Neuroscience 285 (2015) 269–280

THE VOLATILE ANESTHETIC METHOXYFLURANE PROTECTS MOTONEURONS AGAINST EXCITOTOXICITY IN AN *IN VITRO* MODEL OF RAT SPINAL CORD INJURY

A. SHABBIR, E. BIANCHETTI AND A. NISTRI *

Neuroscience Department, Scuola Internazionale Superiore di Studi Avanzati (SISSA), Trieste, Italy

Abstract—Neuroprotection of the spinal cord during the early phase of injury is an important goal to determine a favorable outcome by prevention of delayed pathological events, including excitotoxicity, which otherwise extend the primary damage and amplify the often irreversible loss of motor function. While intensive care and neurosurgical intervention are important treatments, effective neuroprotection requires further experimental studies focused to target vulnerable neurons, particularly motoneurons. The present investigation examined whether the volatile general anesthetic methoxyflurane might protect spinal locomotor networks from kainate-evoked excitotoxicity using an *in vitro* rat spinal cord preparation as a model. The protocols involved 1 h excitotoxic stimulation on day 1 followed by electrophysiological and immunohistochemical testing on day 2. A single administration of methoxyflurane applied together with kainate (1 h), or 30 or even 60 min later prevented any depression of spinal reflexes, loss of motoneuron excitability, and histological damage. Methoxyflurane per se temporarily decreased synaptic transmission and motoneuron excitability, effects readily reversible on washout. Spinal locomotor activity recorded as alternating electrical discharges from lumbar motor pools was fully preserved on the second day after application of methoxyflurane together with (or after) kainate. These data suggest that a volatile general anesthetic could provide strong electrophysiological and histological neuroprotection that enabled expression of locomotor network activity 1 day after the excitotoxic challenge. It is hypothesized that the benefits of early neurosurgery for acute spinal cord injury (SCI) might be enhanced if, in addition to injury decompression and stabilization, the protective role of general anesthesia is exploited.

Key words: spinal locomotor network, fictive locomotion, motoneurons, spinal reflex.

*Corresponding author. Address: SISSA, Via Bonomea 265, 34136 Trieste, Italy. Tel: +39-0403987718; fax: +39-0403987702.
E-mail addresses: ashabbir@sissa.it (A. Shabbir), eb2985@columbia.edu (E. Bianchetti), nistri@sissa.it (A. Nistri).
Abbreviations: 5HT, 5-hydroxytryptamine; ChAT, choline acetyltransferase; CPG, central pattern generator; CV, coefficient of variance; DAPI, 4′,6-diamidino-2-phenylindole; DR, dorsal root; DR-VRPs, dorsal root ventral root potentials; FL, fictive locomotion; FP, field potential; KA, kainic acid (kainate); MF, methoxyflurane; NMDA, *N*-methyl-D-aspartate; ROI, region of interest; VR, ventral root.

http://dx.doi.org/10.1016/j.neuroscience.2014.11.023
0306-4522/

INTRODUCTION

Although the majority of new cases of spinal cord injury (SCI) are associated with an incomplete lesion, severe locomotor deficit usually ensues with resultant paralysis and uncertain recovery (McDonald and Sadowsky, 2002; Nair et al., 2005; van den Berg et al., 2010). One essential factor for this negative outcome is the extension of the primary lesion over the first few hours and days to surrounding spinal areas (secondary lesion) (Sekhon and Fehlings, 2001; Park et al., 2004; Rowland et al., 2008). Hence, it is important to protect, with various strategies (Boulenguez and Vinay, 2009), initially-spared nervous tissue from further damage which, according to experimental preclinical data, might be already complete within the first 24 h (Mazzone and Nistri, 2014). One major mechanism for lesion progression is excitotoxicity whereby sustained activation of glutamate receptors leads to neuronal degeneration via a delayed cell death process termed parthanatos (Mandir et al., 2000; David et al., 2009; Kuzhandaivel et al., 2010). The realization of the existence of a time lag between primary injury and cell loss should prompt attempts to neuroprotect the lesioned spinal cord (Baptiste and Fehlings, 2008). Investigating these issues demands animal models, though it is difficult to relate the severity of an acute injury to later outcome because *in vivo* animal studies do not readily allow repeated time-related measurements of pathophysiological mechanisms.

In the attempt to circumvent these difficulties, we have developed an *in vitro* model of SCI based on transient (1 h) application of the potent glutamate analogue kainate to mimic a clinical scenario (Taccola et al., 2008; Kuzhandaivel et al., 2011; Nasrabady et al., 2011a) in which intensive care treatment is applied as soon as possible to provide life support, correct metabolic imbalance and, perhaps, restrict the early component of excitotoxic stress (Baptiste and Fehlings, 2008; Munce et al., 2013). One important characteristic of this model is the possibility to analyze the functional activity of locomotor networks by recording their cyclic oscillatory discharges (alternating between flexor and extensor motor pools), and relate them to the topography and extent of the induced lesion. Notwithstanding the intrinsic limitations of this model (e.g. lack of blood supply), several features appear to be reminiscent of the clinical scenario like the limited extent of cell damage which primarily concerns neurons (rather than glia), and motoneurons in particular that are very vulnerable to this type of injury. Furthermore,

there is very high sensitivity of locomotor networks to damage as their electrical activity is suppressed even when substantial cell numbers remain apparently unscathed. These properties may render this model as a simple predictive tool for further testing the *in vivo* effectiveness of novel devices (Nistri, 2012). Our former studies of SCI model neuroprotection have so far obtained limited success (Nasrabady et al., 2011a, 2012) in line with disappointing clinical data (Baptiste and Fehlings, 2008) and should stimulate further work in this field.

The present investigation explored the usefulness of a volatile general anesthetic like methoxyflurane as a protective agent against experimental SCI. Volatile anesthetics are known to be neuroprotective against excitotoxicity in the brain (Popovic et al., 2000; Kudo et al., 2001; Ren et al., 2014) and spinal cord (Nout et al., 2012) although no data are currently available for their efficacy to preserve locomotor function. Methoxyflurane is a convenient drug for such studies because of its physical properties (Andersen and Andersen, 1961; Seto et al., 1992) and its pharmacological activity on synaptic and firing properties has been previously reported (De Jong et al., 1968; Richens, 1969). Hence, we tested the ability of methoxyflurane to contrast the histological and functional damage evoked by kainate. We employed two protocols of administration, namely the anesthetic was started after the application of kainate, or applied together with kainate as a test for proof of principle for the effectiveness of this treatment.

EXPERIMENTAL PROCEDURES

Spinal cord preparations

Thoraco-lumbar spinal cord preparations were isolated from urethane anesthetized (0.2 ml of i.p. of a 10%, w/v solution) neonatal Wistar rats of 0–2 day postnatal age according to the guidelines of NIH and with the approval of the local ethical committee. The experimental set up was as described previously (Taccola and Nistri, 2006a). The isolated spinal cords were superfused with Kreb's solution (in mM: 113 NaCl, 4.5 KCl, 1 $MgCl_2 \cdot 7H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose, gassed with 95% O_2, 5% CO_2, pH 7.4 at room temperature at rate of 7.5 mL/min). In accordance with the three Rs objective, all efforts were made to minimize the number of animals used for the experiments and their suffering. The experiments were performed in accordance with the ethical guidelines for the care and use of laboratory animals of National Institutes of Health (NIH) and the Italian act D. L. 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88). All experimental protocols were approved by the ethical committee of the International School for Advanced Studies.

Electrophysiological recordings

Spinal reflexes and motoneuron field potential (FP). Dorsal root-ventral root potentials (DR-VRPs) were evoked by stimulating a single dorsal root (DR) through a suction electrode with 0.5–10 V stimuli (0.1–0.2 ms duration) and recorded from the homolateral ventral root (VR) by using glass suction electrodes (Taccola and Nistri, 2006a) filled with Kreb's solution as previously reported (Ostroumov et al., 2011). Usually five synaptic responses were averaged for further analysis.

Recent investigations have demonstrated that spinal motoneurons are most vulnerable to excitotoxicity (Mazzone et al., 2010) and sensitive to membrane potential changes evoked by gas anesthetics (Marinc et al., 2012). Thus, to monitor their functional responses after excitotoxic stimulation and methoxyflurane application, motoneuron field potentials (FP) due to synchronous motoneuron firing (Fulton and Walton, 1986) were recorded with an extracellular pipette located in the lumbar ventral horn region of sagitally hemisected (24 h after dissection) spinal cords continuously superfused with Kreb's solution (Ostroumov et al., 2011). Lumbar motoneurons were approached blindly through the medial cut surface of spinal cords and identified (in L3–L5 segments) on the basis of their antidromic response to stimulation (2 Hz) of the corresponding VR. After establishing the stimulus threshold value by

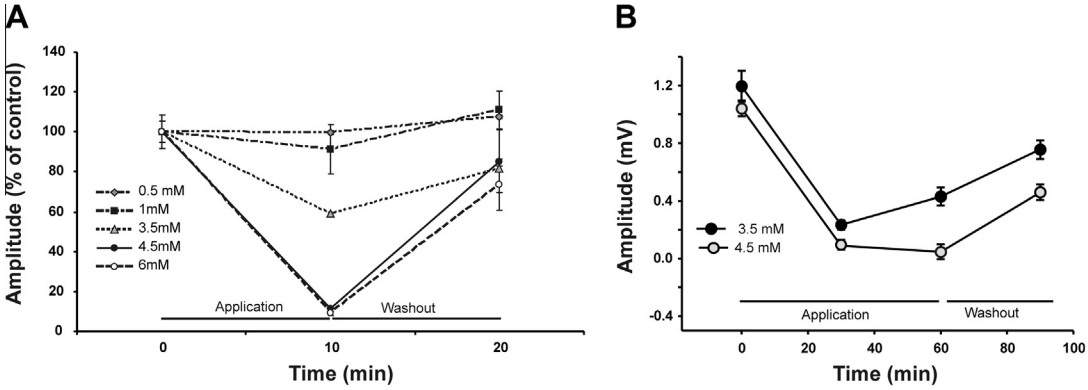

Fig. 1. Changes in DR-VRP amplitude after methoxyflurane treatments *in vitro*. (A) DR-VRP amplitude vs time following application of various concentrations of methoxyflurane. Strong DR-VRP reduction appeared with concentration larger than 1 mM and became very strong at 4.5–6 mM with recovery on washout. Data are from three experiments for each anesthetic concentration. (B) Acute effect of methoxyflurane (MF; 4.5 mM) applied for 1 h at 3.5 mM or 4.5 mM concentration ($n = 3$ for both): complete depression of polysynaptic reflex response was apparent with gradual recovery. Data are represented as mean ± SEM.