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# MODULATORY EFFECTS BY CB1 RECEPTORS ON RAT SPINAL LOCOMOTOR NETWORKS AFTER SUSTAINED APPLICATION OF AGONISTS OR ANTAGONISTS

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**Abstract—Sustained administration of cannabinoid agonists acting on neuronal CB1 receptors (CB1Rs) are proposed for treating spasticity and chronic pain. The impact of CB1Rs on mammalian locomotor networks remains, however, incompletely understood. To clarify how CB1Rs may control synaptic activity and locomotor network function, we used the rat spinal cord *in vitro* which is an advantageous model to investigate locomotor circuit mechanisms produced by the local central pattern generator. Neither the CB1 agonist anandamide (AEA) nor the CB1R antagonist AM-251 evoked early (<3 h) changes in mono or polysynaptic reflexes or in locomotor rhythms. Application of AEA (24 h) significantly decreased the ability of dorsal root (DR) afferents to elicit oscillatory cycles, and left synaptic responses unchanged. Similar application of LY 2183240, or JZL 184, inhibitors of endocannabinoid uptake processes, produced analogous results. Application of the antagonist AM-251 (or rimonabant) for >3–24 h largely impaired locomotor network activity induced by DR stimuli or neurochemicals, and depressed disinhibited bursting without changing reflex amplitude or inducing neurotoxicity even if CB1R immunoreactivity was lowered in the central region. Since CB1R activation usually inhibits cyclic adenosine monophosphate (cAMP) synthesis, we investigated how a 24-h application of AEA or AM-251 affected basal or forskolin-stimulated cAMP levels. While AEA decreased them in an AM-251-sensitive manner, AM-251 per se did not change resting or stimulated cAMP. Our data suggest that CB1Rs may control the circuit gateway regulating the inflow of sensory afferent inputs into the locomotor circuits, indicating a potential site of action for restricting peripheral signals disruptive for locomotor activity.** 

**Key words: cannabinoid receptor-1, fictive locomotion, anandamide, AM-251, cAMP, endocannabinoids.**

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*Abbreviations:* 2 AG, 2-arachidonoylglycerol; 5-HT, 5-hydroxytryptamine or serotonin; AEA, N-arachidonoylethanolamine or anandamide; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; cAMP, cyclic adenosine monophosphate; CB1R, cannabinoid receptor-1; DMSO, dimethyl sulfoxide; DR, dorsal root; DRP, dorsal root potential; Frsk, forskolin; GABA, γ-aminobutyric acid; NMDA, N-methyl-D-aspartate; VR, ventral root.

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## INTRODUCTION

There is increasing interest in cannabinoid research as this field may yield important therapeutic tools against a range of diseases. A recent report has summarized the North American guidelines for the medical use of cannabinoids, highlighting the principal goals of decreasing chronic pain and spasticity especially in multiple sclerosis patients (Koppel et al., 2014). Conversely, use of endocannabinoid antagonists has been investigated to treat obesity and food craving (Poncelet et al., 2003; Howlett et al., 2004; Pagotto et al., 2005): however, early antagonists have not received approval by drug regulatory agencies because of severe central nervous system effects in man (Jones, 2008; Bifulco and Pisanti, 2009). A large body of evidence supports a critical role of cannabinoid receptors type 1 (CB1Rs; Pertwee et al., 2010) that are physiologically activated by endocannabinoids like anandamide (AEA) and 2-arachidonoylglycerol (2 AG), as pharmacological targets to control aberrant nociceptive transmission in the spinal cord and the maladaptive processing of afferent inputs underlying spasticity (Piomelli, 2005; Manzanares et al., 2006). Nevertheless, the role of CB1Rs in spinal locomotor networks is incompletely understood. *In vivo* observations indicate that activation of CB1Rs usually produces less locomotor activity, although the precise mechanisms remain unknown and the site of action unclear (Lee et al., 2006; Bosier et al., 2010). Previous studies of the lamprey spinal cord *in vitro* have shown that acute CB1R activation by AEA speeds up chemically-stimulated locomotor network function, while the antagonist AM-251 depresses locomotor cycles in a time-dependent fashion (Kettunen et al., 2005). Data on mammalian locomotor networks are currently unavailable. Thus, it seems important to investigate how CB1Rs may modulate mammalian circuits because the pharmacological consequences of CB1R activity largely depends on their coupling to distinct G-proteins, in turn regulating various intracellular effectors that may be species, tissue and cell specific (Hudson et al., 2010).

Most experimental studies have employed short-term application of CB1R agonists or antagonists. Nonetheless, these drugs are usually expected to be administered for a prolonged time, raising the possibility that they might induce adaptive changes in their receptors. To explore the functional consequences of sustained application of such drugs, we used the rat

spinal cord preparation *in vitro* as a model. This is an advantageous system to study locomotor network activity that is generated by a central pattern generator (namely a local neuronal circuitry with intrinsic rhythmicity) located in the lumbar spinal and readily activated by either neurochemicals (N-methyl-D-aspartate (NMDA) and serotonin (5-HT); Grillner, 2003, 2006; Kiehn, 2006, 2011) or trains of electrical stimuli applied to one dorsal root (DR; Marchetti et al., 2001). This pattern, which comprises alternating ventral root (VR) discharges among extensor and flexor motoneuron pools located on both sides of the spinal cord, is termed fictive locomotion because the rhythm is expressed in the absence of hind limbs. One important advantage of the isolated spinal cord is its 24-h survival *in vitro* with intact histological profile (Cifra et al., 2012) and fully preserved locomotor rhythms (Taccola et al., 2008). This property enables studies of delayed consequences of drug action with a timecourse resembling the *in vivo* administration protocols as CB1R ligands are not intended for brief effects only.

The aims of the present study were to clarify the effects of activation or block of CB1Rs on synaptic transmission in the rat spinal cord both acutely or after a 24-h application of CB1R ligands, their role in locomotor networks, any plasticity in the CB1R expression and potential neurotoxicity, and any preferential modulation of CB1R effectors.

## EXPERIMENTAL PROCEDURES

### *In vitro* spinal cord preparation

Spinal cords were isolated from neonatal Wistar rats (0–2 days old) in accordance with the guidelines of the National Institutes of Health and the Italian act D.Lgs. 27/1/92 no.116 116 (implementing the European Community directives n. 86/609 and 93/88). All efforts were made to minimize the number of animals used for the experiments and their suffering. Preparations were continuously superfused (7.5 ml $min^{-1}$) with standard Kreb's solution of the following composition (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% $O_2$ and 5% $CO_2$ (pH 7.4) at room temperature during electrophysiological recording. All the experimental procedures were carried out as previously described (Bracci et al., 1996; Beato and Nistri, 1999; Taccola et al., 2008) with ethical permission granted by the ethics committee of our School.

### Experimental protocols for early or delayed effects of CB1R ligands

Reflex and locomotor network activity were first tested in all preparations before any ligand application. On the first day of experimentation, preparations were superfused with cannabinoid ligands (AM-251, AEA), while any changes in electrophysiological responses were continuously monitored. Ligands were then washed out and the preparations were kept in Kreb's solution (24 h) for further electrophysiological testing. In a separate batch of experiments, preparations were treated continuously for 24 h with ligands (AM-251, rimonabant, AEA, LY 218324, 2 AG, or JZL 184) that were washed out before recording on the second day. All experiments were done in parallel with untreated "sham" preparations kept for 24 h in Kreb's solution to be used as control. The same protocol of drug application was used for quantifying basal cyclic adenosine monophosphate (cAMP) levels: these experiments also included an additional experimental group treated with AM-251 for 24 h, washed out and then treated with AEA for 30 min to analyze whether any sustained block of CB1Rs was reversed by short-term application of AEA. Finally, we measured changes in cAMP levels induced by forskolin (Frsk) (1 μM; 30-min application; Steffens et al., 2004) in preparations treated for 24 h with AM-251 or AEA: in this paradigm, CB1R ligands were also co-applied with Frsk.

Drug concentrations used for the present experiments were obtained from previously validated studies with the appropriate references provided for each one of the chemicals listed below. To detect changes in locomotor rhythms by blocking CB1Rs, two antagonist/inverse agonists were employed, namely AM-251 (5 μM; Kettunen et al., 2005; Gonzalez-Islas et al., 2012) or SR 141716A, (rimonabant; 5 μM; Kettunen et al., 2005). CB1Rs were activated by exogenously applying AEA (5–10 μM; Eljaschewitsch et al., 2006) or 2 AG (2–5 μM; Iannotti et al., 2014). We also used the AEA uptake inhibitor; LY 218324 (1–5 μM; Gonzalez-Islas et al., 2012) and the 2 AG uptake inhibitor JZL 184 (1–5 μM; Pan et al., 2009). All these drugs were dissolved in dimethyl sulfoxide (DMSO) except AEA (which was dissolved in ethanol) to reach a 10 mM stock from which final dilutions were made with Kreb's. In all cases, the final concentrations of DMSO or ethanol were also added to the Kreb's solution for control or sham preparations without inducing any detectable change in electrophysiological parameters.

### Electrophysiology

Full details of these methods were previously reported (Marchetti et al., 2001; Taccola et al., 2008). In brief, to study reflexes and rhythmicity (flexor and extensor cyclic discharges) of the spinal cord, VRs of the lumbar region segments (L2 and L5) were tightly sucked into monopolar suction electrodes connected to Ag/Ag–Cl micropellets in glass micropipettes. To evoke VR responses, electrical square pulses (0.1-ms duration) were given through a single ipsilateral DR using a bipolar suction electrode. We graded the DR stimulus intensity to evoke either low-threshold monosynaptic responses when the stimulus was just above threshold to induce a detectable response (Fulton and Walton, 1986; Marchetti et al., 2001) or polysynaptic responses when the stimulus was three times higher. Our previous experiments with intracellular recording from motoneurons have validated the functional identification of these responses as either mono or polysynaptic (Ostroumov et al., 2007).