Original Article

Role of NMDA receptor activation in serotonin agonist-induced air-stepping in paraplegic mice

PA Guertin*,¹

¹Department of Anatomy and Physiology, Research Centre of the Laval University Medical center, Laval University, Quebec, Canada

Study design: Experimental laboratory investigation of the effects of serotonergic and glutamatergic drugs in early paraplegic mice.

Objectives: To examine whether NMDA and 5-HT receptors synergistically participate to generate basic stepping movements in paraplegic mice.

Setting: Laval University Medical Center, Quebec, Canada.

Methods: Adult mice completely spinalized at the low-thoracic level 1 week earlier were suspended in harnesses for experiments. Acute drug-induced effects were examined on hindlimb movements filmed with a digital video camera. Detailed kinematic analyses included stick diagrams reconstructions of hindlimb movements and analysis of bilateral coordination, angular excursion, stepping amplitude and frequency.

Results: A single treatment with the 5-HT₂ agonist quipazine (>0.7 mg/kg, i.p.) induced episodes of air-stepping movements in the hindlimbs of paraplegic mice. In contrast, injection of the glutamatergic agonist NMDA (1–45 mg/kg i.p.) failed to induce rhythmicity, although nonlocomotor rhythmic movements were observed with higher doses (45–60 mg/kg i.p.). Subthreshold doses of NMDA (22–30 mg/kg) could induce episodes of hindlimb air-stepping if combined with subthreshold doses of quipazine (0.3–0.7 mg/kg). Air-stepping was entirely blocked by administration of the selective NMDA antagonist MK-801.

Conclusion: A single treatment with quipazine can trigger episodes of locomotor-like movements in early chronic spinal mice. Even though NMDA alone could not generate bilaterally coordinated air-stepping, NMDA receptor activation was nonetheless critical for spinal locomotor rhythmogenesis induced by 5-HT agonists in awake behaving animals.

Spinal Cord (2004) 42, 185–190. doi:10.1038/sj.sc.3101580; Published online 3 February 2004

Keywords: locomotion; rehabilitation; spinal cord injury

Introduction

Specific pharmacological treatments can help restore basic locomotor functions after spinal cord injury presumably via an activation of the central pattern generator located in the lumbar spinal cord.¹ For instance, 5-HT applied intrathecally or 5-HT agonists such as quipazine or 2,5-Dimethoxy-4-iodoamphetamine (DOI) delivered intraperitoneally (i.p.) can trigger episodes of basic locomotor movements in chronic spinal rats² and rat pups.³ In spinal cats, NMDA has been shown not to trigger stepping movements but to improve spontaneous locomotor movements partially restored on treadmill in chronic animals.^{4,5} In contrast, NMDA was reported to steadily induce fictive locomotion in curarized decerebrate or spinal cats.⁶ In *in vitro* isolated spinal cord

preparations, NMDA can steadily induce rhythmic locomotor-like activity.⁷ However, stable and well-organized fictive locomotor rhythms are best induced when NMDA and 5-HT receptor agonists are combined at least in *in vitro* preparations obtained from neonatal rats^{3,8,9} and mice.^{10–12} Based on results reported *in vitro* and because no studies have tested this combination of drugs *in vivo*, the aim of the present study was to examine whether NMDA and 5-HT receptors synergistically interact also *in vivo* to generate basic spinal stepping movements in awake behaving paraplegic mice. Part of this work has been published in abstract form.¹³

Methods

Subjects and surgical procedures

Surgical procedures A total of 119 ($n = 119$) male CD1 mice initially weighing 33–37 g were used for this study.

*Correspondence: PA Guertin, Research Centre of the Laval University Medical Center, 2705 Bld Laurier, RC-9800, Quebec City, (Quebec), Canada G1V4G2

Complete spinalization between the 9th and 10th thoracic vertebrae was performed under general anesthesia with isoflurane (2.5%). Spinalization was done intervertebrally, without a dorsal laminectomy, to minimize damage of the vertebral structure and bleeding. To ensure that complete transection was achieved, the inner-vertebral walls were explored and entirely scraped with scissor tips in order to disrupt any small fibers that had not been severed. The opened skin area was sutured and animals were placed for few hours on heated pads. Mice typically received postoperative care for 3 days that included injection of lactate-Ringer's solution (2 ml/day, s.c.), an analgesic (buprenorphine 0.2 mg/kg/day, s.c.) and an antibiotic (Baytril 50 mg/kg/day, s.c.). Bladders were emptied manually twice daily and animals were housed in groups of five per cage with food and water *ad libitum*. Complete spinalization was confirmed by (1) initial full paralysis of the hindlimbs, (2) postmortem examination of the spinal cord lesion, and (3) transverse or midsagittal spinal cord sections stained with luxol fast blue/cresyl violet for myelinated axons and for Nissl substance, respectively. Sections were analyzed microscopically for evidence of tissue sparing. Since previous observations from our laboratory have revealed that, in some cases (5–10%), mice progressively lose all signs of sublesional spinal cord viability/functionality few days after spinalization, flexion reflexes were tested in the hindlimbs by pinching the toes 1 week after surgery. Only results from complete spinal animals confirmed histologically and tested positive for flexion reflexes were kept for further experimental observations. Mice were tested at 6–7 days postspinalization to better distinguish drug-induced effects from other types of hindlimb movements (spasms, jerks, etc.) that start occurring spontaneously after 1–2 weeks postspinalization in this animal model (unpublished observations). Experimental procedures were in accordance with the Canadian Council for Animal Care guidelines and accepted by the Laval University Research Hospital Animal Care and Use Committee.

Experimental protocol

Mice received an injection of quipazine (0.2–7.5 mg/kg i.p., Tocris, Ellisville, MO, USA), NMDA (1–60 mg/kg, i.p., Tocris, Ellisville, MO, USA) or both. This was carried out 6–7 days postspinalization to (1) allow full recovery from surgery, (2) to precede the onset of spontaneous reflex hindlimb movements that begins 1–2 weeks postspinalization (unpublished). Hindlimb movements were monitored immediately before and several times 30 min after drug injection. The effects of subsequent injection of MK-801 (4–8 mg/kg, i.p., Sigma, Oakville, Ontario), cyproheptadine hydrochloride (4–8 mg/kg, i.p., Sigma, Oakville, Ontario), or vehicle (1 ml lactate-Ringer's) were also examined. All drugs were diluted in lactate-Ringer's solution. To specifically examine drug-induced effects on the CPG, pinching the tail (which can also activate the CPG *in vivo*)³ or

other body parts was not performed. Movements were examined in air-stepping conditions rather than on treadmill to (1) facilitate detection of bilateral movements that would otherwise be difficult to analyze in mice, and (2) eliminate overground constraints to maximize detection of even small drug-induced effects on the CPG.

Data acquisition and analysis

Movements were monitored with a digital video camera (3Com, HomeConnect). Marks put on the iliac crest, femoral head, knee joint, lateral malleolus, and the toes of the left hindlimb were used for digitization (30 frame/sec) and movement reconstruction. Stick diagrams allowed measurements of joint angular excursion, and the excursion amplitude is defined as the difference between the min/max joint angle reached at each cycle. Direction and amplitude of foot displacement were measured and plotted for both feet to examine bilateral coordination. Angular excursion and foot displacement graphs were made with Excel 2000 (Microsoft, WA, USA). Air-stepping was defined as repeated cycles constituted of a 'swing' phase (flexion) and a 'stance' phase (extension) produced alternatively in both hindlimbs. Average step frequency was based on total number of step cycles displayed during a 30-s bout of air-stepping. Statistical analyses (SPSS BI, Chicago, IL, USA) were performed by means of Student's *t*-tests or one-way ANOVA.

Results

Induction of air-stepping by quipazine in adult spinal mice

One of the main results of the present study is that a single injection of quipazine could acutely induce hindlimb air-stepping for 1–2 h in adult paraplegic animals. With doses ranging from 1–2 mg/kg, air-stepping movements were found in 58% of the mice tested (Figure 1h). A typical example is illustrated in Figure 1 where air-stepping movements occurred spontaneously 25 min after injection of 1.2 mg/kg of quipazine in a mouse spinalized 6 days earlier. Stick diagrams of the left hindlimb shows rhythmic alternating flexions and extensions after quipazine injection (Figure 1b). Joint angles at the hip, knee, and ankle levels (Figure 1e) show rhythmic movements involving all three joints although greater effects were found at the ankle level (Figures 1d, 2h). In this case, the amplitude of joint angular excursion was 13 ± 6 , 17 ± 4 and $33 \pm 8^\circ$ (4 cycles) at the hip, knee, and ankle levels, respectively (Figure 1d). On average ($n=10$), similar values were found for stepping episodes induced in other mice by 0.8–1.5 mg/kg of quipazine (Figure 2h). These effects (Figure 1b, d) are in clear contrast with the lack of movement observed immediately prior to quipazine injection (Figure 1a, c).

Air-stepping induced by quipazine was characterized by coordinated alternating movements in both hindlimbs. Bilateral coordination was examined by analyzing

Figure 1 Induction of air-stepping with quipazine. Before (a, c) and after injection of quipazine (1.2 mg/kg) induced four consecutive cycles (b) characterized by angular changes mainly at the knee and the ankle (d) and bilaterally alternating foot displacement (g). Markers were put on the iliac crest, hip, knee, ankle, and toes (e). Foot displacement calculated from most extended (a) and flexed (a') positions of the hindpaw (f). Time between sticks in (a) and (b) is 0.1 s. Same time scale and direction for (a–d, g). Number of step cycles calculated in 30 s and reported in Hz (i), accompanied by the number of mice (incidence expressed in %) in which air-stepping activity was detected (h). Doses are in mg/kg

the displacement of both feet as shown in Figure 1f. In this mouse, displacement amplitude of 8 ± 4 mm was found for both feet on average (Figure 1g). Movements were bilaterally coordinated so that when one foot was moving up/forward, the other foot was moving down/backward (Fig. 1g). At these doses, average air-stepping frequency was 0.22 ± 0.10 Hz ($n = 10$, Figure 1i). While similar step frequencies were found at higher doses (Figure 1i), the incidence (ie, number of animals) of induced air-stepping progressively decreased with higher doses of quipazine (Figure 1h). This dose-dependent decrease in efficacy coincided with an increase occurrence of side effects constituted of noncoordinated large amplitude movements, spasms, twitches, toe spanning, and fast-paw shaking. These movements were clearly not locomotor like and were not counted as such as they lacked bilateral coordination. They were typically unilateral although sometimes evoked in both hindlimbs simultaneously. Doses of 0.7 mg/kg or less were considered subthreshold since no air-stepping episodes were induced in all cases tested ($n = 24$, Figure 2i, Table 1). At these subthreshold doses, however,

hindlimbs usually adopted a more flexed position (Figure 2e) compared to predrug (Figure 2d). It is worth mentioning that subthreshold doses of quipazine can induce spinal locomotion in other experimental conditions if combined with intense afferent inputs such as tail pinching² or if given daily in chronic spinal animals.¹⁴

Nonlocomotor movements induced by NMDA

Administration of NMDA alone (ie, without quipazine) at doses ranging from 1 to 45 mg/kg never induced air-stepping activity ($n = 10$, Figure 2i, Table 1). However, at doses above 45 mg/kg, large nonlocomotor movements were occasionally observed ($n = 2/6$). These movements mostly consisted of large amplitude kicks occurring in one hindlimb or in both simultaneously. In Figure 2, no movements could be detected after injection of 24 mg/kg of NMDA (Figure 2a). An additional injection of 24 mg/kg of NMDA induced repeated flexions (Figure 2b), which occasionally were seen in both hindlimbs at the same time (arrow, Figure 2c).

Synergistic effects induced by subthreshold doses of quipazine and NMDA

Synergistic effects were observed by combining subthreshold doses of NMDA (< 45 mg/kg) with subthreshold doses of quipazine (≤ 0.7 mg/kg). NMDA ranging from 22 to 30 mg/kg combined with 0.3–0.7 mg/kg of quipazine triggered spontaneous episodes of air-stepping in 50% of the cases ($n = 5/10$, Table 1). In Fig. 2, 0.7 mg/kg of quipazine alone produced no detectable hindlimb movements (Figure 2e). However, given in combination with subthreshold doses of NMDA (30 mg/kg, Fig. 2i), it induced air-stepping movements (Figure 2f) with bilaterally alternating foot displacement (Figure 2g). Each increased displacement (flexion) of the left foot (black trace) coincided with a decreased displacement (extension) of the right foot (gray trace) for each of the two cycles displayed in 10 s (Figure 2g). Rhythmicity induced by quipazine/NMDA (Figure 2f, g) was slower than that induced by suprathreshold doses of quipazine (Figure 1b, d, g). In fact, average air-stepping frequency induced by NMDA/quipazine was significantly ($P < 0.01$, Student's *t*) slower than that induced by just above threshold doses of quipazine (0.8–1.5 mg/kg, Figure 2i). Movement amplitudes, however, were not dissimilar since angular excursion amplitudes at the hip, knee, and ankle levels for quipazine/NMDA closely resembled those found, respectively, with 0.8–1.5 mg/kg of quipazine (Figure 2h).

Role of endogenous glutamate release in quipazine-induced rhythmicity

MK-801, a selective noncompetitive NMDA receptor antagonist, was used to further examine the involvement of the glutamatergic system in spinal locomotion. Injected alone, MK-801 (4–8 mg/kg, i.p.) did not induce

Table 1 Effects induced by serotonergic and glutamatergic drugs on hindlimb movements in paraplegic mice

Drugs	Doses (mg/kg)	Rhythmic movements	Frequency (Hz)	Bilateral coordination	Comments
Quipazine	< 1.0	2/26	0.1–0.2	Alternated	≤0.7 mg/kg never induced air-stepping Induced spontaneous air-stepping. Blocked by 4–8 mg/kg MK-801 (5/5), and by cyproheptadine (4/4) but unchanged in vehicle-treated mice (4/4)
	1.0–3.0	20/36	0.1–0.5	Alternated	
NMDA	≥3.0	11/26	0.1–0.6	Alternated	Induced air-stepping but also spasms, fast paw-shaking, twitches, toe spanning Doses <45 mg/kg never induced any sort of hindlimb movements Rhythmic movements but nonlocomotor (i.e. no bilateral alternation) and very fast large amplitude movements and kicks
	1–45	0/10	—	—	
Quipazine + NMDA	>45	2/6	>0.4	Synchronous	In 5/10, 22–30 mg/kg of NMDA induced air-stepping when added to subthreshold doses of quipazine (≤0.7 mg/kg) In only 1/21 did NMDA <20 mg/kg induced air-stepping when added to subthreshold doses of quipazine (<0.8 mg/kg)
	0.3–0.7 + 22–30	5/10	0.02–0.1	Alternated	
Quipazine + NMDA	0.3–0.7 + 1–20	1/21	0.2	Alternated	
MK-801	4–8	0/7	—	—	No hindlimb movements
Saline	1 ml	0/8	—	—	No hindlimb movements

Figure 2 Effects of NMDA with or without quipazine on induction of air-stepping. A measure of 24 mg/kg of NMDA (subthreshold) did not induce hindlimb movement (a), while 48 mg/kg induced disorganized movements (b) that were mostly unilateral (c) but occasionally bilaterally simultaneous (arrow). Before (d) and after subthreshold quipazine (0.7 mg/kg, e). Addition of NMDA (30 mg/kg) induced slow rhythmic bilaterally alternating air-stepping (f, g). Angular excursion amplitudes induced by quipazine (black) and quipazine-NMDA (white h, i). Same time scale and direction for (a–g). All doses are in mg/kg

Figure 3 Effects of NMDA or 5-HT₂ antagonists on quipazine-induced air-stepping. Quipazine air-stepping was abolished by 4–8 mg/kg of MK-801, a noncompetitive NMDA antagonist (a, *n* = 5) or by 4 mg/kg of cyproheptadine, a nonselective 5-HT₂ antagonist (b, *n* = 4), but not by saline (c, *n* = 4)

air-stepping or perturb in any way the immobilized and pendant hindlimbs of all paraplegic mice tested (*n* = 7). However, when administered 30 min after quipazine, similar doses of MK-801 completely abolished the quipazine-induced air-stepping (*n* = 5/5, see Figure 3a, Table 1). In other mice, similar blocking effects were observed following administration of cyproheptadine (4 mg/kg, i.p.), a nonselective 5-HT₂ antagonist (*n* = 4/4, Figure 3b). In contrast, mice that instead received corresponding volumes (1 ml) of lactate-Ringer's solution, continued to display air-stepping movements (*n* = 4/4, vehicle-treated Figure 3c). Note that lactate-

Ringer's solution alone never induced hindlimb movements, and that movements induced by quipazine/NMDA or quipazine lasted for 1–2 h (ie, although not systematically examined).

Discussion

The present study shows that the 5-HT₂ agonist quipazine can acutely induce air-stepping movements in the hindlimbs of adult paraplegic mice. This nicely complements results obtained in other species where 5-HT or 5-HT₂ agonists injected daily have been reported to increase treadmill locomotor recovery in adult spinal rats^{2,14} and in spinal rats with transplanted 5-HT neurons,¹⁵ and to increase weight-support in chronic spinal cats.¹⁶ Most recently, another 5-HT₂ agonist DOI was shown to initiate air-stepping in neonatal rats spinalized 1 week earlier.³ The present results further extend these previous observations by showing that (1) locomotor movements can be induced by a single injection of quipazine in adult untrained and nonstimulated animals (eg, no treadmill training, no tail-pinching, no grafts) demonstrating the potency of quipazine treatment alone to activate CPG neurons; (2) doses of 1–2 mg/kg of quipazine are optimal for initiation of air-stepping movements in these conditions; (3) high doses of quipazine induce deleterious side effects on spinal locomotion (spasms, twitches, toe spanning, paw-shaking) as has been reported in intact animals.¹⁷

Another important finding in this study is the failure of NMDA used separately to induce bilaterally alternating locomotor movements. Higher doses of NMDA did induce rhythmic movements in the hindlimbs, but usually without bilateral coordination and never with alternation of the hindlimbs. Similar effects have been found in other preparations where NMDA was reported to typically trigger fast-paw shaking in noncurarized decerebrate or spinal cats⁴ and unilateral rhythms in spinal marmoset monkeys.¹⁸ NMDA was also found to induce fast-paw shaking in early spinal cats and to improve spontaneous locomotor performances partially restored already by daily treadmill training in more chronic spinal cats.⁶ On the other hand, NMDA was clearly shown to induce fictive locomotion in curarized decerebrate cats.⁶ Altogether, this suggests that effects of NMDA on locomotor genesis is best achieved in curarized preparations for unclear reasons and/or in decerebrate animals⁶ perhaps due to spontaneous monoamine release in the spinal cord from supraspinal monoaminergic descending fibers. It also suggests that NMDA alone cannot trigger *per se* locomotor rhythms *in vivo* and may require the use of other drugs to properly activate the CPG at least in noncurarized acute or early spinal animals.

The present study reports for the first time *in vivo* synergistic effects from NMDA and 5-HT receptor agonists on locomotor rhythmogenesis. These results are supported by earlier observation from *in vitro* isolated spinal cord preparations. Indeed, although NMDA can trigger rhythmic ventral root activity in *in vitro*

preparations, stable and well-coordinated locomotor-like patterns are known to be best induced by combining NMDA and 5-HT in the neonatal rat^{8,9,19} and mice preparations.^{10,11,20} 5-HT₂ agonists have also been shown to be involved in pattern generation since reversal of disorganized NMDA-induced rhythms was found in *in vitro* preparations obtained from early spinal neonatal rats.³ Furthermore, appearance of NMDA-induced fictive locomotion is delayed in MAOA-deficient mice, suggesting an important role for 5-HT in spinal network maturation and pattern formation.²¹ The mechanism underlying the synergistic action of NMDA and quipazine or 5-HT remains unclear. However, fictive locomotion induced *in vitro* by 5-HT/NMDA was shown to depend upon an interplay between 5-HT and NMDA receptor actions and functions^{22,23} involving modulation of I_h and I_{kir} currents and induction of 'pacemaker' cell properties.²⁴

Results obtained in this study from blocking NMDA receptor activation with MK-801 provide additional details regarding the mechanism underlying spinal locomotor rhythmogenesis. Complete abolition by MK-801 of quipazine-induced air-stepping provides evidence of a role for endogenous glutamate release in spinal locomotion. This is supported by immunohistological evidence of glutamatergic CPG candidate neurons²⁵ and by results with microdialysis showing an important increase of glutamate concentration in the lumbar area of normal or complete spinal rats during stepping.²⁶ These results are in apparent contrast with those observed in the neonatal mouse-isolated spinal cord showing that AP5 or MK-801 do not abolish but only reduce fictive locomotion induced by dopamine and 5-HT.¹⁰ However, nearly complete blocking effects of MK-801 have been reported on slow locomotor rhythms induced by bicuculline.¹² *In vivo*, MK-801 and AP5 can interfere with L-DOPA-induced fictive locomotion in curarized spinal rabbits²⁷ and block MLR-induced fictive activity in decerebrate curarized cats.⁶ It appears from these results that the detailed role of NMDA receptor-channel activation in rhythmogenesis may depend on several factors such as (1) the drug or combination of drugs used to activate the CPG, (2) the intensity (ie, strength and speed) of locomotor activity, (3) the experimental condition (*in vitro* isolated vs *in vivo* preparations). Altogether, the present results strongly support the idea of an important synergistic interactions between spinal 5-HT and NMDA receptors in locomotor rhythmogenesis *in vivo* and indicate that small doses of 5-HT₂ agonists could constitute valuable therapeutic tools to enhance locomotor recovery soon after extensive spinal cord injury (see also Giroux *et al.*).⁵

Acknowledgements

This study was supported by the Andre-Senecal Foundation (Fondation pour la Recherche sur la Moelle Épineuse). I thank Drs M-C Perreault and M Tresch for comments on the manuscript.

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