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CANNABINOIDS INHIBIT EXCITATORY NEUROTRANSMISSION IN THE SUBSTANTIA NIGRA PARS RETICULATA

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Abstract—The substantia nigra pars reticulata belongs to the brain regions with the highest density of CB_1 cannabinoid receptors. Since the level of CB_1 receptor messenger RNA is very low in the pars reticulata, most of the receptors are probably localized on terminals of afferent axons. The hypothesis was tested that terminals of glutamatergic afferents of substantia nigra pars reticulata neurons possess CB_1 cannnabinoid receptors, the activation of which presynaptically modulates neurotransmission.

Rat midbrain slices were superfused and the electrophysiological properties of substantia nigra pars reticulata neurons were studied with the patch-clamp technique. Focal electrical stimulation in the presence of bicuculline evoked excitatory postsynaptic currents mediated by α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate glutamate receptors. The excitatory postsynaptic currents were reduced by the metabotropic glutamate receptor agonist (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (trans-ACPD; 10^{-4} M). The mixed CB_1/CB_2 cannabinoid receptor agonists R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]-pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone (WIN55212-2; 10^{-8}–10^{-5} M) and (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940; 10^{-6} M) also produced inhibition. The maximal inhibition by WIN55212-2 was 54 ± 6%. The CB_1 cannabinoid antagonist *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A; 10^{-6} M) prevented the effect of WIN55212-2, but had no effect when superfused alone. WIN55212-2 (10^{-6} M) increased the amplitude ratio of two excitatory postsynaptic currents evoked with an interstimulus interval of 100 ms. Currents evoked by short ejection of glutamate on to the surface of the slices were not changed by WIN55212-2.

The results show that activation of CB_1 cannabinoid receptors inhibits glutamatergic synaptic transmission between afferent axons and neurons in the substantia nigra pars reticulata. The lack of effect of the cannabinoids on glutamate-evoked currents and the increase of the paired-pulse ratio indicate that the mechanism of action is presynaptic inhibition of transmitter release. © 2000 IBRO. Published by Elsevier Science Ltd.

Key words: cannabinoid receptor, catalepsy, EPSC, extrapyramidal motor system, glutamate, patch clamp.

Two G-protein-coupled receptors have been identified as primary targets of the active components of *Cannabis sativa*. The CB_1 cannabinoid receptor is located mainly on neurons, whereas the CB_2 receptor is located on peripheral non-neuronal cells.[5,16,28] The distribution of CB_1 receptors in the CNS is well characterized.[12,15,18,23,47] Nuclei of the extrapyramidal motor system, i.e. corpus striatum, globus pallidus, substantia nigra pars reticulata (SNR) and the subthalamic nucleus, all contain medium to high densities of CB_1 receptors. In accordance with the presence of CB_1 receptors in these nuclei, cannabinoids can impair the function of the extrapyramidal motor system, causing catalepsy.[5] The mechanism of the cataleptic effect is not known.

The aim of the present study was to clarify the function of CB_1 receptors in the SNR, a nucleus with an especially high density of CB_1 receptors. In contrast to the high density of receptors, the level of CB_1 receptor mRNA is very low in the SNR,[23,25] indicating that the majority of the receptors is not synthesized in the SNR itself, but is brought there by axonal transport. Most of the CB_1 receptors in the SNR are probably located on endings of striatonigral GABAergic neurons, since the perikarya of these neurons, the medium spiny neurons in the striatum, produce CB_1 receptor mRNA,[23,25] and since the CB_1 receptor level in the SNR decreases strongly when medium spiny neurons are damaged experimentally or during the disease process of Huntington's chorea.[13,14] The likely role of these receptors is presynaptic inhibition of GABA release from terminals of striatonigral neurons.[3]

Another part of the CB_1 receptors in the SNR may be located on terminals of afferent glutamatergic axons. The SNR receives its excitatory neuronal input from the subthalamic nucleus and, to a lesser degree, the cortex,[8] and subthalamic nucleus neurons and cortical projection neurons produce CB_1 mRNA, indicating synthesis of the receptor.[23,25] It is possible that, in analogy to the striatonigral GABAergic neurons, these receptors are transported to the axon endings in the SNR, where their activation can change the release of glutamate. In the present experiments we tested the hypothesis that activation of CB_1 cannabinoid receptors presynaptically influences excitatory neurotransmission between SNR neurons and their afferent input. Since the knowledge on excitatory neurotransmission in the SNR is limited,[26] the experiments were begun by characterizing this process.

EXPERIMENTAL PROCEDURES

The experiments conformed to the rules of the German law regulating

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Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; AP5, DL-2-amino-5-phosphonopentanoic acid; BSA, bovine serum albumin; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CP55940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; DMSO, dimethylsulfoxide; EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; EPSC, excitatory postsynaptic current; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; NMDA, *N*-methyl-D-aspartate; PRE, initial reference value; QX-314, *N*-ethyl-lidocaine Br; SNR, substantia nigra pars reticulata; SR141716A, *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; *trans*-ACPD, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid; *trans*-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; WIN55212-2, R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone.

the use of animals in biomedical research (Tierschutzgesetz). All efforts were made to minimize both the suffering and the number of animals used.

Patch-clamp recording from neurons in brain slices was performed as described by Edwards and Konnerth[7] and Stuart *et al.*[43] (for further details see Ref. 45).

Superfusion of brain slices

The medium for storage and superfusion of brain slices was artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 126, NaH_2PO_4 1, KCl 3, $MgCl_2$ 1, $CaCl_2$ 2.5, $NaHCO_3$ 26.2, glucose 10, pH 7.3–7.4 (after the solution was gassed with 95% O_2/5% CO_2). Three hundred-micrometer-thick coronal slices were cut with a vibrating tissue slicer from brains of eight- to 15-day-old Wistar rats; the substantia nigra was identified in three or four coronal slices, obtained rostrally from the pons. The slices were stored at 20–24°C until superfusion started up to 8 h later. For patch-clamp recording, slices were fixed at the glass bottom of a superfusion chamber with a nylon grid on a platinum frame, and superfused at 20–24°C at a flow rate of 1.3 ml/min.

Patch-clamping

The slices were trans-illuminated with red light ($\lambda_{max} = 780$ nM) and viewed with a Zeiss Axioskop FS microscope (Zeiss, Göttingen, Germany) equipped with a water-immersion lens (×40, 0.75 W Ph 2) and differential interference-contrast optics. The microscope was connected to a video camera sensitive to infrared light (Newvicon C 2400-07-C; Hamamatsu, Herrsching, Germany). Pipettes were pulled from borosilicate glass (2 mm outer diameter, 0.3 mm wall thickness) and had a resistance of 3–5 MΩ when filled with intracellular solution. Patch-clamp recordings were obtained with an EPC-9 amplifier (List, Darmstadt, Germany) and evaluated with the TIDA for Windows software (HEKA Elektronik, Lambrecht, Germany). Series resistance compensation of 50–60% was applied. Voltage values were not corrected for liquid junction potential, except where indicated. In most cases, data were filtered at 2.9 kHz and recorded at a sampling rate of 5 kHz. For characterization of neurons, an intracellular solution of the following composition was used (mM): K gluconate 145, $CaCl_2$ 0.1, $MgCl_2$ 2, HEPES 5, EGTA 1.1, ATP–Mg 5, GTP–Tris 0.3, pH 7.4 (adjusted with KOH).

Recording of excitatory postsynaptic currents and glutamate-evoked currents

ACSF containing bicuculline 20 μM was used for superfusion in order to suppress spontaneous and evoked fast GABAergic neurotransmission. The composition of the intracellular solution was (mM): CsCl 107, CsF 35, $MgCl_2$ 1, HEPES 10, EGTA 10, ATP–Na_2 4, *N*-ethyl-lidocaine Br (QX-314) 2, pH 7.4 (adjusted with CsOH). Cs^+ and *N*-ethyl-lidocaine were used to prevent potassium and fast sodium currents, respectively. Excitatory postsynaptic currents (EPSCs) were elicited at a holding potential of −60 mV with a stimulating pipette (filled with superfusion buffer) inserted 20–40 μm into the slice at a horizontal distance of 30–100 μm from the recorded neuron. Rectangular electrical pulses were delivered by an isolated stimulator. The strength of stimulation (20–50 μs pulse width, ≤100 V pulse amplitude) was adjusted to obtain EPSCs of 50–100 pA amplitude. In some experiments, currents were evoked by pressure ejection of glutamate (1 mM) from a pipette positioned about 20 μm above the surface of the slice. Pressure pulses (100 ms pulse width, 35–70 kPa amplitude) were delivered by a Picopump 820 (World Precision Instruments, Berlin, Germany). Series resistance was measured before and after recording of EPSCs and glutamate-evoked currents, and experiments were discarded when input resistance increased by more than 100% or decreased by more than 50%.

Experimental protocol, data evaluation and statistics

Recordings started 15 min after establishment of the whole-cell configuration. Zero time in the figures is the time when recording began. EPSCs were elicited every 15 s. Glutamate-evoked currents were elicited every 60 s.

Recordings lasted up to 30 min. Slices were superfused initially with control solution for 5 min. Either drugs or control solutions were then superfused. Because it is difficult to wash out cannabinoids from brain slices, no further recording was carried out in a brain slice after a cannabinoid experiment. Values were evaluated in three steps. At first, currents measured during the initial 5 min were averaged and this average is referred to as PRE (initial reference value). In the second step, currents measured in consecutive 1-min periods (four EPSCs), 2.5-min periods (10 EPSCs) or 5-min periods (20 EPSCs in paired-pulse experiments, five glutamate-evoked currents) were averaged. In the third step, these averages were expressed as percentages of PRE.

Means ± S.E.M. are given throughout. Non-parametric statistical tests were used. Differences between groups and within groups (only in a few experiments) were evaluated with the two-tailed Mann–Whitney and Wilcoxon signed rank tests, respectively. Where necessary, the Bonferroni correction was applied. $P < 0.05$ was taken as the limit of significance and only this level is indicated even if P was <0.01 or 0.001.

Materials

Drugs were obtained from the following sources. Alamone (Jerusalem, Israel): *N*-ethyl-lidocaine Br (QX-314); Pfizer (Groton, CT, USA): (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940); RBI (Köln, Germany): R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate (WIN55212-2); Sanofi (Montpellier, France): *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide HCl (SR141716A); Sigma (Deisenhofen, Germany): ATP–Mg, ATP–Na_2, bicuculline, bovine serum albumin (fatty acid free), tetrodotoxin and GTP–Tris; and Tocris Cookson (Bristol, England): (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (trans-ACPD), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), DL-2-amino-5-phosphonopentanoic acid (AP5) and L-*trans*-pyrrolidine-2,4-dicarboxylic acid (trans-PDC).

The cannabinoid ligands WIN55212-2, CP55940 and SR141716A were dissolved in dimethylsulphoxide (DMSO). Stock solutions were stored at −20°C. Dilutions were made with superfusion buffer containing 1 g/l bovine serum albumin (BSA; in order to keep cannabinoids in solution and to prevent adsorption to the tubing); the final concentration of DMSO was 1 ml/l. Control solutions always contained the appropriate concentration of DMSO and BSA. CNQX and bicuculline were dissolved in DMSO. Baclofen, AP5, glutamate, quinpirole, trans-PDC and tetrodotoxin were dissolved in distilled water, whereas trans-ACPD was dissolved in 0.05 M NaOH. Stock solutions were stored at −20°C. Further dilutions were made with superfusion buffer.

RESULTS

Characterization of substantia nigra pars reticulata neurons

The substantia nigra was identified in three or four coronal slices obtained rostrally from the pons. At low microscopic magnification, the substantia nigra pars compacta appeared as a narrow band running in a ventromedial-dorsolateral direction. The SNR was considered to be the ovoid region ventrolateral from the pars compacta. The regions could be easily identified with infrared video microscopy at high magnification. The neurons in the pars compacta were densely packed and had a round-ovoid shape (Fig. 1, lower right panel). In the SNR, the density of neurons was much lower, and usually the neurons had the form of an elongated triangle or were fusiform (Fig. 1, upper left panel).

Electrophysiological properties of SNR neurons were studied using pipettes filled with the potassium gluconate-based intracellular solution. Series resistance and cell resistance were 22 ± 2 MΩ and 561 ± 96 MΩ, respectively ($n = 10$). When recorded in the current-clamp mode (at 0 pA holding current), the majority of SNR neurons fired spontaneously and regularly (see Fig. 1, lower left panel); the mean firing rate of all neurons was 3.1 ± 0.8 Hz ($n = 10$). The membrane potential was calculated as the mean of the potentials measured at peak afterhyperpolarization and at action potential threshold; it was -58 ± 2 mV

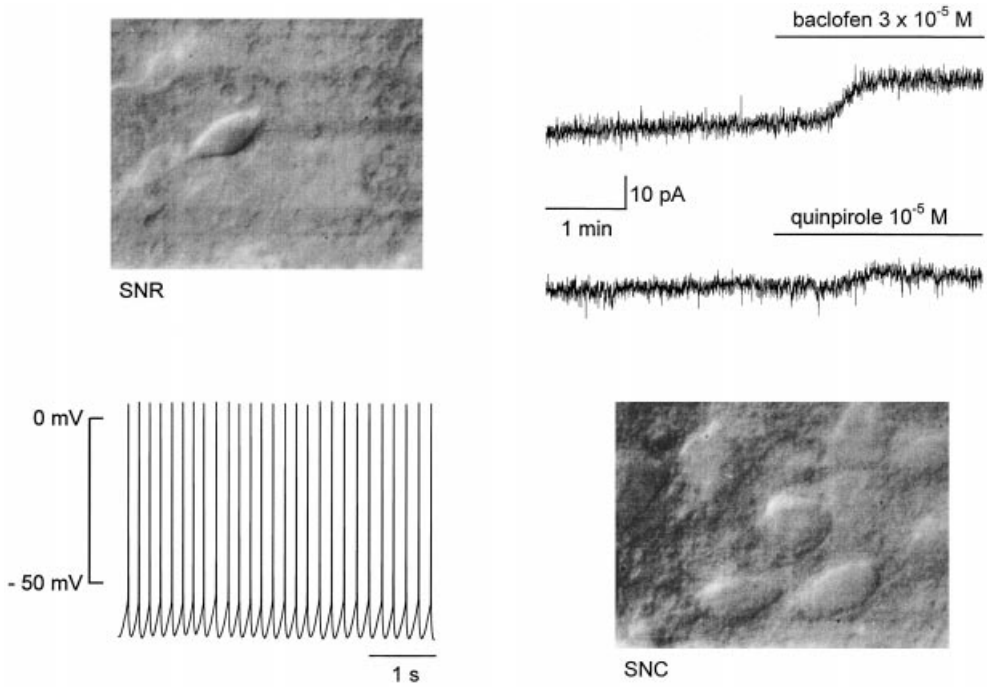

Fig. 1. Properties of substantia nigra pars reticulata (SNR) neurons. (Upper left panel) Infrared video microscopic image of an SNR neuron. (Lower left panel) Membrane potential recording (current-clamp mode; holding current 0 pA) in the SNR neuron shown in the upper left panel. (Upper right panel) Effect of quinpirole (10^{-5} M) and baclofen (3×10^{-5} M) on the SNR neuron shown in the upper left panel (voltage-clamp mode; holding potential −50 mV). (Lower right panel) infrared video microscopic image of pars compacta (SNC) neurons is shown for comparison.

($n=10$) (corrected for liquid junction potential). When studied in the voltage-clamp mode (at −50 mV holding potential), superfusion with the D_2/D_3 dopamine receptor agonist quinpirole (10^{-5} M) had no effect on the resting membrane current in most neurons (Fig. 1, upper right panel); on average, a weak outward current of 7 ± 2 pA amplitude ($n=10$) was evoked. Superfusion with the $GABA_B$ receptor agonist baclofen (3×10^{-5} M) elicited an outward current with a mean amplitude of 19 ± 5 pA ($n=10$) (Fig. 1, upper right panel). The observed properties of SNR neurons resemble those described previously.[38,42]

Characterization of evoked postsynaptic currents

EPSCs were elicited every 15 s by applying single electrical pulses of 20–50 μs width and ≤100 V amplitude with a pipette in the vicinity of the recorded neuron. This stimulation evoked EPSCs of 59 ± 3 pA amplitude ($n=153$) during the initial reference period (PRE) (see original tracings in Figs 2–5). The amplitude of EPSCs remained constant or decreased slightly during superfusion, for up to 30 min, with buffer containing different solvents (see solvent groups in Figs 2–5). The sodium channel blocker tetrodotoxin (10^{-6} M) strongly reduced the amplitude of EPSCs (not shown): the effect was apparent 1–2 min after the beginning of tetrodotoxin superfusion; after 10 min the EPSC amplitude had decreased to $6\pm3\%$ ($n=8$) of the initial reference value (PRE) (the change was significant; $P<0.05$). The α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate glutamate receptor antagonist CNQX (10^{-5} M) rapidly and completely suppressed the EPSCs; additional superfusion of the *N*-methyl-D-aspartate (NMDA) glutamate receptor antagonist AP5 (2.5×10^{-5} M) had no effect (Fig. 2). In experiments in which AP5 was given alone, it also did not change the peak amplitude of EPSCs (not shown). *Trans*-ACPD (10^{-4} M), an agonist of metabotropic glutamate receptors, lowered the amplitude of EPSCs by approximately 50% (Fig. 3). The selective glutamate uptake inhibitor *trans*-PDC (10^{-4} M) slightly reduced the amplitude of EPSCs (Fig. 4). The EPSC decay rate was not changed by *trans*-PDC (see original tracings in Fig. 4): in the presence of *trans*-PDC, the time constant of EPSC decay was 5.9 ± 1.6 ms ($n=11$), not different from the value determined during PRE, 5.6 ± 1.2 ms ($n=11$) ($P>0.05$).

Effects of cannabinoids on evoked postsynaptic currents

The mixed CB_1/CB_2 cannabinoid receptor agonists WIN55212-2 (10^{-6} M) and CP55940 (10^{-6} M) inhibited EPSCs (Fig. 5). In contrast to the rapid effects of tetrodotoxin, CNQX and *trans*-ACPD, the effect of the cannabinoids developed slowly, at least 15 min was necessary for the full effect. Reversibility of the effect of the cannabinoid agonists was not studied, because the recording would have become too long (note that at the end of the application of the cannabinoids in Fig. 5, at $t=30$ min, the neurons were in the whole-cell configuration already for 45 min). The decrease in EPSC amplitude in the cannabinoid groups was certainly not due to run-down, since there was no run-down in the appropriate solvent control group (Fig. 5). Generally, the effect of cannabinoid agonists is slow in brain slices and the effects are not readily reversible;[1,22,45] the high lipophilicity of the drugs is thought to be responsible for these phenomena.

Figure 6 shows the effects of increasing concentrations of WIN55212-2 (10^{-8}–10^{-5} M). Significant inhibition appeared at 10^{-7} M. The maximum effect was observed at 10^{-6} M; it was an inhibition of the EPSC amplitude by 54%.

Effects of the CB_1 cannabinoid receptor antagonist

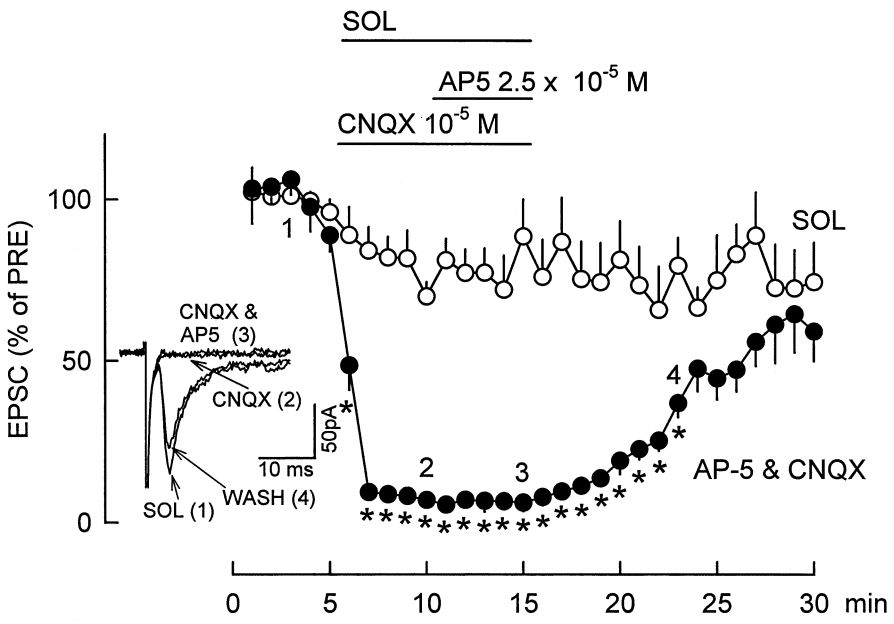

Fig. 2. Effects of CNQX and AP5 on excitatory postsynaptic currents (EPSCs). EPSCs were evoked every 15 s by a single pulse. CNQX (10^{-5} M) was applied from $t = 5$ to $t = 10$ min. CNQX (10^{-5} M) and AP5 (2.5×10^{-5} M) were applied together from $t = 10$ to $t = 15$ min. Slices of the control group were superfused with solvent [SOL; buffer containing DMSO (1 ml/l)] from $t = 5$ to $t = 15$ min. Values measured during the first 5 min were averaged (PRE). Values were averaged every minute (four EPSCs) and these averages were expressed as percentages of PRE. Means ± S.E.M. of six (CNQX and AP5) and seven (SOL) experiments. Significant difference from SOL: $^*P < 0.05$. (Inset) Original tracings from an experiment with CNQX and AP5; tracings were obtained at time-points 1–4.

SR141716A are shown in Fig. 7. Given alone, SR141716A (10^{-6} M) did not change the amplitude of EPSCs (Fig. 7, upper panel). In slices receiving SR141716A (10^{-6} M), the inhibitory effect of WIN55212-2 (10^{-6} M) on EPSC amplitude was abolished (Fig. 7, lower panel).

Effects of cannabinoids on postsynaptic currents evoked by paired-pulses

In order to study the mechanism of the inhibition of EPSCs by cannabinoids, effects on EPSCs elicited by pairs of electrical pulses were studied. As a first step, the influence of the interpulse interval on EPSC amplitudes was studied. At intervals of 10, 30, 100, 300, 1000 and 3000 ms, the ratio of currents elicited by the second and first pulses (paired-pulse ratio) was 1.32 ± 0.16, 1.70 ± 0.18 (responses to the second and first pulses were significantly different; $P < 0.05$), 1.39 ± 0.11 (responses to the second and first pulses were significantly different; $P < 0.05$), 1.09 ± 0.08, 0.91 ± 0.05 and 0.95 ± 0.04 ($n = 24$), respectively. Thus, facilitation was observed when the second pulse followed the first pulse within 30 and 100 ms.

The effect of WIN55212-2 was studied using an interpulse interval of 100 ms (Fig. 8). During the initial reference period (PRE), the first and second pulses elicited EPSCs with 53 ± 8 pA and 70 ± 11 pA amplitude, respectively; the paired-pulse ratio was 1.51 ± 0.20 ($n = 19$). In control experiments in which solvent was superfused, the amplitudes of the first and second pulses (Fig. 8, panel A) and the paired-pulse ratio (Fig. 8, panel B) decreased slightly. In experiments in

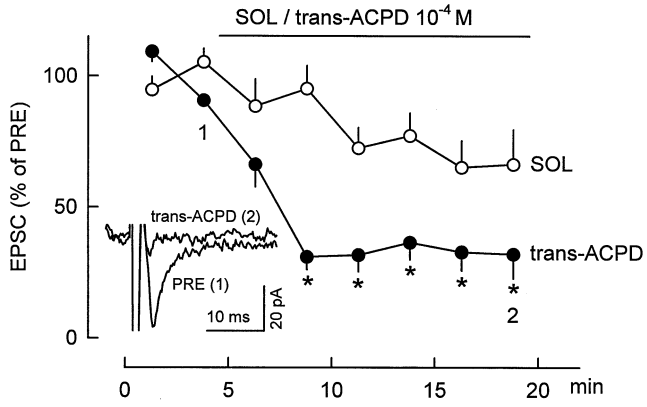

Fig. 3. Effect of *trans*-ACPD on excitatory postsynaptic currents (EPSCs). EPSCs were evoked every 15 s by a single pulse. *Trans*-ACPD (10^{-4} M) was applied from $t = 5$ to $t = 20$ min. Slices of the control group were superfused with solvent (SOL; buffer containing NaOH [10^{-4} M]) from $t = 5$ to $t = 20$ min. Values measured during the first 5 min were averaged (PRE). Values were averaged every 2.5 min (10 EPSCs) and these averages were expressed as percentages of PRE. Means ± S.E.M. of 11 (*trans*-ACPD) and 15 (SOL) experiments. Significant difference from SOL: $^*P < 0.05$. (Inset) Original tracings from an experiment with *trans*-ACPD; tracings were obtained at time-points 1 and 2.

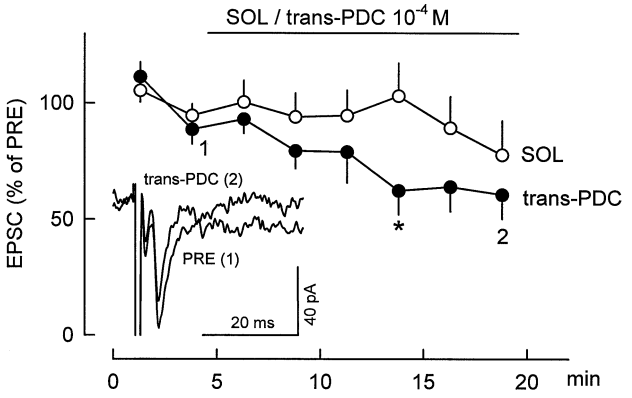

Fig. 4. Effect of *trans*-PDC on excitatory postsynaptic currents (EPSCs). EPSCs were evoked every 15 s by a single pulse. *Trans*-PDC (10^{-4} M) was applied from $t=5$ min to $t=20$ min. Slices of the control group were superfused with solvent [SOL; buffer containing distilled water (10 ml/l)] from $t=5$ min to $t=20$ min. Values measured during the first 5 min were averaged (PRE). Values were averaged every 2.5 min (10 EPSCs) and these averages were expressed as percentages of PRE. Means ± S.E.M. of 11 (*trans*-PDC) and seven (SOL) experiments. Significant difference from SOL: $^*P<0.05$. (Inset) Original tracings from an experiment with trans-PDC; tracings were obtained at time-points 1 and 2.

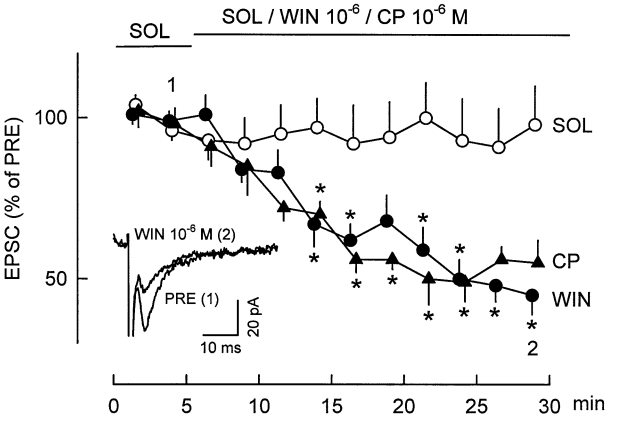

Fig. 5. Effect of WIN55212-2 (WIN) and CP55940 (CP) on excitatory postsynaptic currents (EPSCs). EPSCs were evoked every 15 s by a single pulse. Slices of the WIN and CP groups were superfused with solvent [SOL; buffer containing DMSO (1 ml/l) and BSA (1 g/l)] until $t=5$ min; WIN (10^{-6} M) and CP (10^{-6} M) were applied from $t=5$ to $t=30$ min. Slices of the control group were superfused with SOL during the whole experiment. Values measured during the first 5 min were averaged (PRE). Values were averaged every 2.5 min (10 EPSCs) and these averages were expressed as percentages of PRE. Means ± S.E.M. of six (WIN), six (CP) and 10 (SOL) experiments. Significant difference from SOL: $^*P<0.05$. (Inset) Original tracings from an experiment with WIN; tracings were obtained at time-points 1 and 2.

which WIN55212-2 (10^{-6} M) was superfused, the amplitudes of the first and second pulses decreased significantly (Fig. 8, panels A and C), confirming the observations shown in Fig. 5. More importantly, WIN55212-2 significantly increased the paired-pulse ratio (Fig. 8, panels B and D).

Effects of cannabinoids on currents evoked by ejection of glutamate

Currents were elicited by pressure ejection (35–70 kPa for 100 ms) of glutamate (10^{-3} M) from a pipette in the vicinity of the recorded neuron. During the initial reference period

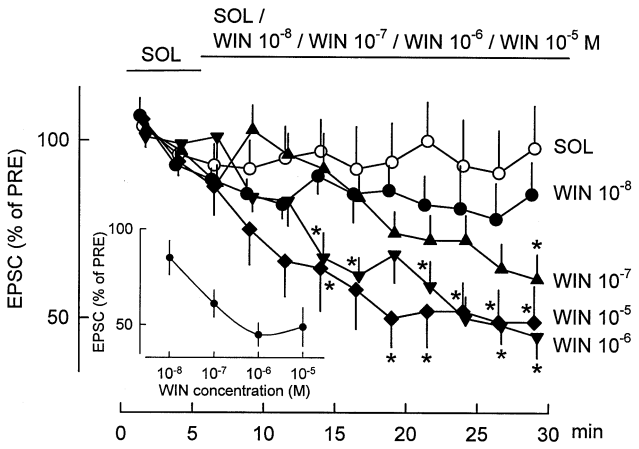

Fig. 6. Concentration-dependent effect of WIN55212-2 (WIN) on excitatory postsynaptic currents (EPSCs). EPSCs were evoked every 15 s by a single pulse. Slices of the WIN groups were superfused with solvent [SOL; buffer containing DMSO (1 ml/l) and BSA (1 g/l)] until $t=5$ min; WIN (10^{-8} M, 10^{-7} M, 10^{-6} M or 10^{-5} M) was applied from $t=5$ to $t=30$ min. Slices of the control group were superfused with SOL during the whole experiment. Values measured during the first 5 min were averaged (PRE). Values were averaged every 2.5 min (10 EPSCs) and these averages were expressed as percentages of PRE. Means ± S.E.M. of 10 (WIN 10^{-8} M), eight (WIN 10^{-7} M), seven (WIN 10^{-6} M), eight (WIN 10^{-5} M) and 10 (SOL) experiments. Significant difference from SOL: $^*P<0.05$. (Inset) Concentration–response relationship; values measured at the end of the 30-min superfusion were used.

(PRE), the amplitude of the glutamate-evoked currents was 87 ± 6 pA ($n=32$). The NMDA glutamate receptor antagonist AP5 (2.5×10^{-5} M) reduced the amplitude of glutamate-evoked currents by about 50% (Fig. 9). Combined superfusion with AP5 (2.5×10^{-5} M) and the AMPA/kainate glutamate receptor antagonist CNQX (10^{-5} M) nearly abolished the glutamate-evoked currents (Fig. 9). The selective glutamate uptake inhibitor trans-PDC (10^{-4} M) caused an approximately four-fold increase in the amplitude of glutamate-evoked currents (Fig. 10).

The cannabinoid receptor agonist WIN55212-2 (10^{-6} M), superfused for 25 min, did not change the amplitude of currents evoked by ejection of glutamate (Fig. 11).

DISCUSSION

Two major groups of neurons have been identified in the SNR with neuroanatomical and electrophysiological techniques.[8,26,38,42] The more numerous population consists of GABAergic neurons which are the main output neurons of the extrapyramidal motor system. The smaller population (less than 15% of all neurons) consists of dopaminergic neurons belonging functionally to the neighbouring pars compacta. Our experimental conditions were optimized for measuring EPSCs (potassium and sodium channels in the recorded neurons were blocked), and this prevented classification of the individual neurons before or after recording EPSCs. Therefore, the results refer to all SNR neurons. Since the GABAergic and the dopaminergic SNR neurons receive excitatory innervation from the same sources, of which the subthalamic nucleus is the most important,[8,26] it is likely that presynaptic modulatory effects on the glutamatergic input of the GABAergic and dopaminergic SNR neurons are similar.

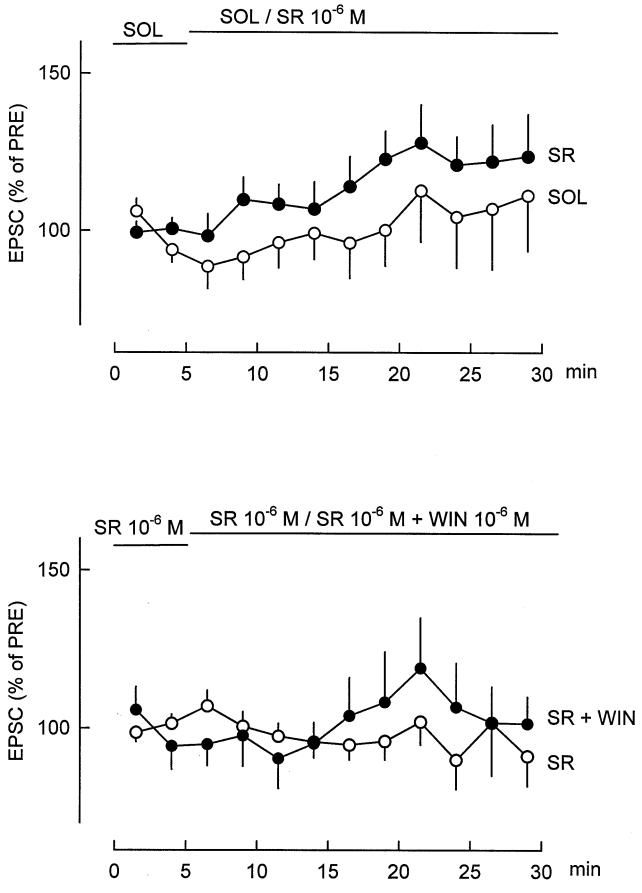

Fig. 7. Effect of SR141716A (SR) and interaction of WIN55212-2 (WIN) with SR on excitatory postsynaptic currents (EPSCs). EPSCs were evoked every 15 s by a single pulse. (Upper panel) One group of slices was superfused with solvent [SOL; buffer containing DMSO (1 ml/l) and BSA (1 g/l)] until $t = 5$ min; SR (10^{-6} M) was applied from $t = 5$ to $t = 30$ min. Slices of the control group were superfused with SOL during the whole experiment. (Lower panel) One group of slices was superfused with SR (10^{-6} M) until $t = 5$ min; SR (10^{-6} M) and WIN (10^{-6} M) were applied together from $t = 5$ to $t = 30$ min. Slices of the control group were superfused with SR (10^{-6} M) during the whole experiment. Values measured during the first 5 min were averaged (PRE). Values were averaged every 2.5 min (10 EPSCs) and these averages were expressed as percentages of PRE. Means ± S.E.M. of 10 (SR, upper panel), 10 (SOL), eight (SR + WIN) and eight (SR, lower panel) experiments. There was no significant difference between the groups in the upper panel and in the lower panel, respectively.

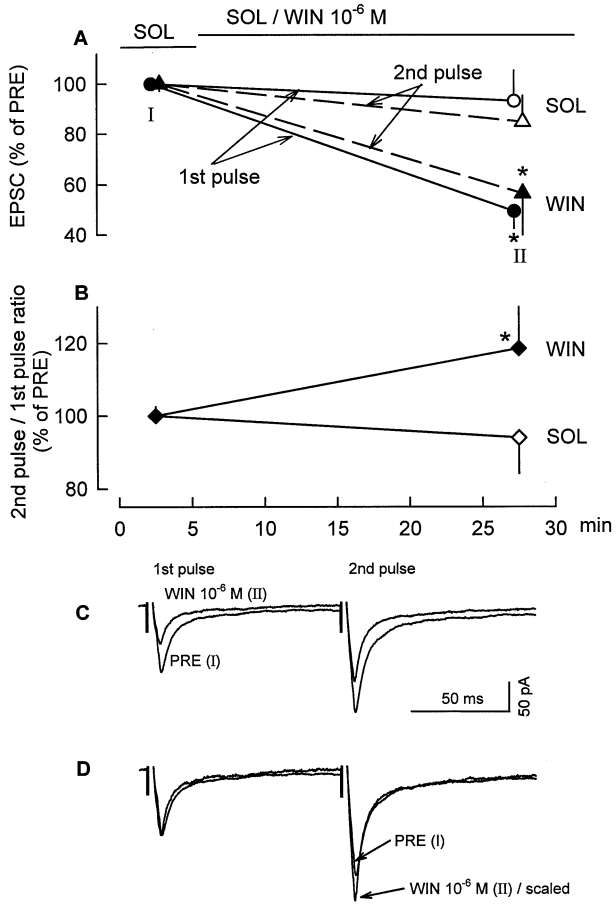

Fig. 8. Effect of WIN55212-2 (WIN) on excitatory postsynaptic currents (EPSCs) evoked by pairs of electrical pulses. From $t = 0$ to $t = 5$ min and from $t = 25$ to $t = 30$ min, EPSCs were evoked every 15 s by pairs of pulses; the interpulse interval was 100 ms. Slices of the WIN group were superfused with solvent [SOL; buffer containing DMSO (1 ml/l) and BSA (1 g/l)] until $t = 5$ min; WIN (10^{-6} M) was applied from $t = 5$ to $t = 30$ min. Slices of the control group were superfused with SOL during the whole experiment. (A) Amplitude of the currents elicited by the 1st and 2nd pulses. (B) Ratios of currents elicited by the 2nd and 1st pulses. Values measured between $t = 0$ and $t = 5$ (20 EPSCs) were averaged (PRE). Values measured between $t = 25$ and $t = 30$ min (20 EPSCs) were also averaged and the averages were expressed as percentages of PRE. Means ± S.E.M. of 11 (WIN) and eight (SOL) experiments. Significant difference from SOL: $^*P < 0.05$. (C) Original tracings from an experiment with WIN; tracings were obtained at time points I and II (see panel A); in the presence of WIN, the current elicited by the first pulse was 58% of the current elicited by the first pulse during PRE. (D) The curve obtained in the presence of WIN was scaled with the factor 1/0.58 so that the peak currents elicited by the first pulse during PRE and in the presence of WIN appeared identical; the difference between the amplitudes of the second pulses illustrate the enhancement of the paired-pulse ratio by WIN.

Glutamatergic neurotransmission in the substantia nigra

The electrically-evoked synaptic currents studied by us in the substantia nigra had the typical properties of glutamatergic synaptic currents. The evoked currents were dependent on action potential propagation. Under our recording conditions (−60 mV holding potential, 1 mM extracellular Mg^{2+} concentration), they were mediated by AMPA/kainate glutamate receptors, as in many other brain regions (see e.g., Araki *et al.*[2] and Keller *et al.*[20]; for review see Collingridge and Lester[4]). In experiments in which glutamate was pressure-ejected, the contribution of NMDA receptors to the current increased to about 50%; one possible reason is that glutamate simultaneously activated AMPA/kainate and NMDA receptors in regions of the neurons in which voltage control was insufficient.

The metabotropic glutamate receptor agonist *trans*-ACPD depressed glutamatergic transmission in the substantia nigra, as it did in several other brain regions.[6,31] The mechanism is probably presynaptic inhibition of glutamate release due to activation of metabotropic glutamate autoreceptors. Such receptors are likely to be present on terminals of glutamatergic afferents in the SNR, since subthalamic nucleus neurons, which are the major source of the glutamatergic innervation of SNR neurons, synthesize mRNA for group I (mGluR1, mGluR5) and group II (mGluR2, mGluR3) metabotropic glutamate receptors.[46]

Glutamate uptake mechanisms seem to play a similar role in glutamatergic neurotransmission in the substantia nigra as in the hippocampus and cerebellum.[24,35] *Trans*-PDC, an inhibitor of several types of glial and neuronal glutamate

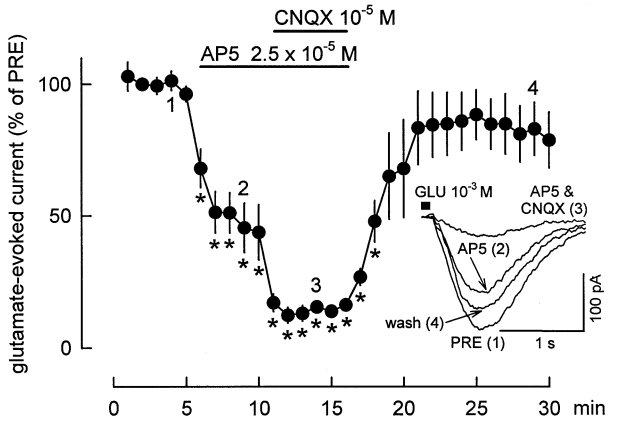

Fig. 9. Effects of AP5 and CNQX on currents evoked by pressure ejection of glutamate. Currents were evoked every 60 s by ejection of glutamate (10^{-3} M) from a pipette in the vicinity of the recorded neuron. AP5 (2.5×10^{-5} M) was applied from $t=5$ to $t=10$ min. AP5 (2.5×10^{-5} M) and CNQX (10^{-5} M) were applied together from $t=10$ to $t=15$ min. Values measured during the first 5 min were averaged (PRE) and values were expressed as percentages of PRE. Means ± S.E.M. of six experiments. Significant difference from PRE: $^*P<0.05$. (Inset) Original tracings obtained at time-points 1–4.

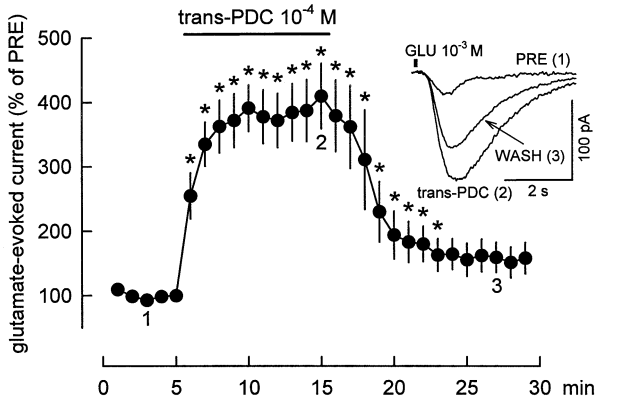

Fig. 10. Effect of *trans*-PDC on currents evoked by pressure ejection of glutamate. Currents were evoked every 60 s by ejection of glutamate (10^{-3} M) from a pipette in the vicinity of the recorded neuron. *Trans*-PDC (10^{-4} M) was applied from $t=5$ min to $t=15$ min. Values measured during the first 5 min were averaged (PRE) and values were expressed as percentages of PRE. Means ± S.E.M. of nine experiments. Significant difference from PRE: $^*P<0.05$. (Inset) Original tracings obtained at time-points 1–3.

transporters,[10] did not enhance the amplitude and duration of EPSCs but decreased the amplitude slightly. Possibly, glutamate transporters are not localized within the synapse or they are too slow in comparison with the kinetics of AMPA/kainate receptors (which mediate EPSCs in the SNR, see above); therefore, they do not influence the maximal activation of synaptic AMPA/kainate receptors by released glutamate and the decay rate of EPSCs. The mechanism for the small decrease in EPSC amplitude produced by *trans*-PDC is probably the one proposed by Maki *et al.*:[24] *trans*-PDC increases the extrasynaptic glutamate concentration which in turn leads to activation of inhibitory presynaptic metabotropic glutamate receptors. *Trans*-PDC strongly enhanced the current evoked by pressure ejection of glutamate, verifying the effectivity of *trans*-PDC in our preparation. More importantly, this latter observation indicates that uptake is important at determining the concentration of glutamate (and hence

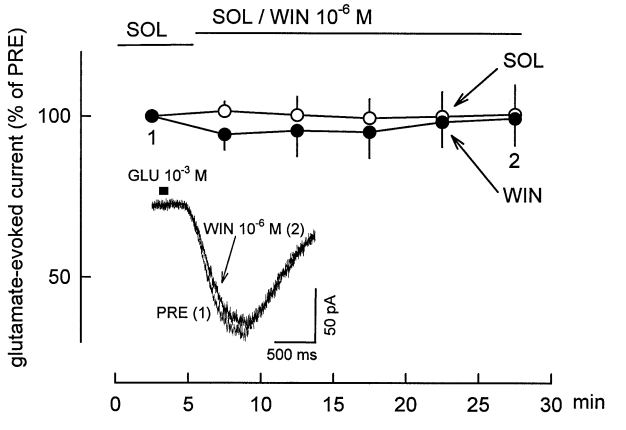

Fig. 11. Effect of WIN55212-2 (WIN) on currents evoked by pressure ejection of glutamate. Currents were evoked every 60 s by ejection of glutamate (10^{-3} M) from a pipette in the vicinity of the recorded neuron. Slices of the WIN group were superfused with buffer containing solvent [SOL; buffer containing DMSO (1 ml/l) and BSA (1 g/l)] until $t=5$ min; WIN (10^{-6} M) was applied from $t=5$ to $t=30$ min. Slices of the control group were superfused with SOL during the whole experiment. Values measured during the first 5 min were averaged (PRE). Values were averaged every 5 min (five glutamate-evoked currents) and these averages were expressed as percentages of PRE. Means ± S.E.M. of eight (WIN) and nine (SOL) experiments. (Inset) Original tracings from an experiment with WIN; tracings were obtained at time-points 1 and 2.

the response to glutamate) in the vicinity of extrasynaptic glutamate receptors.

Cannabinoids presynaptically inhibit glutamatergic neurotransmission in the substantia nigra

WIN55212-2 and CP55940 belong to different chemical classes, and the common property of the two drugs is their agonist activity at CB_1 and CB_2 cannabinoid receptors.[9,28,41] WIN55212-2 is devoid of affinity for a great number of neurotransmitter receptors and ion channels.[21] The reduction of EPSC amplitudes by WIN55212-2 and CP55940, therefore, points to an activation of cannabinoid receptors. Antagonism of the effect of WIN55212-2 by the selective CB_1 cannabinoid receptor antagonist SR141716A[9,28,32,41] indicates that activation of CB_1 cannabinoid receptors was responsible for the inhibition of EPSCs. Given alone, SR141716A had no effect on the EPSC amplitude, ruling out the possibility that CB_1 receptors were activated by an endogenous agonist under our experimental conditions.

Several observations and arguments support the view that the reduction in EPSC amplitude caused by activation of CB_1 receptors is due to presynaptic inhibition of glutamate release from terminals of afferent axons. First, WIN55212-2 did not change the effects of pressure-ejected glutamate on SNR neurons. Second, WIN55212-2 increased the paired-pulse ratio, which is usually considered to support a presynaptic mode of action of drugs (see e.g., Levenes *et al.*,[22] Shen and Johnson,[38] and Stuart and Redman[44]). Third, since cesium was included in the intracellular solution, a postsynaptic cannabinoid effect on SNR neurons, mediated by potassium currents, is unlikely. Fourth, the presynaptic inhibition of glutamate release from terminals of afferent axons is in accord with the localization of cannabinoid receptors. As already mentioned in the Introduction, CB_1 cannabinoid receptors are synthesized in subthalamic nucleus neurons and in cortical projection neurons, the sources of the glutamatergic afferents

of the SNR. On the other hand, no or very few cannabinoid receptors are synthesized in the SNR neurons themselves. The exact mechanism of the presynaptic inhibition by cannabinoids was not identified in the present study. A likely mechanism is inhibition of voltage-dependent calcium channels, which is the best documented electrophysiological effect of cannabinoids (for references see Ref. 48). At the concentrations used in the present study, cannabinoids do not inhibit voltage-dependent sodium channels, and hence, action potential propagation.[1,22,45]

The results of a recent study suggest that cannabinoids may inhibit glutamatergic neurotransmission in the SNR also *in vivo*: in animals in which the firing rate of SNR neurons was enhanced by microinjection of bicuculline into the subthalamic nucleus, systemically injected WIN55212-2 lowered the firing rate of SNR neurons.[34]

Presynaptic inhibition: a general effect of cannabinoids

It has recently been shown that cannabinoids presynaptically inhibit glutamatergic synaptic transmission between hippocampal neurons in culture[39,40] and between parallel fibers and Purkinje cells in the cerebellum.[22] Our observation is the second example that cannabinoids presynaptically inhibit neurotransmission in an identified *in situ* glutamatergic synapse.

Evidence is accumulating that cannabinoids act presynaptically to inhibit the release of several other neurotransmitters, in addition to glutamate. Thus, in the CNS, cannabinoids suppress GABAergic neurotransmission in the corpus striatum[45] and in the rostral ventromedial medulla oblongata.[49] The electrically-evoked release of [^{3}H]acetylcholine[11] and [^{3}H]GABA in the hippocampus,[19] the electrically-evoked release of [^{3}H]noradrenaline in several brain regions[37] and the evoked release of dopamine in the retina[36] are inhibited as well. Cannabinoids decrease neurotransmitter release also in the peripheral nervous system. Thus, presynaptic inhibition was observed in the vas deferens and ileum,[30] urinary bladder[29] and heart.[17] The plasma noradrenaline concentration was strongly lowered by cannabinoids in pithed rabbits with electrically stimulated sympathetic outflow, indicating that the noradrenaline release was inhibited in many peripheral tissues.[27] It seems that presynaptic inhibition of neurotransmitter release is a typical cannabinoid action in the central and peripheral nervous system.

CONCLUSION

The present study shows that activation of CB_1 cannabinoid receptors presynaptically inhibits glutamatergic neurotransmission in the SNR. This identifies the role of a subpopulation of the large number of cannabinoid receptors found in this nucleus. The major source of the glutamatergic input of SNR neurons, the subthalamonigral pathway, plays an inhibitory role in motor control (see Ref. 50). Therefore, inhibition of this pathway in the SNR by cannabinoids cannot explain the catalepsy elicited by these drugs *in vivo*. On the contrary, it was suggested that inhibition of the subthalamonigral pathway by cannabinoids could be beneficial in Parkinson's disease.[33]

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