Dopamine D3 Receptor Antagonist SB-277011A Influences Cell Firing in the Rat Ventral Tegmental Area, Parallel Role with the Cannabinoid System in Addiction and Neuropsychiatry Disorders?

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Abstract: SB-277011A is a compound entering the brain with high affinity and selectivity for the dopamine (DA) D3 receptor. Recent electrophysiological study has shown that acute oral administration of SB-277011A significantly alters the spontaneous activity of DA neurons in the ventral tegmental area (VTA) but that intravenous administration has no effects. In that electrophysiological study hypotheses to explain this discrepancy involved either administration route-dependent timing for the compound to reach its active site or the formation of an active metabolite following oral administration that would sustain the activity of SB-277011A on DA cell firing. In an attempt to assess whether formation of a metabolite may account for the activity of the parent compound we conducted electrophysiological multi-unit field recordings of DA neurons in the VTA of anaesthetised rats following treatment with SB-277011A either systemically or locally in the VTA. The local dose (2.5µg) was selected based on brain exposure achieved following systemic i.p. administration of 10mg/kg. Results show that both administrations increased VTA neurons firing compared to vehicle administration. However, local injection of SB-277011A in the VTA induced a more rapid and higher increase of neuronal activity than systemic treatment. These results suggest that the increased VTA cell firing occurring following systemic administration of SB-277011A is likely due to the compound itself and not to a putative metabolite. Finally, since the growing evidence that cannabinoids (CBs) modulate DA release in the brain and in view of the fact that CB1 receptors are widely distributed over DA neurons, the interplay between these two systems in the context of the current findings is discussed.

Keywords: *In vivo* cell firing, rat brain, VTA, SB-277011A, dopamine, cannabinoids.

INTRODUCTION

Dopamine D3 receptor has been identified as a target of choice to treat psychiatric diseases associated with an alteration of dopamine (DA) dynamics [1]. DA D3 receptors distribute in the limbic regions of the brain including the substantia nigra, nucleus accumbens, olfactory tubercle and, to a lesser extent, amygdala. This suggests its implication in mediating centrally some aspects of emotions, cognition and addictive behaviours. Indeed, antagonists at the D3 receptors proved effective in affecting the behaviour of several animal models of drug dependence [2-4].

Several lines of evidence suggest that SB-277011A reduces behaviours associated with drug intake and relapse. In particular, SB-277011A proved effective in diminishing nicotine [5] cocaine [6] and alcohol [2] self-administration and reinstatement of drug taking behaviours in rodents. Furthermore, pharmacological MRI studies have demonstrated that SB-277011A induces a disruption of the functional connectivity that occurs after amphetamine administration, in the brain areas delineating the mesolimbic pathway [7]. In addition, recent voltammetric *in vivo* analysis [8] demonstrated that SB-277011A potentiates cocaine-induced DA release in the nucleus accumbens. This nucleus is a major component of the mesocorticolimbic system that is constituted by the dopaminergic neurons located in the VTA area mainly projecting to the nucleus accumbens and cortical structures such as the medial prefrontal and motor cortices [9]. Together, these results suggest that the selective D3-receptor antagonist SB-277011A interferes with the mechanisms underlying drug taking and reinstatement of drug taking behaviours.

In an attempt to unravel the mechanism of action sustaining the behavioural effect of the compounds, several authors have investigated its neurochemical and electrophysiological effects. SB-277011A had no effect *per se* on basal DA levels but reduced the D2/D3 agonist (+/-) 7-OH-DPAT-induced inhibition of DA efflux in the nucleus accumbens core and shell. Furthermore, electrophysiological studies have demonstrated that D3 receptor blockade by acute oral (p.o.) administration of SB-277011A significantly alters the spontaneous activity of DA neurons in the ventral tegmental area (VTA or A10 [10]). In contrast to p.o. administration, the same authors report that intravenous (i.v.) administration of SB-277011A does not affect VTA cell firing. Such a discrepancy was hypothesized by these authors to relate to the different timing of drug distribution in both experimental procedures or to the possibility that a metabolite of SB-277011A instead of the parent compound could be responsible for the alteration of DA neuronal activity. On the basis of all these reports, together with the observations that the tool compound SB-277011A was

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1876-5238/09

shown to effectively penetrate the brain as it displays a good brain penetration (cerebral to blood ratio of 3.6:1 [11] and binds with high affinity and selectivity at the dopamine D3 receptor [12], the present work has been undertaken to verify whether SB-277011A itself can affect cell firing in the VTA when given directly into the VTA. Hence, field electrophysiology *in vivo* experiments have been performed by means of dedicated carbon based biosensors and in particular carbon fibre micro-electrodes (mCFE) whose main characteristic is the low noise when compared to classic glass electrodes (*i.e.* tip resistance approximately 0.2-0.5 MΩ versus approximately 1-2 MΩ [13, 14]. The mCFE, prepared as described previously [15-17], were inserted in the VTA of anaesthetised rats in order to perform DA neuronal multi-unit recordings. Indeed, the feasibility of using carbon based electrodes for field electrophysiological studies has already been demonstrated by various authors [15, 16, 18, 19] as they have a lower noise-ground (approx. 2μVRMS: root mean square value) compared to the tungsten or platinum based electrodes, the most noisy being the glass electrodes.

DA neurons were discriminated following the identification criteria described in the literature; briefly, the following properties were considered: a) action potential width >2.5 ms with a distinct initial segment and late positive component; b) slow regular, or bursting firing pattern; c) spontaneous firing rate of 2 to 9 Hz [20-22]. Furthermore, the electrophysiological characteristics identifying non-DA neurons were taken into consideration [and avoided] on the basis of the criteria described in the literature [22-25]. In addition, the sensor was targeted towards the middle part of VTA as this is the part containing mainly DA cells, while non-DA cells are localised in the lateral part of VTA [22]. It is also well known that DA neurons make up the majority of the neuronal population in the VTA [22, 26, 27] *i.e.* the VTA region contains a large proportion (up to 80%) of DA neurons and a smaller population of GABAergic neurons [27, 28].

In particular, here we compared the effect of SB-277011A on VTA cell firing in animals treated systemically (i.p.) with that of local, intra-VTA administration. The i.p. route of administration was preferred to the p.o. route used by Ashby and coll. [10] as we were working with anaesthetised rats and as both ways are comparable parentelar treatments *i.e.* both following the "first pass effect" hepatic metabolic route [29].

Recently, evidence that cannabinoids (CBs) modulate dopamine release in the brain is raised. CB1 and CB2 constitute the CB receptor family [30]. However, only CB1 receptors are widely distributed in the CNS, so they are believed to be responsible for the majority of the effects in the CNS elicited by CBs. Pre-synaptic inhibition of neurotransmitter release is one of the most frequently observed and best characterized effects of CB1 receptor activation in the CNS. In particular, CB1 receptors are [also] widely distributed over dopaminergic neurons [31]. Thus, there is the possibility that the endocannabinoid system influences dopaminergic transmission by controlling dopamine release from the pre-synaptic nerve terminal. In particular, recent observations indicated that CB1 receptors are antagonistic to D2 receptors [32 and for a review see ref. 33]. In a recent review Moore *et al*. [34] have proposed sufficient evidence for connections between dopaminergic activities, the use of cannabis and the incidence of psychotic illness. In addition, the endogenous cannabinoid system has recently been implicated, together with the DA system, in the modulation of addictive behaviour and in the mechanism of action of different drugs of abuse [for a review see ref. 35]. Also, the participation of the CB system in the patho-physiology of neuropsychiatric disorders involving DA and other neurotrasmitters has been introduced [for a review see refs. 36, 37].

MATERIALS AND METHODS

Experiments were performed on adult male Wistar rats (260-300 g, Harlan, Italy). All procedures were carried out in accordance with the Italian law (Legislative Decree no.116, 27 January 1992), which acknowledges the European Directive 86/609/EEC, and were fully compliant with GlaxoSmithKline policy on the care and use of laboratory animal and codes of practice.

Rats were anaesthetised using urethane (2 g/kg i.p.) and held in a Kopf stereotaxic frame (tooth bars at -3.3 mm) throughout the experiments. *In vivo* electrophysiological measurements were performed with carbon fibre micro-electrodes (mCFE, Fig. **1a**) prepared as described previously [15, 17]. The active tip of the mCFE (diameter 10μm, length approximately 50μm, Fig. **1c**) was positioned in the VTA under a stereomicroscope at the following coordinates: AP, -5.0; ML, 1.0; DV, -8.0 mm from bregma [38]. The mCFE was then connected to an instrument amplifier (A-M Systems, USA). The specific "electrophysiological signal" [20, 21, 22] was then visualised and selected on an oscilloscope, "quantified" in spikes/sec that were averaged over 15s bins *via* a Multi Spike Detector, (MSD, Alpha-Omega, Israel) as shown in Fig. (**1e**).

Following insertion of the recording electrode, local injection of vehicle (1μl cavasol 10%) or SB-277011A (2.5μg in 1μl vehicle) was performed with an injection needle (32G) positioned at the upper margin of the VTA nucleus and approximately 200-250μm apart from the mCFE on the sagittal line. Following 30min of basal recordings, the group of rats receiving systemic administration was treated either with an acute dose of SB-277011A (*i.e.* 10mg/kg i.p., n = 5) or vehicle (cavasol 10%, control, n = 5). The group of rats receiving local treatment into the VTA was injected either with vehicle (cavasol 10%, 1μl in 18-20 sec, n = 5) or with SB-277011A at 2.5μg/μl (n = 5).

The acute systemic dose of SB-277011A used here has been selected as it has been reported to significantly increase VTA cell firing [10].

The amount of SB-277011A infused locally was defined based on the reported observation that peripheral treatment with 10mg/kg SB-277011A resulted in a brain concentration of 1700μg/g at 1 hour (ex vivo pharmacokinetic analysis, not shown).

Histology - At the end of each experiment a direct current (DC) of 5 Volt was applied through the active tip of the

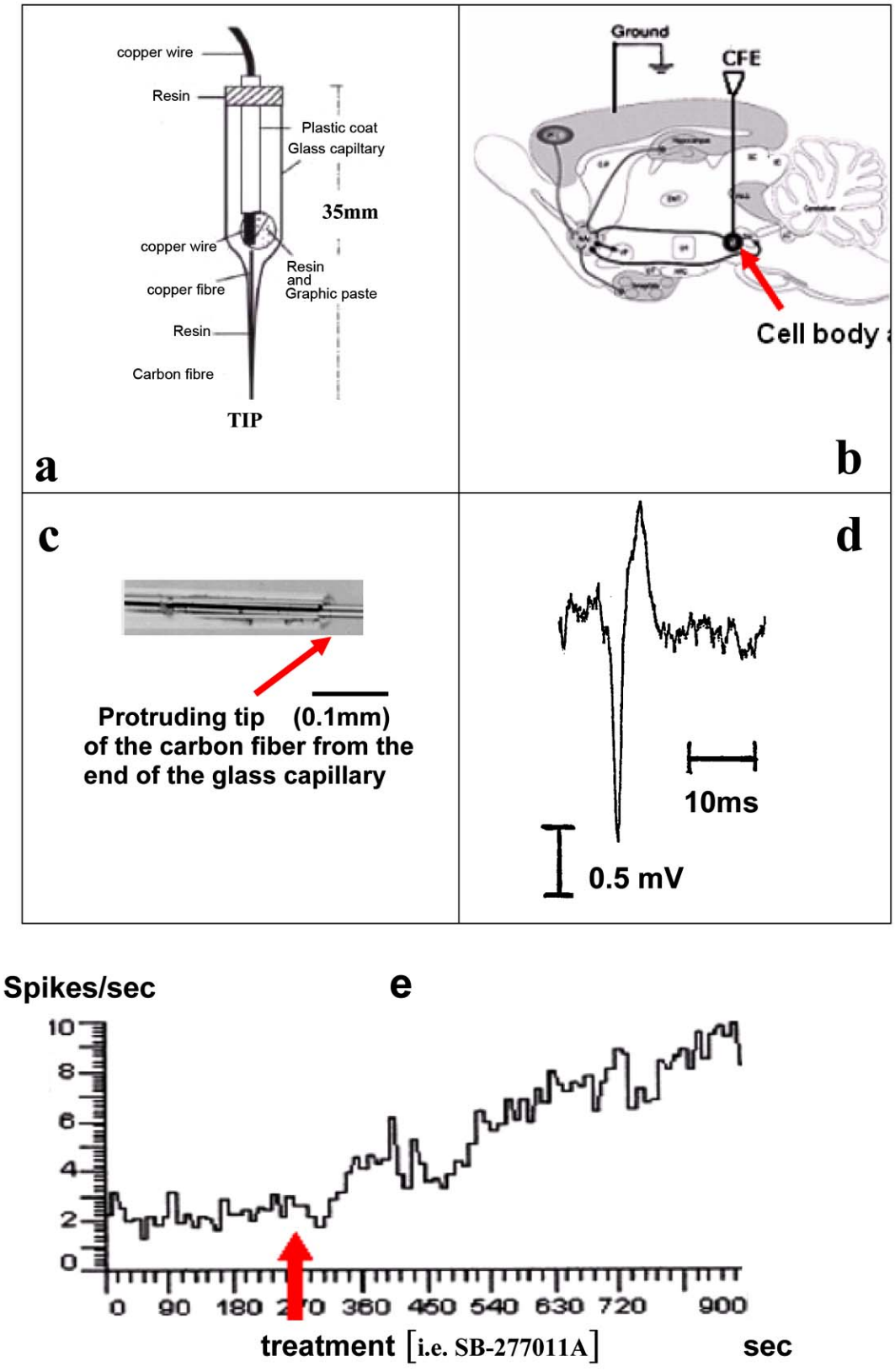

Fig. (1). (**a**, **b**) Scheme of a carbon fibre micro-electrode (mCFE) prepared for field electrophysiology in VTA. (**c**) Particular of the tip of the mCFE used for *in vivo* monitoring of cell firing. (**d**) Scheme of an action potential monitored with mCFE in VTA. (**e**) Example of an *in vivo* field electrophysiology firing rate tachogram: *i.e.* numbers on the ordinate refer to number of spikes/sec that were averaged over 15s bins *via* a Multi Spike Detector, (MSD, Alpha-Omega, Israel). First 270 sec: baseline levels showing an average firing rate of 2 to 3 Hz. Treatment [arrow]: *i.e.* local injection of SB-277011A in VTA.

mCFE in order to produce a lesion of the surrounding brain tissue. The brain was then rapidly removed and sectioned using a cryostat. The brain slices were 60µm in thickness and stained using the NISSL solution. Under light microscopy the lesion (coagulated brain tissue) was observed in order to confirm the correct position of the active tip of the mCFE in VTA. Rats showing a lesion outside VTA were discarded from final analysis.

Data analysis - Data were converted in percent from basal values and analysed with STATISTICA software version 6.0. Analysis of variance (ANOVA, see results section) was used for statistical analyses. In the case of statistically significant differences between mean values produced by drug treatments versus controls (vehicle treatment) main factor Dunnet *post-hoc* test was applied. Statistical significance was set at $p<0.05$.

RESULTS

Peripheral treatment with SB-277011A significantly increased VTA cell firing up to 175±20% of basal values within 15min. Local treatment with SB-277011A also resulted in a significant increase of cell firing up to approximately 290±50% of controls. In particular, this increase started earlier than the peripheral treatment (see Fig. **2**). Statistical analysis using a 3-way ANOVA with between subjects factors of treatment route (systemic *vs* local), treatment (vehicle *vs* SB-277011A) and repeated measurements factor of time, indicated a significant effect of treatment [$F_{(1,14)} = 30.1$, $p<0.001$]. Treatment route appeared only marginally significant with $F_{(1,14)} = 3,99$, $p = 0.06$. Further, post hoc analyses showed that the difference between routes of administration is significant for treated rats ($p = 0.01$) but not for vehicle rats ($p = 0.8$, Fig. **2**).

DISCUSSION

Systemic administration of the selective D3 receptor antagonist SB-277011A was shown to alter the activity of midbrain DA neurons. In particular, Ashby *et al*. [10] have shown that the acute administration of 10 mg/kg SB-277011A significantly increased the number of spontaneously active DA neurons in the VTA. Moreover, the same authors have reported that efficacy of SB-277011A treatment at altering the firing rate of VTA neurons may depend on its administration route. Indeed, intravenous (i.v.) dosing had no effects on firing, whereas dosing *per os* (p.o.) increased the number of spontaneously active DA neurons. The hypothesis of these authors to explain the efficacy of SB-277011A following p.o. dosing and not i.v. is the supposed formation of an active metabolite that would directly stimulate DA VTA neurons. This assumption derives, as again proposed by these authors, from the time length of the experiments performed. In particular, these authors selected a time frame of 2 to 3 hours for the experiments where animals received SB-277011A p.o. as opposed to 15-20min within the experiments where animals received SB-277011A i.v. In support [or in alternative] of that supposition Ashby *et al*. [10] argued also the possibility that under i.v. dosing conditions an adequate amount of brain concentration of SB-277011A may not be attained within the selected time frame of 15-20min so that no effect on DA neuronal activity could be monitored [39].

In the present *in vivo*, real time study performed in anaesthetised rats, the i.p. route of administration was preferred to the p.o. route used by Ashby and coll. [10] as both ways are comparable systemic treatments *i.e.* both following the "first pass effect" metabolic route [29]. Essentially, our results show that both systemic and local injection of SB-277011A induced a comparable increase of neuronal activity in VTA, indicating that this D3 receptor antagonist can be active by itself within this brain region. Even so, we cannot totally rule out the possibility that an active metabolite of SB-277011A could be formed in the brain area. However, in general the rate at which compounds are metabolized in the brain is lower than that in the periphery. Furthermore, the concentration of metabolic enzymes in the brain is in general lower than that in the liver [29]. Thus it is expected that the concentration of the hypothetical metabolite of SB-277011A in VTA could be lower and rising slowly following intra-cerebral injection than following systemic administration. As a consequence, if the effect of SB-277011A on VTA cell firing was only mediated through its metabolism we would expect a slower effect of lower magnitude than that obtained following peripheral administration. We show here that local administration of SB-277011A increased quicker and to a higher level the VTA neurons firing rate; this data further supporting the direct influence of SB-277011A upon VTA cell firing.

An easier approach to investigate if active metabolite of SB-277011A is involved in mediating the firing regulation is to employ thick brain slice preparation. However, the *in vivo* approach used here helps in discriminating the regulation of dopamine neurons by systemic and local administrations of SB-277011A. In addition, a major advantage of *in vivo* extracellular recordings when compared with intracellular recordings is that stable healthy extracellular measurements can be made over long periods (hours). These characteristics are desirable within studies on the physiology and pharmacology of the VTA, given that the great majority of studies on the physiology and pharmacology of the VTA have utilized intracellular microelectrode or patch clamp recordings.

Recently, extracellular field potential recordings have been made from the VTA in horizontal brain slices and by using 6-hydroxydopamine lesions, a large reduction of the field potentials was obtained, further supporting the findings that VTA field potentials are largely generated by the postsynaptic responses of dopamine neurons [40]. Thus, in accord with another recent *in vitro* work from Nugent and coll. [14] the present data propose extracellular field potential recording as a technically straightforward approach to analyse electrophysiological properties of VTA neurons. Furthermore, the present data support previous proposals that extracellular field potentials recorded *in vivo* from the VTA are also possible, and that this may make it feasible to link more directly *in vivo* and *in vitro* findings [13, 41]. Hence, while not excluding the possibility that an active metabolite of SB-277011A could be present in the brain area studied, our *in vivo* data propose that the effect of SB-277011A on VTA neurons cell firing seems to be not [only] mediated by a putative metabolite but is probably mainly related to the presence of SB-277011A itself in brain. This is in accord with the good blood brain barrier penetration of SB-

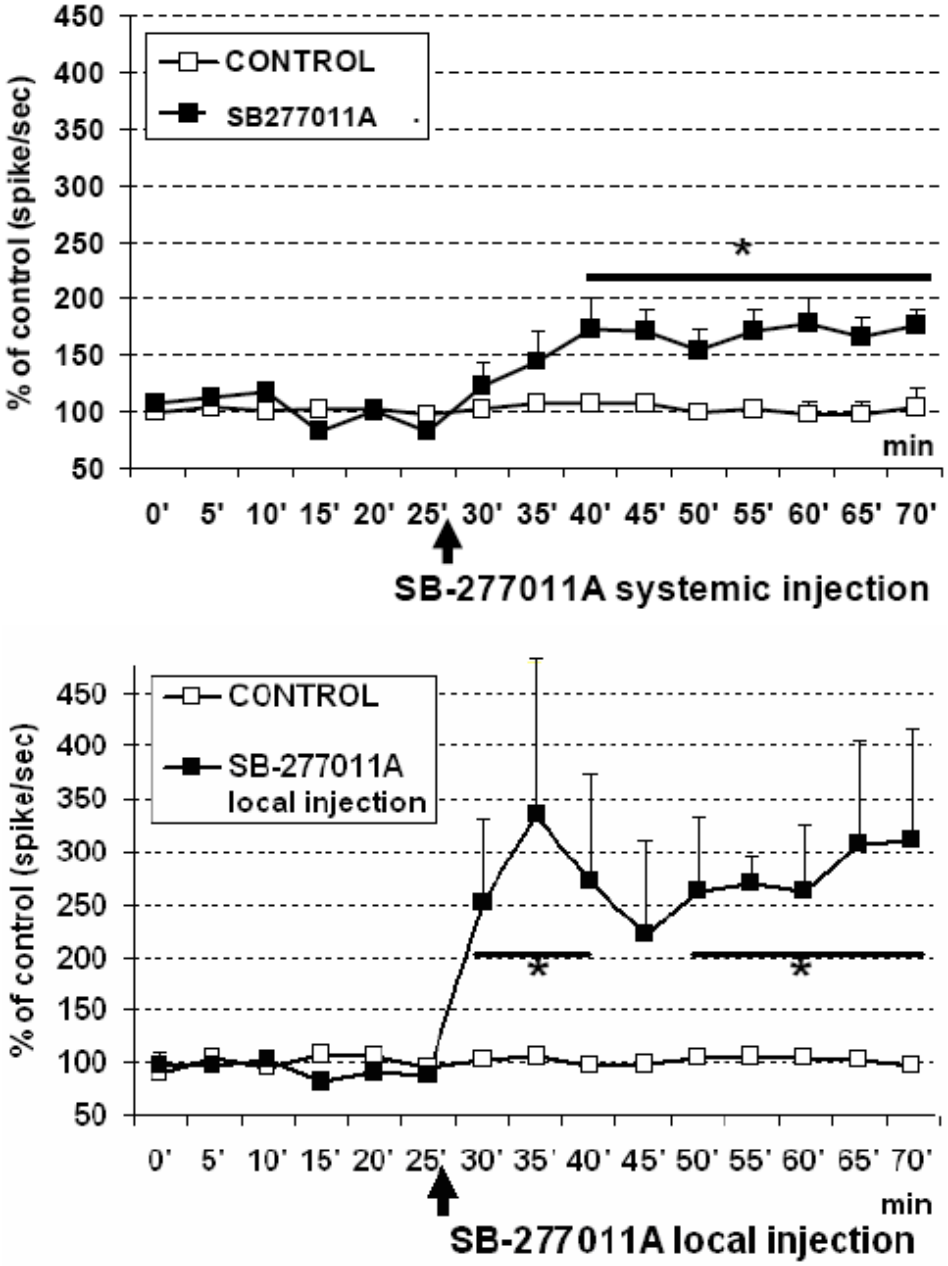

Fig. (2). Effect of systemic (10mg/kg, i.p., upper panel) and local intra-VTA (2.5µg/µl) treatment with SB-277011A. Close symbols show treatments with SB-277011A whereas open symbols are vehicle-treated animals (cavasol 10%, 2ml/kg i.p. or 1µl in VTA, respectively). Data are presented as percent of control (pre-injection values) n = 5 each treatment ± S.D. *Post-hoc* Dunnet t-test was applied: * $p<0.05$ versus vehicle.

277011A (cerebral to blood ratio of 3.6:1 [11]) therefore supporting the findings that its action is mainly mediated by central antagonist at the D3 receptor either in man [39] and rat [11].

Together with the DA system, the endogenous cannabinoid [CB] system has been recently implicated in the modulation of addictive behaviour and in the mechanism of action of different drugs of abuse [35]. In addition, the participation of the CB system in the patho-physiology of neuropsychiatric disorders involving DA and other neurotrasmitters has been introduced [37].

Among the various functions modulated by the endocannabinoid system [42], the control of emotions and the regulation of motivational behaviour appear to be of particular importance for the possible implication of this system in the pathogenesis of mental disorders such as drug addiction, depression, anxiety, and psychoses [for a review see ref. 43]. Furthermore, there is considerable evidence that endogenous cannabinoids can directly modulate the cerebral DA system [44] and it has been shown that CB1 receptor activation increases the activity of dopaminergic neurons in the ventral tegamental area (VTA), thereby increasing DA release in the nucleus accumbens [45]. Thus, there appears to be a direct link between dopaminergic activity in the nucleus accumbens and activation of the cannabinoidergic signaling system. It has also been demonstrated that the common property of drugs of abuse to facilitate the mesolimbic

dopaminergic transmission involves activation of CB_1 receptors [46]. Consequently, CB1 antagonists seem to exert their suppressive effects on abuse by blocking the effects of endogenously released endocannabinoids from mesencephalic neurons [47]. In line with this hypothesis, ethanol-induced dopamine release is lacking in CB1 receptor knock-out mice [48, 49]. Additionally, these mice showed reduced alcohol self-administration [12], alcohol-induced place preference [50] and alcohol-induced DA release in the nucleus accumbens [49]: *i.e.* the target region of the mesolimbic DA system originating in the VTA and involved in the neural processing triggering drug addiction [51].

Regulation of DA functions by cannabinoids is further supported by several biochemical and behavioural studies. *In vivo* experiments suggest that chronic treatment with D2-receptor antagonists up-regulate CB1receptor expression in the rat striatum [52]. Furthermore, a DA D2-receptor antagonist has been shown to attenuate the alcohol-induced formation of 2-arachidonoyl glycerol, (2-AG: the second CB discovered in brain and non-neural organs [53, 54]) and in cerebellar granular neurons [55]. In addition, the hyperactivity associated with postsynaptic D2-receptor activation is accompanied by a dramatic increase in the fatty acid amide, N-arachidonoyl ethanolamide (AEA or anandamide, the first endocannabinoid [EC] to be identified [56]) output within the striatum and this effect is potentiated by the CB1 receptor antagonist SR141716A [57].

Intriguingly, the present data parallel a recent work showing that the effect of the endogenous cannabinoid anandamide that increases neurotransmission in the mesolimbic DA reward system *via* cannabinoid CB1 receptor activation, can be markedly potentiated by enzymatic blockade of its metabolism [58].

Accordingly, the finding that the endocannabinoid 2-AG is released by midbrain DA neurons under both physiological synaptic activity to modulate afferent inputs and pathological conditions such as ischemia [for a review see ref 59] supports the direct correlation of these two systems in brain functions and dysfunctions and in particular in DA system dysfunctions involved in neuropsychiatric disorders such as schizophrenia, psychoses, and drug addiction.

It has also been shown that the endocannabinoid system can alter DA transmission through trans-synaptic mechanisms involving gamma-aminobutyric acid (GABA)-ergic and glutamatergic synapses and by assembling signal transduction cascades of the cannabinoid and DA receptors. In particular, CB signalling may lead to release of DA, which can operate *via* DA D1-like receptors as a negative feedback mechanism to offset the effects of activation of the CB1 receptor. Conversely, dopaminergic signalling *via* DA D2-like receptors may lead to up-regulation of CB signalling, which may correspond to a negative feedback on DA signalling [for a review see ref. 36]. All these considerations taken together with our present results and with our previous data [8] strongly support the proposed mutual control between dopamine and endocannabinoid systems [36] and imply the possibility of an influence of D3 antagonism upon endogenous cannabinoids. Indeed, it has been proved effective in diminishing nicotine [5], cocaine [6] and alcohol [2] self-administration and reinstatement of drug taking behaviours in rodents, *i.e.* ending in suppressive effects on abuse as well as CB1 antagonists.

Further work is needed to evaluate such a possibility as therapeutics that regulate CB receptors or interfere with the synthesis and degradation of endocannabinoids, or interfere with the post receptor signalling events would be of considerable interest in the potential treatment of abuse.

In conclusion, all these findings indicate that parallel selected pharmacological manipulations involving CB1 receptor functions and DA central system activities may open new therapeutic avenues in the treatment of addiction [*i.e.* alcoholism] as well as neuropsychiatric disorders.

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Received: April 17, 2009 Revised: July 12, 2009 Accepted: July 14, 2009