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Methodological optimization of applying neuroactive agents for the study of locomotor-like activity in the mudpuppies (*Necturus Maculatus*)

Igor Lavrov[a] and **Jianguo Cheng**[b]

a*Department of Physiological Science and Brain Research Institute University of California Los Angeles, California 90095*

b*Departments of Pain Management and Neurosciences, Cleveland Clinic, Ohio 44195*

Abstract

We compared the effects of mode of delivery of neuroactive agents and the effects of Dimethyl sulfoxide (DMSO), a vehicle for dissolving neuroactive agents, on locomotor-like activity *in vitro*. By superfusion, D-glutamate (0.3 – 0.9 mM) produced robust walking-like activity at superfusion rates 10–25 ml/min. In contrast, bolus application of the same or higher doses of glutamate (0.1–1.5 mM) failed to induce any rhythmic activity. Superfusion with AP-5, a NMDA receptor antagonist, produced dose-dependent inhibition of the ongoing walking-like activity induced by D-glutamate and completely blocked the activity at 20 μM. In contrast, bolus application of AP-5 did not block the walking-like activity at concentrations up to 120 μM. Similarly, superfusion of AP-5 inhibited the initiation of walking-like activity and completely blocked the initiation at 20 μM, while bolus application of AP-5 failed to do so at concentrations up to 120 μM. Superfusion of strychnine, a glycine receptor antagonist, blocked the walking-like activity at concentrations of 3–5 μM, while its bolus application altered NMDA-induced, but not glutamate-induced, walking-like activity to a synchronized pattern. DMSO significantly affected the walking-like activity in a dose-dependent manner at concentrations ranging 1–10% (v/v). These results demonstrate that the way by which the neuroactive agents are applied is a significant factor that determines the outcome of experiments on the neural control of locomotion. Also, the dose-dependent effects of DMSO on the activity of neural networks for locomotion should be taken into account in data interpretation.

Keywords

Drug application; Locomotion; Central pattern generator; Spinal cord; Mudpuppy; NMDA; Glutamate; Strychnine; DMSO; Neuromodulation

INTRODUCTION

Much of the knowledge about the neural control of rhythmic behaviors has been gained by the utilization of applying neuroactive agents into the bath of *in vitro* spinal cord or brainstem

Correspondence: Jianguo Cheng, MD, PhD, Department of Pain Management/C25, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195, Email: chengj@ccf.org Phone: 216 444-0237.

preparations. Locomotor-like activities were induced by bolus application of N-methyl-D-aspartic acid (NMDA) in *in vitro* preparations from the chick embryo (Barry and O'Donovan, 1987), frog embryo and tadpole (Roberts et al., 1983), neonatal rat (Kudo and Yamada, 1987; Smith and Feldman, 1987), neonatal mouse (Hernandez et al., 1991; Bonnot et al., 1998; Jiang et al., 1999), lamprey (Grillner et al., 1981), and the mudpuppy (Wheatley and Stein 1992; Wheatley et al,. 1992; Wheatley et al., 1994; Jovanovic et al., 1996; Cheng et al. 1998, 2002, Fok and Stein, 2002; Igor and Cheng 2004). Bath application of 5-HT has also induced locomotor-like activities in preparations from the lamprey (Grillner et al., 1991) and the neonatal rat (Cowley and Schmidt, 1997; Kiehn and Kjaerulff, 1996). For all these studies, bolus application was sufficient to produce stable locomotor-like activities. However, although bolus application of D-glutamate, L-glutamate or DL-homocysteate produced fictive locomotion in lamprey (Poon 1980; Cohen and Wallén, 1980) and chick embryo (Barry and O'Donovan, 1987), such application has not been successful in inducing locomotor-like activity in the mudpuppy and other preparations, whereas the same substance can induce robust walking-like pattern when applied to the bath with superfusion (Brodin and Grillner, 1985 Lavrov and Cheng, 2004). Clearly, the means by which the neuroactive agents are delivered can be an important determinant in the outcomes of locomotor behavior. We thus compared effects of continuous superfusion of the agonists and antagonists of the excitatory and inhibitory neurotransmitter receptors on the initiation and maintenance of locomotor-like activity in comparison to bolus applications of these agents.

A second issue concerns the use of DMSO as a vehicle to facilitate the application of neuroactive agents for medicine and experimental practices, as many of the agents are poorly water-soluble (Jacob and Herschler, 1986; Bralow et al., 1973). An ideal vehicle should be inert, highly penetratable through biological membranes, and have no biological action on the nervous and muscular systems. However, such vehicles rarely exist. DMSO, a vehicle commonly used for dissolving water insoluble substances, may have a wide range of actions on different tissues (Bralow et al., 1973; Jacob and Herschler, 1986; North and Mark, 1989; Sams and Carroll, 1966; Jourdon et al., 1986; Winmill and Hedrick, 2003; Hedric and Morales, 1999). For instance, effects of DMSO were noted on the rhythmicity of the heart (Kramer et al., 1995; Bazil et al., 1993) and respiration (de la Torre et al., 1974, 1975). Superfusion of 1% DMSO enhanced the duration and amplitude of burst complex without affecting the rhythmicity of respiration (Hedric and Moralis, 1999). It is therefore important to quantify the effects of DMSO on the locomotor behavior for a better understanding of its impact on the study of neural control of locomotion. We thus investigated the effects of DMSO on the walking-like activity induced by NMDA or Glutamate in the mudpuppy. Part of this study was published in an abstract (Cheng and Lavrov 2004).

MATERIALS AND METHODS

Experiments used 40 adult mudpuppies (body length 20–30 cm). The experimental protocols were approved by the Animal Care and Use Committee of the University of Louisville.

The spinal cord-forelimb preparation

The dissection was performed as described in detail elsewhere (Wheatley et al. 1992). Briefly, animals were first anesthetized with application of 3-aminobenzoic acid ethyl ester (1–1.5 g/l) (Sigma, St. Louis, MO) to the water in which mudpuppy was placed. A longitudinal incision was made and paravertebral muscles were removed. A dorsal Laminectomy was performed from the first to the fifth cervical segments, which are then isolated along with the brachial nerve plexuses and the forelimbs. The preparation is placed in a Petri dish containing 100% oxygenate Ringer's solution (NaCl 115mM, KCl 2mM, CaCl 2mM, MgCl2 1.8mM, HEPES 5mM and glucose 1 gm/l, pH 7.35). While in the Petri dish, the brachial plexus was exposed,

the paraspinal muscles were removed, and the dura mater covering the spinal cord was opened. The dissection took about 45 min to complete. After dissection, the preparation was transferred to a recording chamber (120 ml) and perfused with cooled (15°C) and oxygenated Ringer's solution throughout the experiment at a flow rate of 4–5 ml/min. The spinal cord and forelimbs were stabilized by the pinning the vertebral column to the Sylgard resin (Dow Corning) coating the base of the bath. Teflon-coated silver wires (75μm) were inserted into the elbow flexor (*Brahialis*) and extensor (*Extensor ulnae*) muscles for electromyography (EMG) recording, which was amplified, high-pass (10 Hz) and low-pass (300 Hz) filtered, and stored on a computer's hard disk. Commercially available programs (Axon Instruments/Molecular Devices, Sunnyvale, CA; Origin, Northampton, MA and SPSS, Chicago, IL) were used for recording and processing. After a recovery period of 1 hour, all preparations showed a withdrawal reflex to pinching of the limbs.

Application of neuroactive agents

The neuroactive agents were applied either directly to the bath as a bolus or by continuous superfusion. The spinal cord compartment of the recording chamber contains 15 ml of artificial CSF solution and the superfusion rates were 4–5ml/min when bolus application is used and 5–35 ml/min when continuous superfusion with neuroactive agents is intended. Induction of walking-like activity was attempted by bolus bath application of D-glutamate (0.3–0.9 mmol/L, Sigma, St. Louis, MO) together with D-serine (10 μmol/L, Sigma, St. Louis, MO) or by continuous superfusion of known concentrations of D-glutamate. D-serine was co-applied with D-glutamate for more stable walking-like activity, presumably through saturating the coagonist glycine binding site (Wheatly et al. 1992). However, it was not required as application of D-glutamate alone induced robust walking-like activity in all 4 preparations tested. A 3-tank system interconnected by a 3-way stopcock was used for (i) washout with Ringer' s solution, (ii) delivery of a glutamate receptor agonist, or (iii) simultaneous delivery of a glutamate receptor agonist and an antagonist. Only 1 of the 3 solutions was delivered to the preparation at any given time. The agonist was either D-glutamate or NMDA (20–80 μmol/L, Sigma, St. Louis, MO) and the antagonist was AP-5 (1–120 μmol/L), CNQX (1–100 μmol/L), Strychnine (1–120 μM) (Sigma, St. Louis, MO). All drugs were dissolved in 100–200 mL of Ringer's solution immediately before use. Each application was followed by a period of washing-out with Ringer's solution. The walking-like activity was monitored, recorded, and analyzed with the use of Axoscope 8.1 (Axon Instruments). Measurements of cycle duration of flexor and extensor bursts were made before and after each application of agonist and antagonist and quantified as means ± SE of at least 20 cycles. DMSO (1–10% v/v, Sigma, St. Louis, MO) was applied through continuous superfusion in the presence of NMDA or glutamate.

Dorsal root reflex testing

To test effects of neuroactive and DMSO on reflex amplitude, the dorsal root (C3) was stimulated with constant cathode current of 0.5 ms pulse duration through a stimulator (S88; Grass-Telefactor, Astro-Med Inc, West Warwick, RI, USA) and stimulus isolation unit (PSIU6; Grass-Telefactor, Astro-Med Inc, West Warwick, RI, USA). The stimulus intensity was up to 1.5 times the motor threshold. The amplitude of the short latency reflex was evaluated from *Extensor ulnae*.

Analysis

Measurements of cycle duration of flexor and extensor bursts were made of greater that 20 cycles and averaged. The amplitude of EMG bursts was calculated as area of rectified EMG and reported as percentage of the control for each preparation. All data are reported as mean ± SEM. Statistically significant differences were determined using a one-way repeated

measures analysis of variance (ANOVA). The confidence level of statistical significance was 95% for all comparisons.

RESULTS

Initiation and maintenance of walking-like activity

Bolus application of D-glutamate produced tonic activity in a wide range of concentrations (0.5–100 mM). Short bouts of irregular rhythmic pattern (5–10 sec) were observed in 2 out of 12 animals. In contrast, stable and long-lasting walking-like activity was induced by continuous superfusion of glutamate (0.5 mM) at a rate of 20 ml/min for all tested animals. The walking-like pattern became unstable and irregular and came to a termination when the superfusion rate was reduced to < 5ml/min. Strong tonic activity was induced at superfusion at rates > 30 ml/min and the rhythmic activity was terminated shortly after such rate was reached. Upon termination of glutamate application, rhythmic walking-like activity continued for a period of 5–100 seconds. The duration of this period was superfusion rate dependent with the longest duration (100 seconds) obtained at superfusion rates of 10–20 ml/min, as shown in Figure 1A. Also shown in this Figure is the relationship between the frequency of the walking-like activity and the superfusion rate. Clearly, the frequency was also dependent on the superfusion rate, albeit to a lesser extent. The highest frequency (0.8 Hz) was achieved at superfusion rates of 10– 20 ml/min.

Both bolus application and continuous superfusion NMDA produced robust, long-lasting (> 30 min) rhythmic patterns in all preparations, beginning within a few seconds after NMDA was applied (Figure 1B). Bolus application induced walking-like activity at a wide range of concentrations. In contrast, continuous superfusion showed a window of concentrations (around 60 μM) at which the best locomotor-like pattern was achieved. Beyond this window of concentrations, NMDA lost its efficacy in producing locomotor-like activity. Also shown in this panel is the linear relationship between the frequency of the rhythmic activity and the concentration of glutamate superfusion solution within the range of 0.3 to 0.9 mM (established at a superfusion rate of 20ml/min). The rhythmic activity was disrupted with tonic activity at glutamate concentrations greater than 0.9 mM. In comparison, NMDA bolus application produced stable walking-like activity at concentrations ranging 20–300 μM (data not shown).

Inhibitory effects of AP-5 and strychnine applied as a bolus or with superfusion

Both bolus application and continuous superfusion of AP-5 reduced the frequency of walking-like activity induced by D-glutamate in a dose-dependent manner (Figure 2). However, much lower concentrations of AP-5 (5–20 μM) were required for this inhibitory effect when applied as continuous superfusion (open circles), in contrast to bolus application (20–120 μM, closed circles) (Figure 2A). Similar effects were observed on the initiation phase of walking-like activity induced by glutamate superfusion (Figure 2B). In contrast to bolus application (20–120 μM, closed circles), continuous superfusion of much lower concentrations of AP-5 (5–20 μM) was required to suppress the frequency of the locomotor-like activity. Complete block of the activity was achieved at 20 μM AP-5 in 86% of preparations (6/7).

Bolus application of glycine receptor antagonist strychnine had profound effects on the walking-like activity (Figure 3). At low concentrations (1–20 μM), strychnine increased its frequency without affecting the alternating patterns of flexor-extensor activation (data not shown). At higher concentrations (20–100 μM), in contrast, Strychnine converted the alternating pattern to a synchronized pattern and reduced the frequency dramatically (Figure 3A, B). The slow, synchronized rhythm was long-lasting (>30 minutes) and was stable without additional NMDA administration. Restoration of the regular patterns was only possible after prolonged washout (2–3 h) or did not occur at all in 3 out of 8 preparations. In contrast,

continuous superfusion of strychnine (3–5 μM) completely blocked the walking-like activity induced by NMDA (50 μM) in all preparations (n=8) (Figure 3C). A slow and synchronous pattern, similar to that produced by bolus strychnine application, emerged within 10–15 minutes after termination of the superfusion. Continuous superfusion of NMDA converted this synchronous pattern to tonic activities and abolished the rhythmicity. The synchronous rhythm was also terminated with superfusion of Ringer's solution at rates 15–25 ml/min and reappeared after the superfusion was stopped.

The walking-like activity induced by glutamate superfusion was completely terminated by strychnine application either as a boluses or by continuous superfusion at 1–2 μM (Figure 3D). In comparison with NMDA-induced rhythm, even prolonged washout (> 9 hours) did not restore the ability to produce rhythmic activity with glutamate superfusion.

Effects of DMSO on walking-like activity

Dose-dependent effects of DMSO on the walking-like activity were observed. The frequency and amplitude of the locomotor-like activity induced by NMDA were not significantly affected by DMSO at concentrations up to 8% (v/v) (Figure 4A, B). The activity was depressed in both frequency and amplitude when DMSO concentration reached 12%. In contrast, the locomotor-like activity induced by glutamate was more sensitive to DMSO. The frequency and amplitude were depressed when the concentrations of DMSO were as low as 2%. The glutamate induced locomotor-like activity was completely blocked at DMSO concentration of 8% while the NMDA induced pattern was depressed, but not blocked, at 12% of DMSO. In higher concentrations (> 14%), DMSO produced complete block in the first seconds of superfusion. The inhibitory effects of DMSO were reversible with washout (data not shown). However, it took 6 hours of washout before locomotor-like activity could be evoked in 4 of 5 preparations and higher concentrations of NMDA or glutamate were required. In contrast to the locomotor-like activity, the amplitude of the dorsal root reflex was not affected significantly by DMSO at concentrations up to 12% ($p < 0.05$; n=8) (Figure 4A). In a few preparations (3/8), an excitatory effect of DMSO was observed as the frequency and amplitude of the walking-like activity were increased when 1–2% of DMSO was applied. On average, however, this effect did not reach statistical significance.

DISCUSSION

The *in vitro* spinal cord-forelimb preparation from the mudpuppy allows neuropharmacological and electrophysiological studies of locomotion in a mature spinal cord. (Wheatley et al., 1992, 1994; Jovanovic et al., 1996; Shik 1997; Jovanovic et al., 1999; Cheng et al. 1998, 2002; Fok and Stein, 2002; Lavrov and Cheng 2004). Bath application of neuroactive agents (neurotransmitters and neuromodulators) was able to produce long-lasting and stable locomotor-like patterns. In this study, we systematically investigated the methodological factors that affect locomotor-like activity in the mudpuppy and identified parameters that are useful to optimize the experimental conditions for future studies.

Consistent with our earlier report (Lavrov and Cheng, 2004), the results of this study demonstrated that glutamate is only effective in inducing locomotor-like activity when it is applied by superfusion. In contrast to bolus application of NMDA, glutamate delivered as boluses failed to induce rhythmic walking-like activity regardless of the concentrations used. This difference may be accounted for, at least in part, by pharmacodynamics of drug-receptor interactions. The mode of applications may be less important for agents with high affinity, such as NMDA, to activate sufficient number of receptors. In contrast, bolus application may be not adequate for agents with low affinity, such as glutamate, to activate sufficient receptors. This is particularly relevant when rapid uptake of amino acid neurotransmitters takes place in the spinal cord, as suggested by experiments with amino acid uptake inhibitors in the lamprey

(Brodin and Grillner, 1985). Other potential disadvantages of bolus application include an unequal distribution of the neuroactive agent in the chamber, rapid desensitization of neurotransmitter receptors, and inability to determine the exact concentrations for the initiation and maintenance of locomotor-like activity.

The optimal superfusion rates for glutamate-induced walking-like activity are around 10– 20 ml/per minutes (Figure 1). At this range, the frequency of the walking-like activity was highest (0.8 Hz) and the duration of the activity upon termination of glutamate superfusion was longest (9–100 seconds) (Figure 1A). It is note worthy that this optimal superfusion rate was determined at a glutamate concentration of 0.5mM. Clearly, the frequency of the walking-like activity was dependent on the concentration within the range of 0.3–0.9 (Figure 1B). The optimal superfusion rate may shift slightly if a different concentration of glutamate is used. High doses of glutamate were able to induce fictive locomotion without fluid circulation in the lamprey (Brodin and Grillner, 1985), while superfusion rate is critical even with high concentrations of glutamate in the mudpuppy (Lavrov and Cheng, 2004).

In contrast to glutamate, bolus application of NMDA is more effective than NMDA superfusion in the induction of walking-like activity. The effective range of NMDA concentrations (20–300 μM) was much wider when applied as boluses, compared to application by superfusion (20–80 μM). Faster and more robust walking-like activity was induced when NMDA was applied as boluses at any given concentrations, compared to superfusion (Figure 1B). Superfusion of NMDA at concentrations greater than 60 μM depressed the locomotor-like activity. These data suggest that bolus application is a preferred means of inducing locomotor-like activity when NMDA is used.

NMDA receptor antagonist AP-5 produced dose-dependent inhibition of the initiation and maintenance of the locomotor-like activities (Figure 2). Notably, superfusion of AP-5 produced more profound inhibition of the frequency of walking-like activity induced by glutamate, compared to bolus application. This applies to both the initiation phase and the maintenance phase of the walking-like activity. The alternating pattern and the amplitude of the activity were not significantly changed by either mode of AP-5 application.

Interestingly, superfusion of glycine receptor antagonist strychnine produced complete and irreversible block of the walking-like activity, while bolus application changed the alternating pattern to a slow and synchronized pattern (Figure 3). The inhibitory effects of strychnine applied as boluses have been studied *in vitro* preparations from neonatal rats (Cowley and Schmidt, 1995;McClellan, 1996;Kremer and Lev-Tov 1997) and mudpuppies (Jovanovic et al., 1999). A consistent finding is a change from a reciprocal to a synchronized pattern between the flexor and extensor muscles. The present data showed that application of strychnine by superfusion can not reproduce the results of bolus application, regardless the concentrations employed. This result was not changed whether the walking-like activity was induced by NMDA or by glutamate.

We demonstrated for the first time that DMSO has a profound impact on the activity of neural networks for locomotion. DMSO caused dose-dependent inhibition of the walking-like activity both in frequency and amplitude (Figure 4). The activity was blocked at concentrations above 8% (v/v). The inhibitory effects were reversible with adequate washout. The block can not be explained by non-specific toxic effects of DMSO as the dorsal root reflexes were not blocked at concentrations up to 12% (v/v). A further observation is that the walking-like activity induced by glutamate was more sensitive to the inhibitory effects of DMSO, compared to that induced by NMDA. The mechanisms of DMSO action on the nervous system may be related to its effects on cell membrane ion channels and neurotransmitter receptors. It has been shown that the repolarizing phase of the action potentials is prolonged by 8% DMSO as it blocks the active

increase in potassium permeability (Sawada and Sato, 1975). This change suppresses the high frequency discharge, because the refractory period of each spike is increased. In concentrations less than 20%, DMSO may make neurons more excitable by depolarizing resting membrane potentials through inhibiting neuronal membrane permeability to potassium and chloride ions channels (Jourdon et al., 1986). DMSO in concentrations less than 1% may facilitate cholinergic transmission via blocking cholinesterase activity (Sawada and Sato, 1975). It may also produce dose-dependent inhibition of GABA-induced inward current at concentrations of 0.3–3% (Nakahiro et al., 1992). Both inhibitory and facilitate effects have been observed at concentrations of 1–10% (McLarnon, 1986;Sawada and Sato, 1975). In cholinergic synapses, excitatory transmission is more susceptible to DMSO than inhibitory transmission. This is because the activity of the excitatory receptor is blocked more readily than that of the inhibitory receptor. The depressing effects of DMSO are not specific to the cholinergic system and the activities of GABA and glutamate receptors are similarly depressed (Sawada and Sato, 1975). Our data suggest that the rhythmicity of the locomotor-like behavior is largely uninterrupted when the concentration of DMSO is below 4% and that the amplitude is essentially stable when the concentration of DMSO is below 2% in the mudpuppy. The walking-like activity induced NMDA is more resistant to the effects of DMSO than that induced by glutamate.

Taken together, this study demonstrates that way by which neuroactive agents are delivered may have a profound impact on the results of *in vitro* neurophysiological experiments. This principle applies to both excitatory and inhibitory neurotransmitters and their antagonists. The vehicle for dissolving neuroactive agents, DMSO, can also have significant effects on the activities of neural networks for locomotion. It is therefore essential to take into account of these factors when interpreting data of this kind of experiments.

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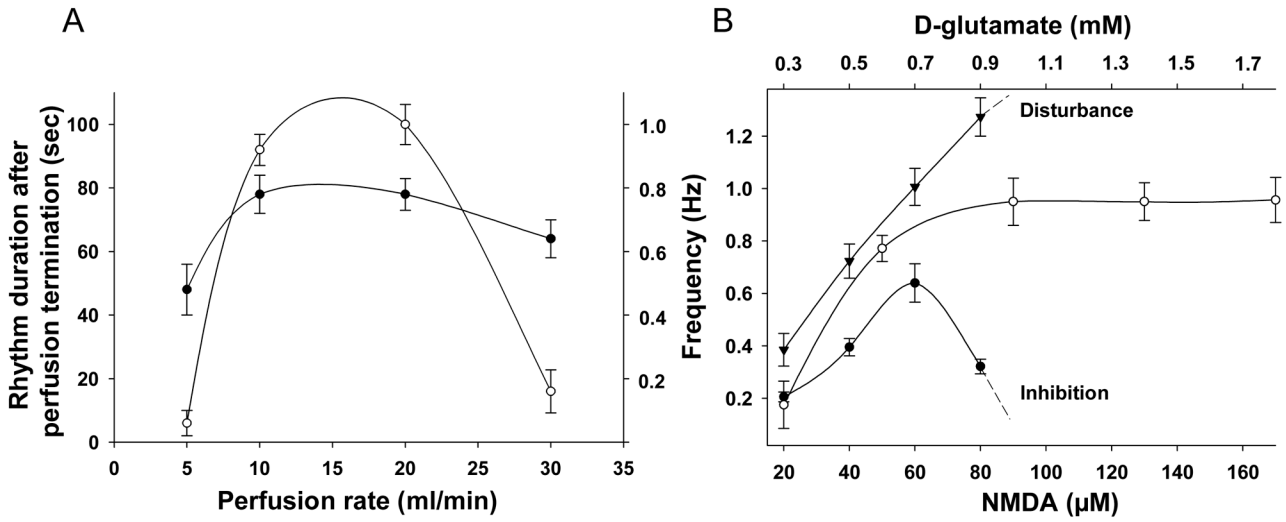

Figure 1. Optimal superfusion flow rate and comparisons of delivery modes

A: Frequency (●) and duration (○) of walking-like activity were dependent on the superfusion flow rate of D-glutamate (0.5 mM) (mean ± SEM, n=5). The optimal range of superfusion rate was between 10 and 20 ml/min. In this range, peak frequency (0.8 Hz) was achieved and the du-ration of the activity upon termination of the superfusion was longest (90–100 seconds). ***B***: Effects of mode of delivery on the walking-like activity (mean ± SEM, n=12). Superfusion of D-glutamate (▼) induced stable walking-like activity, the frequency of which was dose-dependent in the range of 0.3–0.9 mM. Beyond 0.9 mM, superfusion of glutamate caused tonic activity of flexor and extensor muscles. Superfusion of NMDA (●) induced walking-like activity in a small effective range of concentrations (40–80 μM), with the highest frequency of 0.6 Hz at a concentration of 60μM. Beyond 80 μM, superfusion of NMDA induced tonic activities. Bolus application of NMDA (○) was effective in a much wider range of concentrations (50–300 μM). The peak frequency was higher than that induced by superfusion.

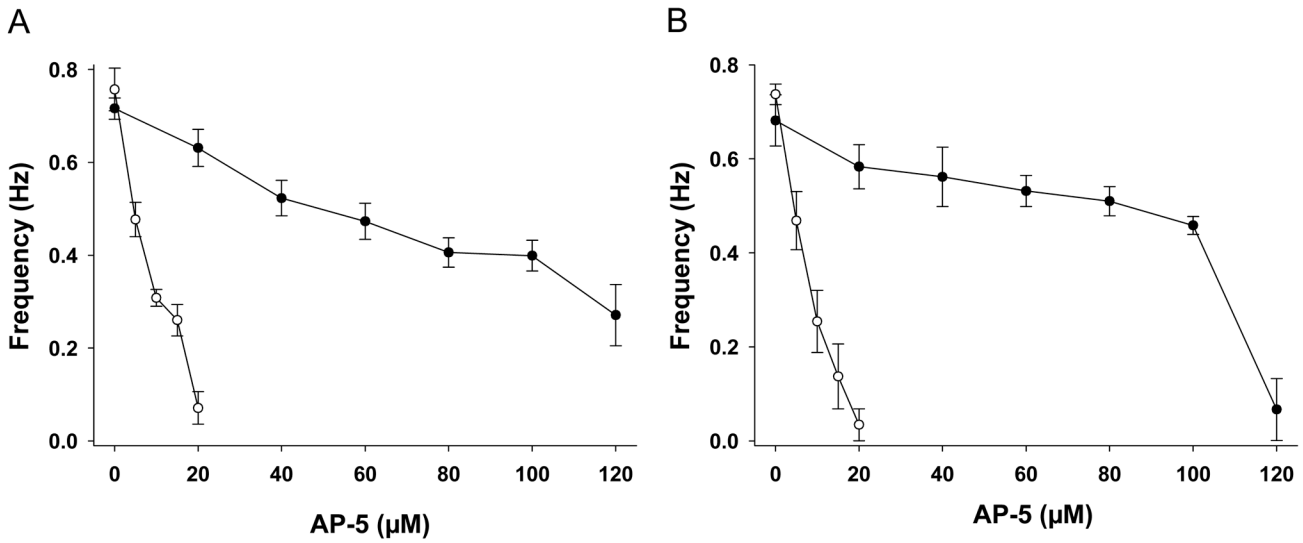

Figure 2. Mode of application of AP-5 affects the efficacy of its inhibitory effects

A. Compared to bolus application (●), continuous superfusion (○) of AP-5 was more effective in inhibiting the ongoing walking-like activity induced glutamate (mean ± SEM, n=7).

B: Continuous superfusion of AP-5 (○) was similarly more effective in inhibiting the initiation of walking-like activity by glutamate, compared to bolus application (●) (mean ± SEM, n=7).

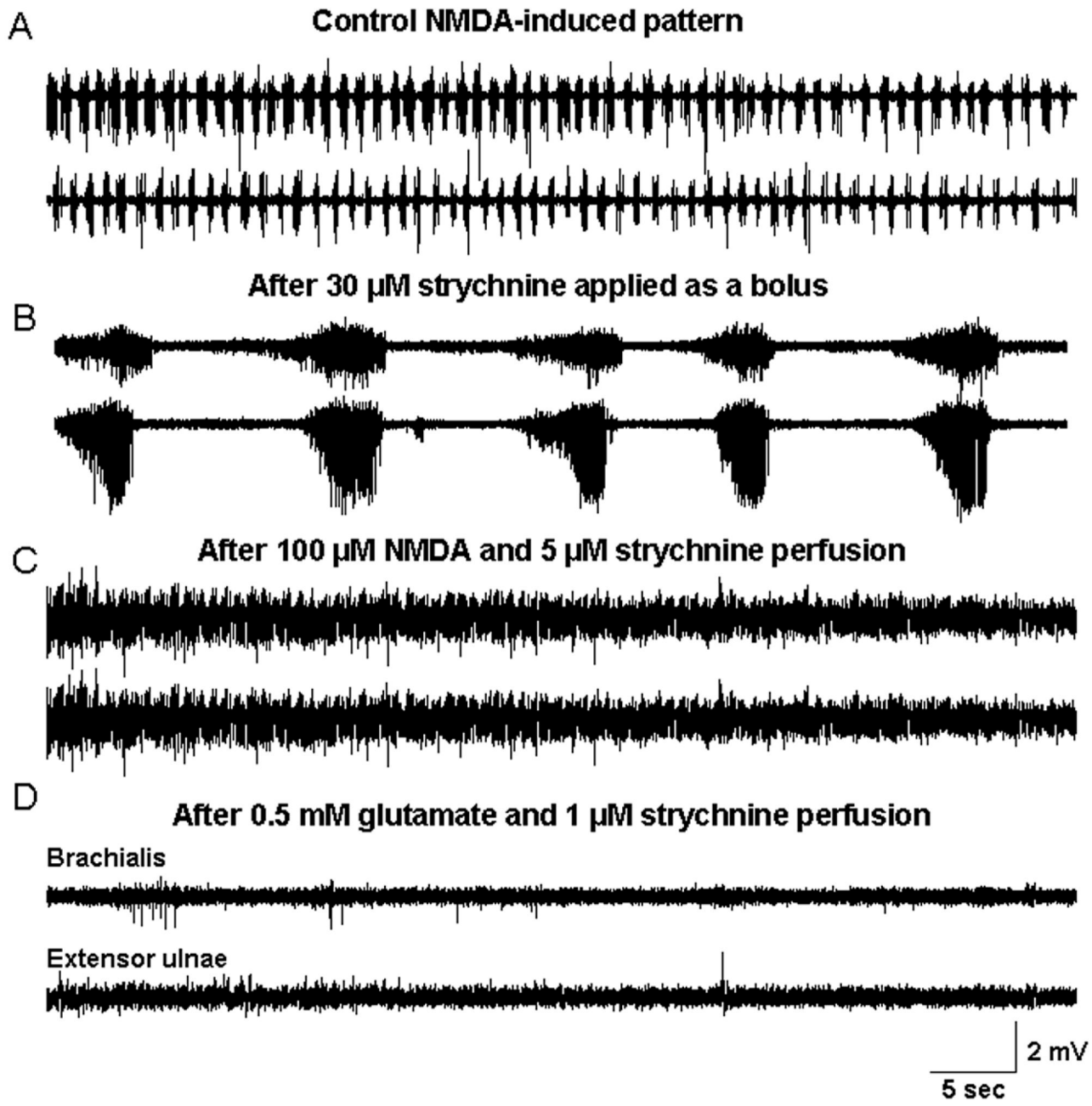

Figure 3. Inhibitory effects of strychnine on walking-like activity

Bolus application of strychnine changed the rhythmic, alternating the pattern of walking-like activity (A) to a slow and synchronized pattern (B). In contrast, superfusion of strychnine blocked completely the walking-like activity induced by NMDA (C) or glutamate (D).

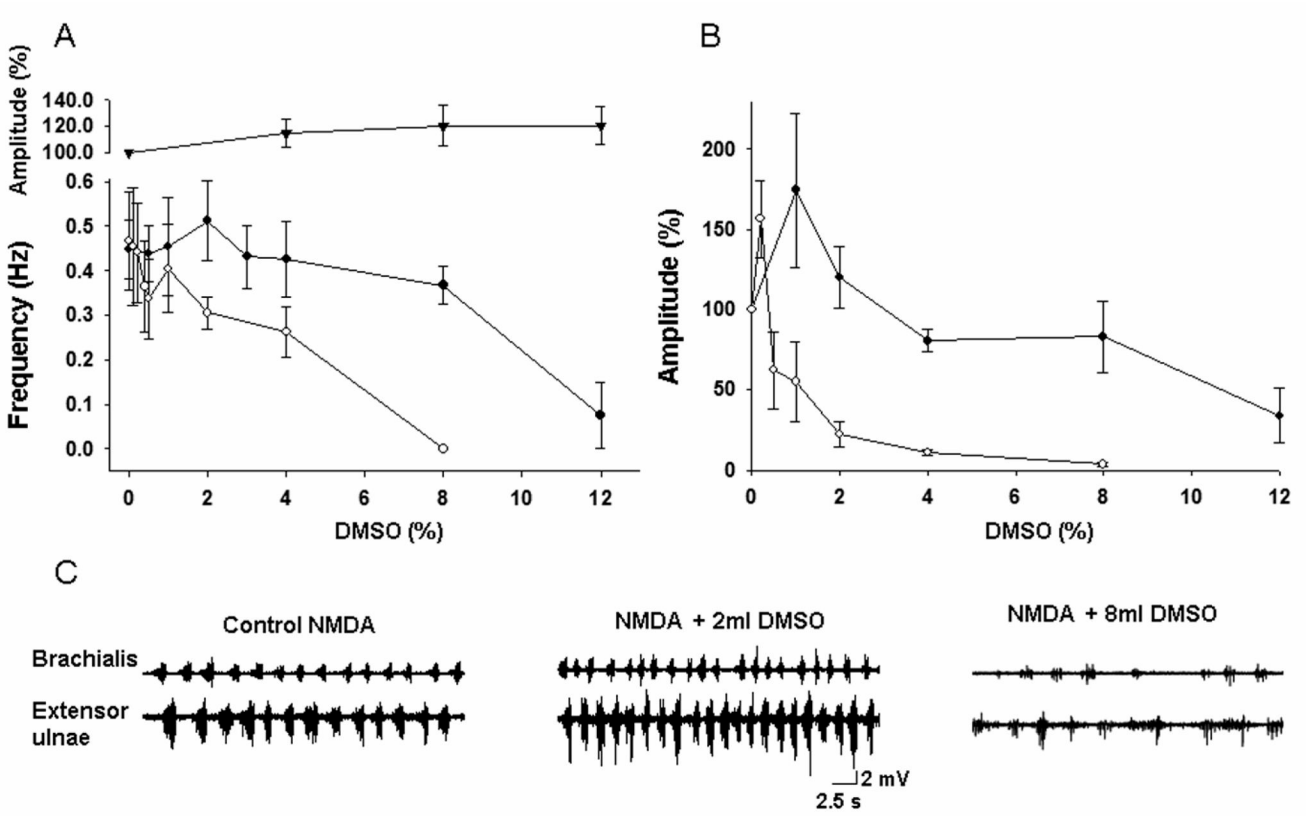

Figure 4. Effects of DMSO on locomotor-like activity

A: The mean frequency of the walking-like activity was decreased dose-dependently with superfusion of DMSO (mean ± SEM, n=8). The activity induced by glutamate (○) was more sensitive to the inhibitory effects of DMSO than that induced by NMDA (●). In contrast, the amplitudes of the dorsal root reflexes were not significantly affected by DMSO (top curve, mean ± SEM, n=8).

B: The mean amplitude of the walking-like active was more profoundly depressed in a dose-dependent manner. Again, the activity induced by glutamate was more sensitive to the inhibition compared to that induced by NMDA (mean ± SEM, n=8).

C: Examples of dose-dependent inhibition of the walking-like activity by DMSO. Note that depression of the amplitude was more profound than the inhibition of the frequency, consistent with the pooled data shown in panels A and B.