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AMPA Receptor Mediated Behavioral Plasticity in the Isolated Rat Spinal Cord

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Abstract

Previous research has demonstrated that the spinal cord is capable of a simple form of instrumental learning. Spinally transected rats that receive shock to a hind leg in an extended position quickly learn to maintain the leg in a flexed position, reducing net shock exposure whenever that leg is flexed. Subjects that receive shock independent of leg position (uncontrollable shock) do not exhibit an increase in flexion duration and later fail to learn when tested with controllable shock (learning deficit). The present study examined the role of the ionotropic glutamate receptor L-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) in spinal learning. Intrathecal application of the AMPA receptor antagonist CNQX disrupted performance of a spinal instrumental learning in a dose dependent fashion (Experiment 1). CNQX also disrupted the maintenance of the instrumental response (Experiment 2) and blocked the induction of the learning deficit (Experiment 3). Intrathecal application of the agonist AMPA had a non-monotonic effect, producing a slight facilitation of performance at a low dose and disrupting learning at a high concentration (Experiment 4). Within the dose range tested, intrathecal application of AMPA did not have a long-term effect (Experiment 5). The results suggest that AMPA-mediated transmission plays an essential role in both instrumental learning and the induction of the learning deficit.

Keywords

Spinal cord; AMPA; CNQX; instrumental learning; plasticity

1. Introduction

Neurons within the spinal cord are plastic and can support a range of behavioral phenomena (Patterson & Grau, 2001). Using traditional learning tasks, the isolated spinal cord has been found to support single stimulus learning (Groves & Thompson 1970), Pavlovian conditioning (Durkovic, 1975; Joynes & Grau, 1996), and instrumental learning (Grau, Barstow, & Joynes, 1998). Our laboratory has focused on the last form of learning, in part because instrumental learning contributes to the recovery of function after spinal cord injury (SCI) (Edgerton, Kim, Ichiyama, Gerasimenko, & Roy, 2006; Hook & Grau, 2007a).

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Evidence that spinal neurons are sensitive to instrumental (response-outcome) relations has been obtained using animal subjects that have undergone a complete transection of the thoracic spinal cord, which blocks all communication between the brain and neurons below the injury. In a typical experiment (Grau et al., 1998; Grau et al., 2006), a response-outcome relationship is instituted by administering shock to the tibialis anterior muscle of one hind leg whenever that leg is extended (controllable shock). Over time, subjects in this condition exhibit a progressive increase in flexion duration that minimizes net shock exposure. Other subjects receive shock at the same time and for the same duration, but independent of leg position (uncontrollable shock). Uncontrollably shocked subjects do not exhibit an increase in flexion duration and later fail to learn when given controllable shock to either the pretreated (ipsilateral) or opposite (contralateral) leg. Further work has shown that just 6 minutes of intermittent, uncontrollable, shock to the leg or tail impairs learning for up to 48 hours (Crown, Ferguson, Joynes, & Grau, 2002b).

Both the acquisition of spinal instrumental learning and the induction of the learning deficit depend on a glutamatergic signal (Joynes, & Grau, 2004; Ferguson Crown, & Grau, 2006). The N-methyl-D-aspartic acid receptor (NMDAR) and α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA) are part of the same family of ionotropic glutamate receptors (Palmer, Cotton, & Henly, 2005). Engaging the AMPAR, through the binding of glutamate, results in rapid depolarization of the cell and delayed activation of the NMDAR (Watt et al., 2004). Activation of the NMDAR allows Ca^{++} ions to flow freely into the cell (Bliss & Lomo 1973; Watkins & Jane 2006). A strong Ca^{++} influx initiates intracellular mechanisms that modify synaptic communication, altering components thought to contribute to learning and memory (Yang et al., 2004; Blair, Schafe, Bauer, Rodrigues, & LeDoux, 2001), including the open probability of NMDARs, activating AMPARs and AMPAR trafficking at the synaptic cleft (Palmer et al., 2005; Lau, & Zukin, 2007). Prolonged high frequency stimulation has been linked to an NMDAR-dependent enhancement of synaptic function (long-term potentiation [LTP]) mediated by an up-regulation of AMPARs (Yang et al., 2004; Palmer et al., 2005). Conversely, stimulation parameters that lead to an overall reduction in synaptic efficacy (long-term depression [LTD]) produce a reduction of AMPAR function (Palmer et al., 2005).

Enumerable studies have examined the role of the NMDAR in behavioral plasticity, both within the brain and spinal cord (Watkins & Jane, 2006; Ji, Kohn, Moore, & Woolf, 2003; Yang et al., 2004). In contrast, while much is known about the AMPAR at the cellular level (Fleming & England, 2010; Santos, Carvalho, Caldeira, & Duarte, 2009; Song & Hanganir, 2009), relatively few studies have examined how pharmacological manipulations that target this receptor impact behavior (excluding studies designed to examine AMPA-mediated neurotoxic effects). At the level of the spinal cord, the limited work that has been done indicates that manipulations that impact AMPAR function affect nociceptive reactivity (Kong & Yu, 2006; Imamachi, Saito, Hara, Sakura, & Kosaka, 1999). Also as found within the brain, chronic spinal administration of AMPAR agonists can induce a lasting effect (excitotoxicity) that results in tissue damage and a loss of plasticity (Yezierski, 2005).

In prior studies, we have assumed that alterations in AMPAR function play a pivotal role in both instrumental learning and the induction of the learning deficit (Ferguson et al., 2008; Grau et al., 2006; Joynes et al., 2004), but this has not been tested. Instead, we have assumed that evidence that the NMDAR plays an essential role implicates, through association, the AMPAR. From this perspective, instrumental learning reflects a NMDAR/AMPA-dependent alteration in a spinal circuit that increases flexion duration. The learning deficit was then explained by positing uncontrollable stimulation saturates glutamate-dependent plasticity (Ferguson et al., 2008; Moser et al., 1998). While this reasoning seems sound given the standard view, derived from studies of hippocampal LTP, NMDAR's within the

spinal cord may act in a distinct manner. For example, it is generally assumed that NMDAR antagonists affect the induction, but not the maintenance, of amygdala-dependent plasticity (Ikegaya, Sato, & Abe, 1995). However, administration of an NMDAR antagonist disrupts both the induction and maintenance of instrumental learning and C-fiber dependent windup (Joynes et al., 2004; Woolf & Thompson, 1991). The latter suggests that both effects may depend on slow NMDAR-mediated synaptic potentials and the sensitization of the NMDAR (Woolf & Thompson, 1991). The presumption AMPA-signaling plays a pivotal role is also called into question by studies linking spinal LTP to C-fiber activity and the neurokinin (substance P) receptor (Baumbauer, Young, Hoy, & Joynes, 2007; Liu & Sandkühler, 1997). A final complexity stems from the observation that approximately a third of the NMDARs within the spinal cord are presynaptic, which suggests that the receptor may also regulate transmitter release (Liu, Wang, Shen, Jan, Jan, & Basbaum, 1994).

Thus, while our theorizing has assumed that the AMPAR plays a central role in spinal plasticity, we lack any direct evidence that the AMPAR is involved and current data implicate other neural signals. We address this issue by testing the impact of the AMPAR antagonist CNQX on instrumental learning (Exps. 1 and 2) and the induction of the learning deficit (Exp. 3). We also examine whether pretreatment with the agonist AMPA has an acute (Exp. 4), and/or long-term (Exp. 5), effect on instrumental learning. We hypothesize that the AMPAR signal is a necessary component of spinal instrumental learning.

2. Materials and Methods

2.1 Animals

All subjects, male Sprague-Dawley rats (100-120 days old; 300-450 g), were obtained from Harlan Laboratories (Houston, TX). Subjects were individually housed with water and food *ad libitum*, and maintained on a 12-hour light dark cycle. Behavioral testing and surgeries were performed during the light portion of the cycle. All experiments were carried out in accordance with the NIH standards for the care and use of laboratory animals (NIH publications No. 80-23), and were approved by the University Laboratory Animal Care Committee at Texas A&M University. Every effort was made to minimize suffering and limit the number of animals used.

2.2 Surgery

The surgical procedure consisted of a complete transection of the spinal cord at the second thoracic vertebra (T2). Anesthesia was induced using a concentration of 5% isoflurane, which was maintained at a 2% concentration during surgery. The T2 vertebra was located and an incision was made rostral-caudal to the vertebra. A laminectomy was performed to expose the cord rostral of T2. Heat cautery was used to transect the exposed cord and the cavity formed was filled with gelfoam (Harvard Apparatus, Holliston, MA). A 25 cm catheter (PE-10, VWR International Bristol, CT), held rigid with a 0.9 mm stainless steel wire (Small Parts Inc. Miami Lakes, FL), was inserted 9 cm into the subarachnoid space on the dorsal surface of the spinal cord (Yaksh & Rudy, 1976). Following insertion, the wire was gently removed and the exposed tubing adhered to the skin externally using cyanoacrylate. Following testing catheter placement was verified during a post-mortem inspection. The incision was closed using Michel Clips (Fine Science Tools, Foster City, CA). All subjects received injections of 0.9% saline (2.5 ml i.p.) immediately following surgery and the subject's legs were taped using a piece of porous tape (Ortholetic 1.3 cm width). The tape maintained the legs in a secure natural position and was removed prior to testing. Subjects were then allowed to recover for 18-24 hours before testing in a temperature-controlled room (25-27! C) with free access to food and water. During the recovery period, subjects were able to move about within their home cage (via their

forelimbs). Bladders were expressed twice daily and immediately before any behavioral procedures were conducted. When behavioral testing was complete, all animals were euthanized with a lethal dose of pentobarbital (100 mg/kg).

The surgical transections were verified by 1) observing behavior during the recovery period to confirm complete paralysis and a lack of vocalization to leg shock, 2) visual inspection of the transection site during surgery, and 3) post-mortem cord examination in a random sample of subjects.

2.3 Instrumental learning apparatus and procedure

Instrumental training/testing was conducted while rats were loosely restrained in tubes (23.5 cm [length]_8 cm [internal diameter]). Two slots in the tube, (5.6 cm [length]_1.8 cm [width]), 4 cm apart, 1.5 cm from the end of the tube, allowed both hind legs to hang freely. To minimize the effects of upper body movement on leg position, a wire belt was used to secure the rat's trunk within the tube. Hind limbs were shaved and marked for electrode placement prior to testing. A wire electrode was then inserted through the skin over the distal portion of the tibialis anterior (1.5 cm from the plantar surface of the foot), and one lead from a BRS/LVE (Laurel, MD, USA) constant current (60 Hz, AC) shock generator (Model SG-903) was attached to this wire. A 7-cm long, 0.46-mm diameter stainless steel contact electrode was secured to the foot between the second and third digits with a piece of porous tape. The last 2.5 cm of the electrode was insulated from the foot with heat shrink tubing. A fine wire (0.01 sq mm [36 AWG], 20 cm) attached to the end of the contact electrode extended from the rear of the foot and was connected to a digital input monitored by a Macintosh computer. A plastic rectangular dish (11.5 cm [w]_19 cm [l]_5 cm [d]) containing an NaCl solution was placed approximately 7.5 cm below the restraining tube. A drop of soap was added to the solution to reduce surface tension. A ground wire was connected to a 1-mm wide stainless steel rod, which was placed in the solution. The shock generator was set to deliver a 0.4-mA shock, and the proximal portion of the tibialis anterior (approximately 1.7 cm proximal to the wire electrode) was probed with a 2.5-cm stainless steel pin attached to a shock lead to find a robust flexion response. The pin was then inserted 0.4 cm into the muscle.

To standardize response parameters across subjects, we adjusted the intensity of electrical shock necessary to generate a 0.4-Newton flexion force for each subject. Flexion force was measured by attaching a monofilament plastic line ("4 lb test" Stren, DuPont, Wilmington, DE, USA) to the rat's foot immediately behind the plantar protuberance. The 40-cm length of line was threaded through an eyelet attached to the apparatus directly under the paw, 16 cm beneath the base of the tube. The end of the line was attached to a strain gauge (Fort-1000, World Precision Instruments, New Haven, CT, USA) fastened to a ring stand. After the line was connected to the rat's paw, the ring stand was positioned so that the line was taut, just barely registering on the gauge. The strain gauge was calibrated by determining the relationship between voltage and force in Newtons. These data revealed a linear relation, which allowed us to convert voltage to force.

To minimize lateral leg movements, a 20-cm piece of porous tape was wrapped around the leg and attached to a bar extending across the apparatus directly under the front panel of the restraining tube. The tape was adjusted so that it was taut enough to slightly extend the knee. Finally, three short (0.15 s) shock pulses, given at the intensity previously determined to elicit a 0.4-N flexion response, were applied and the level of the salt solution was adjusted so that the tip of the contact electrode (attached to the rat's foot) was submerged 4 mm below the surface. Recognizing that some experimental manipulations could potentially affect the subject's capacity to perform the target (flexion) response, we recorded two measures of baseline behavioral reactivity: the shock intensity required to elicit a flexion

force of 0.4 N and the duration of the first shock-elicited flexion response. Independent ANOVAs showed that there were no group differences on either measure across all experiments, all $F_s < 1.40$, $P < .05$.

Upon completion of this setup phase, the 30-min instrumental training/testing began. An instrumental (response-outcome) contingency was established by applying electrical stimulation (at the intensity previously determined to elicit a 0.4-N flexion force) to the tibialis anterior muscle each time the contact electrode touched the underlying salt solution. The shock stimulation typically elicited a flexion response, raising the contact electrode above the salt solution, which terminated the shock. The state of this circuit was sampled at a rate of 30 times/s.

2.3.1 Measures of Instrumental learning and performance—Three behavioral measures were used to assess a subject's capacity to perform the instrumental response: response number, response duration, and time in solution (see Grau et al., 1998). Performance was measured over time in 30 1-min time bins. The computer monitoring leg position recorded an increase in response number whenever the contact electrode left the salt solution. Response duration was derived from time in solution and response number using the following equation: $\text{Response Duration}_i = (60 \text{ s} - \text{time in solution}_i) / (\text{Response Number}_i + 1)$, where i is the current time bin. As discussed elsewhere (Grau et al., 1998), a system capable of instrumental learning should exhibit an increase in response duration as a function of training. Because this measure avoids some interpretative problems that plagued earlier studies, and because we have shown that this measure is sensitive to a variety of manipulations known to impact learning (reviewed in Grau et al., 2006), it is used as our primary index of learning.

Prior work has also shown that treatments that disrupt learning do not normally interfere with the performance of a flexion response. Indeed, subjects that fail to learn (that do not exhibit an increase in response duration) tend to exhibit the highest rates of responding (i.e. the greatest response number). This is important because it indicates that the failure to learn does not reflect a performance deficit. In the present experiments, we analyzed both response duration and response number and found the inverse relationship reported in prior studies. Because no unexpected results were obtained, we focus on our measure of learning, response duration.

2.4 Uncontrollable Shock Administration

To examine the learning deficit in Experiment 3 we applied uncontrollable shock prior to instrumental testing. Uncontrollable shock was administered while the subjects were loosely restrained in black Plexiglas tubes (22 cm [l] $\times$ 6.8 cm [w]) with holes drilled in them for ventilation (Crown et al., 2002b). A flat floor was attached 5.3 cm below the top of the tube that is 5.5 cm wide. Shocked subjects received 80-msec shocks on a variable schedule that ranged from 0.2–3.8 s (a rectangular distribution with a mean of 2 s). Stimulation was applied for 6 min. Prior work has shown that this shock treatment produces a robust learning deficit (Crown et al., 2002b). Tailshock was delivered using a modified fuse clip, coated with ECG gel (Harvard Apparatus, Holliston, MA) and gently secured with porous tape approximately 6 cm behind the base of the tail. Attaching the clip does not elicit a behavioral response, suggesting it is innocuous. A constant current 1.5 mA shock was delivered using a 660-V transformer (Crown et al., 2002b).

2.5 Drug administration

In all experiments a 25 cm polyethylene catheter (PE-10, VWR International, Bristol, CT, USA) was threaded 9 cm down the vertebral column, into the subarachnoid space between

the dura and the white matter so that it lies on the dorsal surface of the spinal cord. Prior work has shown that this places the catheter tip just rostral to the lumbar-sacral enlargement (Grau et al., 2006). The exposed end of the catheter was secured to the skin with cyanoacrylate. Drugs were dissolved into 10 μ l of vehicle (0.9% saline or DMSO) followed by a 20 μ l 0.9% saline flush. CNQX and AMPA were purchased from Sigma Aldrich (St. Louis, MO). Drugs were administered caudal to the injury at a rate of 15 μ l / min through an intrathecal catheter.

2.6 Statistics

All data were analyzed using an analysis of variance (ANOVA). Alpha values of .05 or below were considered statistically significant. *Post hoc* comparisons were made using Duncan's new multiple range test.

3. Results

3.1 Experiment 1: CNQX Disrupts Acquisition of Instrumental Learning

Experiment 1 examined whether 40-80 nmol of CNQX affects instrumental learning. If AMPAR activation is necessary for instrumental learning, then blocking the action of the AMPAR with an antagonist (CNQX) should inhibit instrumental learning.

Spinally transected rats ($N = 18$, 6 per group) were placed in the instrumental learning apparatus and the exterior portion of the catheter was threaded through a hole in the tube to administer the drug. Subjects received CNQX (40 nmol or 80 nmol) or its vehicle (DMSO). Based on prior work (Ferguson et al., 2008), twenty minutes after drug injection, the instrumental testing session was initiated. All subjects received 30 minutes of controllable shock.

To ensure drug treatment did not affect baseline reactivity, the shock intensity needed to induce a 0.4 N flexion force and initial flexion duration were analyzed. Mean shock intensities ($\pm$ standard error of the mean [SE]) ranged from 0.60 ($\pm$ 0.02) to 0.70 ($\pm$ 0.03) mA. A between-subjects ANOVA revealed no significant differences in baseline reactivity, $F(2,15) = 1.00$, $p > .05$. Mean initial responses ranged from 0.15 ($\pm$ 0.01) to 0.16 ($\pm$ 0.02) seconds and were not significantly different, $F(2,15) < 1.00$, $p > .05$.

The effect of CNQX on instrumental learning is depicted in Figure 1 (representative data from individual subjects are shown in Supplemental Material S1). Subjects that received the vehicle (DMSO) exhibited a progressive increase in flexion duration indicative of learning (Figure 1.). A low dose of CNQX (40 nmol) had little effect whereas the high dose (80 nmol) disrupted learning. A two-way mixed (between/within subjects) ANOVA for response duration revealed a significant main effect of drug treatment, $F(2,15) = 6.47$, $p < .01$, and a Time $\times$ Drug interaction $F(29, 58) = 1.85$, $p < .01$. *Post hoc* comparisons showed that subjects that received the highest dose of CNQX (80 nmol) differed from the groups that received 40 nmol of CNQX or its vehicle (DMSO) ($p < .05$).

As found in past studies (e.g., Grau et al., 1998), subjects that failed to learn exhibit the highest rate of responding (see Supplemental Materials, S2). The mean responses per minute ($\pm$ SE) were 7.95 ($\pm$ 3.49), 40.17 ($\pm$ 22.91), and 185.85 ($\pm$ 20.45), for the 0, 40, and 80 nmol treated groups, respectively. A mixed ANOVA confirmed that drug treatment, and the Drug $\times$ Time interactions were statistically significant, both $F_s > 5.49$, $p < .0001$. *Post hoc* comparisons showed that the 80 nmol treated group exhibited a significantly higher level of responding ($p < .01$). A similar pattern was observed in all of the subsequent experiments.

3.2 Experiment 2: CNQX Disrupts the Maintenance of the Learned Response

Prior research has shown that administering an NMDAR antagonist after the instrumental response has been acquired disrupts the maintenance of the acquired response (Joynes et al., 2004). Experiment 2 examined whether intrathecal treatment with an AMPAR antagonist has the same disruptive effect.

A 2×2 factorial design was used for this experiment ($N = 32$). Half the subjects received 30 minutes of training with response contingent shock (pretrained), and the other half received nothing. Twenty-five minutes into the session, half of the subjects in each condition received CNQX (80 nmol) while the remaining subjects received the vehicle (DMSO). Five minutes later, both the pretrained and untrained subjects were tested for 30 minutes with response-contingent shock.

Analyses of baseline behavioral reactivity did not reveal any group differences. The shock intensity required to elicit a 0.4 N change in flexion force ranged from 0.58 (± 0.03) to 0.69 (± 0.04) mA. Initial flexion response means ranged from 0.14 (± 0.01) to 0.23 (± 0.07). These differences did not approach statistical significance, both $F_s < 1.10$, $p > .05$.

As shown in Figure 2, the 2 pretrained groups exhibited a progressive increase in response duration and did not differ prior to drug treatment, $F_s < 1.0$, $p > .05$.

After drug treatment, untrained rats that received the vehicle exhibited an increase in flexion duration and this learning was blocked by pretreatment with CNQX. Pretrained rats given the vehicle maintained the instrumental response whereas CNQX treated rats exhibited a decline in response duration. A mixed ANOVA performed on the data collected after drug treatment (min 31-60) revealed a significant main effect of drug treatment $F(1, 28) = 21.96$, $p < .01$ and training condition $F(1, 28) = 10.26$, $p < .01$, with no other significant relationships. *Post hoc* comparisons showed that the CNQX-untrained group was significantly different from all other groups ($p < .05$), verifying that CNQX blocks the acquisition of learning (Experiment 1). The CNQX-pretrained group was significantly different from the vehicle-pretrained group ($p < .05$), showing that CNQX also disrupts the maintenance of learning. Finally, the vehicle-untrained group was significantly different from the vehicle-pretrained group ($p < .05$) showing that pretraining has a significant effect on learning.

Our results confirm that the maintenance of the instrumental response depends on the AMPAR. What is somewhat surprising is that CNQX produced a gradual decline in response duration (from minute 25 to 50). This observation implies that a residual component contributes to the maintenance of the response.

3.3 Experiment 3: CNQX Blocks the Induction of the Learning Deficit

Spinally-transected rats that have previously received 6-minutes of tail-shock (uncontrollable shock) failed to learn when subsequently tested with response-contingent leg-shock (Crown et al., 2002b). Experiment 3 examined whether CNQX would block the induction of the behavioral deficit when administered prior to uncontrollable shock. The highest concentration (80 nmol) was used in this experiment. If the AMPAR is required for the induction of the learning deficit, then blocking the action of the AMPAR with CNQX should eliminate the learning deficit when subjects are tested with response-contingent shock 24 hours later.

A 2×2 factorial design was used in this experiment ($N = 40$). Subjects were pretreated with CNQX or its vehicle, DMSO. Thirty-minutes later, subjects were placed in Plexiglas tubes and the tail electrodes were attached. Half the subjects in each drug condition received 6

minutes of uncontrollable shock, while the other half remained unshocked. The animals were returned to their home cages and, 24-hours later, tested for 30-minutes with response-contingent shock.

The shock intensity needed to elicit a 0.4N response ranged from 0.65 ($\pm$ 0.03) to 0.71 ($\pm$ 0.04) mA. Mean initial flexion duration ranged from 0.14 ($\pm$ 0.01) to 0.24 ($\pm$ 0.05). These differences were not statistically significant, all F s $<$ 2.07 $p >$.05.

Previously unshocked subjects, regardless of drug condition, exhibited a progressive increase in response duration during testing (Figure 3). Vehicle-treated subjects exposed to uncontrollable shock failed to learn. This learning deficit was blocked by pretreatment with CNQX. An ANOVA revealed a significant effect of drug treatment $F(1, 36)=8.21$, $p <$.01, shock condition $F(1, 36)=9.07$, $p <$.01, a Drug $\times$ Shock condition interaction $F(1, 36)=4.99$, $p <$.05, and a Time $\times$ Drug interaction $F(29,1044)=1.77$, $p <$.01. *Post hoc* comparisons showed that the vehicle-shock group differed from the other three ($p <$.05).

3.4 Experiment 4: Pretreatment with the Agonist AMPA Disrupts the Acquisition of Instrumental Learning

We have suggested that uncontrollable shock may disrupt learning because it induces a diffuse over-excitation that saturates glutamate-dependent plasticity (Joynes et al., 2004). Experiment 3 provided indirect support for this hypothesis by demonstrating that pretreatment with an AMPA receptor antagonist prevents the induction of the learning deficit. Experiment 4 examined the converse issue, whether pharmacologically saturating AMPAR function, using the agonist AMPA, inhibits instrumental learning.

Subjects were given either AMPA: 0.125 nmol, 0.50 nmol, and 2.0 nmol, or vehicle ($N = 40$, 10 per group) 30 minutes before testing. Drugs were dissolved into 10 μ l of vehicle (0.9% saline) followed by a 20 μ l 0.9% saline flush. They were then tested for 30 min with response-contingent legshock as described above.

The shock intensity needed to induce a 0.4 N change in flexion force ranged from 0.60 ($\pm$ 0.03) to 0.70 ($\pm$ 0.03) mA. Mean initial flexion durations ranged from 0.11 ($\pm$ 0.01) to 0.14 ($\pm$ 0.01). These differences did not approach statistical significance, all F s $<$ 1.14, $p >$.05.

Subjects that received the highest dose of AMPA (2.0 nmol) prior to instrumental learning showed a marked learning deficit (Figure 4). An ANOVA yielded a significant main effect of drug treatment, $F(3, 36)=6.22$, $p <$.01. *Post hoc* comparisons showed that subjects that received the highest dose of AMPA (2.0 nmol) were significantly different from the groups that received lower doses (0.125 nmol & 0.5 nmol) or the vehicle, $p <$.05.

While saturating AMPAR function is expected to have a disruptive effect, low concentrations of the agonist could have a depolarizing effect that enhances NMDAR-mediated alterations in synaptic function. For this reason, pretreatment with an AMPAR agonist could have a non-monotonic effect (quadratic), fostering learning at a low concentration but disrupting learning at high concentrations (that saturate AMPAR function). To explore this possibility, a trend analysis was performed. This analysis revealed that both the linear, $F(1, 36)=6.75$, $p <$.05, and quadratic contrast, $F(1, 36)=9.11$, $p <$.01, trends were statistically significant.

Our results confirm that a high concentration of the agonist AMPA disrupts instrumental learning. Further, the significant quadratic relation between performance and drug

concentration, which indicates one significant inflection, suggests that drug treatment had a non-monotonic effect that fosters learning at low concentrations.

3.5 Experiment 5: Pretreatment with AMPA did not have a Long-Term Effect on Instrumental Learning

A high dose of AMPA could have disrupted learning in Experiment 5 because the resultant over-excitation has a non-reversible (e.g. neurotoxic) effect that permanently abolishes the capacity for instrumental learning (Gorman et al., 2001). Experiment 5 examined this possibility by testing whether a high dose of AMPA produces a lasting impairment.

Twenty-four hours post-surgery, subjects received drug treatment (AMPA 2.0 nmol or saline) ($N = 30$). A positive control group received AMPA (2.0 nmol) administration 30 minutes prior to instrumental testing ($n=10$). The next day (48 hours post surgery), all subjects received instrumental testing.

The shock intensity needed to induce a 0.4 N change in flexion force ranged from 0.60 (± 0.05) to 0.70 (± 0.05) mA. Mean initial flexion durations ranged from 0.14 (± 0.01) to 0.13 (± 0.01). Again, these differences were not significant, both $F_s < 1.00$, $p > .05$.

Subjects that received AMPA treatment 24 hours prior to testing (Delayed) displayed response durations similar to vehicle treated controls (Figure 5). Subjects that received AMPA 30 minutes prior to instrumental testing failed to learn. A repeated measures ANOVA revealed a significant effect of drug $F(2,27) = 6.23$, $p < .01$ and a Time $\times$ Drug interaction $F(2, 58) = 1.82$, $p < .01$. *Post hoc* comparison of the groups showed significant differences between the AMPA-positive control group and both groups that received treatment 24 hours prior to instrumental testing ($p < .05$).

We found that acute AMPA (2.0 nmol) administration blocked the acquisition of learning, replicating the results from Experiment 4. A high dose of AMPA (2.0 nmol), at a 24 hour delay, did not have a long-term effect on our primary measure of learning, response duration.

4. General Discussion

The present experiments confirmed that the AMPAR is involved in spinal learning. The AMPAR antagonist CNQX produced a dose-dependent disruption in instrumental learning (Experiment 1). CNQX also disrupted the maintenance of the instrumental response (Experiment 2) and the induction of the learning deficit (Experiment 3). Administration of the agonist AMPA impaired learning when subjects were tested soon after drug treatment (Experiment 4), but did not affect learning when subjects were tested 24 hrs later (Experiment 5).

CNQX affected instrumental learning and the induction of the learning deficit in the same manner as the NMDAR antagonist APV and, in this way, the results are consistent with the claim that spinal plasticity co-depends upon AMPAR/NMDAR function. Further, while past work suggested that the induction of the learning deficit was linked to C-fiber input and the release of substance P (Baumbauer et al., 2007, 2009), it is clear that AMPA-dependent neural transmission also plays an essential role (Experiment 3). In addition, the results provide indirect support for our claim that the learning deficit arises because uncontrollable stimulation saturates AMPA-dependent neural plasticity (Ferguson et al., 2006). Supporting this, pretreatment with a high dose of AMPA impaired instrumental learning (Experiment 4). It did not, however, produce a long-term impairment (Experiment 5), implying that other processes contribute to the lasting effect of uncontrollable shock.

Elsewhere, we suggested that instrumental learning and the induction of the learning deficit reflect a NMDAR-dependent modification of AMPAR function (Ferguson et al., 2008; Joynes et al., 2004). The current results are consistent with this claim. At the same time, it is recognized that there are multiple ways in which AMPAR-dependent neural transmission could contribute to synaptic plasticity. While we assume that AMPAR function is modulated by NMDAR activation, this would not explain why NMDAR antagonism disrupts the maintenance of instrumental behavior (Joynes et al., 2004). The latter observation suggests that a NMDAR-dependent process contributes to on-going performance through a parallel process.

The idea that instrumental learning depends upon parallel processes may help to explain an observation that seems otherwise difficult to understand. The finding arose in Experiment 2 where we tested the effect of CNQX on the maintenance of instrumental behavior. If an intervening AMPAR dependent process was responsible for maintaining the increase in flexion duration, administration of CNQX should rapidly (within a few minutes) disrupt the performance of the instrumental response. Indeed, intrathecal CNQX administration affects nociceptive reactivity within 2 minutes of application (data not shown). Yet, CNQX had a gradual effect on the maintenance of instrumental performance that emerged slowly over a 10-15 min interval. Such an outcome would be expected if a parallel process (e.g., the build-up of NMDAR-dependent slow synaptic potentials [Woolf & Thompson, 1991]) contributes to instrumental performance. Alternatively, the key AMPAR-dependent process may be related to a sensory input indicative of leg position. If this input encodes the effective response (R), and the reinforcing outcome (O) is linked to C-fiber input, removing the AMPAR-dependent process may be equivalent to placing the subject on extinction (due to breaking the R-O relation). Under these conditions, the response would be expected to gradually wane. A final alternative is that a peripheral motor process, that would be unaffected by intrathecal drug delivery, contributes to the maintenance of the instrumental response. We are currently exploring these alternative explanations.

Our hypothesis that uncontrollable shock interferes with learning because it produces a diffuse state of over-excitation suggested that this phenomenon might reflect a form of central sensitization (Ferguson et al., 2008). Central sensitization refers to the increase in neural excitability observed in response to neural injury or inflammation and is thought to underlie the development of neuropathic pain (Ji et al., 2003; Latremoliere & Woolf, 2009; Willis, 2002). Central sensitization causes neurons within the dorsal horn to exhibit a lower threshold of response to tetanic stimulation and heightened excitability that outlives the initial injury (Ji et al., 2003, Sotgiu & Biella, 2000). This heightened state of excitability is associated with increased phosphorylation of NMDARs, trafficking of Ca^{++} permeable AMPARs and the release of pro-inflammatory cytokines (Latremoliere & Woolf, 2009; Ji et al., 2003; Beattie et al., 2010).

If intermittent shock induces a central sensitization-like state, it too should induce allodynia. Conversely, manipulations that induce central sensitization (e.g., treatment with intradermal carrageenan) should inhibit learning. Supporting this, Ferguson et al. (2008) showed that intermittent shock induces allodynia and that intradermal carrageenan impairs learning. Similarly, Hook et al. (2008) showed that peripheral capsaicin, which induces a robust central sensitization, produces a lasting learning impairment. Further, just as prior exposure to controllable stimulation blocks the induction of the learning deficit by uncontrollable shock (Crown & Grau, 2001), prior exposure to controllable shock blocks both capsaicin-induced allodynia and its effect on instrumental learning (Hook et al., 2008).

Both the induction of the learning deficit, and central sensitization, have been linked to the release of the cytokine TNF! (Huie et al., 2012), which is known to enhance neural

excitability by fostering the trafficking of AMPA receptors and by increasing the proportion of Ca^{++} permeable AMPA receptors (Beattie et al., 2010). Supporting this, we have shown that pretreatment with $\text{TNF}\alpha$ induces a lasting inhibition of instrumental learning (Huie et al., 2012) conversely, pretreatment with a $\text{TNF}\alpha$ inhibitor blocks both the induction and the expression of the learning deficit. Further, the expression of the learning deficit can be blocked by pretreatment with NASPM, a selective antagonist of Ca^{++} permeable AMPA receptors (Huie et al., 2012).

5. Conclusions

The present experiments demonstrate that manipulations that impact AMPAR-mediated communication within the spinal cord can affect both instrumental learning and the long-term consequences of uncontrollable stimulation, which may be characterized as adaptive and maladaptive plasticity, respectively. The demonstrated role in learning suggests glutamatergic systems play a pivotal role in the abstraction of instrumental relations and/or the maintenance of instrumental behavior (memory). The fact that AMPAR-mediated transmission is also involved in maladaptive plasticity suggests that drug-treatments that inhibit AMPAR-mediated transmission could have therapeutic value. Elsewhere, we have shown that exposure to uncontrollable stimulation impairs recovery after a contusion injury (Grau et al., 2004). By reducing AMPA-mediated over-excitation, AMPA antagonists may help preserve long-term adaptive plasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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We further examine the role of the glutamatergic system on instrumental learning The role of the AMPA Receptor on instrumental learning is highlighted Extends our hypothesis that the plasticity of the spinal cord can be saturated

Figure 1.

CNQX blocked the acquisition of instrumental learning in a dose dependent fashion. Mean flexion duration over the 30 min test session is indicated to the left and the group means (+SE) are provided to the right. Vehicle treated rats (open circles) exhibited a progressive increase in flexion duration. Learning was disrupted by the 80 nmol dose of CNQX (filled diamonds). The intermediate dose (40 nmol; filled squares) had no effect. An asterisk indicates that the 80 nmol dose of CNQX was significantly different from all other groups ($p < .05$).

Figure 2.

CNQX disrupted the maintenance of spinal learning task. The left side graph illustrates response duration over initial training (first 30 min) and testing (last 30 min). Mean performance (+SE) during the last 30 min of testing is illustrated to the right. Vehicle treated rats (open symbols) exhibited an increase in flexion duration independent of whether training began immediately (at 0 min) or after 30 min. Untrained subjects (Control) that received CNQX prior to the onset of training at 30 min (filled circles) failed to learn. Subjects treated with CNQX after training (filled squares) exhibited a gradual decline in response duration. An asterisk (*) indicates that CNQX had a significant effect ($p < .05$) relative to the vehicle controls.

Figure 3.

CNQX blocked the induction of the learning deficit. The left-most graphs illustrate response duration over time. Mean test performance (+SE) is displayed to the right. Unshocked rats given the vehicle (open squares) or CNQX (filled squares) 24 hrs earlier exhibited an increase in flexion duration. Previously shocked rats that received the vehicle (open circles) failed to learn. Subjects given CNQX prior to shock treatment (filled circles) did not exhibit a learning deficit. An asterisk (*) indicates that the vehicle treated shock group was significantly different from all other groups ($p < .05$)

Figure 4.

AMPA blocked the acquisition of instrumental learning in a dose dependent fashion. The left-most graph illustrates response duration over time. Mean test performance (+SE) is illustrated to the right. Vehicle treated rats (open circles) exhibit an increase in flexion duration. An asterisk (*) indicates significant group differences ($p < .05$).

Figure 5.

AMPA did not have a long-term influence on instrumental learning. The left-most panel depicts response duration over time. Mean test performance (+SE) is depicted to the right. Vehicle treated controls (open triangles) exhibited an increase in flexion duration. Treatment with AMPA immediately prior to testing (open squares) disrupted learning (Immediate; open squares). Treatment with AMPA 24 hrs. prior to testing (Delayed; filled squares) had no effect. An asterisk indicates significant group differences ($p < .05$).