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DO SILENT DOPAMINERGIC NEURONS EXIST IN RAT SUBSTANTIA NIGRA *IN VIVO*?

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Abstract—A subpopulation of inactive or "silent" dopaminergic neurons has been reported to exist *in vivo* in rat substantia nigra, comprising up to 50% of nigral dopaminergic neurons. The existence of this large proportion of silent neurons has been inferred from various experimental manipulations, but never demonstrated directly. In the present study, striatal or medial forebrain bundle stimulation was used to activate antidromically substantia nigra dopaminergic neurons *in vivo*. Antidromic spikes of dopaminergic neurons observed by extracellular single-unit recordings in the absence of spontaneous activity were employed as indicators of the presence of a silent cell. A total of 312 dopamine neurons were recorded, including 190 neurons that could be antidromically activated from the striatum and/or the medial forebrain bundle. All neurons exhibited spontaneous activity. The firing rates were unimodally distributed about the mean of 4 spikes/s, and very few cells were observed to fire at less than 0.5 spikes/s. The numbers of spontaneously active and antidromically activated dopaminergic neurons per track were recorded and compared with the number of antidromically responding silent dopaminergic neurons per track after systemic apomorphine administration. Under control conditions, 0.80 ± 0.10 or 1.36 ± 0.13 spontaneously active neurons per track could be antidromically activated at 1.0 mA by striatal or medial forebrain bundle stimulation, respectively. After apomorphine completely suppressed spontaneous activity, 0.69 ± 0.08 and 1.39 ± 0.14 antidromic neurons per track were detected by stimulating the striatum or medial forebrain bundle respectively at 1.0 mA, demonstrating that silent dopaminergic neurons can be reliably identified through antidromic activation. In sharp contrast to previous reports, these data suggest that silent neurons do not comprise a substantial proportion of the total number of dopaminergic neurons in the substantia nigra. Reverse χ^2 analysis revealed that, if they exist at all, silent dopaminergic neurons make up less than 2% of the dopaminergic cells in the substantia nigra.

These findings are related to current theories of the mechanisms of action of antipsychotic drugs and the maintenance of near-normal levels of dopamine in the striatum following large-scale loss of nigral dopaminergic neurons. © 1998 IBRO. Published by Elsevier Science Ltd.

Key words: substantia nigra, silent dopamine neurons, striatum, medial forebrain bundle, antidromic activation, apomorphine.

The mesencephalic dopaminergic system has been the focus of considerable research for several decades. Dysfunction of this system has been associated with several disease states, such as Parkinson's disease and schizophrenia.[11,34] A large fraction of the mesencephalic dopaminergic neurons are located in the substantia nigra pars compacta, which projects via the medial forebrain bundle (MFB) to the neostriatum, where dopaminergic terminals synapse on medium spiny neurons as well as on interneurons.[18,28,39,40,45]

Although most dopaminergic neurons appear to be spontaneously active, it has been widely reported that a significant fraction are silent *in vivo* under normal conditions.[6,9,10,30,33,43,55] This concept was originally introduced in an *in vivo* extracellular recording study of the effects of the neuroleptic drug, haloperidol, on substantia nigra dopaminergic neurons.[6] In that study, the number of cells per track that possessed waveforms and firing rates characteristic of dopaminergic neurons was counted by passing a recording microelectrode 12 times through a stereotaxically defined area of the substantia nigra. Acute administration of haloperidol, a dopamine (DA) receptor antagonist, was found to increase the number of cells per track. It was hypothesized that administration of haloperidol blocked the inhibitory somatodendritic D_2 autoreceptors, causing a number of silent, hyperpolarized dopaminergic neurons to depolarize and begin firing. By comparing the number of cells per track under control conditions with that observed after haloperidol, silent neurons were estimated to make up about one-third of the dopaminergic neurons in the substantia nigra *in vivo*.[6]

Similar findings were obtained in subsequent studies measuring cells per track, and led to hypotheses regarding the mechanisms underlying maintenance of striatal DA levels in Parkinson's disease, as

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Abbreviations: DA, dopamine; IS, initial segment (spike); MFB, medial forebrain bundle.

well as the therapeutic effects of L-DOPA and antipsychotic drugs.[8,30,33,55] For example, one study reported that, four to eight days after severe (>96% cell loss) 6-hydroxydopamine depletion of midbrain dopaminergic neurons, the relative proportion of spontaneously firing dopaminergic neurons was increased.[33] It was suggested that a lesion-induced activation of the normally silent dopaminergic neurons may be involved in the compensatory mechanisms attempting to maintain striatal levels of DA in the face of massive loss of dopaminergic neurons where biochemical compensatory mechanisms[1,3,32,57] may fail.[33] Another study reported that although there was no significant change in the proportion of spontaneously firing neurons in animals with moderate midbrain dopaminergic neuron losses, three weeks of L-DOPA treatment caused a substantial increase in the relative proportion of dopaminergic neurons exhibiting spontaneous firing.[30] This phenomenon was suggested to contribute to the long-term effects of L-DOPA in treating parkinsonian patients. The proportion of silent dopaminergic neurons from these studies was estimated to be as high as 50% (see Ref. 10 for review).

However, all these studies used essentially the same "cells per track" method to estimate the proportion of silent dopaminergic neurons.[6,10,30,33,43,55] Because the criteria used to identify dopaminergic neurons in these studies were the waveforms, firing rates and firing patterns, i.e. parameters of spontaneously active neurons, silent dopaminergic neurons could not be directly observed in these studies. Thus, the conclusion that up to 50% of substantia nigra dopaminergic neurons are silent was only inferred from the change in the numbers of cells encountered in two groups of animals, and not observed or demonstrated directly.

Silent dopaminergic neurons have also been reported to exist based on *in vivo* intracellular recordings from dopaminergic neurons that did not display spontaneous activity,[23] and from *in vivo* extracellular recordings made by iontophoresing glutamate[23] or cholecystokinin[44] as a micropipette was advanced through the midbrain. In the latter studies, some putative dopaminergic neurons were found to fire only when glutamate or cholecystokinin was administered iontophoretically. However, only a relatively small number of these pharmacologically activated "silent" neurons was described and no data on the frequency of their occurrence relative to spontaneously active neurons were available in these studies.[23,44]

The current study was designed to test for the existence and proportion of silent dopaminergic neurons in the substantia nigra *in vivo*. Dopaminergic neurons can be antidromically activated by stimulation of either the striatum or the MFB.[13,24,29,40,48,49] If silent dopaminergic neurons exist and are hyperpolarized, as reported previously,[6,9,30,44] then it should be possible to identify and observe them antidromically, even in the absence of spontaneous activity (e.g., Ref. 27). In the present study, stimulation of the striatum or MFB was used to elicit antidromic responses in dopaminergic neurons to determine if there exists a significant population of dopaminergic neurons that can be antidromically activated but that is not firing spontaneously.

EXPERIMENTAL PROCEDURES

General surgical procedures

In vivo experiments were performed on 36 male Sprague–Dawley rats (Zivic-Miller) weighing between 225 and 350 g. Animal care and surgical procedures were performed in accordance with the guidelines of the USPHS manual "Guide for the Care and Use of Laboratory Animals". Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and maintained by supplementary doses of chloral hydrate via a chronically implanted intraperitoneal cannula. The left femoral vein was cannulated for administration of drugs and the animals were mounted in a stereotaxic frame. All wound margins and points of contact between the animal and the stereotaxic apparatus were infiltrated with lidocaine solution (2%) or xylocaine ointment (5%). The animal's electrocardiogram was continuously monitored on an oscilloscope. In order to minimize pulsation, the atlanto-occipital membrane was punctured to release some cerebrospinal fluid. Body temperature was maintained at 37 ± 1°C using a thermostatically controlled solid-state heating pad.

After exposing the skull, small burr holes were drilled over the striatum (0.5 mm anterior to bregma and 3.7 mm lateral to the midline) and the medial forebrain bundle (4.2 mm anterior to lambda and 1.75 mm lateral to the midline). Bipolar stimulating electrodes were lowered into the striatum (4.0 mm below the cortical surface) and MFB (7.9 mm below the cortical surface), and fixed in place with cyanoacrylate glue and dental cement. A 3.0-mm-diameter recording hole was drilled above the substantia nigra at coordinates 2.1 mm anterior to lambda and 2.0 mm lateral to the midline.

Stimulating and recording

Bipolar stimulating electrodes were made from 100-μm-diameter enamel-coated stainless steel wires (California Fine Wire) with a tip separation of approximately 150 μm. The stimulating electrodes had an *in vitro* impedance of 10–30 kΩ. Monophasic square wave pulses of 0.1–4.0 mA intensity and 500 μs duration were generated by a Winston A-65 timer/stimulator and a Winston SC-100 constant-current stimulus isolation unit and delivered at a rate of 0.67 Hz to either the striatum or MFB.

Extracellular recording electrodes were constructed from 2.0-mm o.d. glass capillary tubing (World Precision Instruments) on a Narishige PE-2 vertical puller. Recording electrodes were filled with 1 M NaCl and had an initial impedance of 20–30 MΩ, which was lowered to between 4 and 10 MΩ by passing 500-ms alternating positive or negative 150-V d.c. pulses (Grass stimulator, model S-48).

Extracellular single-unit activity was amplified by a Neurodata IR183 preamplifier, displayed on a Tektronix 5113A storage oscilloscope (high filter at 30 kHz and low filter at 100 Hz) and a Nicolet 4094C digital oscilloscope, and simultaneously monitored with a Grass audio monitor. Filtered spike waveforms were digitized by the Nicolet 4094C on-line and stored on disk. All other data were recorded on to magnetic tape for off-line analysis on a Macintosh Quadra 950 equipped with a

National Instruments MIO16L multifunction board and custom-designed software, as described previously.[51]

Identification of dopaminergic neurons

The recording microelectrode was first lowered to 6.5 mm below the cortical surface by a piezoelectric microdrive (Burleigh Co.). After this, microelectrodes were advanced at a constant speed of 2 μm/s while stimulating either the striatum or the MFB at 1.0 mA, 0.67 Hz until a dopaminergic neuron was encountered. Spontaneously firing dopaminergic neurons were identified by their characteristic extracellularly recorded electrophysiological properties. Briefly, these include a long-duration action potential (>2 ms) sometimes displaying a notch on the initial positive component, slow spontaneous firing rate (<10 Hz) and antidromic activation from the striatum or/and MFB at long latency, usually consisting of the initial segment spike only.[7,13,29] When a spontaneously firing dopaminergic neuron was encountered, if no antidromic activation was observed, the stimulating current was increased for a few seconds until an antidromic spike was induced. The threshold current, defined as the minimum current that elicited antidromic responses in 100% of the non-collision trials,[48,49] was noted and the stimulation was stopped. The maximal current was never increased beyond 4.0 mA to minimize tissue damage. In spontaneously active neurons, responses were considered to be antidromic only if they exhibited collision extinction with appropriately timed spontaneous spikes.[19] If a spontaneously active neuron could not be antidromically activated, it was still considered to be a dopaminergic neuron when all other characteristics described above were met.

Silent dopaminergic neurons were identified by their antidromic response to striatal or MFB stimulation in the absence of spontaneous activity. Once such a silent neuron was encountered, the evoked response was tested with double pulse stimuli, with a 5-ms interstimulus interval corresponding to 200-Hz stimulation. If the evoked spike could follow the 200-Hz stimulus, antidromic activation was confirmed. Otherwise, the neuron was considered to be orthodromically activated.

Pharmacologically induced silent cells

In some experiments, apomorphine was administered (400 μg/kg, i.p. or 50 μg/kg, i.v.) to inhibit the spontaneous activity of dopaminergic neurons by activating somatodendritic D_2-like autoreceptors.[2,29,35,52] Supplementary doses of apomorphine were administered periodically to maintain the complete inhibition of spontaneous activity throughout the subsequent recording period, which typically lasted about 1 h. In some experiments, apomorphine was continuously infused through a femoral catheter at 20 μg/kg/min to maintain the inhibition. During the period of effective inhibition, recording electrodes were advanced at 2 μm/s to search for dopaminergic neurons identified by antidromic responses elicited by either striatal or MFB stimulation at 1.0 mA, a value chosen on the basis of previous experiments (e.g., Refs 48, 49 and 53) to be sufficient to activate antidromically a large proportion of dopaminergic neurons.

Cells per track experiments

In some experiments, a modification of the "cells per track" method[6,30,33,55] was employed. At the beginning of the experiment, the recording electrode was placed at predetermined mediolateral coordinates (see below) at −6.5 mm from the cortical surface. Starting at these coordinates, the electrode was advanced at 2 μm/s until −8.0 mm was reached. The electrode was then withdrawn and repositioned 50 μm anterior or posterior at −6.5 mm from the cortical surface and the procedure repeated. A series (four to eight) of similar recording tracks was made anterioposteriorly with 50 μm between adjacent tracks, and the number of spontaneously firing dopaminergic neurons in each track was recorded, in addition to the number of antidromically responding neurons and their thresholds. The anterior and posterior boundaries of the substantia nigra pars compacta were determined when two consecutive tracks were made in which no dopaminergic neurons were encountered. The last track in which a dopaminergic neuron was encountered was taken as the boundary of the pars compacta.

Apomorphine was then administered as described above and the procedure repeated at the same set of anterioposterior coordinates, with the mediolateral coordinates shifted 50 μm medially. Either striatal or MFB stimulation at 0.67 Hz with 1.0-mA intensity and 0.5-ms duration was maintained throughout the entire procedure. An antidromic spike in the absence of spontaneous activity indicated the existence of a silent dopaminergic neuron. The number of antidromically responding neurons and the number of tracks were recorded to calculate the number of "antidromic cells per track" after apomorphine application.

The predetermined mediolateral coordinates were designed in a manner that, although in each animal only two mediolateral coordinates were recorded (one control and one after apomorphine), overall these experiments (n=16) sampled a large region of the substantia nigra pars compacta (1.0–3.0 mm lateral from the midline and 1.5–2.5 mm anterior from lambda).

Materials

Apomorphine hydrochloride was obtained from Sigma Co. and was dissolved freshly each day in physiological saline containing 0.1% ascorbic acid. Apomorphine was administered either intravenously via a femoral catheter (50 μg/kg bolus or continuous infusion at 20 μg/kg/min) or intraperitoneally via an indwelling intraperitoneal cannula (400 μg/kg). Doses are expressed in terms of the hydrochloride salt.

Data analysis

All extracellularly recorded dopaminergic neurons encountered were recorded on magnetic tape for off-line analysis. At least 2 min of spontaneous activity was recorded for each neuron and was analysed off-line, as described previously.[51] The numbers and percentage of spontaneously firing and silent dopaminergic neurons were reported and compared to previous reports (χ^2 contingency analysis). Student's two-tailed t-test was used to test for a difference between the number of antidromically activated neurons per track under control conditions and after apomorphine administration in the cells per track experiments. Numbers in the text are expressed as mean ± S.E.M.

Histology

At the end of the experiment, animals were perfused transcardially with 0.9% saline followed by 10% formalin. Brains were postfixed and sectioned into 70-μm slices which were stained by Neutral Red to determine the recording and stimulating sites.

RESULTS

Spontaneous activity and antidromic responses under control conditions

Examples of extracellularly recorded waveforms of spontaneous spikes and typical antidromic responses of dopaminergic neurons from the striatum and MFB are shown in Fig. 1. The waveforms of the recorded neurons were biphasic, normally exceeded

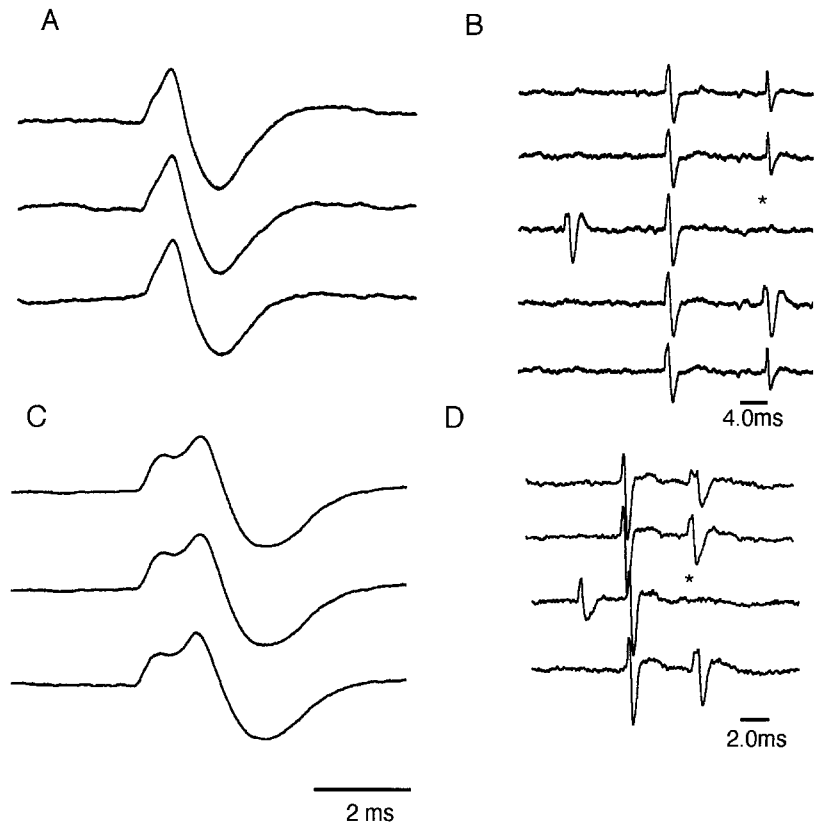

Fig. 1. Identification of dopaminergic neurons recorded extracellularly. (A) Digitally averaged spontaneous spikes from a dopaminergic neuron antidromically activated from the striatum. (B) Consecutive traces representing the antidromic activation of the dopaminergic neuron shown in A by 0.8-mA striatal stimulation. Trace marked by an asterisk shows collision extinction of antidromic spike with spontaneous discharge. (C) Digitally averaged spontaneous spikes from a different dopaminergic neuron antidromically activated from the MFB. Note the long duration and prominent IS–somatodendritic notch. (D) Consecutive traces representing the antidromic activation of the dopaminergic neuron in C by 0.4-mA MFB stimulation. Trace labeled by an asterisk shows the collision extinction of the antidromic response with a spontaneous discharge. Positivity is up in this and all subsequent figures.

2 ms in duration and often exhibited a notch on the rising phases. The antidromic latencies were long (MFB: 4.97 ± 0.11 ms, n=82; striatum: 14.16 ± 0.46 ms, n=78) and the antidromic spikes usually consisted of the initial segment (IS) spike only, with occasional full IS–somatodendritic spikes encountered. These properties have been shown to be characteristic of substantia nigra dopaminergic neurons recorded extracellularly *in vivo*.[2,7,10,13,23,24,29,48] A total of 312 spontaneously active dopaminergic neurons was recorded extracellularly. The mean firing rate was 3.94 ± 0.11 spikes/s (n=312), with a range of 0.22–11.99 spikes/s. The firing rates were unimodally distributed about the mean, with very little skew (skewness=0.488), as shown in Fig. 2.

About 44% (75/171) of the neurons tested exhibited antidromic activation when the striatum was stimulated at 1.0 mA. If the stimulating intensity was increased to 4.0 mA, the percentage of antidromically activated dopaminergic neurons increased to 63.2% (108/171). The mean antidromic threshold for striatal stimulation was 1.12 ± 0.08 mA (n=108). Stimulation of the MFB at 1.0 mA antidromically activated 74.2% (115/155) of the dopaminergic neurons tested. By increasing the stimulating current to 4.0 mA, 81.3% of dopaminergic neurons were found to respond antidromically to MFB stimulation (126/155). The mean antidromic threshold from the MFB was 0.70 ± 0.05 mA (n=126). Combining these two populations, 190 spontaneously firing, antidromically activated dopaminergic neurons and no silent dopaminergic neurons were identified in the substantia nigra when a constant stimulation of either the striatum or MFB with 1.0 mA was maintained during the course of recording. In other words, no antidromically responding neurons were encountered that did not also exhibit spontaneous activity. These data are summarized in Table 1.

Pharmacologically induced silent cells

To exclude the possibility that silent, hyperpolarized dopaminergic neurons could not be detected antidromically, a positive control experiment was conducted in which the spontaneous activity of

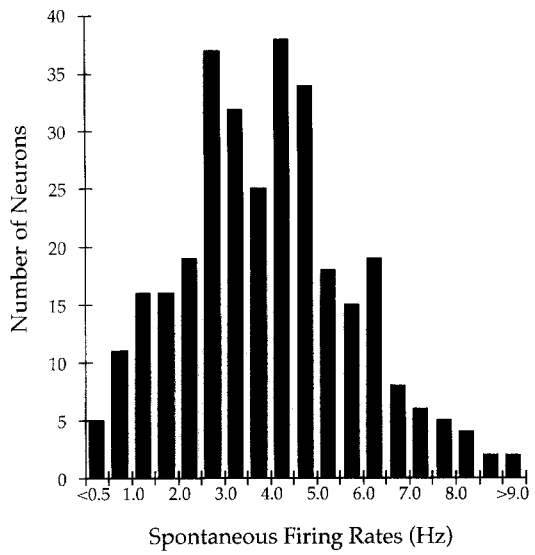

Fig. 2. Histogram illustrating the spontaneous firing rates of the dopaminergic neurons studied. Note that the firing rates are unimodally distributed about the mean.

dopaminergic neurons in the substantia nigra was suppressed by systemic administration of the DA receptor agonist, apomorphine, while maintaining striatal stimulation (0.67 Hz, 1.0 mA). The effect of apomorphine on a typical dopaminergic neuron is shown in Fig. 3. The neuron was antidromically activated from the striatum (Fig. 3A). Within a few seconds after intravenous apomorphine administration (50 μg/kg), the spontaneously activity was completely suppressed (Fig. 3B, C). Supplementary doses of apomorphine (20 μg/kg, i.v., every 10 min) were supplied to maintain the inhibition for the duration of the experiment. Even in the complete absence of spontaneous activity, dopaminergic neurons could still be antidromically activated, as shown in Fig. 3B. Almost 100% of the antidromic responses recorded following apomorphine administration consisted of the IS spikes only, which could nevertheless be reliably detected (cf. Figs 3 and 4).

During apomorphine-induced suppression of spontaneous activity, recording electrodes were advanced as before and silent dopaminergic neurons could be detected by their antidromic responses to striatal or MFB stimulation at 1.0 mA. As shown for one typical example in Fig. 4, as the recording electrode was slowly advanced, an antidromic response consisting of the IS spike only gradually appeared and increased in amplitude, indicating the presence of a silent dopaminergic neuron, thus demonstrating that antidromic activation can identify hyperpolarized silent dopaminergic neurons.

"Cells per track" experiments

To explore the reason(s) why the current study generated results in sharp opposition to previous reports, the "cells per track" method, which was the primary technique in these previous studies,[6,9,30,33,55] was also employed in a specially designed control experiment. Usually, four to eight tracks of recordings were obtained for each animal between the anterior and posterior boundaries of the pars compacta at the predetermined coordinates prior to apomorphine administration. Either striatal or MFB stimulation was maintained at 1.0 mA during these tracks, and the numbers of spontaneously active and antidromically activated cells per track were counted as described above. Apomorphine was then administered systemically to inhibit the spontaneous activity of dopaminergic neurons, and the number of antidromically responding neurons per track was counted as described above.

As shown in Table 2, under control conditions, 1.84 ± 0.15 (n=44 tracks) spontaneously active dopaminergic neurons per track were recorded during striatal stimulation. If only those neurons that responded antidromically at 1.0 mA were counted, 0.80 ± 0.10 (n=44 tracks) neurons per track were recorded. If silent, hyperpolarized dopaminergic neurons cannot be antidromically identified as readily as normally polarized spontaneously active neurons, one would expect to see a smaller number of antidromically activated neurons per track after apomorphine administration. On the other hand, if silent, hyperpolarized dopaminergic neurons can be antidromically detected as easily as normally polarized, spontaneously active dopaminergic neurons, then one would expect to observe a similar number of antidromically responding dopaminergic neurons per track with a 1.0-mA striatal stimulus after apomorphine administration, as in the control condition. The actual observed number of antidromically activated cells recorded per track with apomorphine was 0.69 ± 0.08 (n=39 tracks), which did not differ significantly from that observed under control conditions.

In control experiments in which the MFB was stimulated, 1.82 ± 0.14 (n=39 tracks) spontaneously active dopaminergic neurons per track were recorded. Among them, 1.36 ± 0.13 (n=39 tracks) dopaminergic neurons per track had antidromic threshold less than or equal to 1.0 mA. The observed number of apomorphine-induced silent dopaminergic neurons by 1.0-mA MFB stimulation was 1.39 ± 0.14 (n=31 tracks), which was in precise accordance with the predicted number.

These latter experiments demonstrate that antidromic responses can be reliably used to detect dopaminergic neurons whose spontaneous activity is completely suppressed due to somatodendritic hyperpolarization, and that even after all dopaminergic neurons are hyperpolarized by systemic administration of apomorphine, the observed numbers of antidromically responding neurons per track from the striatum and MFB do not differ from those observed in the control condition.

Table 1. Summary information of the dopaminergic neurons studied

Stimulus site	Total number of neurons	Number of silent neurons	Number of neurons with AD threshold ≤4 mA	Percentage of neurons with AD threshold ≤4 mA	Number of neurons with AD threshold ≤1 mA	Percentage of neurons with AD threshold ≤1 mA	Mean AD threshold	Mean AD latency
Striatum	171	0	108	63.2	75	43.9	1.12±0.08 (n=108)	14.16±0.46 (n=78)
MFB	155	0	126	81.3	115	74.2	0.70±0.05 (n=126)	4.97±0.11 (n=82)

Mean antidromic (AD) threshold refers to the minimum current sufficient to elicit antidromic responses in 100% of the stimulus trials on which a collision did not occur. Antidromic threshold and latency are expressed as mean±S.E.M. Numbers in parentheses refer to numbers of cells measured.

DISCUSSION

Silent dopaminergic neurons were not observed in control rats

The present study directly tested the existence and proportion of silent dopaminergic neurons in rat substantia nigra *in vivo*. In addition to recording spontaneously firing dopaminergic neurons, attempts were made to identify silent dopaminergic neurons by their antidromic spikes elicited by striatal or MFB stimulation. Of the 312 dopaminergic neurons studied, 190 could be antidromically activated from the striatum and/or MFB when the stimulus intensity was maintained at 1.0 mA. All of these cells exhibited spontaneous activity. The ratio between silent and spontaneously firing dopaminergic neurons (0:190) differed significantly from previous studies in which silent dopaminergic neurons were reported to comprise up to 50% of the total number of substantia nigra dopaminergic neurons.[6,8,10,30] By conducting a χ^2 test to determine the maximum proportion of silent cells that could exist in order to make the comparison to a distribution of 0:190 found in the present study non-significant, the proportion of silent dopaminergic neurons had to be reduced to below 1.98% to reach statistical non-significance using an α level of 0.05. Thus, it appears that silent dopaminergic neurons, if they exist at all, do not account for more than 2% of the population of dopaminergic neurons in the substantia nigra. This disagreement with the previously reported figures of up to 50% cannot be explained simply by differences in experimental conditions such as anesthesia or animal strains, because these were identical to those in the previous reports.[6,9,30,33]

One might argue that the failure to find silent dopaminergic neurons in the present study resulted from the striatal and/or MFB stimulation activating "normally" silent neurons. Although there is a well-known monosynaptic GABAergic pathway from the striatum to nigral dopaminergic neurons,[5,21,41,46,54] the majority of the striatonigral fibers terminate in the pars reticulata,[5,21,25] which contains GABAergic projection neurons that have been shown to project via axon collaterals to dopaminergic neurons.[12,14,51] Thus, one could argue that the striatal stimulation inhibits reticulata GABAergic neurons, thereby disinhibiting the silent dopaminergic neurons and causing them to fire. Indeed, a reciprocal relation between the spontaneous firing rates of nigral dopaminergic neurons and non-dopaminergic neurons has been observed,[22] and an increase in the spontaneous firing rate of dopaminergic neurons has been reported when the striatum was stimulated at extremely low intensity (20–50 μA, 50 μs duration).[24] However, when a higher stimulus intensity (>500 μA) was used, dopaminergic neurons were inhibited,[24] consistent with the majority of reports on the effects of striatal stimulation on identified dopaminergic neurons.[15,16,29,37,38,42,50,52] Since the stimulus parameters used in the present study were 1.0 mA with a duration of 500 μs, the stimulus would be expected to inhibit, not excite, dopaminergic neurons, and is thus unlikely to account for the failure to detect any silent dopaminergic neurons.

Apomorphine-induced silent dopaminergic neurons

To confirm that silent nigral dopaminergic neurons could be detected by antidromic activation, apomorphine was used to hyperpolarize and thereby suppress the firing of dopaminergic neurons. As reported previously,[4,7,10,17,29] systemic administration of apomorphine could completely inhibit the spontaneous activity of dopaminergic neurons. During the period of suppressed activity, an identical recording procedure was conducted to search for these artificially silenced dopaminergic neurons based on their antidromic responses. Since antidromic stimulation was maintained at 1.0 mA throughout the recording, one would predict that the same number of dopaminergic neurons which had antidromic thresholds less than or equal to 1.0 mA in the absence of apomorphine should be recorded. This is precisely what was observed with MFB stimulation.

Although not statistically significant, a slightly smaller number of neurons per track antidromically activated from the striatum was recorded after apomorphine administration. This is likely due to the

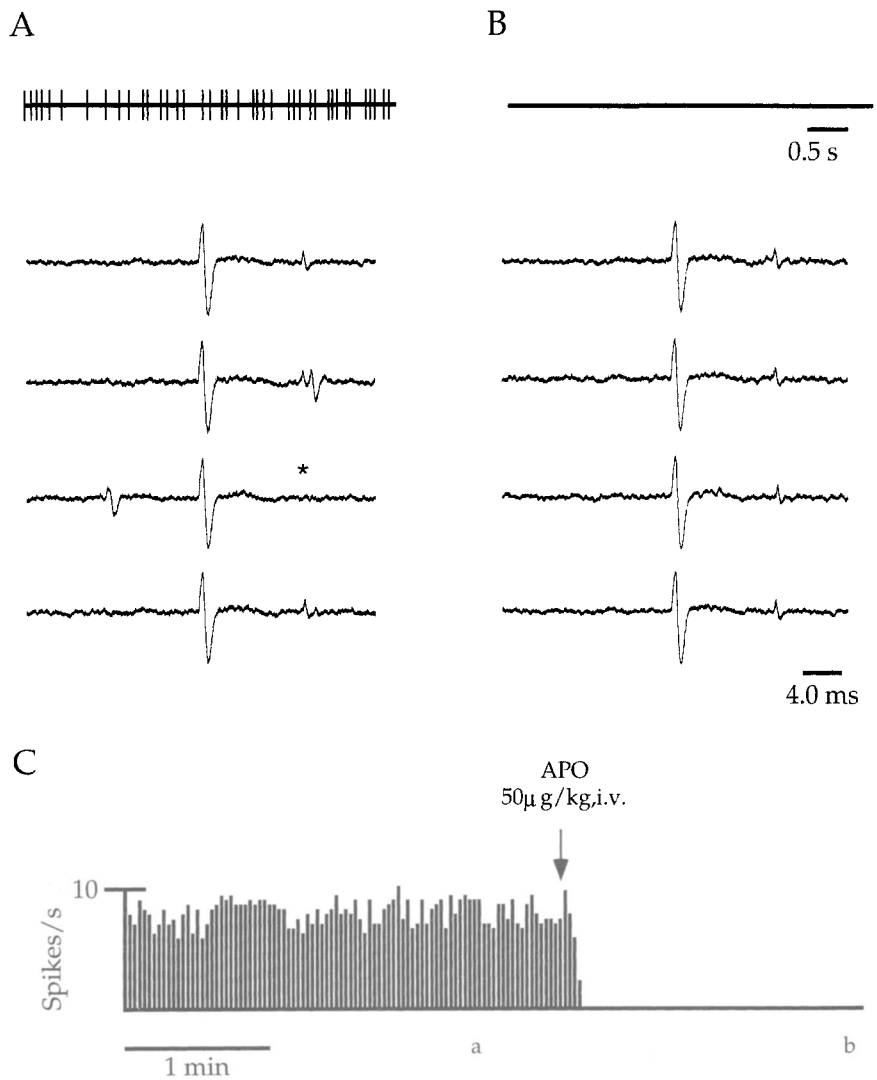

Fig. 3. The effect of apomorphine on the spontaneous and antidromic activity of a dopaminergic neuron. (A) Extracellular recording of a spontaneously firing dopaminergic neuron. Top: spike train shows spontaneous activity at 8.54 Hz. Bottom: four consecutive traces show the antidromic activation of this dopaminergic cell by 1.0-mA striatal stimulation. Asterisk shows the collision extinction of the antidromic spike with a spontaneous discharge. Note the presence of a full spike antidromic response in the third trace. (B) The same neuron 2 min after the intravenous administration of apomorphine (50 μg/kg, i.v.). Top: spike train shows the neuron is completely silent. Bottom: striatal stimulation with the same current still elicits antidromic spikes, which now consist entirely of the IS spike only. (C) Ratemeter record illustrating the inhibition of the spontaneous firing of this dopaminergic neuron by apomorphine administration (i.v.). Letters "a" and "b" indicate the approximate times at which A and B were recorded.

presence of D_2 autoreceptors on the axon terminals of dopaminergic neurons (see Ref. 47, for review). Apomorphine has been shown to stimulate axon terminal autoreceptors on dopaminergic neurons, thereby increasing the nigrostriatal antidromic threshold by about 20%.[48,49] Therefore, a proportion of neurons that had thresholds below 1.0 mA and were antidromically activated prior to apomorphine administration would be expected to have their thresholds increased above 1.0 mA and no longer be antidromically activated and observed. This interpretation is supported by the lack of effect of apomorphine on the number of cells per track antidromically activated from the MFB. As reported previously,[48,49] since there are no autoreceptors located at pre-terminal regions of the axon in the MFB, apomorphine does not affect the antidromic thresholds from this region.

One might argue that for some reason other than a hyperpolarized membrane potential dopaminergic neurons that are silent *in vivo* under normal conditions have elevated antidromic thresholds and are thus more difficult to activate antidromically than spontaneously firing neurons. Although this is a possibility that is difficult to refute experimentally, it should be noted that it is not consistent with what

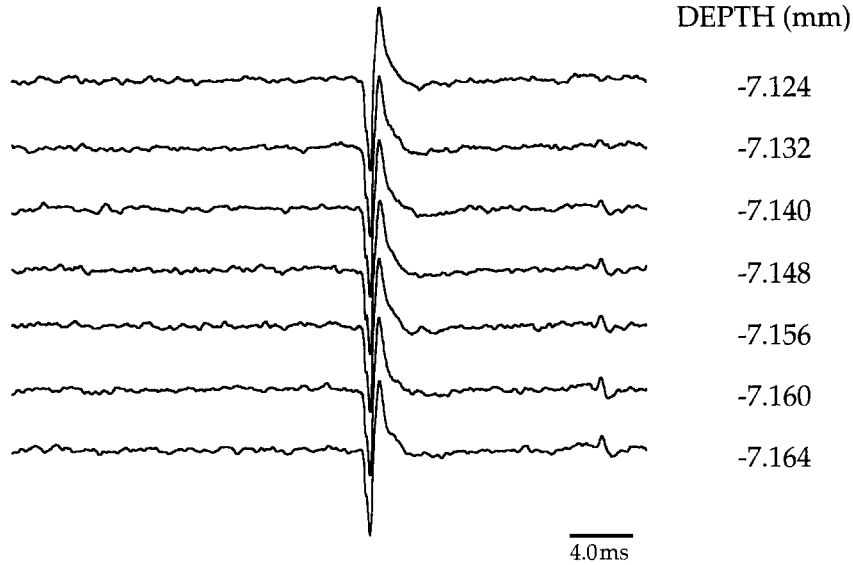

Fig. 4. An example of the antidromic identification of an apomorphine-induced (50 μg/kg, i.v.) silent dopaminergic neuron. Striatal stimulation at 1.0 mA was maintained during recording and traces were obtained at the depth marked on the right. As the recording electrode was advanced, a silent dopaminergic neuron was readily identified by its antidromic response occurring at a fixed onset latency. Note that only IS spikes were elicited.

Table 2. Effect of apomorphine on the number of antidromically activated cells per track

Stimulus site	Spontaneously active Neurons/track	Antidromic neurons/track Control	Antidromic neurons/track Apomorphine
Striatum	1.85 ± 0.15 (44)	0.80 ± 10 (44)	0.69 ± 0.08 (39)
MFB	1.82 ± 0.14 (39)	1.36 ± 0.13 (39)	1.39 ± 0.14 (31)

Antidromic neurons/track refers to the number of neurons that could be antidromically activated with a stimulating current of 1.0 mA under control conditions (Control) or after all spontaneous activity was suppressed by systemic administration of apomorphine (Apomorphine). Data are expressed as the mean ± S.E.M. Numbers in parentheses refer to the numbers of tracks studied.

is known about the relation between spontaneous activity and antidromic excitability. Antidromic thresholds of dopaminergic neurons are directly correlated with firing rate.[20,29,49] This is due, at least in part, to the increasing activation of terminal autoreceptors with increased release of DA.[48,49] Thus, one would expect silent dopaminergic neurons to have lower, not higher, thresholds.

Thus, taken together, the data in the present study do not support the existence of a large population of silent dopaminergic neurons in the substantia nigra and suggest instead that most, if not all, nigral dopaminergic neurons are spontaneously active *in vivo* under chloral hydrate or urethane anesthesia,[49] and by inference, in unanesthetized rats as well.

Silent dopaminergic neurons and the distribution of spontaneous firing rates

The putative silent dopaminergic neurons reported in previous studies were thought to be hyperpolarized and could be activated when they were depolarized by iontophoresis of excitatory neurotransmitters or neuromodulators.[6,23,44] If silent dopaminergic neurons comprise up to 50% of the total dopaminergic neurons in the substantia nigra, one would expect to see a significant proportion of neurons that were almost silent or firing at very low rates. As a result, the firing rate histogram should have had a left skewed or biphasic distribution, with one peak at very low firing rates and another around 3–4 Hz. This is a reasonable assumption, since the firing rates of dopaminergic neurons can be manipulated in a continuous fashion by intracellular injection of current (Iribe and Tepper, unpublished observations, and Ref. 23), suggesting that dopaminergic neurons can continue to fire spontaneously at low rates when hyperpolarized. Although the pacemaker-like firing pattern disappears when enough current is injected to slow the neurons below about 0.75 spikes/s, cells still continue to fire intermittent spikes at very low rates (Iribe and Tepper, unpublished observations). However, as reported previously[23,49] and in the current

study, dopaminergic neurons with very low firing rates are rarely encountered *in vivo*. Also, similar to those previously published results,[23] the spontaneous firing rates of dopaminergic neurons in the present study were unimodally distributed around the mean, with very little skew. These observations seem inconsistent with the existence of a significant population of silent neurons. In addition, if silent dopaminergic neurons exist, one might expect to see silent dopaminergic neurons switch to firing neurons or vice versa, similar to the transitions observed between the single-spike firing patterns and burst firing. However, such transitions were not observed in the present study, nor have they been reported elsewhere.

Alternative explanations for the changes in number of cells per track

As mentioned previously, the quantitative estimates that 30–50% of dopaminergic neurons are silent *in vivo* under normal conditions were inferred from increases in the number of spontaneously active dopaminergic neurons per track after acute haloperidol administration or other experimental manipulations.[6,9,10,30,33,43,55] However, the results obtained with antidromic activation in the present study, which allowed direct observation of both spontaneously active as well as silent dopaminergic neurons, argue strongly against the existence of a significant population of silent dopaminergic neurons *in vivo* under control conditions. This conclusion is not necessarily at odds with the previous cells per track data, since explanations other than the existence and recruitment of silent dopaminergic neurons could account for the changes in the numbers of cells per track observed after acute administration of D_2-like antagonists and other manipulations in some of the previous studies.

For example, since acute haloperidol and other D_2 receptor antagonists have been shown to increase the firing rate of dopaminergic neurons,[7–10,17,26] one might expect to detect more spontaneously firing dopaminergic neurons while advancing an electrode through the substantia nigra, since these cells normally fire relatively slowly and since the drug increased the average number of spikes per second. It is possible that the elevation of firing rate of dopaminergic neurons induced by acute haloperidol administration simply increased the chances of finding dopaminergic neurons using the typical cells per track method. The rate of advancement of the microelectrode is a critical variable for these measurements and lack of standardization of the rate of electrode advance may account in part for the large (approximately two-fold) differences in the number of cells per track in mesencephalic DA nuclei recorded under control conditions reported in various papers (e.g., Refs 6, 9, 17, 33 and 55). Along these lines, it is interesting to note that the mean number of spontaneously active cells per track found in the present experiment (1.84), while using a very slow, controlled rate of advance (2 μm/s), is somewhat higher than for controls in many other studies, and is similar to the average reported for the substantia nigra after acute administration of DA receptor antagonists in several studies using the conventional cells per track measurement.[6,8,9]

In addition, it is conceivable that D_2-like receptor antagonists affect the extracellular field potential generated by action potentials in dopaminergic neurons. Several lines of evidence, including increases in firing rate,[6–10,26] alterations in firing pattern[56] and changes in somatodendritic excitability[52] of dopaminergic neurons after blockade of somatodendritic autoreceptors, suggest that under normal conditions *in vivo* these autoreceptors are tonically activated. This leads to the opening of potassium channels, which not only hyperpolarizes the somatodendritic region, but also decreases its membrane resistance and increases the electrotonic length of the neuron.[35] This would be expected to decrease the invasion of the dendrites by the spike, whether active[31] or passive. Since the size of the field potential around a neuron depends in large part on the extent to which dendrites distal to the cell body are depolarized,[36] autoreceptor blockade by D_2-like antagonists might increase the size of the extracellular field as a consequence of increased membrane resistance and decreased electrotonic length of the cell. Similar effects could also result from actions of haloperidol on nigral afferents. Either would result in the ability to record neurons extracellularly from a greater distance away and could increase the number of cells per track observed in extracellular recordings.

CONCLUSIONS

In summary, the current study employed antidromic stimulation to investigate the existence and proportion of silent dopaminergic neurons in the substantia nigra of normal rats. It was demonstrated that antidromic activation is an effective and reliable method for detecting silent dopaminergic neurons in animals in which the spontaneous firing of dopaminergic neurons was suppressed by systemic administration of apomorphine. However, no silent dopaminergic neurons were identified under control conditions, contrasting sharply with prior studies which inferred that 30–50% of dopaminergic neurons in the substantia nigra were silent *in vivo*. Although the possibility of the existence of these silent dopaminergic neurons could not be completely excluded by this study, it is very likely that the silent dopaminergic neurons constitute at most only a small proportion (<2%) of the substantia nigra dopaminergic neuron population. These data suggest that previous reports of increases in the number of spontaneously active cells per track after acute haloperidol or other manipulations may have

been due to factors other than the recruitment and activation of silent dopaminergic neurons.

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