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Title

Nigral GABAergic inhibition upon mesencephalic dopaminergic cell groups in rats.

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Abstract

Synaptic inhbition from the substantia nigra pars reticulata (SNr) to the mesencephalic dopaminergic neurons which was mediated by gamma (γ)-amino-butyric acid (GABA) was investigated in the midbrain slice preparation of Wistar rats. Whole-cell patch-clamp recording was used to record synaptic potentials/currents from the dopaminergic neurons (n = 120) located in the retrorubral field (n = 23), the substantia nigra pars compacta (n =68), and the ventral tegmental area (n = 29). In the presence of ionotrophic glutamate receptor antagonists, electrical stimulation of the SNr induced inhibitory postsynaptic potentials (IPSPs) and/or currents (IPSC) in 105 neurons. The IPSPs/IPSCs were composed from early and late components. The early IPSCs were mediated by chloride currents through $GABA_A$ receptors. The late IPSCs were mediated by potassium currents through $GABA_B$ receptors. Both $GABA_A$- and $GABA_B$-IPSPs were amplified by repetitive stimuli with frequencies between 50 and 200 Hz, which are equivalent to firing frequencies of SNr neurons *in vivo*. It was observed that an application of $GABA_B$ receptor antagonist increased the amplitude of the $GABA_A$-IPSPs. The amplification was followed by a rebound depolarization that induced transient firing of dopaminergic neurons. These properties of the IPSPs were common in all the three dopaminrgic nuclei.

These results suggest that postsynaptic $GABA_A$- and $GABA_B$-inhibition contribute to transient and persistent alternations of the excitability of dopaminergic neurons, respectively. These postsynaptic mechanisms may be, in turn, regulated by presynaptic $GABA_B$-inhibition. We conclude that nigral GABAergic input may provide the temporospatial regulation of the background excitability of mesencephalic dopaminergic systems.

Introduction

Mesencephalic dopaminergic (DA) neurons are located in the substantia nigra pars compacta (SNc), the ventral tegmental area (VTA) and the retrorubral field (RRF) (Hillarp *et al.*, 1966). The DA neurons in the SNc contributed to the control of movements via nigrostriatal pathway (Beckstead *et al.*, 1979; Lynd-Balta & Haber, 1994a). On the other hand, DA neurons in the VTA are thought to be involved in the cognitive (Gurden *et al*., 1999) and emotional functions (Proshansky *et al*., 1974; Broekkamp *et al*., 1979; Le Moal & Simon, 1991) through mesocorticolimbic pathways (Beckstead *et al*., 1979; Swanson, 1982). DA neurons in the RRF project to the striatum (Vertes, 1984; Gerfen *et al*., 1987; von Krosigk *et al.*, 1992). Neurons in the RRF also project to the pontomedullary reticular formation, and they are assumed to contribute to the orofacial movements (von Krosigk *et al*., 1992).

It has been demonstrated that an application of GABA receptor agonists or antagonists altered the activity of the mesencephalic DA neurons (Engberg *et al*., 1993; Nissbrandt & Engberg, 1996; Paladini & Tepper, 1999b; Erhardt & Engberg, 2000). These results suggest that GABAergic inputs would play an important role as a regulatory system of the mesencephalic DA neurons. Neuroanatomical studies have shown that the DA neurons in the SNc receive GABAergic inputs from the basal

ganglia via the striatonigral (Somogyi *et al*., 1981) and pallidonigral (Smith & Bolam, 1990) pathways. On the other hand, the VTA and RRF receive inputs predominantly from the nucleus accumbens (Nauta *et al*., 1978; Groenewegen & Russchen, 1984; Haber *et al*., 1990) and the pallidum (von Krosigk et al., 1992; Groenewegen et al., 1993). Electrophysiological studies (Grace & Bunney, 1979, 1985a, c; Hajos & Greenfield, 1994; Häusser & Yung, 1994; Paladini *et al*., 1999a) have suggested that DA neurons in the SNc also received GABAergic inhibitory input from the substantia nigra pars reticulata (SNr). Häusser & Yung (1994) have shown, in the guinea pig slice preparation, that the intranigral (SNr-SNc) pathway predominantly activates $GABA_A$ receptors, while the striatonigral and the pallidonigral fibers activate both $GABA_A$ and $GABA_B$ receptors.

However, following two points have not yet been substantiated. First, whether DA neurons in the VTA and RRF receive the GABAergic inhibition from the SNr as has been demonstrated in DA neurons in the SNc (Hajos & Greenfield, 1994; Häusser & Yung, 1994; Paladini *et al*., 1999a). Second, whether $GABA_A$ and $GABA_B$ receptors contribute to nigral inhibition in DA neurons of these nuclei. The present study is designed to answer the above two questions. A whole-cell patch-clamp recording was applied in DA neurons in *in vitro* rat brain slice preparations, which contained both the

SNr and mesencephalic DA neurons. The effects of electrical stimulation applied to the SNr were investigated on DA neurons in each of the SNc, the VTA and the RRF. Preliminary results have been reported as an abstract (Saitoh *et al*., 2001).

Materials and methods

Experiments were approved by the Committees for Animal Experimentation at the Asahikawa Medical College and at Okazaki National Institute.

Slice preparations

A total of 53 Wistar rats (postnatal day 9 to 23) were deeply anesthetized with diethylether and then decapitated. The brain was quickly removed and submerged immediately in an ice-cold cutting solution which was bubbled with a gas mixture of 95% O_2 and 5% CO_2 from 5 to 8 minutes. The cutting solution contained (in mM): 234 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 10 $MgSO_4$, 0.5 $CaCl_2$, 26 $NaHCO_3$, and 11 glucose. Midbrain slices (400 μm thick) were then prepared as described previously (Isa *et al*., 1998) along the parasagittal and coronal plane so as to contain the SNr and mesencephalic dopaminergic nuclei. A microslicer (Microslicer, DTK 2000, Dosaka EM, Kyoto, Japan) was used to cut the slices, which were then incubated in artificial cerebrospinal fluid (ACSF) at room temperature for more than 1 hour before recording. Parasagittal sections were used for recordings of all the RRF-, SNc- and VTA-DA neurons (Fig. 3A, B), and coronal sections for recordings of both the SNc- and VTA-DA neurons (Fig. 3C). The ACSF contained (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 25 glucose. The ACSF was continuously

bubbled with a gas mixture of 95% O_2 and 5% CO_2, and the pH was adjusted to 7.4. After incubation, a slice was mounted in a recording chamber and continuously superfused with the ACSF at the rate of 2 to 3 ml/min by peristaltic pumps (Minipulse 3, Gilson, Villiers, France) at room temperature.

Whole-cell patch-clamp recording

Activity of DA neurons were recorded using a whole-cell patch-clamp technique (Edwards *et al*., 1989). The patch pipettes were guided with micromanipulators under visual control to contact neurons in the slice using video images. Video images were obtained with an IR-CCD camera (C2400-79H, Hamamatsu photonics, Hamamatsu, Japan) attaced to an upright microscope (Axioskop FS, Zeiss, Gottingen, Germany or Eclipse E600FN, Nikon, Tokyo, Japan) with the use of a 40 × water immersion objective. An EPC-7 (HEKA, Lambrecht, Germany) or Axoclamp 200B (Axon Instruments, Foster City, CA) patch-clamp amplifier was used. Patch pipettes were prepared from borosilicate glass capillaries (GC150TF-15, Harvard, Kent, UK) with a micropipette puller (P-97, Sutter Instrument, Novato, CA). The pipettes were filled with a solution containing (in mM): 160 K-gluconate, 0.2 EGTA, 2 $MgCl_2$, 2 Na_2-ATP, 10 HEPES, 0.1 spermine, and 0.5 Na-GTP (the pH was adjusted to 7.3). Biocytin (0.5 %) was also dissolved in the solution just before recording. Because the liquid junction

potential between the ACSF and the pipette internal solution was estimated to be -15 mV, the actual membrane potential was corrected by this value. The osmolarity of the internal solution was 280-290 mOsm/l. The resistance of the electrodes was from 2.5 to 4.0 MΩ in the bath solution. The series resistance during recording was from 6 to 15 MΩ and was routinely compensated by 80-90 %. Bath temperature was maintained at around 27°C (room temperature). In some experiments, the temperature was raised up to 37°C by a temperature controller (Warner instrument corporation, Connecticut, USA). Data were acquired using a pClamp system (Axon Instruments).

Because GABAergic afferents from the striatum (Haber *et al*., 1985; von Krosigk *et al*., 1992; Lynd-Balta & Haber, 1994b; Parent & Hazrati, 1995) and the pallidum (Nauta *et al*., 1978; Haber *et al*., 1985; Groenewegen *et al*., 1993) pass through the area adjacent to the SNr, stimulation of the SNr may activate not only SNr neurons but also these fiber passages by current spread. Therefore we employed six cathodal, concentric bipolar electrodes (Clark Electromedical Instruments, Pangbourne, UK) which were placed in a linear array so that both the inside and outside of the SNr could be stimulated, and we examined the optimal sites for evoking IPSPs in 38 DA neuron as illustrated in Fig. 3. We also determined the appropriate stimulus strength for every neuron (Fig. 2A). The electrical stimulation consisted of a single pulse, and 2 to 50

pulses, with a duration of 0.2 ms and a frequency of 10 to 200 Hz. If stimuli with an intensity of 1 mA failed to induce any inhibitory effects, we ceased any further investigation on that neuron.

Histological procedure

Recorded neurons were visualized by staining with biocytin (Horikawa & Armstrong, 1988). After recording DA neurons, the slices were fixed with 4% paraformaldehyde in 0.12 M phosphate buffer at pH 7.4 for 2 to 3 days at 4°C. The slices were then rinsed in 0.05 M phosphate buffered saline (PBS) at pH 7.4, and incubated in methanol containing 0.6% H_2O_2 for 30 min. After washes with PBS, the slices were incubated in avidin-biotin peroxidase complex solution (1%) (Vector Laboratories, Burlingame, CA) containing 0.3% Triton X-100 for 3 hr. After washes with PBS and with 0.05 M Tris-buffered saline (the pH was 7.6), they were incubated in the Tris-buffered saline solution containing 0.01% diaminobenzidine tetrahydrochloride (DAB) and 0.0003% H_2O_2 for 30 min. These procedures were performed at room temperature. The slices were mounted on gelatin-coated slides and were coverslipped.

Drugs

The DL-2-amino-5-phosphonovaleric acid (AP-5), bicuculline methiodide and biocytin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). The 6-cyano-2,

3-dihydroxy-7-nitro-quinoxaline-2, 3, dione (CNQX) and

P-3-aminopropyl-P-diethoxymethyl phosphinic acid (CGP 35348) were from

TOCRIS/Nacalai (Kyoto, Japan).

Statistics

Statistical significance was examined using Kruskal-Wallis one-way ANOVA. Significance was accepted at *P* value <0.05.

Results

Identification of mesencephalic dopaminergic neurons

Whole cell recordings were made from 120 DA neurons. Among them, 23 cells were obtained from the RRF, 68 cells from the SNc, and 29 cells from the VTA. DA neurons were identified by the following electrophysiological membrane properties (Kita *et al*., 1986; Grace & Onn, 1989; Johnson & North, 1992; Nedergaard & Greenfield, 1992): i) a prominent voltage sag caused by hyperpolarization-activated current (I_h) in response to hyperpolarizing current pulses, ii) a ramp-like depolarization caused by A-current (I_A), iii) action potentials with a long duration (spike width of more than 2 ms), and iv) a prominent afterhyperpolarization (more than 15 mV) (Fig. 1A). Even membrane potential was artificially depolarized up to around –25 mV, spike discharges were not induced in 27 neurons. However we identified these neurons also as dopaminergic because of the presence of prominent I_h and I_A. As shown in Table 1, there was no significant difference in these electrophysiological properties of DA neurons among the three nuclei. Figure 1B shown an example of a biocytin-labeled DA neuron in the SNc. Generally, DA neurons had a soma of round or fusiform in shape with several primary dendrites as previously reported (Kita *et al*., 1986; Grace & Onn, 1989).

General features of inhibitory effects on mesencephalic dopaminergic neurons induced by SNr stimulation

In the presence of ionotropic glutamate receptor antagonists, CNQX (5 μM) and AP-5 (25 μM), stimulation of the SNr induced inhibitory postsynaptic effects on DA neurons. A representative example is shown in Fig. 1C. Single stimuli induced the inhibitory postsynaptic potential (IPSP) in an SNc-DA neuron. As the number of stimulus pulses applied in the SNr was increased up to 20 pulses, amplitude of the IPSPs was increased. Moreover, the repetitive stimulus trains also induced the second component of IPSP with a late time to peak (denoted by open arrowhead). Among 120 neurons, stimulation of the SNr induced both the early and late inhibitory effects in 105 neurons.

As can be seen in Fig. 2Aa, the amplitude of the inhibitory postsynaptic currents (IPSCs), which were recorded in the voltage clamp condition, increased as the stimulus strength was increased. An onset latency and a time to peak of the IPSCs were 5 ms and 8 ms, respectively. The amplitude of the IPSCs was mostly saturated if a stimulus strength of more than 400 μA was delivered (Fig. 2Ab). Thus the stimulus strength less than 400 μA was used for the analysis of this neuron. The onset latency of the IPSCs (denoted by filled bar) remained constant in response to each stimulus pulse when either the strength (Fig. 2Aa) or the frequency of the SNr stimuli was altered (Fig. 2B).

Therefore, the inhibitory effects were considered to be monosynaptically evoked from the SNr.

Then we estimated the optimal nigral stimulus sites for evoking inhibitory postsynaptic effects. The analysis was made on 38 DA neurons, in which the intranigral stimuli induced both early and late IPSPs (Fig. 3). Among them, 12 neurons were obtained from the RRF, 12 neurons from the SNc, and 14 neurons from the VTA. As illustrated in the left column (a) in Fig. 3A-C, a linear array of six electrodes was used to stimulate the inside and outside of the SNr (denoted by closed and open arrowheads, respectively). The distance between the tip of each adjacent electrode was 500 μm. Three types of slices were prepared. (Fig.3, left column a): the medial plane of parasagittal slices for RRF-DA neurons (A), the lateral plane of parasagittal slices for SNc-DA neurons (B), and the coronal slices for VTA-DA neurons (C). There are dual pathways from the striatum and pallidum to the mesencephalon (Haber et al., 1985). One pathway stems from the caudate, putamen and external segment of the globus pallidus, and descends through the cerebral peduncle. (Haber *et al.*, 1985; von Krosigk *et al*., 1992; Lynd-Balta & Haber, 1994b; Parent & Hazrati, 1995). Another pathway from the ventral striatum and the ventral pallidum passes through the region dorsal to the SNc (Nauta *et al*., 1978; Haber *et al*., 1985; Groenewegen *et al*., 1993; Lynd-Balta

& Haber, 1994b). Each pathway descends through the internal capsule, and then separate at the rostral part of the SNr. The former advances into the cerebral peduncle and turn dorsally to the SNc, and reach to the RRF. The latter passes through the region dorsal to the SNc and reaches to the VTA, the SNc and the RRF. Thus the array of electrodes was arranged so as to stimulate not only intranigral neurons but also striatal and pallidal efferent fibers to the mesencephalic DA neurons. In the medial parasagittal slices (A) and the coronal slices (C), the electrodes were placed on the cerebral peduncle (#1), the middle part of the SNr (#2), the dorsal part of the SNr (#3), and the mesencephalic reticular formation (#4, #5 and #6). In the lateral parasagittal slices (B), the electrodes were placed: the inside of the SNr (#1 and #2), along the internal capsule (#3, #4 and #5), and on the pallidum (#6). Representative sets of recordings are shown in the middle column (Fig. 3Ab, Bb, Cb). It was observed that stimulation of the SNr induced both early and late IPSPs in all three DA neuronal groups. However, extranigral stimulation did not induce prominent inhibitory effects in each example. As shown in the right column (Fig. 3Ac, Bc, Cc), the amplitude of both the early (filled circles) and the late (open squares) IPSPs evoked by intranigral stimuli are larger than those evoked by extranigral stimuli. These findings suggest that optimal sites for evoking both the early and late IPSP in DA neurons in each nucleus were located inside

the SNr. The following examinations were, thus, made on the inhibitory effects which were evoked by stimuli applied to the middle part of the SNr.

Involvement of $GABA_A$ and $GABA_B$ receptors in the SNr-induced inhibitory effects

Next, we examined how $GABA_A$ and $GABA_B$ receptors were involved in the generation of early and late inhibitory effects. In Fig. 4A, both early and late IPSCs were evoked by train pulses of stimuli in a DA neuron recorded from the VTA. (Fig. 4Aa-1). An application of a selective $GABA_A$ receptor antagonist, bicuculline (20 μM), abolished the early IPSC (Fig. 4Aa-2), but the late IPSC remained. The late IPSC was, however, diminished by a further application of a selective $GABA_B$ receptor antagonist, CGP 35348 (100 μM) (Fig. 4Aa-3). Accordingly net early IPSC was obtained by subtracting a trace 2 from a trace 1, and net late IPSC was obtained by subtracting a trace 3 from a trace 2 (Fig. 4Ab). In another DA neuron recorded in the SNc, an application of CGP 35348 (100 μM) diminished the late IPSC (Fig. 4Ba). These findings suggest that the early and late IPSCs are mediated by $GABA_A$ and $GABA_B$ receptors, respectively.

It is worth noting that the early IPSC was increased in amplitude (Fig. 4Ba). The IPSC evoked by single stimuli also increased after the application of CGP 35348 (Fig. 4Bb). Such an amplification of the early IPSCs after CGP 35348 application was

observed in all DA neuron groups (n = 25: 4 in RRF, 15 in SNc, 6 in VTA). The amplification could be induced by a blockage of $GABA_B$ receptors which were located at the presynaptic GABAergic fibers terminating on DA neurons.

The locations of the DA neurons, which received the nigral inhibitory effects, were plotted on parasagittal (n = 65, Fig. 5Aa) and coronal slices (n = 40, Fig. 5Ab). Frequency of occurrence of $GABA_A$- and $GABA_B$-inhibition induced by the SNr stimulation is shown in Fig. 5B. In 105 DA neurons, $GABA_A$-inhibition was observed in every neuron. Among them, 22 were in the RRF, 59 in the SNc and 24 in the VTA. On the other hand, $GABA_B$-inhibition was detected in 17 neurons in the RRF, 45 in the SNc and 19 in the VTA. There was no DA neuron exhibiting only $GABA_B$-inhibition. It was observed that DA neurons exhibiting only $GABA_A$-inhibition and those exhibiting both $GABA_A$- and $GABA_B$-inhibition were intermingled within each nucleus (Fig. 5A). Following analyses were restricted to neurons exhibiting both $GABA_A$- and $GABA_B$-inhibition.

Characteristics of GABAA- and $GABA_B$-inhibition in DA neurons

(1) Reversal potentials

Reversal potentials of the early and late IPSCs were examined (Fig. 6). Single stimuli in the presence of CGP 35348 (100 μM) induced early IPSCs (Fig. 6A). The polarity of

the IPSCs was reversed at the membrane potential between –75 mV and –85 mV (Fig. 6Aa). On the other hand, late IPSCs, which were induced by repetitive stimuli in the presence of bicuculline (20 μM), were reversed in polarity at the membrane potential between –100 mV and –110 mV (Fig. 6Ab). Figure 6B shows the current-voltage relationship of early (denoted by filled circles) and late IPSCs (denoted by open circles) obtained from these neurons. The reversal potentials of the early and late IPSCs were estimated to be –83.9 ± 5.4 mV (mean ± S.D; n =13), and –104.4 ± 4.1 mV (mean ± S.D; n = 7), respectively (Fig. 6B). According to the Nernst equation in our experimental condition, the values of the equilibrium potential for Cl^- ions and K^+ ions are calculated to be -88.4 mV (a filled arrowhead) and -104.5 mV (an open arrowhead), respectively. These results suggest that the early IPSCs are mediated by the Cl^- ion conductance linked to $GABA_A$ receptors, and the late IPSCs are attributed to the K^+ ion conductance linked to $GABA_B$ receptors. In the rest of this text, we refer to the early IPSC/IPSP/inhibition as $GABA_A$-IPSC/IPSP/inhibition and the late IPSC/IPSP/inhibition as $GABA_B$-IPSC/IPSP/inhibition.

(2) Changes in SNr stimulus frequency

Next we investigated the effects of the changes in stimulus frequency on $GABA_A$- and $GABA_B$-IPSPs (Fig. 7). Repetitive stimulus pulses were applied to the SNr for a period

of 600 ms with various frequencies between 10 and 200 Hz (Fig. 7Aa, from the top to bottom traces). In a DA neuron obtained from the SNc, postsynaptic potentials were recorded in the control condition (5 μM CNQX and 25 μM AP-5; Fig. 7Aa-1 in each trace), in the presence of 20 μM bicuculline (Fig. 7Aa-2), and in the presence of both 20 μM bicuculline and 100 μM CGP 35348 (Fig. 7Aa-3). Net $GABA_A$- and $GABA_B$-IPSPs were arithmetically isolated, and were superimposed in each set of recordings (Fig. 7Ab). Figure 7B shows the changes in the peak amplitude of each IPSP of 24 trials from four DA neurons in relation to the changes in stimulus frequency. The amplitude of $GABA_A$-IPSPs increased as the stimulus frequency was increased up to 200 Hz. However, the amplitude of the $GABA_B$-IPSPs maximally increased at a stimulus frquency of 50 Hz. A further increase in the stimulus frequency rather reduced the amplitude (Fig. 7B).

(3) Changes in bath temperature

It is worth noting that isolated $GABA_A$-IPSPs induced by high frequency repetitive stimulation sometimes showed biphasic trajectories as demonstrated Fig. 7Ab. This phenomenon was prominent when the stimulus frequency was increased up to 50 Hz and a period of stimulation was over 300 ms (data not shown). One of possible mechanisms for this phenomenon is following: after $GABA_A$-IPSPs faded, remaining

excessive GABA without being took in by GABA transporter could activate $GABA_A$ receptors again. Because GABA uptake mechanism strongly depends on the temperature (Iversen & Neal, 1968), we tested the effect of bath temperature in five neurons to determine whether the $GABA_B$-IPSPs were caused by the reduction of the GABA transporter activity because of the low bath temperature. Representative results are illustrated in Fig. 8, where $GABA_B$-IPSPs were recorded in the control condition with bath temperature of 27°C (A) and heated bath temperature up to 37°C (B). In control, $GABA_B$-IPSPs had an amplitude of 5 mV, a peak latency of 400 ms, and a duration of about 800 ms. When the bath temperature was heated up to 37°C, the amplitude increased to 7 mV. However, time course of the IPSPs was unchanged. This result suggests that reduction in activity of GABA transporters was not the major cause of induction of the $GABA_B$-IPSPs.

SNr-induced modification of the excitability of DA neurons

As a last step of this study, we tried to elucidate how SNr stimulation modified the excitability of DA neurons through $GABA_A$ and $GABA_B$ receptors.

(1) Contribution of pre- and postsynaptic $GABA_B$ receptors to SNr-induced IPSPs

As we demonstrated in Fig. 4B, an application of CGP 35348 (100 μM) revealed the effects of presynaptic $GABA_B$ receptors upon SNr-induced inhibition. Thus we tested

how presynaptic $GABA_B$ receptors, in addition to postsynaptic $GABA_B$ receptors, contribute to generate SNr-induced IPSPs in accordance with the changes in stimulus frequency. Repetitive stimulus pulses were applied to the SNr for a period of 600 ms with various frequencies between 10 and 200 Hz (Fig. 9Aa, from the top to bottom traces). As the example shown in Fig. 9A, the peak amplitude (*) and duration (**) of the IPSPs were estimated before (a) and after (b) application of CGP 35348. In each condition, the amplitude of the early IPSP was increased as the stimulus frequency was incresed. However, it is obvious that the amplification was more prominent after CGP application than the control condition. On the other hand, the duration of the IPSP became longer as the stimulus frequency was increased up to 100 Hz in the control (Fig. 9Aa). The duration was, however, greatly reduced after application of CGP 35348 (Fig. 9Ab).

The alterations in the shape of the IPSP described above were observed in all DA neurons examined (n = 9). The results are summarized in Fig. 9B and 9C. Stimulus frequency dependent augmentation of the peak amplitude was more prominent after blockage of $GABA_B$ receptors by CGP 35348 (Fig. 9B). The change of duration, however, according to stimulus frequency was diminished after blockage of $GABA_B$ receptors (Fig. 9C). These findings suggest that presynaptic $GABA_B$ receptors may

regulate the excitability of DA neurons by inhibiting the $GABA_A$-IPSPs. On the other hand, postsynaptic $GABA_B$ receptors contribute to the prolonged suppression of the excitability of DA neurons.

(2) Modulation of firing property

Finally, we examined how $GABA_A$- and $GABA_B$-IPSPs modulate the firing property of DA neurons. An example is shown in Fig. 10. Repetitive stimulation induced an IPSP, which was followed by a rebound depolarization (indicated by an open arrowhead in Fig. 10A). Rebound depolarization is presumably due to I_h and low-threshold calcium spike as described in cerebellum (Aizenman & Linden, 1999). An application of CGP 35348 (100 μM) increased the amplitude but decreased the duration of the IPSP. In addition, the rebound depolarization produced action potentials with a short duration (1.6 to 1.8 ms) and a small amplitude (45 to 50 mV) (Fig. 10B, *1 and *2). Following these spikes, full-action potentials with a long duration (approximatly 3 ms) and a large amplitude (approximately 90 mV) were generated (Fig. 10B, *3). In the presence of bicuculline (20 μM) after washing of CGP 35348, the rebound depolarization following the SNr-induced IPSPs were diminished, and the generation of action potentials was inhibited.

It is important to note that both $GABA_A$- and $GABA_B$-IPSPs were observed in DA

neurons in the RRF, the SNc and the VTA. In addition, following characteristics of SNr-induced IPSPs: reversal potential, responses to the changes in SNr stimulus frequency, and those to the bath temperature, were common to observed in DA neurons of all three nuclei.

Discussion

In the present study, we showed that SNr stimulation induced both $GABA_A$- and $GABA_B$-receptor mediated inhibitory effects on DA neurons not only in the SNc but also in the VTA and the RRF. We first consider the experimental procedures in this study. Secondary, neuronal mechanisms of generating $GABA_A$ and $GABA_B$ IPSPs are discussed. Functional significance of the $GABA_A$ and $GABA_B$ inhibition will be finally considered in view of controlling the mesencephalic DA systems.

Consideration of the experimental procedures

Intranigral stimulations effectively induced both early ($GABA_A$) and late ($GABA_B$) IPSPs in DA neurons in each of the RRF (Fig. 3A) and the VTA (Fig. 3C) in addition to the SNc (Fig. 3B). However, one may consider that intranigral stimuli activate striatonigral and/or pallidonigral fibers by current spread. There is therefore a need to consider that the SNr stimuli selectively activated SNr neurons and not activate fibers of passages adjacent to the SNr. As shown in Fig. 3Bb, stimulation of the SNr (#2) induced late IPSPs, but stimuli of the internal capsule (#3) did not. Even the stimului with a strength of 1 mA was applied through the electrode placed on the internal capsule (#3), the late IPSP was not induced (data not shown). Thus the extent of current spread due to

a maximal stimulus strength (1 mA) was less than 500 μm, which was the distance of adjacent electrode tips. Moreover, we determined an appropriate stimulus strength for every neuron (Fig.2A). Consequently, we deduced that, with this stimulus strength, the current spread was confined mostly to the vicinity of stimulus electrode tip. Based on these findings obtained from detailed mapping studies, we consider that the GABAergic inhibition obtained in our experimental condition would actually originate from the SNr.

The present results demonstrated that $GABA_A$-inhibition was observed in all DA neurons which received inhibitory inputs from the SNr. This finding agrees well that by Häusser & Yung (1994), and that by Paladini *et al.* (1999a), demonstrating that the stimuli of the nigral GABAergic neurons activate $GABA_A$ receptors on the SNc-DA neurons. On the other hand, $GABA_B$-inhibition was observed in approximately 80% of these DA neurons. There was no difference in the occurrence of each inhibitory effect among the three mesencephalic DA nuclei (Fig. 5B). Ng & Yung (2000) have suggested that most DA neurons might have $GABA_B$ receptors in *in vivo* condition. Then, why was $GABA_B$-inhibition not detected in around 20% of DA neurons? Two possibilities come to mind. First, $GABA_B$ receptors could be easily damaged in the process of making slice preparations. In the hippocampus of the rat and guinea pig, Solis & Nicoll (1992) demonstrated that a GABA analogue, nipecotic acid, applied to the soma could evoke

pure $GABA_A$ responses, while that applied to the dendritic region could elicited pure $GABA_B$ responses. This result suggests that $GABA_A$ and $GABA_B$ receptors of hippocampal neurons could localize mainly on soma, and on the dendrites, respectively. If this is the case in the mesencephalic DA neurons, $GABA_B$ receptors on the dendrites of recording cells could be damaged because we visually selected DA neurons which nearly located in the surface of the slice. Alternative possibility is that $GABA_B$ receptors could down- regulated during the postnatal development. Garant *et al*. (1992) showed that nigral $GABA_B$ density was higher in young rats (postnatal day 14 to 17) than that in adult rats. Because we used rats of postnatal day 12 to 24, $GABA_B$ density could be lower in some rats.

Neuronal mechanisms of generating $GABA_A$ and $GABA_B$ inhibition

Häusser & Yung (1994) suggested that $GABA_B$ receptors would operate dominantly as autoreceptors. The present results also argued for the existence of presynaptic $GABA_B$ receptors, i.e., an application of CGP 35348 (100 μM) augmented the early IPSCs as shown in Fig. 4B. Because single stimuli-induced IPSCs were increased in amplitude with an application of CGP 35348 (Fig. 4Bb), the presynaptic $GABA_B$ receptors could be activated so that these might tonically inhibit a release of GABA from the presynaptic terminals.

In our experimental condition, a high frequency repetitive stimulation of the SNr was usually required to evoke $GABA_B$-IPSP, whereas a single stimulation evoked only $GABA_A$-IPSP. In hippocampal neurons, it has been suggested that $GABA_B$ receptors exist mainly on extrasynaptic membrane whereas $GABA_A$ receptors exist under the synaptic cleft, and $GABA_B$-IPSPs may be induced by spillover of GABA from synaptic cleft, which, in turn, activate extrasynaptic $GABA_B$ receptors (Thompson & Gahwiler, 1992; Isaacson *et al*., 1993; Mody *et al*., 1994). We propose that the same mechanism may operate in the mesencephalic DA neurons.

Functional implication of SNr GABAergic inhibition on mesencephalic DA systems

SNr-neurons in freely moving rats discharged with a rate of 20-80 Hz at rest and changed their firing rate between 5 and 120 Hz during movements (Gulley *et al*., 1999). We demonstrated that both $GABA_A$- and $GABA_B$-IPSPs were prominently amplified by SNr stimuli with frequencies between 25-200 Hz (Fig. 7). Such frequency range covers the physiological firing frequency of SNr neurons in vivo. Therefore both $GABA_A$ and $GABA_B$ receptors could operate in *in vivo* condition.

As shown in Fig. 9B, when the SNr was stimulated with a frequency between 10 and 200 Hz, the peak amplitude of IPSPs was gradually increased, and showed maximal value when stimuli with frequencies of more than 50Hz were delivered (control; denoted

by open circles). In the presence of CGP 35348 (100 μM), the peak amplitude of IPSPs increased as the stimulus frequency was increased. This amplification of $GABA_A$-IPSPs in the presence of CGP 35348 could be derived from the inactivation of presynaptic $GABA_B$ receptors. These findings suggest that firing rates of the SNr-GABAergic neurons could regulate the amplitude of the $GABA_A$-IPSPs in the presence of CGP 35348. Following results further suggest that firing frequency of the SNr-GABAergic neurons determine the duration of $GABA_B$-IPSPs. In a control condition, the duration of the IPSPs increases as the stimulus frequency was increased up to 50 Hz. However, such a stimulus frequency dependent changes were subtle in the presence of CGP 35348 (Fig. 9C; denoted by filled circle). Thus the duration of net $GABA_A$-IPSPs would be robust against firing rates of the SNr-GABAergic neurons. The finding that the duration rather decreased when the stimulation with a frequency of 200Hz was applied, could be due to the presynaptic $GABA_B$-inhibition. Based on these considerations, we conclude that $GABA_A$ and $GABA_B$ receptors may play different roles in regulating the excitability of DA neurons. The activation of $GABA_A$ and $GABA_B$ receptors, respectively, would provide the magnitude and the duration of SNr-induced IPSPs according to the firing rate of SNr-GABAergic neurons. Moreover, this temporospatial regulation of the excitability of DA neurons could be modulated by presynaptic $GABA_B$ receptors.

In the alert monkey, a population of SNr neurons ceased firing preceding the initiation of movements (Hikosaka & Wurtz, 1983). Hikosaka et al. (2000) suggest that the movements are initiated by disinhibition from the persistent activity of the SNr neurons. If SNr- GABAergic neurons cease firing, effects of $GABA_A$-IPSPs may be removed quickly and the removal of the $GABA_A$ effects may transiently increase the excitability of DA neurons because of rebound depolarization as shown in Fig. 10B. On the other hand, inhibitory effects via postsynaptic $GABA_B$ receptors may persist for a while even after termination of the firing of SNr neurons. Presynaptic $GABA_B$ receptors, in addition, would partly inhibit excessive $GABA_A$ effects. Thus, $GABA_B$-inhibition possibly contributes to the maintenance of background excitability of the DA neurons at an appropriate level. We propose that the basal ganglia output would regulate the activity of the mesencephalic DA systems by providing these transient and persistent postsynaptic inhibitory effects on DA neurons.

Grace & Bunney (1979, 1985a, b) has suggested that the dual projection system from the striatum to the SNc which control the activity of SNc-DA neurons: one is direct inhibition (striatum-SNc) and the other is indirect activation (striatum-SNr-SNc). The present results provide the evidence of the existence of the dual projection system that control of the excitability of DA neurons in the RRF and

VTA in addition to the SNc. In corporation with GABAergic intranigral connection from the SNr to the SNc, nigral GABAergic projections to the RRF- and VTA-DA neurons would play an important role in the striatal control of mesencephalic DA systems which is thought to be the neural basis of reward system as proposed by Schultz (1998).

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Abbreviations

ACSF, artificial cerebrospinal fluid

AP-5, $_{DL}$-2-amino-5-phosphonovaleric acid

CGP 35348, P-3-aminopropyl-P- diethoxymethyl phosphinic acid

CNQX, 6-cyano-7-nitroquinoxaline-2, 3-dione disodium

DA, dopaminergic

GABA, gamma (γ)-amino-butyric acid

IPSC, inhibitory postsynaptic current

IPSP, inhibitory postsynaptic potential

RRF, retrorubral field

SNc, substantia nigra pars compacta

SNr, substantia nigra pars reticulata

VTA, ventral tegmental area

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Figure legend

Fig.1. Identification of dopaminergic neurons and inhibitory effects on dopaminergic neurons induced by stimulating the substantia nigra pars reticulata. A. Changes in membrane properties of a dopaminergic (DA) neuron in the SNc in response to injection of depolarizing and hyperpolarizing current pulses into the cell. An action potential denoted by an asterisk is also shown in an expanded time scale (inset). B. A lightmicroscopic photograph of a biocytin-stained DA neuron. The neuron had a round shape soma with a diameter of approximately 20 μm and three primary dendrites. C. Inhibitory postsynaptic potentials (IPSPs) induced by stimuli applied to the SNr in the presence of ionotropic glutamate receptors (25 μM AP-5 and 5 μM CNQX). IPSPs induced by single, three, seven and twenty stimulus pulses were superimposed. A single pulse stimulation induced an early IPSP (denoted by filled arrowhead). Trains of stimuli in addition produced late IPSPs, which are indicated by an open arrowhead. The amplitude of the early and late IPSPs was incresed as the number of the stimulus pulses was increased. Each trace is an average of four sweeps.

Fig. 2. Characteristics of the SNr-induced early inhibitory postsynaptic currents (IPSCs). A. Stimulation of the SNr induced early IPSCs in the presence of CNQX (5 μM) and AP-5 (25 μM). (a) The amplitude of the IPSCs increased as the stimulus strength was increased. Each trace is an average of five sweeps. (b) The amplitude of the IPSCs saturated at a stimulation strength of more than 400 μA in this neuron. B. Repetitive pulse stimulation evoked IPSCs with a fixed latency of approximately 5 ms (filled bars). Each trace is an average of four sweeps.

Fig. 3. Effective stimulus sites for evoking inhibitory effects. A-C. The left column (a) illustrated three types of slice preparations and arrangements of the tips of stimulating electrodes. Each type of slices is prepared for the recording of the DA neurons in the RRF (A), SNc (B), and VTA (C). Filled and open arrowheads indicate the locations of electrode tips which were placed on the inside and outside of the SNr, respectively. Open circles in Aa, Ba and Ca show the location of recorded DA neurons in the RRF, SNc and VTA, respectively. The middle column (b) shows a set of membrane responses evoked with stimuli applied to each point (#1 - #6). Amplitudes of early and late IPSP were measured at the latencies indicated by filled circles and open squares, respectively. Each trace is an average of six sweeps. The right column (c) of

A-C shows normalized peak amplitude of early and late IPSPs induced through each electrode (#1-6). Filled circles with broken line indicate a normalized peak amplitude of the early IPSPs, and open squares with solid line indicate a normalized peak amplitude of late IPSPs. Each symbol and bar show a mean value and the standard error, respectively. These are obtained from 12 neurons in the RRF (A), 12 neurons in the SNc (B) and 14 neurons in the VTA (C). Cb, cerebellum; GPe, globus pallidus, external segment; IC, inferior colliculus; RRF, retrorubral field; SC, superior colliculus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; Str, striatum; VP, ventral pallidum; VTA, ventral tegmental area; aot, accesorry optic tract; cp, cerebral peduncle; ic, internal capsule; ml, medial lemniscus; scp, superior cerebellar peduncle.

Fig. 4. Early and late IPSCs induced by stimulation of the SNr. A. (a) Superimposition of the IPSCs in the control condition (5 μM CNQX and 25 μM AP-5; trace 1), after application of 20 μM bicuculline (trace 2), and aplication of both 20 μM bicuculline and 100 μM CGP 35348 (trace 3). Each trace is an average of five sweeps. (b) Subtraction of 1-2 (net early IPSC) and 2-3 (net late IPSC) of records in (a). B. Superimposition of the IPSCs before and after application of CGP 35348. (a) IPSCs

induced by 10 pulses of stimuli at 100 Hz. (b) IPSCs induced by single stimuli. Each trace is an average of three sweeps.

Fig. 5. Location of DA neurons and a frequency of occurrence of $GABA_A$- and $GABA_B$-inhibition in DA neurons. A. Location of 105 DA neurons which presented both $GABA_A$- and $GABA_B$-inhibition (closed circles) and only $GABA_A$-inhibition (shaded circles) in parasagittal slices (a) and a coronal slice (b). B. Frequency of observation of both $GABA_A$- and $GABA_B$-inhibition (black column) and only $GABA_A$-inhibition (shaded column) in DA neurons in the RRF (n = 22), SNc (n = 59) and VTA (n = 24).

Fig. 6. Reversal potentials of the early and late IPSCs. A. (a) Early IPSCs evoked by single stimulation in the presence of 100 μM CGP 35348. (b) Late IPSCs induced by seven pulses at 100 Hz in the presence of 20 μM bicuculline. In (a) and (b), each trace was recorded at different holding potentials. Each trace is an average of four sweeps. B. Current-voltage relationship of the normalized peak currents of the early (filled circles; n = 13) and late IPSCs (open circles; n = 7). Each circle and bar shows a mean value and the standard error, respectively. Equilibrium potentials of Cl^- ion (-88.4

mV) and K^+ ion (-104.8 mV) are indicated by arrowheads with E_{Cl} (a closed arrowhead) and E_K (an open arrowhead), respectively.

Fig. 7. Effects of changes in stimulus frequency on $GABA_A$ and $GABA_B$ IPSPs. A. (a) From the top to bottom, IPSPs were induced by repetitive stimuli applied with various frequencies (10, 25, 50, 100, 200 Hz) for a period of 600 ms. Each set of superimpositions comprises postsynaptic potentials recorded under the presence of 5 μM CNQX and 25 μM AP-5 (trace 1; Control), after applicaton of 20 μM bicuculline (trace 2; Bic), and after further application of 100 μM CGP 35348 (trace 3; Bic + CGP 35348). Each trace is an average of five sweeps. (b) Superimpositions of net $GABA_A$-IPSP (1 - 2) and net $GABA_B$-IPSP (2 - 3) in each stimulus frequency. B. Relationship between the stimulus frequency and peak amplitude of net $GABA_A$-IPSPs (open circles) and net $GABA_B$-IPSPs (filled circles). Each circle and each bar indicate a mean value and a standard error obtained from four neurons, respectively. The voltage and time calibrations in Aa apply to Ab.

Fig. 8. Effects of changes in the bath temperature on $GABA_B$-IPSPs. $GABA_B$-IPSPs

recorded in the bath temperatures of 27°C (A) and 37°C (B). Each trace is an average of three sweeps.

Fig. 9. Contribution of $GABA_B$ receptors to the effects of changes in stimulus frequency on IPSPs. A. (a) control, (b) after application of 100 μM CGP 35348. In each condition, IPSPs were induced by repetitive stimuli applied with various frequencies (10, 25, 50, 100 and 200 Hz) for a period of 600 ms. Each trace is an average of 6 sweeps. B. Relationship between the stimulus frequency and peak amplitude of SNr-induced IPSPs before (open circles) and after application of CGP 35348 (closed circles). C. Relationship between the stimulus frequency and a duration of SNr-induced IPSPs before (open circles) and after application of CGP 35348 (closed circles). In B and C, each circle and bar indicate a mean value and a standard error obtained from four neurons, respectively. The voltage and time calibrations in Aa apply to Ab.

Fig. 10. Effects of SNr-induced IPSPs on the firing properties of a DA neuron in the SNc. A. Repetitive stimuli with 50Hz for 600 ms applied to the SNr evoked the IPSPs followed by rebound depolarization (denoted by an open arrowhead). B. After

application of CGP 35348 (100 μM), rebound depolarization was augmented (denoted by filled arrowheads) and induced spike discharges (*1-3). C. An application of bicuculline (20 μM) following wash of CGP 35348 attenuated rebound depolarization (denoted by shaded arrowheads) and inhibited the spike discharge.

Table 1 Electrophysiological membrane properties of the mesencephalic dopaminergic neurons.

	RRF (n = 22)	SNc (n = 47)	VTA (n = 24)	All (n = 93)	Statistics	Probability
Spike threshold (mV)	-52.6 ± 6.0	-51.7 ± 5.2	-50.6 ± 5.1	-51.6 ± 5.4	1.326	0.515
Spike amplitude (mV)	77.1 ± 15.6	72.2 ± 9.8	72.3 ± 12.1	73.4 ± 12.0	1.489	0.475
AHP amplitude (mV)	22.2 ± 6.2	20.0 ± 6.2	20.6 ± 6.9	20.7 ± 6.4	1.888	0.389
Spike width (ms)	3.9 ± 1.7	3.7 ± 1.2	4.0 ± 1.0	3.8 ± 1.3	1.986	0.370
Spike half width (ms)	1.9 ± 0.8	1.6 ± 0.5	1.8 ± 0.5	1.8 ± 0.6	4.387	0.112

These values were obtained from 93 neurons (22 neurons from the RRF, 47 from the SNc, and 24 from the VTA), which had spontaneous firing. Among DA neurons in three nuclei, there was no difference in the electrophysiological properties.

Fig. 1

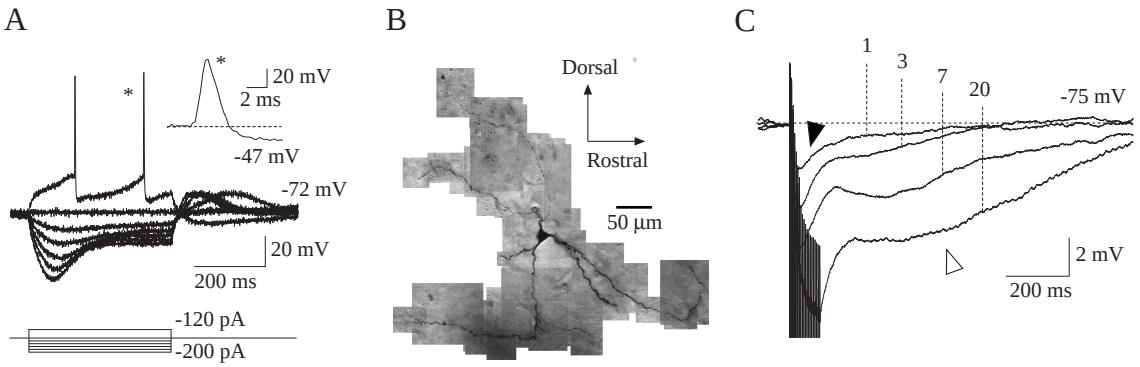

A
*
*
20 mV
2 ms
-47 mV
-72 mV
20 mV
200 ms
-120 pA
-200 pA
B
Dorsal
Rostral
50 µm
C
1
3
7
20
-75 mV
2 mV
200 ms

Fig. 2

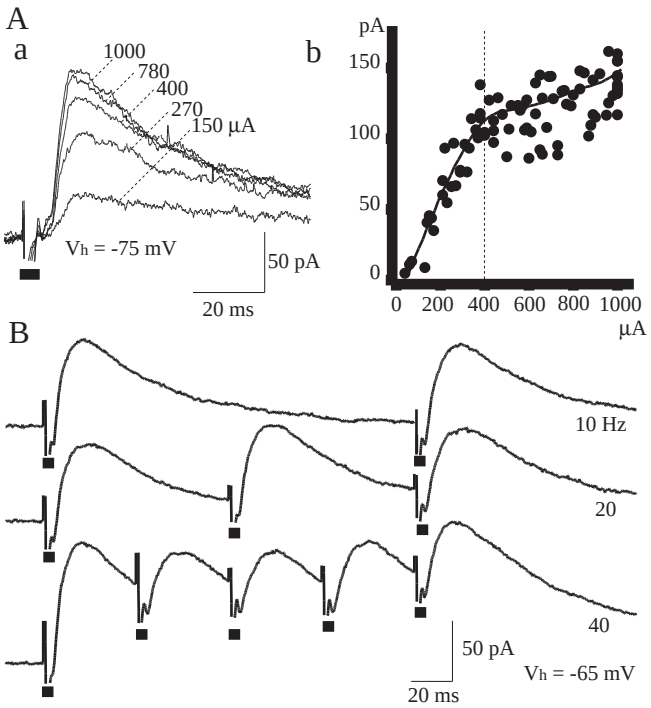

A
a
1000
780
400
270
150 µA
Vh = -75 mV
50 pA
20 ms
b
pA
150
100
50
0
0 200 400 600 800 1000
µA
B
10 Hz
20
40
50 pA
Vh = -65 mV
20 ms

Fig. 3

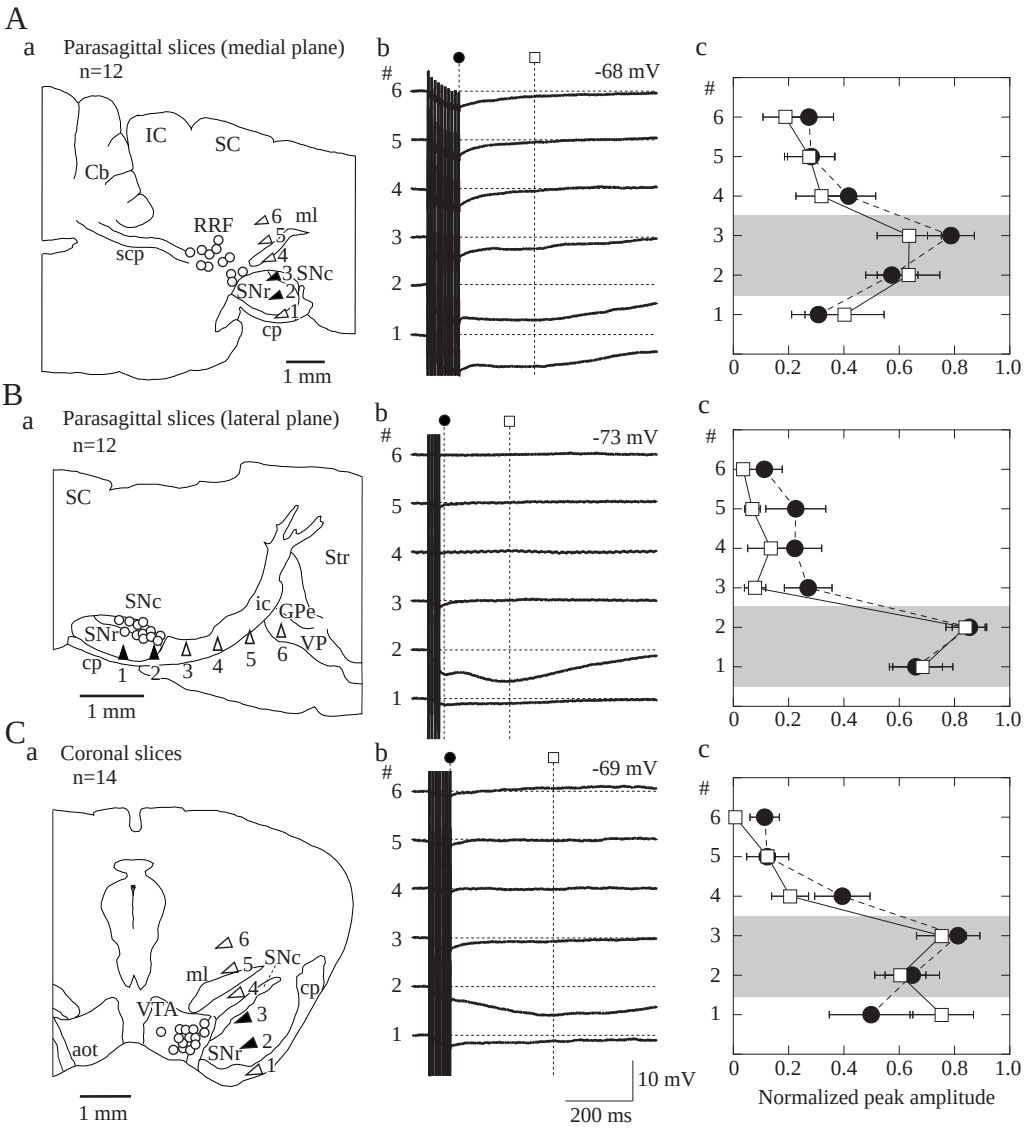

A
a
Parasagittal slices (medial plane)
n=12
IC
SC
Cb
RRF
scp
6
5
4
3
SNc
SNr
2
1
ml
cp
1 mm
b
-68 mV
c
B
a
Parasagittal slices (lateral plane)
n=12
SC
Str
SNc
SNr
ic
GPe
VP
cp
1 mm
b
-73 mV
c
C
a
Coronal slices
n=14
6
5
SNc
ml
4
cp
VTA
3
aot
SNr
2
1
1 mm
b
-69 mV
10 mV
200 ms
c
Normalized peak amplitude

Fig. 4

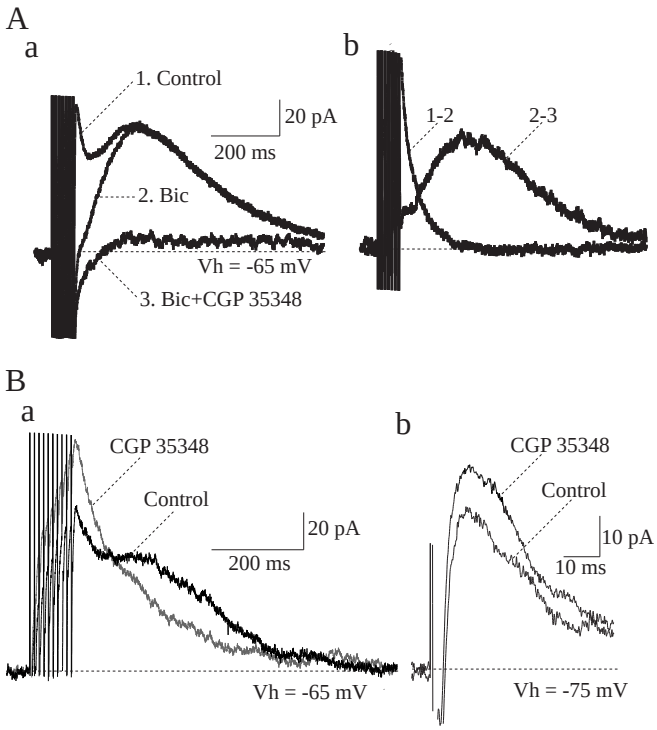

A
a
1. Control
20 pA
200 ms
2. Bic
Vh = -65 mV
3. Bic+CGP 35348
b
1-2
2-3
B
a
CGP 35348
Control
20 pA
200 ms
Vh = -65 mV
b
CGP 35348
Control
10 pA
10 ms
Vh = -75 mV

Fig. 5

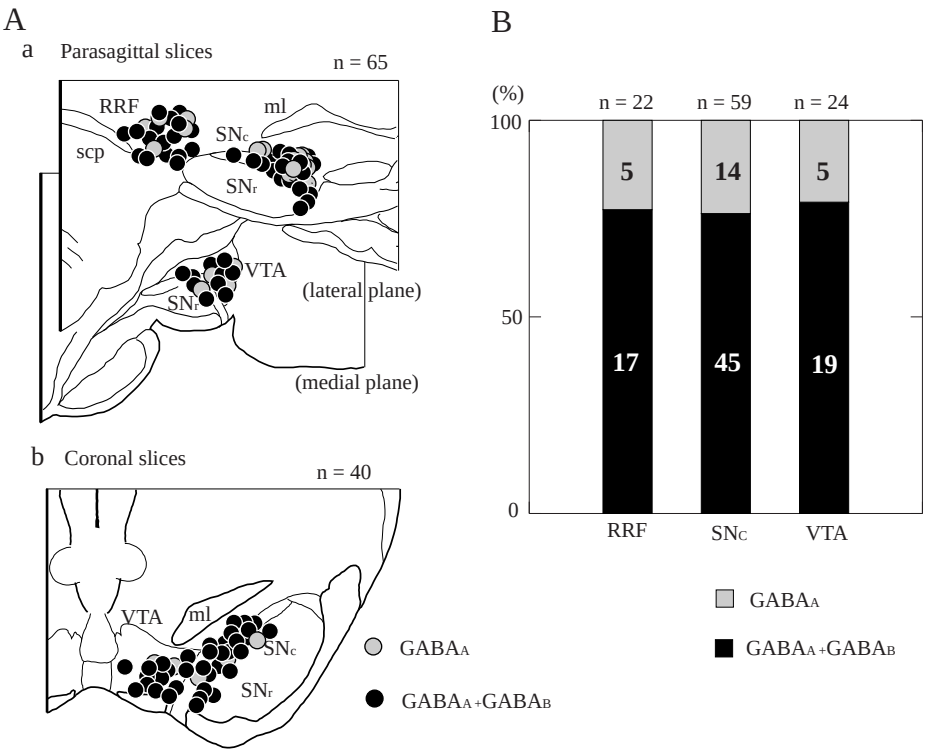

A
a Parasagittal slices
n = 65
RRF
ml
SNc
scp
SNr
VTA
SNr
(lateral plane)
(medial plane)
b Coronal slices
n = 40
VTA
ml
SNc
SNr
GABAA
GABAA+GABAB
B
(%)
n = 22
n = 59
n = 24
100
50
0
5
14
5
17
45
19
RRF
SNC
VTA
GABAA
GABAA+GABAB

Fig. 6

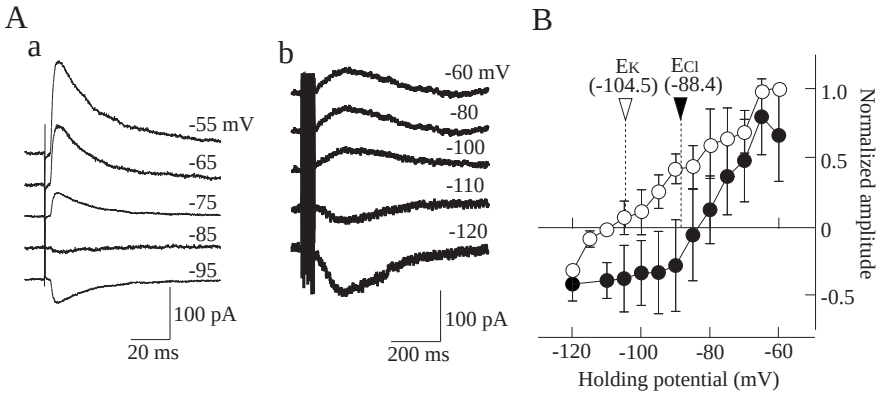

A
a
-55 mV
-65
-75
-85
-95
100 pA
20 ms
b
-60 mV
-80
-100
-110
-120
100 pA
200 ms
B
EK
(-104.5)
ECl
(-88.4)
1.0
0.5
0
-0.5
Normalized amplitude
-120
-100
-80
-60
Holding potential (mV)

Fig.7

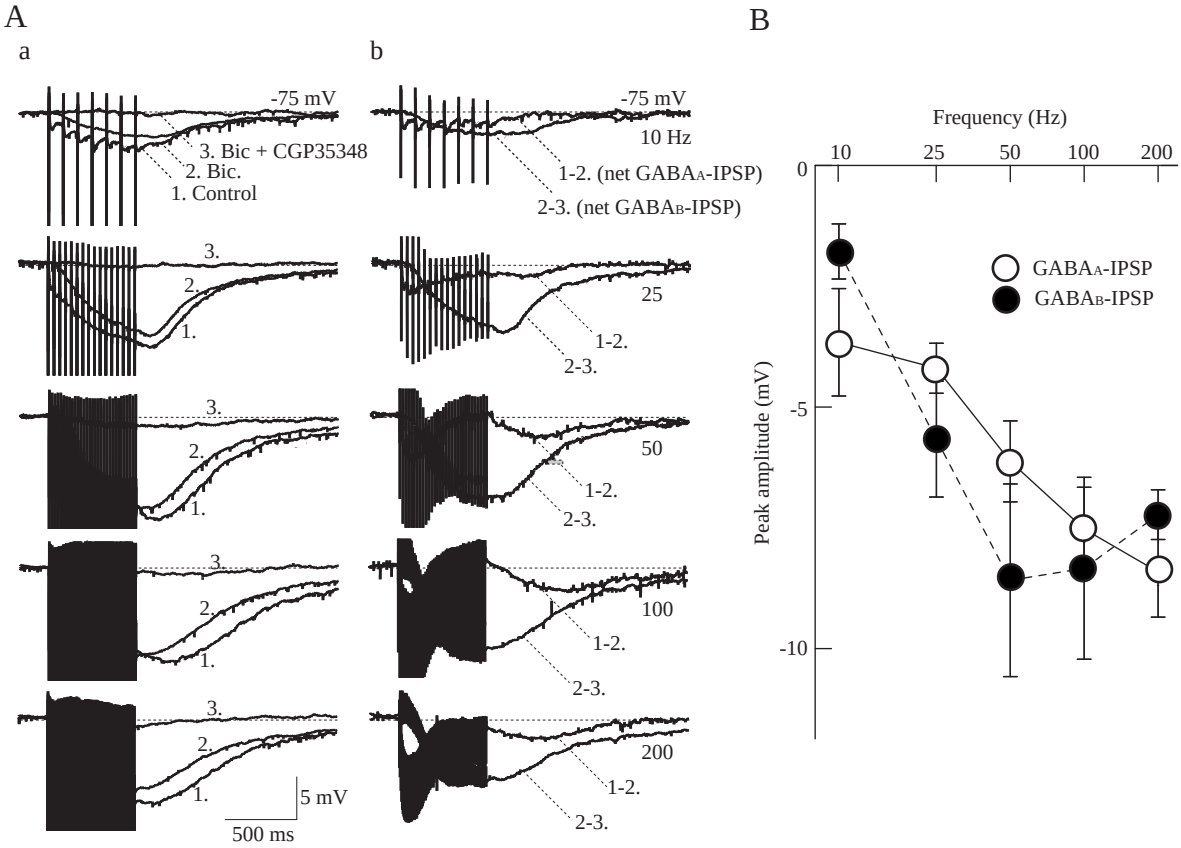

A
a
b
-75 mV
3. Bic + CGP35348
2. Bic.
1. Control
10 Hz
1-2. (net GABAA-IPSP)
2-3. (net GABAB-IPSP)
25
50
100
200
1-2.
2-3.
5 mV
500 ms
B
Frequency (Hz)
10
25
50
100
200
0
-5
-10
Peak amplitude (mV)
GABAA-IPSP
GABAB-IPSP

Fig. 8

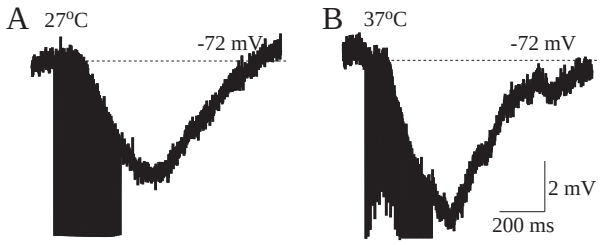


Fig.9

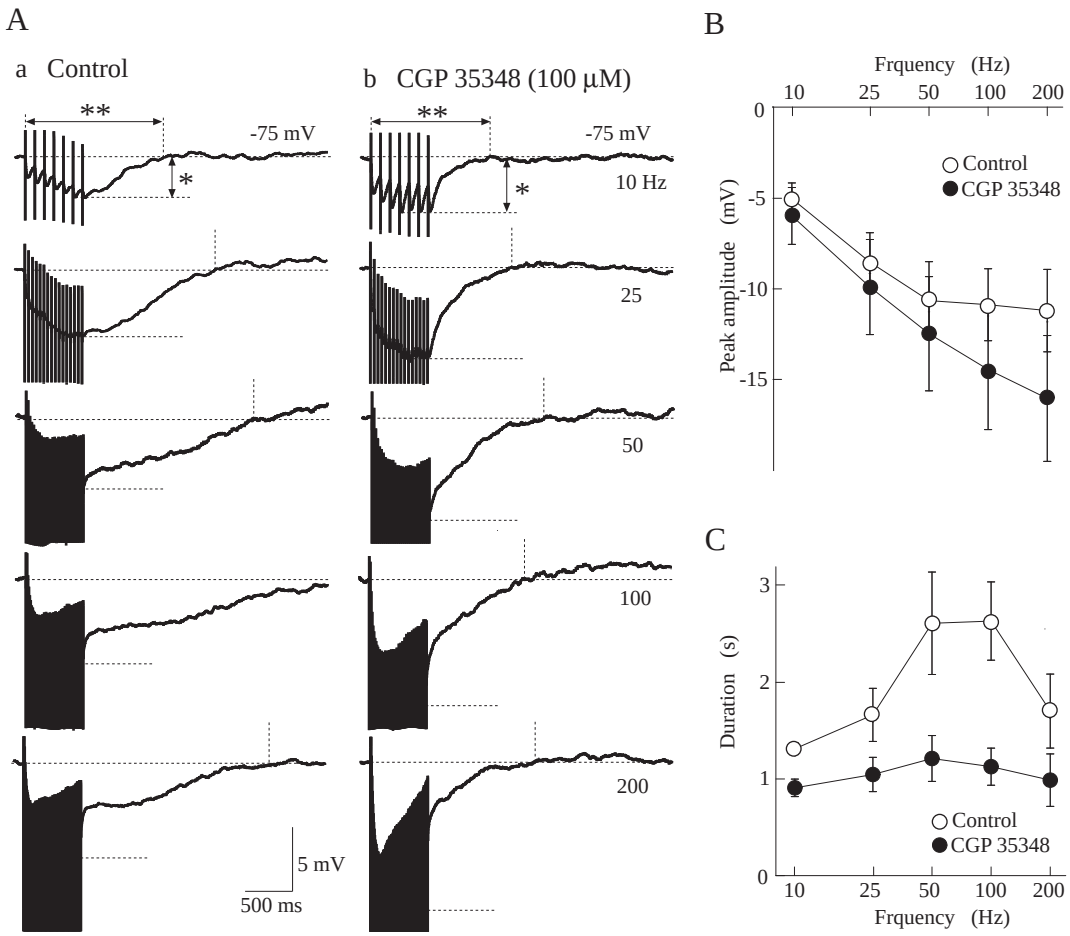
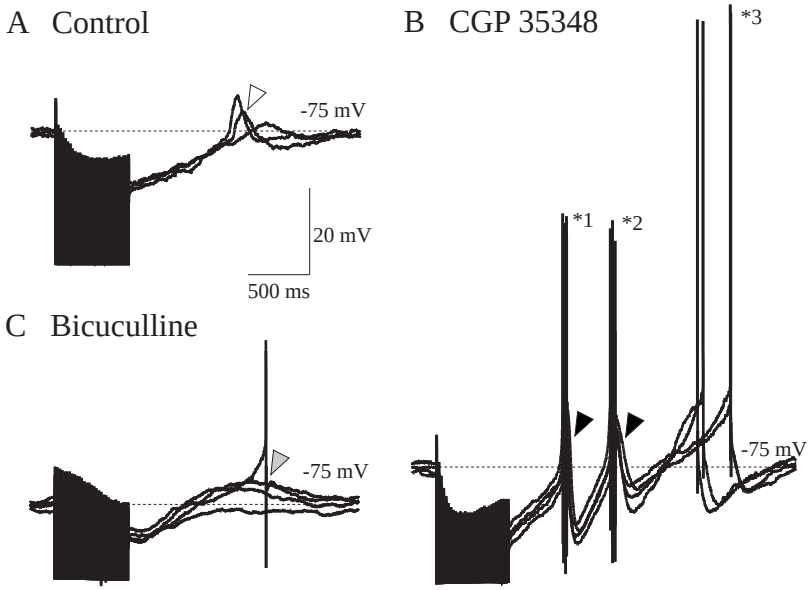

A
a Control
b CGP 35348 (100 μM)
**
*
-75 mV
10 Hz
25
50
100
200
5 mV
500 ms
B
Frquency (Hz)
10
25
50
100
200
0
-5
-10
-15
Peak amplitude (mV)
Control
CGP 35348
C
Duration (s)
3
2
1
0
Control
CGP 35348
Frquency (Hz)

Fig. 10


A Control
-75 mV
20 mV
500 ms
B CGP 35348
*1
*2
*3
-75 mV
C Bicuculline
-75 mV