset of somatic pain and hyperalgesia. Following nerve injury, the expression of ephrinB2 in dorsal root ganglion (DRG) neurons and the EphB1 receptor in the dorsal horn were both enhanced in a time-dependent manner corresponding to the development of thermal hyperalgesia in adult rats (Song et al., 2008a; 2008b). Knock-down of ephrinB2 using specific short interfering RNA decreased expression of ephrinB2 in the DRG, along with attenuation of mechanical allodynia caused by spinal nerve crushing (Kobayashi et al., 2007). Intrathecal administration of ephrinB2-Fc chimera to Wistar rats induced behavioral evidence of thermal hyperalgesia (Slack et al., 2008). Conversely, pharmacological antagonization of EphB1 prevented nerve injury-induced dorsal horn neuron hyperexcitability and neuropathic pain (Song et al., 2008b). Not only in somatic pain caused by injury or inflammation, our study investigating cross talk between pelvic viscera has demonstrated that acute activation of transient receptor potential vanilloid 1 expressing nociceptive afferent fibers coming from the descending colon upregulated spinal ephrinB2 expression and simultaneously induced EphB1/2 phosphorylation in the lumbosacral dorsal horn (Peng et al., 2010). In analogy with this study, results showed spinal administration of ephrinB2 induced lumbosacral EphB1/2 phosphorylation in association with pelvic-urethra reflex potentiation, a phenomenon presumed to participate in the development

of pain and/or hyperalgesia in the lower urinary tract. Pharmacological antagonization of EphB1 and EphB2 receptors prevented EphB1/2 phosphorylation and ephrinB2-dependent reflex potentiation. Together, these results suggest that the ephrinB2-EphBR interactions in the lumbosacral spinal cord are required for the onset of visceral pain in the pelvic area.

In the spinal dorsal horn, NMDARs integrate primary afferent inputs and provide mechanisms to amplify nociceptive signals, leading to the development of neuropathic and/or postinflammatory pain (Dubner and Ruda, 1992, Ma and Woolf, 1995, Wilkinson, 2001). Although the exact role of specific NMDAR subunits is still unclear, functional NMDARs are mostly heteromeric complexes composed of NR1/NR2 subunits in the mammalian central nervous system (McBain and Mayer, 1994). The NR1 subunit is obligatory for NMDARs, whereas NR2 subunits are essential for calcium ion gating (Petralia et al., 1994), which demonstrates the functional diversity of NMDARs (Gogas, 2006, Hayashi et al., 2009). An immunohistochemical study investigating the development of neuropathic pain has demonstrated that, in the superficial lamina of the dorsal horn, NR2B phosphorylation at Tyr1472 is crucial for the development of hyperalgesia (Gu et al., 2009). Using antibody labeling of specific tyrosine residues, our data in the present study showed that the spinal ephrinB2 injection induced NR2B phosphorylation at residues Tyr1336 and Tyr1472, along with urethra reflex potentiation. We propose a possible role of NMDAR NR2B phosphorylation plays a role in the induction of visceral pain. In further support of this proposal, a recent study showed NMDAR NR2B phosphorylation in the lumbosacral spinal cord is essential for cross-organ sensitization caused by acute viscera irritation (Peng et al., 2008). However, our data were obtained from antibody-specific experiments, but there are no other findings to corroborate our results. Further studies are needed to clarify the role of phosphorylation in particular tyrosine residues in spinal reflex potentiation. Moreover, besides Tyr1336 and Tyr1472, there are 23 tyrosine residues in the carboxyl tail of the NR2B subunit that could be phosphorylated (Ali and Salter, 2001). Due to the limitations of this study, it could not be elucidated whether there are other tyrosine residues involved in ephrinB2-dependent reflex potentiation. Through Src-family kinase-dependent modulations of NMDAR, the interaction between EphBR tyrosine kinases and their ephrinB ligands has been shown to play a crucial role in pain processing at the spinal cord level (Battaglia et al., 2003, Slack et al., 2008). Intrathecal administration of exogenous ephrinB2, which activates EphBR, induced behavioral thermal hyperalgesia, and increments in the expression of phosphorylated Src-EphBR complex were both counteracted by pretreatment with NMDAR antagonist (Slack et al., 2008). Conversely, an Src-family inhibitor has been shown to reverse ephrinB2-induced thermal hyperalgesia and NR2B phosphorylation in the spinal dorsal horn (Battaglia et al., 2003). These results are consistent with our finding that intrathecal ephrinB2 induced reflex potentiation in association with Src-family kinases and NMDAR NR2B phosphorylation in the lumbosacral spinal cord. Pharmacological blockage of Src-family kinase activity prevented reflex potentiation and NR2B phosphorylation, indicating NMDAR NR2B phosphorylation downstream of Src-family kinase activation mediated pain-related spinal neural plasticity. However, Srcfamily kinases may target NMDAR subunits other than NR2B (Nakazawa et al., 2001); therefore, the role of other NMDAR subunit(s), e.g., NR2A, in ephrinB2-dependent reflex potentiation, cannot be ruled out.

Studies have demonstrated that an intrathecal EphBR activator can induce thermal hyperalgesia, and mechanical allodynia has been correlated with a reduced long-term spinal potentiation threshold between

nociceptive C afferent fibers and dorsal horn neurons in naive animals. Conversely, pharmacological blocking of EphBR using EphB-Fc prevents nerve injury-induced thermohyperalgesia and mechanical allodynia and reverses enhanced long-term potentiation. This suggests that ephrinB2/EphB effects on neuropathic pain depend on regulation of both neuronal excitability and spinal synaptic plasticity (Song et al., 2008b). In clinical scenarios, syndromes of patients being treated, such as irritable bowel syndrome and chronic pelvic pain, are usually chronic conditions. In the present study, we demonstrated the onset of a spinal EphBR-dependent reflex potentiation 10 min after an ephrinB2 injection, which lasted for at least 30 min. Although this study offers an animal model to investigate the spinal neural mechanism underlying viscero-visceral pain, it is limited by insult acuity and the subsequent measurement interval. On the other hand, behavior studies that investigated neuropathic pain caused by sciatic nerve ligation showed time-dependent upregulation of EphrinB and EphBR in DRG and dorsal horn neurons, which peaks at 7 days and lasts for 21 days after nerve ligation (Battaglia et al., 2003, Song et al., 2008a). Further studies are needed to determine whether the ephrinB2-dependent reflex potentiation plays a role in the induction of acute injury/inflammation pain and eventually contributes to the maintenance of a pain condition or the development of hyperalgesia/allodynia by establishing pathological neural plasticity.

In conclusion, our findings add to the understanding of the ephrin/EphBR system, which is an important player in spinal reflex potentiation, acting as a modulator of NMDAR in the adult spinal cord in vivo. These results help bring to light the mechanisms underlying reflex potentiation in the spinal cord, which is considered essential to the induction of visceral pain.

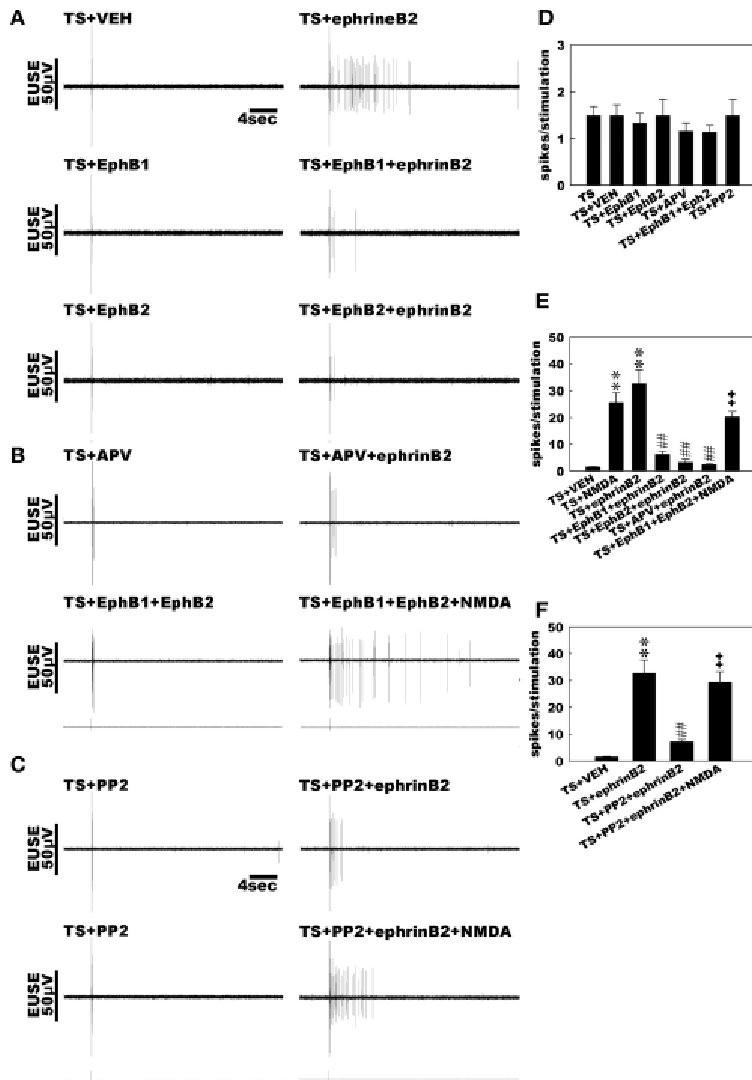

Fig. 1.

EphB1 and EphB2 receptors are involved in ephrinB2-dependent pelvic-urethra reflex potentiation. A: while intrathecal vehicle solution (TS + VEH) exhibited no effect on the baseline EUSE activity evoked by pelvic afferent nerve TS (1 stimulation/30 s for 30 min), intrathecal ephrinB2 administration (TS + ephrinB2) produced reflex potentiation characterized by an elongated firing evoked by each pulse. Neither prior administration of EphB1-Fc (10 ug/rat it) nor EphB2-Fc (10 ug/rat it) affected the baseline reflex activity evoked by the TS (TS + Eph1B and TS + Eph2B, respectively), whereas both reagents prevented ephrinB2-dependent reflex potentiation (TS + EphB1 + ephrinB2 and TS + EphB2 + ephrinB2, respectively). B: intrathecal pretreatment with D-2-amino-5-phosphonovalerate (APV) (10 uM, 10 ul) displayed no effect on the TS-evoked baseline reflex activity (TS + APV), but prevented ephrinB2-dependent reflex potentiation (TS + APV + ephrinB2). Intrathecal NMDA injection provoked reflex potentiation (TS + EphB1 + EphB2 + NMDA), even though EphB1 and EphB2 receptors were both blocked by coadministration of Eph1B-Fc and Eph2B-Fc (TS + EphB1 + EphB2). C: while it exhibited no effects on the TS-evoked baseline reflex activity (TS + PP2), intrathecal pretreatment with PP2 (50 uM, 10 ul) prevented ephrinB2-dependent reflex potentiation (TS + PP2 + ephrinB2). The PP2-induced antagonization on ephrinB2-dependent reflex potentiation was reversed by spinal NMDA administration (TS + PP2 + ephrinB2 + NMDA). D: the mean spike count of EUSE activity evoked by pelvic afferent TS was not affected by all of the test agents, including

vehicle solution (TS + VEH), Eph1B-Fc (TS + EphB1), EphB2-Fc (TS + EphB2), APV (TS + APV), PP2 (TS + PP2), as well as coadministration of Eph1B-Fc and Eph2B-Fc (TS + EphB1 + EphB2, all $P > 0.05$ to TS; N = 7). E: compared with vehicle solution (TS + VEH), intrathecal NMDA (TS + NMDA, $^{**}P < 0.01$ to TS + VEH, n = 7) and ephrinB2 (TS + ephrinB2, $^{**}P < 0.01$ to TS + VEH, n = 7) both significantly increased the mean spike count evoked by TS. The ephrinB2-dependent increment in spike count was reversed by prior administration of Eph1B-Fc (TS + EphB1, $^{\#\#}P < 0.01$ to TS + ephrinB2, n = 7), EphB2-Fc (TS + EphB2, $^{\#\#}P < 0.01$ to TS + ephrinB2, n = 7), and APV (TS + APV, $^{\#\#}P < 0.01$ to TS + ephrinB2, n = 7). Spinal NMDA antagonized the reversal of the ephrinB2-dependent spike increment caused by coadministration of EphB1-Fc and EphB2-Fc (TS + EphB1 + EphB2 + NMDA, $^{++}P < 0.01$ to TS + ephrinB2, n = 7). F: compared with vehicle solution (TS + VEH), intrathecal ephrinB2 (TS + ephrinB2, $^{**}P < 0.01$ to TS + VEH, n = 7) significantly increased the mean spike count evoked by TS. The ephrinB2-dependent increment in spike count was reversed by prior administration of PP2 (TS + PP2 + ephrinB2, $^{\#\#}P < 0.01$ to TS + ephrinB2, n = 7). Spinal NMDA antagonized the reversal of ephrinB2-dependent spike increment caused by PP2 (TS + PP2 + NMDA, $^{++}P < 0.01$ to TS + ephrinB2, n = 7).

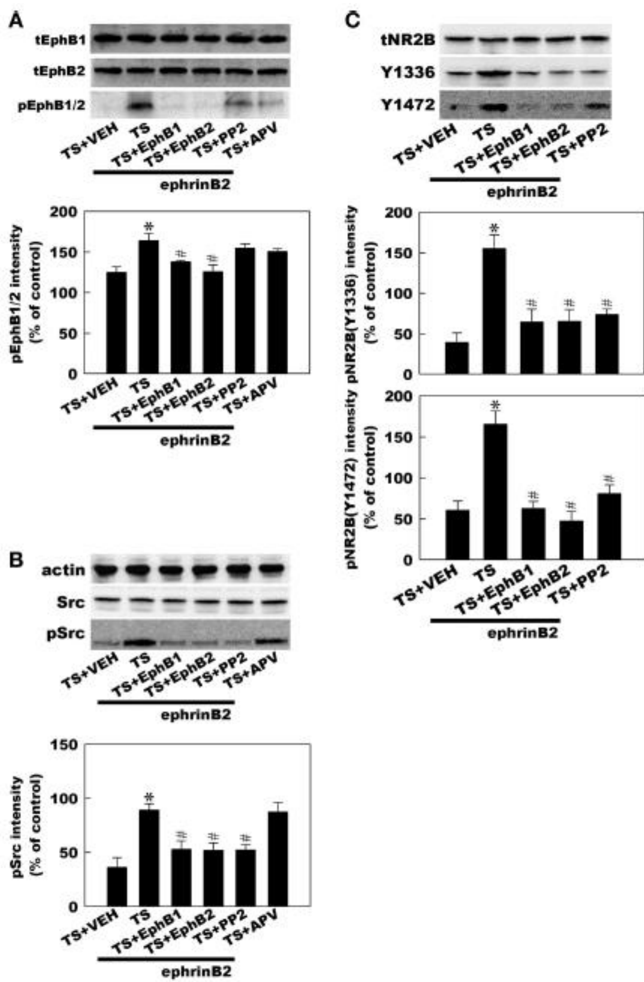

Fig. 2.

Src-dependent NR2B phosphorylation mediates ephrinB2-dependent EphB1/2 phosphorylation in the lumbosacral (L6-S2) dorsal horn. A: compared with vehicle solution (TS + VEH), intrathecal ephrinB2 injection (bar: ephrinB2) statistically enhanced the expression of pEphB1/2, but not tEphB1 and tEphB2, in the lumbosacral dorsal horn obtained from animals that received TS. *$P < 0.05$ to TS + VEH, n = 4. Prior administration of EpB1-Fc (TS + EphB1, #$P < 0.05$ to TS, n = 4) and EphB2-Fc (TS + EphB2, #$P < 0.05$ to TS, n = 4), but not PP2 (TS + PP2, $P > 0.05$ to TS, n = 4) and APV (TS + APV, $P > 0.05$ to TS, n = 4), prevented ephrinB2-dependent EphB1/2 phosphorylation. B: compared with vehicle solution (TS + VEH), intrathecal ephrinB2 injection (bar: ephrinB2) statistically enhanced the expression of phosphorylated (pSrc) but not total Src in the lumbosacral dorsal horn obtained from animals that received TS. *$P < 0.05$ to TS + VEH, n = 4. Prior administration of EpB1-Fc (TS + EphB1, #$P < 0.05$ to TS, n = 4), EphB2-Fc (TS + EphB2, #$P < 0.05$ to TS, n = 4), and PP2 (TS + PP2, #$P < 0.05$ to TS, n = 4), but not APV (TS + APV, $P > 0.05$ to TS, n = 4), prevented ephrinB2-dependent Src phosphorylation. C: compared with vehicle solution (TS + VEH), intrathecal ephrinB2 injection (bar: ephrinB2) statistically enhanced the expression of phosphorylated Y1336 and Y1472 residues of NR2B, but not total NR2B (tNR2B), in the lumbosacral dorsal horn obtained from animals that received TS. *$P < 0.05$ to TS + VEH, n = 4. Prior administration of EpB1-Fc (TS + EphB1, #$P < 0.05$ to TS, n = 4), EphB2-Fc (TS + EphB2, #$P < 0.05$ to TS, n = 4), and PP2 (TS + PP2, $P > 0.05$ to TS, n = 4) prevented ephrinB2-dependent EphB1/2 phosphorylation.

REFERENCES:

1. **Ali DW, Salter MW.** NMDA receptor regulation by Src kinase signaling in excitatory synaptic transmission and plasticity. *Curr Opin Neurobiol* 11: 336–342, 2001.
2. **Battaglia AA, Sehayek K, Grist J, McMahon SB, Gavazzi I.** EphB receptors and ephrin-B ligands regulate spinal sensory connectivity and modulate pain processing. *Nat Neurosci* 6: 339–340, 2003.
3. **Berberich S, Punnakkal P, Jensen V, Pawlak V, Seeburg PH, Hvalby O, Kohr G.** Lack of NMDA receptor subtype selectivity for hippocampal long-term potentiation. *J Neurosci* 25: 6907–6910, 2005.
4. **Birder LA, De Groat WC.** The effect of glutamate antagonists on c-fos expression induced in spinal neurons by irritation of the lower urinary tract. *Brain Res* 580: 115–120, 1992.
5. **Chang JL, Peng HY, Wu HC, Lu HT, Pan SF, Chen MJ, Lin TB.** Acute neurosteroid inhibit the spinal reflex potentiation via GABAergic neurotransmission. *Am J Physiol Renal Physiol* 299: F43–F48, 2010.
6. **Dubner R, Ruda MA.** Activity-dependent neuronal plasticity following tissue injury and inflammation. *Trends Neurosci* 15: 96–103, 1992.
7. **Gogas KR.** Glutamate-based therapeutic approaches: NR2B receptor antagonists. *Curr Opin Pharmacol* 6: 68–74, 2006.
8. **Grunwald IC, Korte M, Wolfer D, Wilkinson GA, Unsicker K, Lipp HP, Bonhoeffer T, Klein R.** Kinase-independent requirement of EphB2 receptors in hippocampal synaptic plasticity. *Neuron* 32: 1027–1040, 2001.
9. **Gu X, Wu X, Liu Y, Cui S, Ma Z.** Tyrosine phosphorylation of the *N*-methyl-D-aspartate receptor 2B subunit in spinal cord contributes to remifentanil-induced postoperative hyperalgesia: the preventive effect of ketamine. *Mol Pain* 5: 76–85, 2009.
10. **Haley JE, Sullivan AF, Dickenson AH.** Evidence for spinal *N*-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. *Brain Res* 518: 218–226, 1990.
11. **Hayashi H, Thomas GM, Huganir R.** Dual palmitoylation of NR2 subunits regulates NMDA receptor trafficking. *Neuron* 64: 213–226, 2009.
12. **Henderson JT, Georgiou J, Jia Z, Robertson J, Elowe S, Roder JC, Pawson T.** The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function. *Neuron* 32: 1041–1056, 2001.
13. **Kobayashi H, Kitamura T, Sekiguchi M, Homma MK, Kabuyama Y, Konno S, Kikuchi S, Homma Y.** Involvement of EphB1 receptor/EphrinB2 ligand in neuropathic pain. *Spine* 32: 1592–15988, 2007.
14. **Kolhekar R, Gebhart GF.** NMDA and quisqualate modulation of visceral nociception in the rat. *Brain Res* 651: 215–226, 1994.
15. **Lin TB.** Dynamic pelvic-pudendal reflex plasticity mediated by glutamate in anesthetized rats. *Neuropharmacology* 44: 163–170, 2003.
16. **Lin TB.** Tetanization-induced pelvic-to-pudendal reflex plasticity in anesthetized rats. *Am J Physiol Renal Physiol* 287: F245–F251, 2004.
17. **Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT.** Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science* 304: 1021–1024, 2004.
18. **Luque JM, Bleuel Z, Malherbe P, Richards JG.** Alternatively spliced isoforms of the N-methyl-D-aspartate receptor subunit 1 are differentially distributed within the rat spinal cord. Neuroscience 63: 629–635, 1994.

19. **Ma QP, Woolf CJ.** Noxious stimuli induce an N-methyl-D-aspartate receptor-dependent hypersensitivity of the flexion withdrawal reflex to touch: implications for the treatment of mechanical allodynia. Pain 61: 383–390, 1995.
20. **Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI.** Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J Neurosci 24: 7821–7828, 2004.
21. **McBain CJ, Mayer ML.** *N*-methyl-D-aspartic acid receptor structure and function. *Physiol Rev* 74: 723–760, 1994.
22. **Moon IS, Apperson ML, Kenedy MB.** The major tyrosine-phosphorylated protein in the post-synaptic density fraction is *N*-methyl-D-aspartate receptor subunit 2B. *Proc Natl Acad Sci USA* 91: 3954–3958, 1994.
23. **Nakazawa T, Komai S, Tezuka T, Hisatsune C, Umemori H, Semba K, Mishina M, Manabe T, Yamamoto T.** Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR epsilon 2 (NR2B) subunit of the *N*-methyl-D-aspartate receptor. *J Biol Chem* 276: 693–699, 2001.
24. **Olivar T, Laird JMA.** Differential effects of *N*-methyl-D-aspartate receptor blockade on nociceptive somatic and visceral reflexes. *Pain* 79: 67–73, 1999.
25. **Peng HY, Chang CH, Tsai SJ, Lai CY, Tung KC, Wu HC, Lin TB.** Protein kinase A-dependent spinal a-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate receptor trafficking mediates the capsaicin-induced colon-urethra cross-organ reflex sensitization. *Anesthesiology.* 114: 70–83, 2011.
26. **Peng HY, Chen GD, Lai CH, Tung KC, Chang JL, Lin TB.** Endogenous ephrinB2 mediates colon-urethra cross-organ sensitization via Src kinase-dependent tyrosine phosphorylation of NR2B. *Am J Physiol Renal Physiol* 298: F109–F117, 2010a.
27. **Peng HY, Chen GD, Lai CY, Hsieh MC, Hsu HH, Wu HC, Lin TB.** PI3K modulates estrogen-dependent facilitation of colon-to-urethra crossorgan reflex sensitization in ovariectomized female rats. *J Neurochem* 113: 54–66, 2010b.
28. **Peng HY, Chen GD, Lai CH, Tung KC, Chang JL, Lin TB.** Endogenous ephrinB2 mediates the colon-urethra cross-organ sensitization via Src kinase-dependent tyrosine prhosphorylation of NR2B. *Am J Physiol Renal Physiol* 298: F109–F117, 2010.
29. **Peng HY, Chen GD, Lee SD, Lai CY, Chiu CH, Cheng CL, Chang YS, Hsieh MC, Tung KC, Lin TB.** Neuroactive steroids inhibit spinal reflex potentiation by selectively enhancing specific spinal GABA(A) receptor subtypes. *Pain* 143: 12–20, 2009.
30. **Peng HY, Chen GD, Tung KC, Chien YW, Lai CY, Hsieh MC, Chiu CH, Lai CH, Lee SD, Lin TB.** Estrogen-dependent facilitation on spinal reflex potentiation involves the Cdk5/ERK1/2/NR2B cascade in anesthetized rats. *Am J Physiol Endocrinol Metab* 297: E416–E426, 2009a.
31. **Peng HY, Chen GD, Tung KC, Lai CY, Hsien MC, Chiu CH, Lu HT, Liao JM, Lee SD, Lin TB.** Colon mustard oil instillation induced cross-organ reflex sensitization on the pelvic-urethra reflex activity in rats. *Pain* 142: 75–88, 2009b.
32. **Peng HY, Chang HM, Chang SY, Tung KC, Lee SD, Chou D, Lai CY, Chiu CH, Chen GD, Lin TB.** Orexin-A modulates glutamatergic NMDA-dependent spinal reflex potentiation via inhibition of NR2B subunit. *Am J Physiol Endocrinol Metab* 295: E117–E1129, 2008a.

33. **Peng HY, Chang HM, Lee SD, Huang PC, Chen GD, Lai CH, Lai CY, Chiu CH, Tung KC, Lin TB.** TRPV1 mediates the uterine capsaicininduced NMDA NR2B-dependent cross-organ reflex sensitization in anesthetized rats. *Am J Physiol Renal Physiol* 295: F1324–F1335, 2008b.
34. **Petralia RS, Wang YX, Wenthold RJ.** The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those of NR1. *J Neurosci* 14: 6102–6120, 1994.
35. **Slack S, Battaglia A, Cibert-Goton V, Gavazzi I.** EphrinB2 induces tyrosine phosphorylation of NR2B via Src-family kinases during inflammatory hyperalgesia. *Neuroscience* 156: 175–218, 2008.
36. **Song XJ, Cao JL, Li HC, Zheng JH, Song XS, Xiong LZ.** Upregulation, and redistribution of ephrinB and EphB receptor in dorsal root ganglion and spinal dorsal horn neurons after peripheral nerve injury and dorsal rhizotomy. *Eur J Pain* 12: 1031–1039, 2008a.
37. **Song XJ, Zheng JH, Cao JL, Liu WT, Song XS, Huang ZJ.** EphrinB EphB receptor signaling contributes to neuropathic pain by regulating neural excitability and spinal synaptic plasticity in rats. *Pain* 30: 168–180, 2008b.
38. **Takasu MA, Dalva MB, Zigmond RE, Greenberg ME.** Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. Science 295: 491–495, 2002.
39. **Urban MO, Gebhart GF.** Supraspinal contributions to hyperalgesia. Proc Natl Acad Sci USA 96: 7687–7692, 1999.
40. **Wilkinson DG.** Multiple roles of EPH receptors and ephrins in neural development. Nat Rev Neurosci 2: 155–164, 2001.
41. **Wu HC, Chiu Tung KC CH, Chen GD, Peng HY, Lin TB.** Dopaminergic D2 receptors activate PKA t5o inhibit spinal pelvic-urethra reflex in rats. Am J Physiol Renal Physiol 299: F681–F686, 2010.
42. **Zhai QZ, Traub RJ.** The NMDA receptor antagonist Dizocilpine attenuates c-Fos expression in the lumbo-sacral spinal cord following repetitive noxious and non-noxious colorectal distention. Pain 83: 321–329, 1999.

國科會補助計畫衍生研發成果推廣資料表

日期:2011/12/01

<table>
<tr><td rowspan="3">國科會補助計畫</td><td>計畫名稱: 肉毒桿菌毒素-A對麻醉大白鼠骨盆-尿道反射增益現象的抑制效應及訊息路徑的調節經由骨盆臟器間對話的去敏感機轉</td></tr>
<tr><td>計畫主持人: 陳進典</td></tr>
<tr><td>計畫編號: 98-2314-B-040-011-MY3　　　學門領域: 婦產科</td></tr>
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98 年度專題研究計畫研究成果彙整表

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<tr><td colspan="3" rowspan="2">成果項目</td><td colspan="3">量化</td><td rowspan="2">單位</td><td rowspan="2">備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）</td></tr>
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<tr><td>專任助理</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
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其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	1. Wu HC, Chang CH, Peng HY, Chen GD, Lai CY, Hsieh MC, Lin TB. EphrinB2 induces pelvic-urethra reflex potentiation via Src kinase-dependent tyrosine phosphorylation of NR2B. Am J Physiol Renal Physiol. 2011 Feb；300(2):F403-11. Epub 2010 Dec 8. 2. Wu HC, Chiu CH, Tung KC, Chen GD, Peng HY, Lin TB. Dopaminergic D2 receptors activate PKA to inhibit spinal pelvic-urethra reflex in rats. Am J Physiol Renal Physiol. 2010 Sep；299(3):F681-6. Epub 2010 Jun 16. 3. Peng HY, Chen GD, Lai CY, Hsieh MC, Hsu HH, Wu HC, Lin TB. PI3K modulates estrogen-dependent facilitation of colon-to-urethra cross-organ reflex sensitization in ovariectomized female rats. J Neurochem. 2010 Apr；113(1):54-66. Epub 2010 Jan 8.

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估 ■達成目標 □未達成目標（請說明，以 100 字為限） 　　□實驗失敗 　　□因故實驗中斷 　　□其他原因 說明：
2. 研究成果在學術期刊發表或申請專利等情形： 論文：■已發表 □未發表之文稿 □撰寫中 □無 專利：□已獲得 □申請中 ■無 技轉：□已技轉 □洽談中 ■無 其他：（以 100 字為限）
3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）