

Original Article

Protective immunity of rAd5/NR2B vaccine against concomitant aversiveness of spontaneous neuropathic pain following spinal nerve ligation injury

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Abstract: Objective: Peripheral nerve injury elicits an aversive state of spontaneous neuropathic pain, and up to now, the modulation of this concomitant aversive state remains a major therapeutic challenge. NMDA receptor subunits NR2B in the rACC are critically involved in the processing of this aversive state and then a strategy targeted at the NR2B subunit might be promising for modulation of the aversive state. Thus, in the present study, using negative reinforcement animal model to reveal spontaneous pain, we investigated the effect of oral immunization with recombinant adenovirus serotype 5-mediated NR2B gene transfer (rAd5/NR2B) on the modulation of the tonic pain. Material and methods: Following oral administration of the rAd5/NR2B vaccine, NR2B-specific antibodies were induced in serum. And the humoral response was involved in the decreased expression of NR2B protein in the rACC. Results: The present study demonstrated that CPP achieved by spinal administration of clonidine in spinal nerve ligation (SNL) rats revealed the presence of aversive state of spontaneous neuropathic pain. Notably, the humoral autoimmune response blocked the CPP by spinal clonidine, suggesting the relief of the concomitant aversive of spontaneous neuropathic pain in the SNL rats. Conclusion: These data proved the feasibility of oral immunization with rAd5/NR2B for modulation of concomitant aversive of spontaneous neuropathic pain due to peripheral nerve injury.

Keywords: Spontaneous neuropathic pain, immunity, anterior cingulate cortex, NR2B

Introduction

Many patients with pain due to nerve injury commonly exhibit ongoing and/or paroxysmal spontaneous pain that is not related to any applied somatic stimulus, which is referred for the aversive state of spontaneous neuropathic pain [1]. Because the measurement of the concomitant aversiveness of spontaneous neuropathic pain in animals has been difficult, this represents a major barrier to the development of effective treatments, and it is often a severe clinical problem for inadequately being treated by current analgesics and [2].

Some evidences implicate the rostral anterior cingulate cortex (rACC) in pain processing. Neuroimaging and electrophysiological studies

in humans and animals revealed that both noxious stimuli and predictive cues of noxious stimulus activate the rACC [3, 4]. Surgical ablation of the rACC attenuated the pain-related depression and unpleasantness in patients suffering from chronic pain [5]. Lesion of the rACC abolished the pain-like aversion in rats [6]. These data strongly suggested that the rACC might be a pivotal brain linked to affective processing of pain. In the rACC, glutamate mediates excitatory synaptic transmission [7]. The forebrain-targeted over expression of the NMDA receptor subunit NR2B significantly increased excitation of the rACC neurons in the transgenic mice. The blockade of NMDA receptors but not of AMPA/KA receptors in the rACC significantly inhibited formalin-induced conditioned place avoidance

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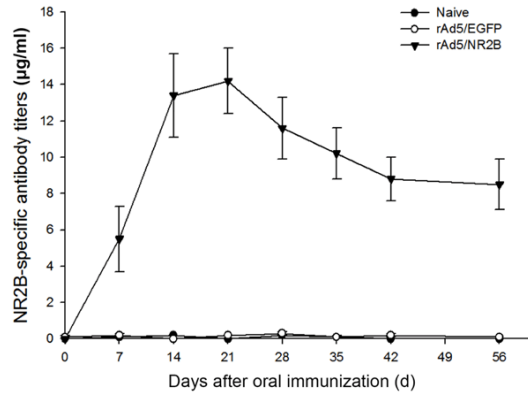


Figure 1. Time course of NR2B-specific antibodies in sera after oral vaccination with rAd5/NR2B vaccine. Each value is the mean antibody titer derived from five rats. The booster immunization was performed by day 14 following the initial immunization. Rats immunized with rAd5/NR2B demonstrated higher titers of NR2B-specific antibody and the control rats immunized with saline or rAd5/EGFP had no anti-NR2B. rAd5/NR2B, recombinant adenovirus serotype 5-mediated NR2B; NR2B, N-methyl-D-aspartate receptor 2B subunit.

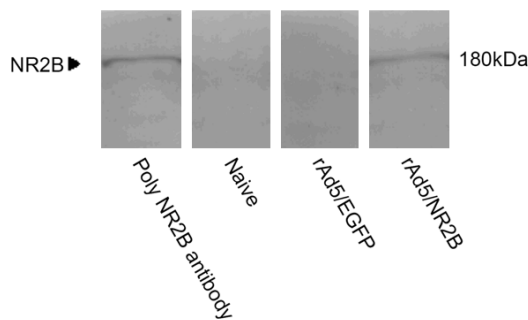


Figure 2. Western blotting analysis suggesting passage of NR2B antibody into the rACC. CSF (1:5) from rats immunized with PBS, rAd5/EGFP or rAd5/NR2B was screened against 30 µg of rACC extract for NR2B antibodies. NR2B antibody is detected in CSF from rats immunized with rAd5/NR2B.

(F-CPA), which reflects the pain-related negative affective state and aversion learning produced by the nociceptive stimulation, and attenuated F-CPA retrieval-induced Fos expression in the rACC [8]. Furthermore, Johansen and Fields [9] demonstrated that glutamatergic activation in the rACC is necessary and sufficient for pain-like aversion. It is therefore suggested that activation of glutamate NMDA receptors in the rACC is required for the induction of the concomitant aversiveness of spontaneous neuropathic pain.

It is believed that brain antigens previously sequestered from the immune system via the blood-brain barrier (BBB) are presented to a naive immune system when ectopically expressed in cancer cells and then elicit a humoral immune response. For example, in paraneoplastic disorders (PND), circulating autoantibodies interact with neuronal antigens. Thus, a vaccination strategy targeting brain protein is feasible and may have therapeutic potential for neurological disorders. Schenk et al have reported that repeated immunization with amyloid- β attenuated Alzheimer's disease-like pathology in the PDAPP mouse model, possibly mediated via an antibody facilitating the clearance of plaques [10]. During et al. demonstrated that immunized rats with an oral vaccine encoding NR1 generated autoantibodies against NR1 to produce strong antiepileptic and neuro-protective activity [11]. Therefore, using conditioned place preference resulting from spinal clonidine in animals with spinal nerve ligation injury, the aim of the present study was to investigate the role of oral immunization with recombinant adenovirus serotype 5-mediated NR2B gene transfer (rAd5/NR2B) for modulation of the aversive state accompanying neuropathic pain.

Materials and methods

Animal models

Male Sprague-Dawley rats weighing 200 ± 20 g were used in experiments which were conducted, with adherence to the guidelines for pain research. The rats were fed a standard laboratory diet and tap water and kept at $23 \pm 1^\circ\text{C}$ with a 12 h light/dark cycle. All animal protocols were approved by the Animal Care and Use Committee of the School of Medicine of Shandong University, Jinan, China. The SNL models were established as described previously [12].

Amplification of rAd5/NR2B and oral immunization

The viral stocks of rAd5/NR2B vaccine targeting at NR2B subunit of ACC neurons were generated by the transient transfection of 293 cells and purified using CsCl ultracentrifugation gradients. The final titer was 5×10^{11} PFU ml⁻¹. Sprague-Dawley rats were orally immunized with rAd5/NR2B, rAd5/EGFP and PBS. Oral

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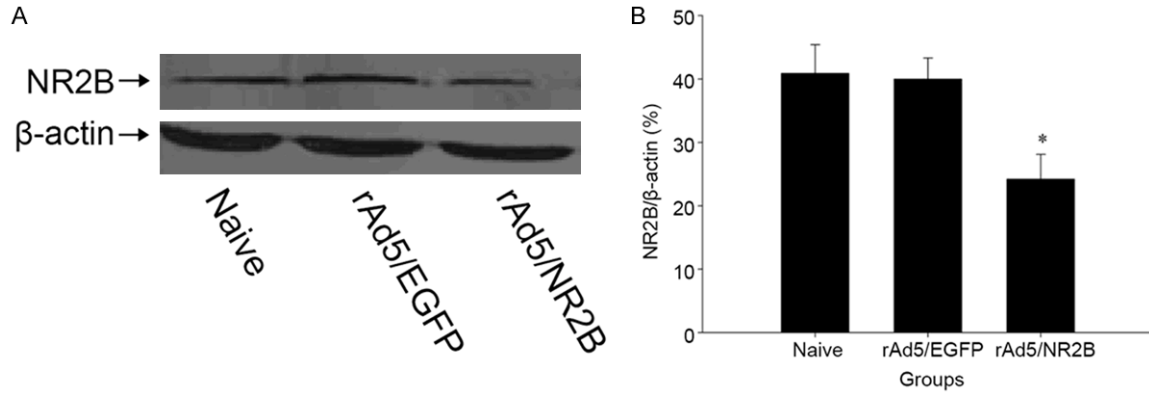


Figure 3. Western blotting analysis showing downregulation of NR2B protein level in the rACC after oral immunization with rAd5/NR2B. A: Each lane represents a sample obtained from one individual rat. B: Data shown are representative of five separate experiments with similar results. * $P < 0.05$ vs. naive or rAd5/EGFP. NR2B, N-methyl-D-aspartate receptor 2B subunit; rAd5/NR2B, recombinant adenovirus serotype 5-mediated NR2B.

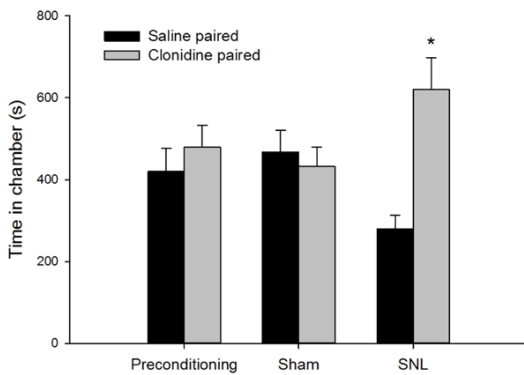


Figure 4. Spinal clonidine alleviates the concomitant aversive state of spontaneous pain due to nerve injury. SNL or sham rats showed equivalent time in the pairing chambers prior to conditioning day. As no differences were observed between SNL and sham operated rats, preconditioning values were pooled for graphical representation. SNL, but not sham-operated rats showed clear preference for the chamber paired with spinal clonidine. *Indicates significant difference from preconditioning time; $P < 0.05$.

inoculation was carried out by administration of 200 ml 5% sodium bicarbonate via an orogastric into the stomach of rats and 10 min later followed by administration of 100 ml of rAd5/NR2B (1×10^8 PFU), with a control group receiving a similar dose of rAd5/EGFP or PBS. For a booster immunization, re-immunization was carried out 2 weeks after the initial immunization.

Assessment of titers of NR2B-specific antibody in sera and in CSF

Enzyme-linked immunosorbent assays were carried out to determine NR2B-specific anti-

body titers in vaccinated rats. Bleeds were collected through the tail every 2 weeks after the initial immunization. After incubation for 3 h at 4°C, the bleeds were centrifuged for 10 min at 1000 g. The sera were collected and stored at -70°C. Microtiter wells were coated with NR2B peptide ($10 \mu\text{g ml}^{-1}$) in 50 mM carbonate buffer pH 9.6 overnight at 4°C and rinsed three times with washing buffer (PBS containing 0.05% Tween 20). Microtiter wells were treated with blocking buffer (10% bovine calf serum in PBS) for 2 h at room temperature. The serum samples were diluted with PBS and added to the microtiter wells. After incubation overnight at 4°C, the plates were washed five times with the washing buffer and incubated for 1 h with an appropriate horseradish peroxidase-conjugated detection antibody. The detection antibodies were diluted at 1:5000 for anti-rat IgG in the blocking buffer. After washing the plates with the washing buffer, the plates were incubated with tetramethylbenzidine for 15 min and the reaction was stopped with the addition of 1 N H_2SO_4 . Optical densities at 450 nm were determined using a microplate reader (Model 680, Finland). Anti-NR2B antibody concentration in rat sera was determined using serial dilutions of anti-NR2B IgG (Abcam, Cambridge, UK) of known concentration as a standard.

To determine passage of NR2B antibodies into the rACC, rats vaccinated with PBS, rAd5/EGFP or rAd5/NR2B underwent non-traumatic sampling of CSF from the cisterna magna on day 7 after the SNL procedure. NR2B-specific antibody in CSF was detected using immunoblot analysis and a highly sensitive chemiluminescence method.

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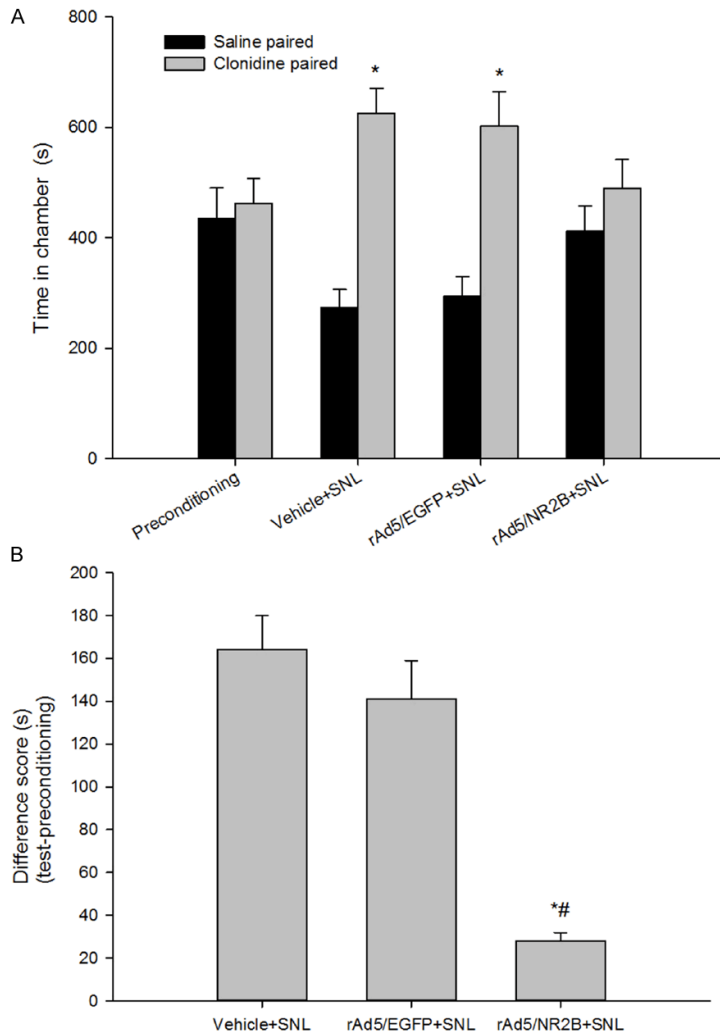


Figure 5. rAd5/NR2B vaccination blocked conditioned place preference (CPP) induced by spinal administration of clonidine. A: SNL-treated rats showed increased time spent in the clonidine-paired chamber, whereas rats vaccinated by rAd5/NR2B did not show preference to the clonidine-paired chamber. *Indicates difference from preconditioning time; $P < 0.05$. B: Difference scores, calculated as test time-preconditioning time spent in the clonidine-paired chamber confirms that SNL rat vaccinated by rAd5/NR2B failed to increase time spent in the clonidine-paired chamber. *Indicates difference from SNL rats with vehicle; #Indicates difference from SNL rats with rAd5/EGFP, $P < 0.05$.

Expressive level of NR2B protein in the rACC of rats

For western blot analysis, rats were killed by an overdose of chloral hydrate (80 mg/kg) on day 42 after surgery, and the rACC tissues were quickly removed. The tissue extracts were prepared following the procedure described in detail previously, with minor modifications. The extract samples (5.0 mg/ml, 10 μ l) were loaded, subjected to 12% SDS-PAGE, and electroblotted

onto polyvinylidene difluoride membranes (Millipore Immobilon-p Transfer Membrane) using Mini-Protean 3 electrophoresis system and Mini Trans-Blot electrophoretic transfer system (Bio-Tanon, Shanghai, China). The membranes were blocked with 5% milk in PBS with 0.1% Tween 20 for 1 h at room temperature and were then incubated with the antibodies against NR2B, or β -actin at working dilutions of 1:1500, overnight at 4°C. The blots were washed, incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (KPL, Gaithersburg, MD, USA) for 2 h at 4°C, and were finally visualized with enhanced chemiluminescence (SuperSignal Wester Femto Maximum Sensitivity substrate, Pierce). For densitometric analysis, blots were scanned and quantified with GeneSnap v6.05 software (England), and the results were expressed as a ratio of NR2B immunoreactivity to β -actin immunoreactivity.

Conditioned place preference

Starting 7 days post-SNL (spinal nerve ligation)/sham surgery, all rats underwent a 3 day pre-conditioning period with behavior recorded on day 3 to verify no pre-conditioning chamber preference as described for the multi-trial conditioning. The following day (day 10 post-SNL), rats received the appropriate control (i.e. vehicle) paired with a randomly chosen chamber in the morning, and the appropriate drug treatment paired with the other chamber 4 hr later (afternoon). Chamber pairings were counterbalanced. On test day, 20 hours following the afternoon pairing, rats were placed in the CPP box with access to all chambers and their behavior recorded for 15 min for analysis for chamber preference. This protocol, in which vehicle was always paired 4 hr prior to the drug, was done to prevent potential confounds of long-lasting drug effects that may

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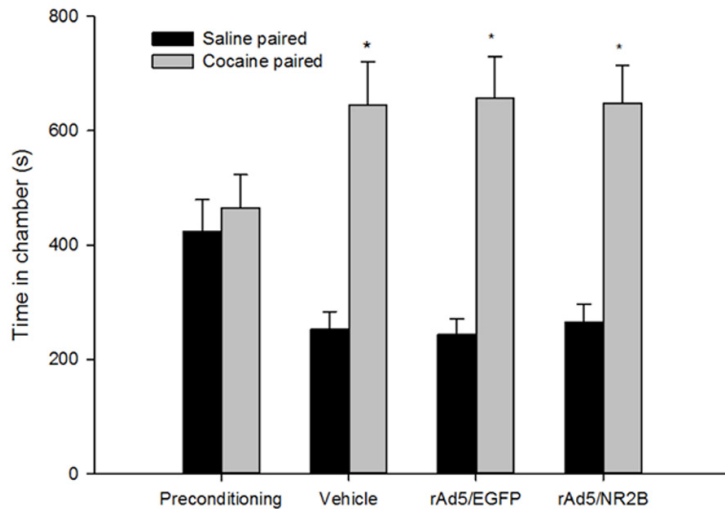


Figure 6. The effect of rAd5/NR2B vaccination on cocaine-induced reward. The rAd5/NR2B vaccination did not block cocaine-induced reward as indicated by increased time spent in the cocaine-paired chamber. *Indicates difference from preconditioning time; $P < 0.05$.

persist for longer than 4 hr (i.e., from the morning exposure). Such a case would interfere with the selective pairing of the drug effect with a specific chamber as the drug effect would be present in both chambers.

Surgical procedures

Rats were anesthetized with isoflurane and placed in a stereotaxic head holder. The atlantooccipital membrane was exposed, cleared and an incision was made in the dura mater. A length of PE-10 tubing was advanced 8 cm caudally to the lumbar spinal cord. The tubing was exteriorized, filled with saline and plugged with wire. The wound was closed, and animals were allowed to recover for 7 days. Animals were not tested prior to the 7 day recovery period. Pilot studies revealed that testing prior to the 7 day recovery period resulted in limited number of crossings and resultant chamber bias, likely due to the invasiveness of the surgery. Drug administration was delivered in a 5 μ l volume followed by a 9 μ l saline flush. Progress was monitored with an air bubble

Nerve injury

Spinal nerve ligation (SNL) injury was produced by tight ligation of the L5 and L6 spinal nerves as previously described [12]. Sham control rats underwent the same surgery and handling as the experimental animals but without SNL. SNL

was done 5-7 days following intrathecal surgery as pilot data demonstrated that doing i.th, and nerve injury surgeries at the same time impaired chamber crossings in baseline CPP measures. Pilot studies revealed that testing prior to the 7 day recovery period resulted in limited number of crossings and resultant chamber bias, likely due to the invasiveness of the surgery. Following surgery the animals were allowed to recover for 7 days as specified above, at which time development of tactile hypersensitivity was verified. SNL rats that exhibit gross motor deficiency or failure to exhibit subsequent evoked pain (less than 10%) were excluded from further testing. Sham operated rats that exhibited tactile hypersensitivity (less than 5%) were excluded from further testing.

Statistical analysis

Data are presented as means \pm standard deviation. The results were evaluated by one-way analysis of variance, followed by the Student-Newman-Keuls test for multiple comparisons with SPSS v12.0. A P -value < 0.05 was considered statistically significant.

Results

Induction of the humoral response following rAd5/NR2B oral vaccination

To evaluate the immunogenicity of rAd5/NR2B vaccine, serum samples were collected from vaccinated rats before and after vaccination and analyzed for anti-NR2B Ab by ELISA. Rats vaccinated with rAd5/NR2B vaccine developed strong levels of anti-NR2B Abs. Anti-NR2B Abs were detected within 7 days after vaccination, with Ab titers peaking at 2-3 weeks and gradually decreasing over a month, as seen in **Figure 1**.

Immunoreactivity of CSF from rats with NR2B protein of rACC

To confirm passage of NR2B-specific antibodies into the brain following spinal nerve ligation injury, a group of vaccinated rats underwent

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nontraumatic sampling of cerebrospinal fluid (CSF) from the cisterna magna. Using western blotting analysis, NR2B autoantibodies were detected at low levels in the CSF, suggesting NR2B-specific antibodies passage into the brain. In contrast, NR2B-specific antibodies were not detected in CSF from control rats vaccinated with PBS or rAd5/EGFP (**Figure 2**).

NR2B protein expressive level in the rACC following rAd5/NR2B oral immunization

To determine if vaccination with rAd5/NR2B decreases NR2B protein expression of rACC, western blot analysis was performed to examine the levels of these subunits in the rACC after rAd5/NR2B vaccination. A significant downregulation of NR2B expression in both sides of the rACC was observed in rats vaccinated with rAd5/NR2B vaccine. In contrast, no significant decrease of NR2B expression was found in rats vaccinated with PBS or rAd5/EGFP (**Figure 3**).

Spinal clonidine alleviates the concomitant aversive state of spontaneous pain in SNL rats

Axotomy of the sciatic nerve results in denervation of the plantar and lateral portions of the hind paw, as well as ectopic discharge from the injured fibers and development of autotomy starting approximately 2 weeks following injury [13, 14]. Therefore, all rats underwent CPP, with conditioning day occurring 8 days following axotomy, a time-point prior to observable autotomy behaviors. Axotomized and sham axotomy rats showed equivalent preconditioning time in the saline- and clonidine-paired chambers, indicating no preconditioning bias for either group, so data were pooled for graphical representation (**Figure 4**). The number of chamber crossings was slightly decreased in the axotomy group, but this difference did not reach statistical significance (15 ± 4 for sham vs 12 ± 3 for axotomized rats, $P > 0.05$). Spinal administration of clonidine ($10 \mu\text{g}$) resulted in a robust increase in time spent in the clonidine-paired chamber in the axotomized rats (**Figure 4**, $*P < 0.05$ vs. preconditioning).

rAd5/NR2B vaccination blocked conditioned place preference (CPP) from spinal administration of clonidine

The week following testing of SNL-induced thermal and tactile allodynia, rats underwent the

single-trial CPP procedure in which spinal administration of clonidine was paired with a distinct chamber. Preconditioning times spent in the saline- or clonidine-paired chambers were equivalent across all treatment groups ($P > 0.05$). As no group differences were observed, data were pooled across groups for graphical representation (**Figure 5A**). Rats with SNL that had received PBS or rAd5/EGFP (SNL/Vehicle or SNL/rAd5/EGFP) showed clear preference for the clonidine-paired chamber (**Figure 5A**, $P < 0.05$ vs. preconditioning). In contrast, SNL rats with rAd5/NR2B vaccination (SNL/rAd5/NR2B) showed no preference for the clonidine-paired chamber. rAd5/EGFP-treated SNL rats had equivalent postconditioning times spent in the saline- and clonidine-paired chambers. Difference from baseline scores further confirmed that rAd5/NR2B vaccination blocked spinal clonidine-induced CPP in the SNL rats. (**Figure 5B**, $P < 0.05$).

The effect of rAd5/NR2B vaccination on cocaine-induced reward

To evaluate that the rAd5/NR2B vaccination did not block the ability of the rats to acquire rewarding stimuli independent of noxious input, we determined whether rAd5/NR2B vaccination block cocaine-induced reward. Systemic administration of 1 mg/kg cocaine (intravenous) produces CPP in animals with vehicle or rAd5/EGFP. Similar levels of CPP were demonstrated in rAd5/NR2B-vaccinated rats, with time spent in the cocaine-paired chamber elevated in both rAd5/EGFP and rAd5/NR2B rats (**Figure 6A**, $*P < 0.05$ vs. preconditioning).

Discussion

An important finding in the present study is that a robust humoral response was induced following oral administration of the rAd5/NR2B vaccine. Of particular interest, the humoral immune-response could decrease NR2B protein expression in the rACC and eliminates the aversiveness of spontaneous neuropathic pain due to peripheral nerve injury.

Pain is defined as a subjective experience [15] and, consequently, animal studies of pain must be indirect. Consequently, pain-related aversive state arising from injured nerve has been difficult to demonstrate in animals. Because relief of pain is rewarding, analgesic agents that are not rewarding in the absence of pain

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should become rewarding only when there is ongoing pain. We used conditioned place preference to concomitantly determine the presence of pain-related aversive state in rats and the efficacy of agents that relieve it. Here, we achieved CPP selectively in nerve-injured rats by spinal administration of clonidine in SNL rats. This approach may have greater predictive power than current methods used to assess the therapeutic potential of new analgesic agents. The present study used it to evaluate the effect of rAd5/NR2B vaccine on the aversive state of neuropathic pain.

Increasing evidence has demonstrated that the rACC plays an important role in the processing of pain affect [3, 4]. In the early clinical reports, patients with surgical ablation of the ACC still felt pain, but experienced obvious decreases in pain-related depression and unpleasantness. A neuro-imaging study clearly revealed that when hypnotic suggestions were used to alter selectively the perceived affective motivational component of the noxious stimuli without changing in the perceived intensity, pain-evoked activity was significantly changed within the rACC, associated with the encoding of perceived unpleasantness [4]. These studies support the role of the rACC in mediating the aversive component of chronic pain. Importantly, the above studies focus on avoidance of the aversive motivational state of evoked pain, rather than on the rewarding properties of relief of spontaneous pain. To date, no studies have directly assessed the role of the rACC in the aversive state of spontaneous pain resulting from nerve injury. Our studies demonstrate that the rACC is required for the reward associated with the aversive relief from nerve-injury induced spontaneous pain.

A number of studies have shown the involvement of excitatory amino acids, especially glutamate in synaptic transmission and signal processing in the rACC [16, 17]. Lei et al, demonstrated that blockade of NMDA receptors but not of AMPA/KA receptors in the rACC significantly inhibited formalin-induced conditioned place avoidance (F-CPA), which reflects the pain-related negative affective state and aversion learning produced by the nociceptive stimulation, and attenuated F-CPA retrieval-induced Fos expression in the rACC [8]. Furthermore, Johansen and Fields [9] demon-

strated that glutamatergic activation in the rACC is necessary and sufficient for pain-like aversion. It is therefore suggested that activation of glutamate NMDA receptors in the rACC is required for the induction of pain-related negative effect. In the present study, rAd5/NR2B vaccination not only decreased NR2B protein expressive level in the rACC, but also blocked CPP from spinal administration of clonidine in SNL rats. This further proved that NR2B subunit of the rACC is the optimal therapeutic target for the aversive state of neuropathic pain.

Both competitive and non-competitive NMDA receptor antagonists produce unacceptable side-effects including psychotomimesis, ataxia, and sedation [18]. A potential advantage of a vaccine or antibody approach to NMDA receptor antagonism is that the receptor blockade is minimal under resting physiological conditions, high serum titers of antibodies do not pass the BBB efficiently. However, after a neuronal insult, the BBB has increased permeability to serum antibodies, and transport and subsequent binding to the target protein can occur. This "on demand" or selective delivery of the neuroprotective agent, limited both spatially to the site of injury and to the precise timing of injury, is one of the most promising features of our approach. In the present study, to confirm whether NR2B-specific antibodies would pass into the rACC region and binding to NR2B protein in rACC, vaccinated rats underwent non-traumatic sampling of cerebrospinal fluid (CSF) from the cisterna magna and we were able to detect NR2B autoantibodies at low levels in the CSF by immunoblotting, suggesting autoantibody passage into the brain. Additionally, NR2B protein level in rACC tissue was to be examined. The results showed that the rAd5/NR2B vaccine decreased significantly the expression of NR2B protein in the rACC.

In addition to the regional distribution of NR2B protein in the pain-related pathway including rACC or spinal cord, it was also abundantly distributed in the other brain region including hippocampus. Therefore, to determine the anatomic extent of the rAd5/NR2B vaccination effect, immunoblot analysis was performed to detect the expression of NR2B protein in these areas. Our previous study showed that rAd5/NR2B vaccination reduced the NR2B protein

level in the spinal cord and not in the hippocampus [19]. In this study, we confirmed that rAd5/NR2B vaccination also decreased NR2B protein in rACC areas. Glutamate has been reported to alter BBB permeability. Hence, in the present study, it is more likely that peripheral nerve injury would increase pain-related areas (such as rACC) extracellular glutamate locally and then increase brain-blood barrier permeability locally, resulting in a facilitated passage of the NR2B antibody and the reduction of NR2B protein in these areas.

The present study showed that rAd5/NR2B vaccination blocked conditioned place preference (CPP) induced by spinal administration of clonidine. It remains possible that these vaccine block the animal's ability to associate the context to the reward induced by pain relief rather than reflecting a reduction in the primary aversive quality of the tonic pain per se. To address this, we found that rAd5/NR2B vaccination did not reduce the animal's ability to acquire place preference to the positive reinforcer, cocaine [17]. These data support the conclusion that rAd5/NR2B vaccination do not have a general disruptive effect on reward learning, but instead reflect a deficit specifically relating to the acquisition or expression of pain-relief-induced negative reinforcement. This is in line with studies indicating that ACC neurons can acquire responses to environmental cues predicting a painful stimulus [20].

Conclusions

In conclusion, it is indicated that NR2B receptors in the rACC are preferentially involved in the aversive state of spontaneous neuropathic pain due to peripheral nerve injury pain. An analgesics vaccine that generates autoantibodies whose access to the rACC and analgesic activity is spatially and temporally regulated may hold promise as a prophylactic measure to treat the aversive state of spontaneous neuropathic pain.

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Disclosure of conflict of interest

None.

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