Effects of Intrathecal Glutamatergic Drugs on Locomotion. II. NMDA and AP-5 in Intact and Late Spinal Cats

Nathalie Giroux, Connie Chau, Hugues Barbeau, Tomás A. Reader,* and Serge Rossignol
Centre de Recherche en Sciences Neurologiques, Faculté de Médecine, Université de Montréal, Montreal, Quebec H3C 3J7, Canada

Submitted 4 September 2002; accepted in final form 8 May 2003

Effects of intrathecal glutamatergic drugs on locomotion. II. NMDA and AP-5 in intact and late spinal cats. J Neurophysiol 90: 1027–1045, 2003; 10.1152/jn.00758.2002. In a previous article, we have shown that, in cats, intrathecal injections of N-methyl-D-aspartate (NMDA) in the first few days after spinalization at T13 do not induce locomotion as in many other spinal preparations. This is in contrast to alpha-2 noradrenergic receptor stimulation, which can trigger locomotion at this early stage. However, it is known that spinal cats do recover spontaneous locomotion in the absence of descending noradrenergic pathways and that the spinal pattern generator must then depend on other neurotransmitters still present in the cord such as excitatory amino acids. In the present paper, therefore we look at the effects of intrathecal NMDA, a glutamatergic agonist, and 2-amino-5-phosphonovaleric acid (AP-5), an NMDA receptor blocker, in both intact and late spinal cats. Low doses of NMDA had no major effect on the locomotor pattern in both intact and late spinal cats. Larger doses of NMDA in the chronic spinal cat initially produced an increase in the general excitability followed by more regular locomotion. AP-5 in intact cats caused a decrease in the amplitude of the flexion reflex and induced a bilateral foot drag as well as some decrease in weight support but it did not prevent locomotion. However, in late spinal cats, the same dose of AP-5 blocked locomotion completely. These results indicate that NMDA receptors may be critical for the spontaneous expression of spinal locomotion. It is proposed that the basic locomotor rhythmicity in cats is NMDA-dependent and that normally this glutamatergic mechanism is modulated by other neurotransmitters, such as 5-HT and NA.

INTRODUCTION

Several studies have shown the importance of the noradrenergic system for locomotion (Rossignol 1996). For instance, L-dihydroxyphenylalanine (L-DOPA), the precursor of noradrenaline (NA), in the presence of the monoamine oxidase inhibitor, nialamide, was found to trigger “fictive” locomotion in immobilized acutely spinalized cats (Grillner and Zangger 1979). Alpha-2-noradrenergic agonists, such as clonidine, can initiate treadmill locomotion soon after spinalization (Forssberg and Grillner 1973) and can modulate the spinal locomotor pattern (Chau et al. 1998), which recovers spontaneously a few weeks after spinalization. However, recent studies in chronic spinal cats have demonstrated that activation of these receptors was not critical for the spontaneous expression of spinal locomotion because their blockade by the antagonist yohimbine does not impair spontaneous spinal locomotion (Giroux et al. 2001) and because spinal cats can recover hindlimb locomotion spontaneously in absence of NA pathways (Barbeau and Rossignol 1987) and without any pharmacological treatment (Bélanger et al. 1996). It seems, therefore that spontaneous spinal locomotion does not depend on the activation of the NA systems but rather on the activation of other transmitter systems, such as excitatory amino acids (EAAs), that are still present in the spinal cord after the spinalization.

EAAs have been studied for their capacity to trigger locomotion in several animal preparations including the lamprey (Cohen and Wallen 1980; Grillner et al. 1981; Poon 1980), amphibians (Dale and Roberts 1984, 1985), and turtles (Currie 1999). In the isolated chick spinal cord, application of the N-methyl-D-aspartate (NMDA) antagonist 2-amino-5-phosphonovaleric acid (AP-5) reduced or blocked both spontaneous and NMDA-induced locomotion (Barry and O’Donovan 1987).

In mammals, the importance of NMDA receptors in the induction of locomotor rhythm was also well documented in the neonatal rat (Cazalets et al. 1990; Kudo and Yamada 1987; MacLean et al. 1998; Smith and Feldman 1987) and confirmed in the rabbit after application of the noncompetitive NMDA antagonist MK801 (Fenaux et al. 1991). In decerebrate cats, it has been shown that intrathecal administration of NMDA-elicited hindlimb fictive locomotion similar to that evoked by the mesencephalic locomotor region (MLR) (Douglas et al. 1993). As shown in a previous paper (Chau et al. 2002), NMDA failed to initiate treadmill locomotion within the first few days (3–5 days) after spinalization. However, when the cat just started to express some rhythmic hindlimb movement (~7–8 days after spinalization), NMDA dramatically improved the locomotor pattern.

It is probable that locomotion in intact and spinal cats is controlled through different neurotransmitter mechanisms. Furthermore, it seems possible that basic mechanisms underlying locomotor rhythmicity in the spinal cat are NMDA-dependent and that the descending system such as NA and serotonin 5-HT (5HT) could modulate this fundamental mechanism. To test the hypothesis that glutamatergic mechanisms are important for spinal locomotion, we investigated the role of NMDA and NMDA antagonist, AP-5. These substances were

*Deceased 7 May 2002.

Address for reprint requests: S. Rossignol, Département de Physiologie, CRSN, Faculté de Médecine, Université de Montréal, C.P. 6128, Succursale Centre-Ville, Montréal, Québec H3C 3J7, Canada (E-mail: serge.rossignol@umontreal.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
injected intrathecally at the lumbar level and locomotion was documented; cutaneous reflexes were tested in the same cats, first while in the intact state and second, after a complete spinal cord transection at the Thoracic 13 (T13) level. Preliminary results have been published in abstract form (Chau et al. 1994; Giroux et al. 1999a).

**METHODS**

**General protocol**

This study was performed on five adult cats weighing 2.3–4 kg. Three cats were trained to walk at different speeds (0.2–0.8 m/s) on a motor-driven treadmill during a period of 3–4 wk. At the end of this initial training, they were chronically implanted with electromyographic (EMG) electrodes in the muscles of the hindlimbs (Bélanger et al. 1996) and with an intrathecal cannula (Chau et al. 1998). Nerve cuff electrodes were also placed on the superficial peroneal nerve of both hindlimbs to test the excitability of the flexion reflex in various conditions. Once baseline values for locomotion in the intact state were made, we started drug injection experiments that lasted for periods of 6 mo to 2 yr. The drugs were first injected in the intact state. Thereafter, the cats were spinalized at T13, and the hindlimbs were trained for several days to walk on the treadmill. When the cat recovered spinal locomotion, the same drugs were injected to allow a comparison of the effects of the drugs in the intact state versus in the spinal state. Figure 1 shows the schedule and dosage of NMDA and AP-5 injections during the intact and spinal states. Cat NG2 was kept for 658 days in the intact state and 158 days after spinalization, whereas cats NG3 and NG5 were kept for 263 and 127 days, respectively, as intact and for 114 and 70 days, respectively, after spinalization. Two other cats were added to this study and were only tested during the postspinalization period (Fig. 1, CC6 and CC7, kept as spinal, for 183 and 230 days, respectively).

**Implantation of cannula, EMG electrodes, and nerve cuffs**

The surgical procedure for implantation of EMGs, nerve cuff electrodes, and intrathecal cannulae were described elsewhere (Chau et al. 1998; Giroux et al. 2001). All surgeries were performed in aseptic conditions and under general anesthesia (isoflurane 1–3%). Experiments were approved by the Deontology Committee of Université de Montréal.

The intrathecal cannula (Telfon 24 WL tubing) was connected to an adaptor that was fixed to the skull using acrylic cement. The other extremity of the tubing was inserted through the atlanto-occipital ligament into the subarachnoid space down to lumbar 2–4 segments. The postmortem location of the tip of the cannula for cats NG2, NG3, and NG5 was described in some detail in the previous companion paper (Giroux et al. 2001). The tip of the cannula for cat NG2 was located dorsally between the L3 and L4 segments. For cat NG3, the tip terminated dorsally on the left side at the L2 segment and for cat NG5, it terminated dorsolaterally, on the right side, just below the L3 ventral root. For cats CC6 and CC7, the cannula terminated ventrolaterally on the right side, at L4 and L5 segments, respectively. To prevent blocking, the cannula was flushed, three to four times/week, with a bolus of 100 μl of saline solution (0.9%).

Two multipin head connectors (TRW Electronic Components Group, Elk Grove Village, IL) were used for connect to the implanted EMG electrodes. Fifteen Telfon-insulated stainless steel wires (AS633; Cooper Wire, Chatsworth, CA) were soldered to each connector and fixed to the skull, using acrylic cement. Pairs of wires were inserted subcutaneously and led to various muscles. The implanted muscle included: ilioptosis (Ip), sartorius anterior (St), semitendinosus (St), tibialis anterior (TA), vastus lateralis (VL), and gastrocnemius medialis (GM) and lateralis (GL). These muscles were implanted in both left (L) and right (R) hindlimbs, but only the left side of the cat facing the video camera was used for illustrations with the kinematics.

Bipolar nerve cuff electrodes, ~1 cm in length with 6 mm between electrodes leads, (Julien and Rossignol 1982) were implanted at the same time as EMG electrodes and used to stimulate the superficial peroneal nerve of both hindlimbs. The connecting leads were soldered to spare pins of the EMG connectors.

**Spinal cord transection and postoperative care**

At the end of the experimental series in the intact period, a laminectomy was performed at the T13 vertebra under general anesthesia. The dura was removed, the intrathecal cannula was localized, and xylocaine (2%) was applied topically before the cord was completely transected at T13, using micro scissors. Sterile absorbable hemostat (Surgicel, oxidized regenerated cellulose) was inserted at the lesion site.

After surgery, cats received appropriate postoperative analgesia (buprenorphine 0.0005–0.01 mg/kg) and other postoperative care. They were attended daily for manual bladder expression, general inspection, cleaning of hindquarters and to flush the cannula with saline when appropriate (Giroux et al. 2001).

**Drug administration**

The excitatory amino acid receptor agonist NMDA and antagonist AP-5, both from RBI, were dissolved in sterile physiological saline solution and administered in concentrations of 1–25 and 15–100 mM, respectively. A single bolus of 100 μl was injected in the cannula and another 100 μl volume of saline, a volume equivalent to the dead space of the cannula, was used to slowly push the drug out of the cannula.

**Recording and analysis procedures**

Recordings of locomotion were done in the intact state before any drug injections, while cats walked freely at different speeds (0.2–0.8 m/s) and tilted (15° up slope or 15° down slope) on a motorized treadmill. Cats were also trained to walk on a horizontal ladder with eight round rungs (3 cm diam) placed ~20 cm apart. This ladder walking, studied in the intact cat, was documented using video tape only. The slopes and ladder tasks were chosen to challenge the locomotor performance of intact cats after drug injections. All these recordings served as a baseline control (intact trials). However, for each drug injection trial, similar recordings were done before (predrug trial) and at different times after the drug injections (postdrug trial). To record spinal locomotion, the forelimbs were placed on a platform while the hindlimbs, separated by a Plexiglas separator to prevent crossing of the hindlimbs, walked on the treadmill belt. The EMG signals were amplified differentially (bandwidth of 100 Hz to 3 kHz) and recorded on a 14-channel tape recorder (Vetter Digital, model 4000A PCM recording adapter) with a frequency response of 1.2 kHz/channel. The EMG recordings were synchronized to the video images by a digital SMPTE (Society for Motion Picture and Television Engineers) time code. This time code was recorded both on the analog EMG tape and on the audio channel of the video tape as well as inserted into the video image itself. The EMG data during locomotion were played back on an electrostatic polygraph (Model ES 2000, Gould Instruments, Valley View, OH), and representative sections of the cat’s performance before and after drug application were selected for analysis. The EMG signals were digitized at 1 kHz.

Video images were captured using a digital camera (Panasonic 5100, shutter speed 1/500 to 1/1,000 s) and recorded on a video cassette recorder (Panasonic, AG 7300). Reflective markers (3M) were glued to the skin of the left hindlimb overlaying the iliac crest, femoral head, knee joint, lateral malleous, metatarso-phalangeal joint (MTP) and the tip of the fourth toe. Calibration markers (10 cm distance) were placed on the trunk of the animals to reduce parallax error.
Kinematic analyses were performed using a Peak Performance system (Peak Performance Technologies, Englewood, CO). Video images were selected and digitized and \( x-y \) coordinates of different joint markers were obtained at 60 fields/s. These coordinates could be displayed as continuous angular displacements or stick diagrams of one step cycle and used to calculate angular joint movements. In this paper, duty cycles are represented by horizontal lines with downward arrows indicating foot contacts and upward arrows indicating foot lifts.

**Reflex testing**

**ELECTRICAL STIMULATION.** The stimulation of the superficial peroneal nerve (single pulse of 250 \( \mu \text{s} \) at 0.45 Hz) was delivered at rest,
when cats were lying down on the treadmill. The threshold of the stimulation was set at the current value necessary to evoke a small short latency (10–12 ms) response in the St muscle half the time. The EMG responses to the electrical stimulation were digitized at 1 kHz and computer-averaged. Quantitative measures of the responses (amplitude and latency) were obtained, using custom-made software.

**FAST PAW SHAKE.** In the spinal cat, fast paw shake (FPS) was elicited by holding the cat in the air and dipping the paw into a bowl of warm water. During the fast paw shake, the video images and EMG signals were recorded. In this study, EMG response to fast paw shakes were shown before and after drug administration.

**Histology**

At the end of the experimental sessions, animals were killed with an overdose of pentobarbital sodium. The spinal cords were removed and frozen rapidly for subsequent autoradiographic studies published in abstract form (Chau et al. 2000; Reader et al. 2000). The lesion site was also removed for histological analysis (Klüver-Barrera method) to ensure the completeness of the spinal cord transection.

**RESULTS**

The results reported in this study are taken from 86 injections of drugs in five chronically implanted cats. The time before or after spinalization, as well as the concentration of the drugs injected intrathecally in boluses of 100 μl, are indicated in Fig. 1. Experiments illustrated in the figures are representative of the effects of the drugs. The changes in EMG characteristics for several representative experiments are summarized in two tables. Detailed video analysis was not performed for all injections but each video was carefully reviewed to verify the similarities or differences between drug effects. In the spinal condition, all drugs were administered in the late postspinalization stage when cats had clearly recovered spontaneous spinal locomotion. We also report an experiment with NMDA injected during the intermediate phase, 7 days after the spinal section, when the cat just started to express some rhythmic hindlimb movement (Fig. 3). This served to establish the dose of NMDA most currently used.

**Effects of NMDA on locomotion**

**INTACT CAT.** Because excitatory amino acids are known to induce excitotoxicity and play a role in spinal nociceptive transmission (Aanonsen and Wilcox 1987; Liu 1994), only a few experiments with low doses of 100 μl of 1 mM of NMDA were performed in the intact period. But those few experiments were necessary to allow comparisons with the effects of similar doses in the spinal state.

The results of an NMDA injection during the intact period are illustrated in Fig. 2 for level walking. Twenty minutes after a bolus injection of NMDA (1 mM), there was no significant change in the locomotor pattern (Fig. 2, E–H) when compared with control locomotion (Fig. 2, A–D). The stick diagrams of the left hindlimb representing one step cycle before and after drug injection (Fig. 2, A and E) are quite similar and so is the angular displacement of the hip, knee, ankle, and MTP joints (Fig. 2, B and F). The raw EMG traces (Fig. 2, C and G) and the duty cycle of the right (R) and left (L) hindlimbs (Fig. 2, D and H) are virtually identical. After NMDA, measurements showed that there were no significant changes in the duration of the step cycles (98–100% of the predrug value) as well as in the amplitude (96–111% of predrug) and duration (91–114% of predrug) of flexor and extensor bursts. Similar observations were made in four trials in two cats. For all the trials, the mean normalized step cycle duration was 99 ± 7% of the predrug value. The mean normalized amplitude and duration of all muscles recorded ranged from 80 to 135 and from 96 to 107% of predrug values, respectively.

After NMDA injections, the locomotor performance on the 15° uphill slope remained unchanged. The kinematics, the step cycle duration (104% of predrug) as well as the amplitude and duration (90–102% and 94–112% of predrug, respectively) of flexor and extensor bursts were similar to uphill predrug session. No significant changes on the locomotor pattern were observed during the 15° downhill task; the kinematics and EMG bursts were similar to the downhill control. All cats were capable of walking on the round rungs of a horizontal ladder before and after NMDA injections.

**SPINAL CAT.** Intermediate spinal cats (7–8 days postspinalization). The ability of NMDA to initiate locomotion in the early and intermediate periods after the spinalization was discussed in more details in a previous paper (Chau et al. 2002). Here, we are focusing on the comparison of the effects of NMDA before and at the late stage after the spinalization. However, it is important to show that a single dose of 1 mM of NMDA, a dose that had practically no effect in the intact state (Fig. 2), produced a marked improvement of the locomotor pattern in a 7-day spinal cat (Fig. 3). Before NMDA (Fig. 3, A–D), a strong peroneal stimulation could induce small steps with occasional foot placement on the treadmill (Fig. 3A) and reduced weight support. This stepping activity was irregular, as shown by the duty cycle of the left/right limbs (Fig. 3D) but was so small that the hip, knee, ankle and MTP joints moved only slightly (Fig. 3B). The EMG traces present a very weak activity in the majority of muscles except for the knee flexor St muscle on both sides (Fig. 3C). After NMDA, there was a dramatic improvement of the locomotor pattern (Fig. 3, E–H). NMDA induced continuous locomotion with good bilateral foot placement and weight support of the hindquarters (requiring only light perineal stimulation) and these effects lasted 24–72 h postinjection. When compared with predrug, there was a marked increase in the step length as shown in the stick diagram (Fig. 3E) as well as by the increase in the total angular excursion (Fig. 3F). A clear rhythmic alternation of flexor and extensor muscles appeared and EMG bursts were more robust and regular than in control (Fig. 3G). This spinal locomotion was sustained and adapted to the varying speed of the treadmill. Indeed, this particular cat could walk ≤0.7 m/s, whereas the maximal treadmill speed before NMDA was 0.3 m/s. These effects of NMDA in the intermediate phase after spinalization were consistently seen in all spinal cats tested, i.e.: NG2, NG3, and NG5 (see also Chau et al. 2002).

**Modulation of locomotion in late spinal cats.** The ability of NMDA (1–25 mM) to modulate spontaneous locomotion was assessed in late spinal cats after they had recovered the ability to walk spontaneously on the treadmill. Figure 4, A–D, illustrates the same cat discussed in Fig. 2 but 61 days after spinalization. The locomotor pattern has now recovered well with correct foot placement and full weight support (Fig. 4, A–D). A low dose of 1 mM that produced no effect in the intact cat but improved markedly the locomotion in the intermediate
phase after spinalization, now induced only minor changes in the locomotor pattern in this late stage. One hour and 25 min after a bolus injection of NMDA, there was no major change in the locomotor pattern, when compared with the locomotion before NMDA, except for a small decrease in the step length (90% of predrug) and in the angular excursions of all joints, in particular the ankle and MTP joints (see stick diagram and angle plots in Fig. 4E and F). The duration of some flexor and extensor bursts decreased significantly but only to 82–92% of predrug values after NMDA (for example, see RTA in Fig. 4G). Variable changes could be seen in burst amplitude (70–121% of predrug values).

These overall observations made in the late spinal cat after NMDA administration were consistently seen in five trials in two cats and summarized in Table 1. Low doses (1 mM) of NMDA caused a decrease (3/5) or no change (2/5) in the mean step length (92 ± 11% of predrug). The normalized EMG showed that the mean duration of most of EMG bursts decreased by 82–95% of predrug values while, on average, slight increases in the mean burst amplitude (105–124%) were seen after NMDA.

Because NMDA produced only slight effects in low doses, higher doses of 5–25 mM were also tested in two late spinal cats (CC6 and CC7). One example of the effect of a high dose of NMDA (cumulative doses of 10 mM) is illustrated in Fig. 5 for cat CC6. The locomotor characteristics of this 183-day spinal cat were very stable as illustrated by the angular displacements, the EMGs, and the successive duty cycles of Fig. 5, A–D. After NMDA, locomotion was temporarily disrupted for ~10–15 min by a marked increase in the general excitability with spontaneous hyperflexions of the hindlimbs, high-frequency tremors and fanning of the toes. The contact of the hindpaw with the treadmill surface was enough to trigger episodes of tonic bilateral hyperflexions of the hindlimbs. When these major effects of excitability dissipated, locomotion could be recorded and illustrated in Fig. 5, E–H. At 65 min post-NMDA administration, the locomotion was brisker than during the predrug condition and the hindlimbs were stiffer, but a regular locomotor pattern was recorded. There was an increase in the step length (113% of predrug), as shown in the stick figure (Fig. 5E) and an increase in the joint angular excursion, in particular the ankle and MTP joints, as shown in the joint angle plots (Fig. 5F). The increase in angular excursion was evident at the end of the stance phase (Fig. 5F) and resulted in a prolongation of the stance phase. The duration of the step cycle did not change significantly in this case. The

**FIG. 2.** Effects of a low dose (1 mM) of NMDA on an intact cat (NG5). A–D: control at 0.4 m/s before drug injection. A: stick diagrams of 1 step cycle representing the swing and the stance phases. B: angular displacement of the left hip, knee, ankle, and metatarso-phalangeal joint (MTP) joints trigger on the left contact. C: raw electromyograms (EMGs) of the hindlimb muscles during treadmill locomotion. D: duty cycles are represented by horizontal lines. †, foot contacts; ‡, foot lifts. E–H: 20 min after NMDA administration. The time base applies to C, D, G, and H. L, left; R, right; Srt, sartorius anterior head; TA, tibialis anterior; VL, vastus lateralis; GL, gastrocnemius lateralis, and GM, gastrocnemius medialis.
duration of muscle bursts did not change dramatically, but the amplitude of the flexor and extensor muscle bursts increased significantly by 107–356% of the predrug values (see TA in Fig. 5G). This may contribute to the more flexed posture at the offset of stance and during swing, observed after NMDA administration.

These observations after high doses of NMDA (5–10 mM) were also seen in all trials (n = 5) in two cats (CC6 and CC7). In the summary table (Table 1), these doses of NMDA caused a major increase in the mean step cycle duration (192 ms ±99% of predrug values). The normalized EMGs showed that the mean duration and amplitude of most of the EMG bursts dramatically increased following NMDA and were within a range of 102–225% and 111–255% of predrug values, respectively.

Effects of NMDA on cutaneous reflex excitability

The amplitude of the reflex responses evoked by electrical stimulation of the superficial peroneal nerve did not change in intact (2/2 injections, ranged from 88 to 100% of predrug) and spinal cats after low doses of 1 mM of NMDA (4/5 injections, ranged from 93 to 111% of predrug). Only high doses of NMDA (5–10 mM) increased the reflex amplitude with values ranging from 133 to 400% of the control (3 of 4 trials). Figure 6, A–F, shows an example of the responses of two flexor muscles after electrical stimulation of the superficial peroneal nerve before and after administration of low and high doses of NMDA. In cat NG5, in both intact (Fig. 6, A and B) and spinal (Fig. 6, C and D) conditions, the stimulating current used before the drug produced similar short-latency responses after NMDA (1 mM) administration, except in the hip flexor muscle (RSrt) where a slight increase was found. However, in the late-spinal cat CC7 (230 days), 15 min after a high dose of NMDA (15 mM), there was a marked increase in the short-latency response in the knee flexor St with the same stimulating current of 350 μA (Fig. 6F).

Similarly, FPS response did not change after NMDA 1 mM (5/5 injections) but increased dramatically at higher doses (4/5 injections). Figure 6, G–J, shows examples of fast paw shake (FPS) responses before and after NMDA in the spinal cats NG5 and CC6. After the administration of NMDA (1 mM), the FPS response was the same in frequency and in duration as in predrug condition (Fig. 6, G and H). However, after NMDA (5
mM), while the frequency of the FPS response was similar, the duration of the episode was always longer (Fig. 6, I and J).

Effects of AP-5 on locomotion

INTACT CAT. The effects of AP-5 injections in intact cat NG2 are illustrated in Figs. 7 and 8. One hour after a bolus injection of AP-5 (25 mM), the figurines of Fig. 7B show that although the cat was still capable of walking regularly (and even of following all treadmill speeds), there was a significant decrease in weight support, represented as a sag of the hindquarters. There was also a quite obvious bilateral foot drag, particularly seen at the beginning of the swing phase. At touchdown, there was also an important yield when the paw touched ground, leading at times to a contact of the ankle itself on the treadmill belt. A more detailed description of these walking abnormalities is provided in Fig. 8. The normal spinal walking behavior...
A percentage of the predrug values of the plantar flexors [semitendinosus (St), sartorius anterior (Srt), R, right; L, left] and extensor [gastrocnemius lateralis and medialis (GL and GM)] muscles are means expressed as a percentage of the predrug values ± the coefficient of variation (CV). The total number of step cycles is enclosed in parentheses. For each individual trial, a Student’s t-test was performed to compare the pre- and postdrug values. 

Table 2 summarizes the effects of AP-5 administration on duration and on amplitude, in 8 trials in intact cats. About 50–60 min after AP-5, the mean step cycle duration was the same as in predrug condition. There was an increase in the mean normalized amplitude (107–136% of predrug) in several flexor and extensor bursts after AP-5, but only small and variable changes could be seen in the mean normalized burst duration, with values ranging from 89 to 109% of predrug.

The time course of the effect of AP-5 administration was evaluated by measuring the duration of drag as a percentage of the total swing phase and is illustrated for two intact cats (NG2 and NG3) in Fig. 9, A and B. The effect started gradually and, at 15 min postinjection, some cats already had a foot drag (see intact cat NG2 at 15 min in Fig. 9B) and only showed slight decreases in weight support of hindquarters. This effect increased and maximal effect was observed ~40–60 min postinjection (peak period). During the peak effect, the drag could occupy 50–60% of the total swing phase. At that time, all cats were capable of walking regularly on the treadmill and of following treadmill speeds as in the control period. After the peak period lasted 20–40 min, the effect of AP-5 gradually dissipated and normal walking resumed ~4 h later.

The effects of AP-5 are dose-dependent. Lower doses of AP-5 such as 10–15 mM only caused foot drag but had remarkably little central effect. Larger doses of 25–100 mM produced an additional important sag of the hindquarters and difficulty in supporting weight. With these doses, secondary central effects such as drowsiness sometimes appeared during the peak period, and cats often slept quietly between recordings. However, even with such central effects and decrease in weight support, cats would generally cooperate with the experimenter and were capable of walking regularly on the treadmill and of following all treadmill speeds as in control. The time course of AP-5 effects was similar to lower doses and lasted for ~4–5 h.

Walking on slopes and on a horizontal ladder. At the smaller doses (7.5–20 mM), all cats were capable of walking on uphill and downhill slopes, and no major changes were seen in the kinematics nor in the EMG activities. High doses of AP-5 (50–100 mM), which produced more pronounced central effects, sometimes prevented the cats from walking uphill, and they tended to remain at the bottom of the treadmill. Walking on a downhill slope was easier for cats even with the highest doses. However, at such doses, some sliding of the hindpaws was often present, and variability in the step cycle length was also observed (62–112% of the predrug values), but these effects did not prevent cats to walk downhill.

During the peak effect of AP-5 (~40–60 min post-AP-5), when cats could walk easily at level and on slopes, they had major difficulties walking on a horizontal ladder. Most of the time, intact cats could correctly place their hindpaws on the rungs but the paws often slipped off. At that time, cats presented an important decrease in weight support and in the cutaneous excitability and this may be responsible for such slippage. In some trials, this decrease was so dramatic that the cats could not even stand on the rungs.

SPINAL CAT. While AP-5 has been shown to induce only some walking perturbations in intact cats, it completely blocked the locomotor pattern in late spinal cats. As illustrated in Fig. 10, A–D, before AP-5 application, the locomotor pattern was well established in this cat 131 days after a complete spinalization (same cat as Fig. 8), and it consisted in a well-organized EMG activity with full weight support and correct placement of the foot (Fig. 10, A–D). About 30 min after a bolus injection of 25 mM of AP-5, the locomotor pattern was completely blocked and both hindlimbs dragged on the treadmill belt. As shown by the consecutive stick diagram of Fig. 10E, no placement of the foot nor weight support could be observed. When a strong perineal stimulation was given, it was possible to evoke some faint limb movement (Fig. 10F) with no real organization, as also illustrated by the EMG traces and the duty cycle (Fig. 10, G and H). Generally, the complete locomotor blockade was
established between 20 and 40 min post-AP-5 and lasted for 40- to 60-min periods. Figure 9C shows the time course of such a locomotor block induced by AP-5 in a late spinal cat (NG2). At 40 min, the locomotion was completely blocked and both hindlimbs dragged on the treadmill for ~60 min. Then the cat gradually recovered some limb movements and spinal locomotion returned to normal at 3 h post-AP-5 application. The time course for AP-5 seemed to be shorter in spinal (3 h) than in intact state (4–5 h).

Several experiments on AP-5 administration were done during the late spinal state. In 17/29 trials, AP-5 induced a total block of locomotion of one or both hindlimbs even when strong perineal stimulation was given. In the remaining experiments (12/29), seven injections of AP-5 caused a partial block of locomotion, where both feet never reached beyond the hip joint at paw contact, whereas six other injections induced only a slight decrease in step length with, occasionally, increases in foot drag.

The total block induced by AP-5 could be restored by an intrathecal injection of the glutamatergic agonist NMDA. Figure 11, illustrates that the well-organized locomotor pattern of the 211-day late spinal cat CC7 (Fig. 11, A–C) was blocked at 48 min after the application of AP-5 (Fig. 11, D and E), even if some rapid rhythmic activity remained in some flexor muscles of the right hindlimb. Thirty-five minutes after NMDA (cumulative dose of 15 mM) and 1 h and 23 min post-AP-5, locomotion similar to predrug now reappeared (Fig. 11, F–H).

Interestingly, in the same spinal cat, the well-organized locomotion blocked by AP-5 was not restored by clonidine (4 mM), the alpha-2 noradrenergic agonists known to initiate locomotion in the early spinal cats (Barbeau et al. 1987; Chau et al. 1998). The blockade of AP-5 was still present even at 3 h
FIG. 6. Responses to electrical stimulation of the superficial peroneal nerve at rest and fast paw shake (FPS) before and after NMDA. A and B: averaged response of 19 and 12 stimuli at a current of 200 μA in the intact cat and the same cat 26 min post-NMDA injection (1 mM), on the right. C and D: averaged responses of 9 and 8 stimuli at a current of 220 μA in a late spinal cat (62 day) and 60 min post-NMDA (1 mM), respectively. E and F: averaged responses of 15 stimuli at a current of 350 μA in a late spinal cat (230 day) and 15 min after a high dose (15 mM) of NMDA, respectively. G and H: FPS responses in a late spinal cat (61 day) before and 1 h 35 min post-NMDA (1 mM). I and J: FPS in the late spinal cat (183 day) before and 23 min after a high dose of NMDA (5 mM).
postclonidine administration. After that period, the effect of AP-5 dissipated rapidly and spinal locomotion similar to control could be recorded 5 h post-AP-5, with strong perineal stimulation.

**Effects of AP-5 on cutaneous reflex excitability**

After AP-5 application, the amplitude of the reflex response evoked by electrical stimulation of the superficial peroneal nerve decreased by 7–50% of predrug values during the intact state (3/3 trials) and by 0–75% of predrug after spinalization (5/6 trials). In the intact cat NG2 (Fig. 12, A and B), the same stimulation current of 160 μA used in the predrug condition (Fig. 12A) abolished the short-latency response in both Srt and TA muscles (Fig. 12B); however, it did not change significantly the response in the St muscle after an AP-5 injection of 25 mM. Similarly, in the same cat but 41 days postspinalization, there was a pronounced reduction in the response for the St muscle and a complete abolition of the Ip response. In late spinal cats, the FPS response disappeared after AP-5 (4/6 injections) or was the same as in predrug condition in 2/6 injections. Figure 12, E and F, shows an example of the FPS response before and after AP-5 administration in the cat NG3, 68 days postspinalization. At 50 min after AP-5, the FPS response was completely abolished (Fig. 12F).

**DISCUSSION**

In this study, we compared the effects of the intrathecal injection of the glutamatergic agonist NMDA and the antagonist AP-5 in the same animal, initially while in the intact state and then at different times after a complete spinal cord section at T13. This allowed us to determine the relative importance of NMDA receptor activation on the expression of the locomotor pattern under conditions where other neuromodulators such as 5-HT or NA were present (intact cat) or absent (spinal cat).

**NMDA on intact and spinal cats**

We have shown here that a low dose of NMDA (100 μl of 1 mM solution) had practically no effect on the locomotor pattern in the intact state. In the companion paper (Chau et al. 2002), we had seen also that, 4 days after the spinal cord section in cat NG2, the same dose of NMDA failed to initiate the locomotor pattern at this early stage, although the spinal cat became hyperexcitable with tremors and toe fanning. However, in the intermediate stage (7 days), when the animal was just starting to recover spinal locomotion, a dramatic improvement of the locomotor pattern was observed (Fig. 3). A few weeks later, when the cat had completely recovered the ability to walk on the treadmill belt, the same dose of NMDA only caused small modifications of the already well-established locomotor pattern. These small changes consisted in a decrease of the step cycle duration and a tendency to have larger EMG bursts. Larger doses of NMDA in the spinal state, however, could have major effects characterized by a state of hyperexcitability, such that could even preclude locomotion for some 10–15 min; locomotion then resumed with longer cycle duration and a marked increase in EMG burst amplitude, in most cases. How can we explain the responsiveness to NMDA as a function of the state of the animal?

The relative ineffectiveness of NMDA in the intact state is also reminiscent of the ineffectiveness of the alpha-2 agonist clonidine in the intact state (Giroux et al. 2001). The lack of effect after NMDA or clonidine in the intact state could be explained by the effective inactivation mechanisms and a better clearance of the agonists that may exist in the intact state. Other efficient compensating mechanisms may be involved, as discussed later, could offset the neurotransmitter imbalance resulting from the injection of the agonists of a particular neurotransmitter.

The ineffectiveness of NMDA to trigger locomotion in
the early spinal cat as well as the marked effects of NMDA in the intermediate-spinal cats were discussed in the preceding paper (Chau et al. 2002). In the early stage, NMDA does not induce locomotion but, instead, increases markedly the general excitability leading to a state where merely touching the treadmill with the feet led to hyperflexions. If we consider that the spinal lesion might by itself induce a release of EAA below the spinal lesion, adding more EAA only leads, apparently, to a nonfunctional state of hyperexcitability that may interfere with the expression of the locomotor pattern. This finding was surprising, considering the activation of NMDA receptors has been found to be efficient in triggering locomotion in several in vitro spinal preparations. Later, however, when signs of functional locomotor recovery reappear, adding NMDA can indeed boost the action of the spinal pattern generator.

Recent autoradiographic studies have shown that NMDA receptor density differs according to the time elapsed after the spinal lesion.
receptors were found mainly in the superficial layers of the dorsal horn or Rexed’s laminae (Rexed 1952) I and II as well as around the central canal or lamina X (Giroux et al. 1997; Reader et al. 2001). At 15–30 days after spinalization, the binding significantly increased in these laminae, and this upregulation was maintained for several months (Reader et al. 2001), contrary to other NA or 5-HT receptors (Giroux et al. 1999b). We cannot explain directly the pharmacological effects observed nor the density of receptors at different times after spinalization. Indeed other changes in receptor functionality can be more even important than changes in density; for instance, preliminary work on alpha-2 noradrenergic receptors has shown a possible mismatch between the functional G protein coupling, measured by GTP-gamma S, and the receptor density, observed by autoradiography (Chau et al. 2001).

Another concern may be the activation of glutamatergic receptor subtypes. EAAs have been reported to induce locomotion in different animal preparations by their action on NMDA but also on non-NMDA receptor subtypes (kainate but not quisqualate). For instance, activation of kainate receptors was found to induce fictive locomotion in both lamprey and tadpole (Brodin et al. 1985; Dale and Roberts 1984). In Xenopus embryos, bath application of kainate caused a sustained motor output similar to swimming (Dale and Roberts 1984). In isolated brain stem-spinal cord preparation of neonatal rats, both kainate and quisqualate were barely effective or ineffective in inducing locomotor activity (Smith et al. 1988). In acute in vivo preparations such as decerebrate cats, non-NMDA drugs failed to produce fictive locomotion (Douglas et al. 1993). In experiments not included in the present study, the use of non-NMDA drugs, such as kainate and AMPA or their antagonists, were not conclusive.

**AP-5 on intact and spinal cats**

The use of the NMDA antagonist AP-5 in the present study was also instructive. In the intact state, AP-5 caused both a reduction in weight support and a foot drag, but, otherwise, the cat could continue to walk at any speed on the treadmill and could cope with even more demanding situations, such as walking on slopes and a horizontal ladder except when higher doses of AP-5 were used. The increase in EMG amplitude, especially in flexor muscles (see Table 2) could represent an attempt to increase swing to compensate for the preceding increased yield occurring during stance. It should be stressed that AP-5 in the normal cat is very well tolerated, whereas in the spinal state, AP-5 completely blocked the locomotor pattern; this is in line with other studies that use NMDA antagonists. In the lamprey, AP-5 has been shown to reduce fictive locomotion elicited by NMDA (Brodin et al. 1985) and to depress spontaneous fictive swimming (Brodin et al. 1985). Similarly, in the in vitro chick spinal cord, AP-5 reduces the locomotor activity evoked by bath-applied NMDA (Barry and O’Donovan 1987). In both decerebrate and spinal rabbits, the noncompetitive NMDA antagonist MK-801 dose dependently suppresses the evoked locomotor activity (Fenaux et al. 1991). In the cat, intrathecal infusion of AP-5 and the non-NMDA antagonist CNQX was found to completely block locomotion induced by electrical stimulation of the MLR of the midbrain (Douglas et al. 1993). In summary, the activation of NMDA receptors in the spinal state appears to be critical.

Such pharmacological experiments performed in vivo are bound to produce some variability of responses, which are being reported in some details in the appended tables. Despite this variability, the general effects were consistent and the intrathecal cannula appears to be an effective delivery system. It decreases systemic side effects and it can remain in place and be effective for 2 yr. (Giroux et al. 2001). One concern can be the localization of the tip of the cannula in the different cats. When this cannula is inserted, the level

---

**FIG. 9.** Time course of the effect of AP-5 in 2 cats. The effect of AP-5 was evaluated by expressing the time of left hindpaw drag as a percentage of the total left swing as a function of time period postadministration. A and B: intact cats NG2 and NG3, respectively. The effect started gradually and dissipated over ~4 h. The maximal effect was around 40–60 min postinjection. C: spinal cat (NG2). Same dose of AP-5 caused a total block of the hindlimb (100% of drag during the stance phase) during 1 h and the overall effect lasted 3.5–4 h.

**AP-5 25 mM**

**Intact (ng3)**

A

**Intact (ng2)**

B

**Spinal 39 d (ng2)**

C

Drag (% of left swing)

Consecutive step cycles for time period

J Neurophysiol • VOL 90 • AUGUST 2003 • www.jn.org
of termination can be controlled fairly well by measurements of external landmarks; however, the path followed by the cannula within the vertebral canal cannot be controlled with our present technique. The tip of the cannula of cat NG3 was found to be the most rostral at L2; in this animal, the pharmacological effects observed on locomotion was the same as in other animals (see effect of 25 mM of AP-5 in intact cats NG3 and NG2, Fig. 9, A and B). It appears that the location of the cannula (L3–L5) did not affect significantly the effects observed on locomotion and reflexes.

It is of interest to note that the location of the tip of the cannula was quite rostral to the main hindlimb motoneuron pools (Vanderhorst and Holstege 1997). Our postmortem dissection of the cannulae and the extent of diffusion of a 100-μl bolus (the volume used throughout) was limited to about one segment because a fibrotic pocket was formed at
the end of the cannula (Giroux et al. 2001). The importance of pharmacological activation of the mid-lumbar segments was further developed in a different context (Marcoux and Rossignol 2000).

**Cutaneous excitability after NMDA and AP-5**

The effects on reflexes were largely predictable based on the effects on locomotion. In this study, NMDA (large doses of 5–25 mM) caused an increase in cutaneous reflex excitability in the spinal cats, while AP-5 reduced these responses in both intact and spinal cats. These results were in line with other studies made on different animal preparations. In pentobarbital-anesthetized rats, intrathecal NMDA (1 mM) increased the flexion reflex, which was induced by electrical stimulation in both intact and spinal states (Moore et al. 1992). This increase was hindered when rats were pretreated with the antagonist MK-801. In our study, 1 mM NMDA did not change cutaneous reflex excitability in either intact or spinal condition. However, larger doses (>5 mM) dramatically increased the response in spinal cats (not tested in intact cats). Furthermore, in the turtle spinal cord, the application of AP-5 in situ to the spinal cord segments of the hindlimb enlargement induced a decrease of the flexion reflex amplitude. This suggests a role for NMDA receptors on sensory interneurons in the processing of cutaneous information (Stein and Schild 1989). Also, in the in vitro turtle spinal cord preparation, the sensory-evoked pocket scratch reflex was greatly reduced by the application of AP-5 (Currie and Lee 1996).

The intrathecal administration of excitatory amino acid agonists, such as NMDA, into the mice spinal cord was found to produce behaviors such as biting and scratching of the hindquarters (Urca and Raigorodsky 1988). In this study, comparable behavior was seen in one intact cat (NG2) after NMDA application of 1 mM. A few minutes after NMDA application, cat NG2 started to lick vigorously its hindquarters for ~5- to 8 min periods. Biting and scratching behaviors were never observed even with larger doses of NMDA except for spinal cat NG2. In this animal, the tip of the canula was located near the site of the spinal transection, which could explain that NMDA could induce such nociceptive effects.

**Pharmacology of locomotion in intact and spinal cats**

The present work should be viewed in the wider perspective of previous work. The results reported here, together with recently published studies from our laboratory (Chau et al. 2002; Giroux et al. 2001) demonstrate that the pharmacology of locomotion is quite different in intact cats from what it is in spinal cats.

In the intact state, noradrenergic agonists such as clonidine (Giroux et al. 2001) and NMDA, as shown in this paper, have no major effect on locomotion. However, administration of the alpha-2 noradrenergic blocker yohimbine curtailed markedly the hindlimb coordination during walking in the intact cat to the point of impeding locomotion (Giroux et al. 2001). While the NMDA antagonist AP-5 caused some deficits (foot drag, decrease in body weight), it did not prevent cats from walking as shown in this study. However, the administration of yohimbine has no effect on spinal locomotion (Giroux et al. 2001), whereas AP-5 completely blocks locomotion in the spinal state. Altogether these findings might suggest that after spinalization, the operation of the spinal pattern generator becomes more dependent on glutamatergic mechanisms alone because other neuromodulators (NA and 5-HT) are absent. The facts that first, a block of noradrenergic receptors has no effect on spinal locomotion and that, second, spinal cats recover locomotion after spinalization in the absence of noradrenergic

---

**FIG. 11.** Locomotion blocked by AP-5 in a late spinal cat CC7 can be restored by NMDA. A-C Locomotion recorded at 0.4m/s in a spinal cat, 211 days postspinalisation, before drug application. A: Stick figures of one step cycle representing the swing and the stance phase. B: Raw EMGs of the hindlimb muscles during treadmill locomotion. C: Duty cycles. D-E 48 min after AP-5 (15 mM) administration, the well-established locomotion is blocked. F-H. Locomotion is restored 35 min following a cumulative dose of NMDA (15 mM).
descending pathways (Barbeau and Rossignol 1987) are compatible with this notion. We might postulate also that the more modest effects of AP-5 in the intact cat result from an effective compensation by other neurotransmitters, ones that can modulate glutamatergic mechanisms and that this mechanism is absent after spinalization.

**Interaction between glutamatergic and monoaminergic system (NA and 5-HT)**

NMDA produces rhythmical oscillations in spinal neurons by inducing changes in intrinsic membrane properties that generate plateau potentials (Hochman et al. 1994; Kiehn et al. 1996; Wallen and Grillner 1987). Antagonists of NMDA receptors such as AP-5 may interfere with this fundamental property and lead to the complete blockage of the locomotor pattern in spinal cats, as seen in this study. However, under normal conditions, this basic mechanism for rhythmogenesis is undoubtedly modulated by other neurotransmitters. Indeed, 5-HT and NA have been found to modulate and potentiate the effects of NMDA. Some of the evidence for such interactions between monoamines and NMDA will be discussed (for review Kiehn et al. 1997; Schmidt and Jordan 2000).

Serotonergic drugs, alone or with NMDA, have been reported to induce or modulate locomotor activity in the in vitro neonatal rat (Beato et al. 1997; Cazalets et al. 1992; Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996; Sgalli-Houssaini et al. 1993; Tresch and Kiehn 2000), leech (Willard 1981), mollusk (Lam and Pearson 2002), and tadpole (Sillar and Roberts 1992). For example, in the mudpuppy, 5-HT dose dependently modulated the NMDA-induced locomotion by increasing the overall cycle duration and enhancing the EMG burst duration. In the lamprey, 5-HT modulated the D-gluta-
mate activated fictive locomotion by reducing the ventral root firing frequency but increasing the intensity of firing (Harris-Warrick and Cohen 1985). Also, the application of NMDA in the presence of 5-HT increased the depolarization in spinal neurons to a higher amplitude than that seen with NMDA alone (Batueva et al. 2002). In the neonatal rat, combined application of 5-HT and NMDA is also more effective in producing a stable and robust locomotor rhythm in than the application of either drug alone (Kiehn and Kjaerulff 1996). At the cellular level, 5-HT1A agonist (8-OH-DPAT) significantly enhanced the NMDA-induced motoneuron depolarizations in the in vitro frog spinal cord (Holopainen et al. 1992).

The unique importance of noradrenergic drugs, especially alpha-2 agonists, in triggering and modulating locomotion in acute and chronic spinal cats has been established (for review Rossignol 1996). In the neonatal rat in vitro preparation, NA alone did not induce locomotion. NA induced an extremely slow rhythm (outside the range of locomotion) between the right and left sides but not between the flexor and the extensor (Sgalli-Houssaini and Cazalets 2000). However, NA was found to modulate consistently the NMDA/5-HT-induced fictive locomotor activity in the neonatal rat by decreasing the cycle frequency and increasing the ventral root burst duration (Kiehn et al. 1999). Intracellular studies on the neonatal rat motoneurons under current-clamp condition showed that NA potentiated the motoneurons response in the presence of NMA (Sgalli-Houssaini and Cazalets 2000). In addition, NA was found to reinstate a well-coordinated locomotor rhythm on the breakdown of the NMDA/5-HT-induced locomotor rhythm in the neonatal rat spinal cord (Kiehn et al. 1999).

Possible mechanisms of the interaction observed between monoamines and NMDA have been shown to involve the modulatory role of monoamines on the active membrane properties in motoneurons and interneurons that play a significant role in the shaping and production of the rhythmic motor output in the mammalian spinal cord (Kiehn 1991; Kiehn et al. 2000; MacLean and Schmidt 1997; Tresch and Kiehn 2000). For example, 5-HT contributed to the generation of the NMDA-induced intrinsic membrane oscillation [tetrodotoxin (TTX) resistant] in motoneurons and interneurons in the lamprey (Sivardt et al. 1985; Wallen and Grillner 1985, 1987), the tadpole (Sillar and Simmers 1994; Woolston et al. 1994), and the cat (Hochman et al. 1994). Evidence is gathering to suggest that 5-HT potentiates the effect of NMDA through the facilitation of the voltage-dependent block of the Mg$^{2+}$ as studied in the Xenopus (Scrypegour-Wedderburn et al. 1997), in the rat spinal cord motoneurons (MacLean and Schmidt 2001) and in trigeminal motoneurons (Hsiao et al. 2002).

In addition, recent studies showed that NMDA and AMPA also stimulated release of [$^{3}$H]NA from prelabeled rat lumbar spinal cord slices (Sundstrom et al. 1998). It is suggested that there are presynaptic NMDA and AMPA receptors on the noradrenergic axon terminals in the spinal cord and that they interact synergistically to evoke the release of NA.

**Conclusions**

We would like to conclude therefore that locomotion in the cat is basically produced by oscillatory mechanisms that are dependent on NMDA receptor activation and that, under normal conditions, this rhythmogenesis is modulated by other neurotransmitters such as 5-HT and NA. In the spinal state, this glutamatergic mechanism is still operating but probably requires time to function optimally in absence of other neurotransmitters. Thus a strategy of combining agonists that could interact, i.e., NA or 5-HT agonists with other drugs acting on glutamatergic receptors could be beneficial and could improve significantly the function of the spinal pattern generator. Eventually, such combination may prove beneficial to patients with spinal cord injuries (Rossignol 2000).

We are grateful to J. Provenercher and F. Lebel for technical assistance and support during experiments, analyses, and preparation of figures. We also acknowledge P. Drapeau for programming, C. Gauthier and D. Cyr for illustrations and photographs and, finally, C. Gagner for electronic support. The contribution of S. Cabana on stylistic corrections of the final draft is acknowledged.

Present address of H. Barbeau: School of Physical and Occupational Therapy, McGill University, Montréal, Québec H3G 1A5, Canada.

**REFERENCES**


