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Phasic Excitation of Dopamine Neurons in Ventral VTA by Noxious Stimuli

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Midbrain dopamine neurons play central roles in reward processing. It is widely assumed that all dopamine neurons encode the same information. Some evidence, however, suggests functional differences between subgroups of dopamine neurons, particularly with respect to processing non-rewarding, aversive stimuli. To directly test this we recorded from and juxtacellularly-labeled individual ventral tegmental area (VTA) dopamine neurons in anesthetized rats, so that we could link precise anatomical position and neurochemical identity with coding for noxious stimuli. Here we show that dopamine neurons in the dorsal VTA are inhibited by noxious footshocks, consistent with their role in reward processing. In contrast, we find that dopamine neurons in the ventral VTA are phasically excited by footshocks. This observation can explain a number of previously-confusing findings that suggested a role for dopamine in processing both rewarding and aversive events. Taken together, our results indicate that there are two functionally- and anatomically-distinct VTA dopamine systems.

Midbrain dopamine neurons of the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) play key roles in reward processing (1, 2). Although dopamine neurons exhibit considerable heterogeneity regarding projection targets and basic pharmacological properties (3-5), it is widely assumed that they exhibit homogenous reward coding across the entire population (1). In addition, we have reported that dopamine neurons are uniformly inhibited by aversive stimuli, which is consistent with reward theories; a separate group of VTA neurons that are excited by noxious stimuli is not dopaminergic (6). However, there are a number of findings that are difficult to reconcile with this view. First, aversive stimuli evoke dopamine release at projection targets as measured with microdialysis, particularly in the medial shell of the nucleus accumbens (mAcSh) and the medial prefrontal cortex (7, 8). Second, dopamine appears to play an important role in fear conditioning (9). For example, dopamine receptor antagonists block fearful behaviour when infused into mAcSh (10). Detailed anatomical work has shown a strong, reciprocal connection between ventro-medial VTA and the mAcSh, suggesting a closed-loop circuit, in contrast to the feed-forward loops proposed for the rest of the meso-striatal system (11-13). We noted that many electrophysiological studies target the dorso-rostral VTA, predominantly the large parabrachial pigmented nucleus (PBP). It is possible, therefore, that ventro-medial dopamine neurons (particularly the paranigral nucleus (PN)) have been relatively neglected by previous studies, and we hypothesized that these neurons might be excited by noxious stimuli. To directly test this, we recorded from and labeled individual neurons in both dorsal and ventral VTA in anesthetized rats, so that we could deliver temporally-controlled, intense noxious stimulus (electric shock to the hind-paw) and determine the precise anatomical location and neurochemical identity of individual neurons (14). In this study we refer to the

footshock as noxious because it is intense enough to activate nociceptors associated with actual or potential tissue damage (15). In the awake, freely-moving animal this stimulus would be aversive and could act as a punisher.

RESULTS

Electrophysiological characteristics of neurochemically-identified VTA neurons. We recorded extracellular activity from single VTA neurons and delivered multiple (20 Hz), intense (5 mA) and prolonged trains (4 s) of electric shocks to the hind-paw. Following this, the individual recorded neurons were labeled with Neurobiotin using the juxtacellular technique (14). This allowed us to precisely map the location of the neuron post-mortem using established cytoarchitectonic features (11, 16), and to neurochemically identify it with immunofluorescence for tyrosine hydroxylase (TH; the rate-limiting enzyme for dopamine synthesis). Based on our previous work, we deliberately searched for VTA neurons with relatively broad action potentials in an attempt to avoid the population of non-dopaminergic neurons that have similar electrophysiological properties to dopamine neurons (but with narrower action potentials (AP)) (6). However, as expected, we still found some non-dopaminergic neurons within our sample (n = 4), which emphasizes the importance of confirming neurochemical identity through single-cell labeling and immunohistochemistry. It has been suggested that using a high-pass filter setting of 50 Hz is important for distinguishing between dopaminergic and non-dopaminergic VTA neurons on the basis of their action potential waveform (17). We have now directly tested this and find, in fact, that this does not help in electrophysiologically distinguishing VTA neurons. Under these conditions (i.e., 50 Hz high-pass filter) dopamine neurons (n = 14) and non-dopamine neurons (n = 4) exhibited similar shape biphasic APs of the

same duration (from start to trough (mean ± SEM): dopamine neurons 1.62 ± 0.08 ms versus non-dopamine neurons 1.75 ± 0.41 ms, $P = 0.62$, t-test). The neurochemical identity of these non-dopaminergic neurons is currently unknown, but it is unlikely that they are γ-aminobutyric acid (GABA)-ergic, because identified GABAergic neurons in the VTA have different electrophysiological characteristics (i.e., very rapid action potentials (full duration < 1.5 ms) and high firing rates (> 10 Hz)) (18, 19). We and others have described a discrete population of putatively-glutamatergic neurons, concentrated in the rostral VTA, which may represent these TH-negative neurons (20, 21).

Phasic responses of VTA dopamine neurons to noxious stimuli. Consistent with our previous work, in the majority of putative dopamine neurons we observed a rapid inhibition, or no significant response, to the noxious footshocks. We successfully labeled 9 of these neurons (5 inhibited; 4 unresponsive) that were TH-positive (Fig. 1 *A*-*C*). These neurons were primarily located in the dorsal part of the VTA (Fig. 1*G*). In addition, we labeled 5 TH-positive neurons that were strongly excited by the footshocks (Fig. 1 *D*-*F*). Strikingly, these neurons were located in the ventral part of the VTA in, or close to, the PN (Fig. 1*G*). It was, typically, harder to find and juxtacellularly-label excited dopamine neurons compared to inhibited dopamine neurons, which may be one reason why many previous studies could have overlooked this population. Their firing rates and AP waveform characteristics were similar to inhibited dopamine neurons (Fig. 1 *A* and *D*; excited dopamine neurons ($n = 5$) (mean ± SEM): firing rate 2.68 ± 0.58 Hz, AP width (start to trough) 1.25 ± 0.15 ms, versus inhibited dopamine neurons ($n = 5$): firing rate 3.70 ± 0.67 Hz, AP width 1.09 ± 0.11 ms; $P = 0.28$ and $P = 0.43$ respectively, t-test). The phasic excitation peaked around

150 ms after the onset of the footshock (Fig. 2), which is similar to the latency of the well-described rapid excitation seen in most putative dopamine neurons in response to unexpected rewards (1). It is not clear which region is driving this rapid response, but one possibility is the lateral habenula which has been implicated as the source of negative reward prediction errors in dopamine neurons (22).

Firing patterns of VTA dopamine neuron subgroups. Midbrain dopamine neurons can fire in single-spike or bursting mode. Bursts are typically defined as starting with an inter-spike-interval (ISI) of less than 80 ms and finishing with an ISI greater than 160 ms (23). Bursts play an important role in dopamine signalling, because at higher frequencies, such as those that occur in a burst, it is thought that the dopamine transporter becomes overwhelmed and extracellular dopamine increases supralinearly (24). We found that dopamine neurons in the ventral VTA that were excited by the footshocks had particularly high levels of bursting compared to dopamine neurons in the dorsal VTA that were inhibited by the footshocks (mean % of spikes in burst ± SEM: excited (n = 5), 28.71 ± 10.47; inhibited (n = 5), 2.58 ± 1.18; P = 0.016; Mann-Whitney U-test; Fig. 3 *A*-*C*). Dopamine neurons that were excited by the footshocks also had a higher coefficient of variation (CV; a measure of regularity) of their ISIs compared to dopamine neurons that were inhibited by the footshocks (mean ± SEM: excited (n = 5) 0.81 ± 0.14; inhibited (n = 5) 0.35 ± 0.57; P = 0.028; Mann-Whitney U-test). Given this relationship between anatomical position and burst firing it is tempting to speculate that it may be related to different synaptic inputs, although it could also involve differences in their intrinsic excitability (e.g., differential expression of ion channels) (25). Interestingly, non-dopamine neurons had relatively low levels of bursting and ISI CVs (Fig. 3*C*), which suggests that by combining

responsivity to footshocks with burst/regularity analysis it is possible to get a good indication of neurochemical identity and location of the recorded neuron. It is often assumed that dopamine neurons can change between tonic and burst firing states. Our results suggest the interesting possibility that dopamine neurons, depending on their location in the VTA, may be more or less likely to be in one of these two states.

Phasic excitation of dopamine neurons at the termination of noxious stimulation. Behavioural experiments show that the termination of an aversive stimulus can act as a reward (26) and therefore might be expected to excite dopamine neurons (27). Previous studies have reported synchronization of firing following termination of a noxious stimulus in SNc (28), but do not report an increase in firing rate in either SNc or VTA dopamine neurons (6, 28). However, stimuli used previously were either extremely brief or not particularly intense. In contrast, we delivered a stimulus that was both intense and prolonged and found that many dorsal dopamine neurons were clearly excited during the first 500 ms following the termination of the footshocks (as exemplified in Fig. 4 *A*-*C*). Of the 9 neurochemically-identified dopamine neurons that were initially inhibited or unresponsive at the onset of the footshocks, 5 showed a significant, phasic excitation at the termination of the footshocks (Fig. 4*D*). This phasic excitation peaked between 100-150 ms after the termination of the footshocks (Fig. 4*E*). This excitation at stimulus offset may contribute to the dopamine release seen in response to aversive stimuli, as measured using microdialysis. However, it is unlikely to be solely responsible because the increased dopamine release is often large and can occur during stimulus presentation (29). Importantly, this observation can help explain why dopamine receptor antagonists interfere with avoidance learning, where the rewarding role of the offset of an aversive stimulus drives behaviour (30).

DISCUSSION

Considerable attention and controversy has been focused on two types of theory of information coding in dopamine neurons (31-33). One states that dopamine neurons are selectively excited by unexpected rewards and reward-predicting stimuli (1), the other that dopamine neurons are activated by all salient stimuli (33). Our results suggest a novel resolution to this controversy, which is that these two types of theory refer to two functionally- and anatomically-distinct VTA dopamine systems. It is well known that subgroups of VTA dopamine neurons have different projection targets (11) and it seems likely that this will relate to the functionally-distinct populations recorded here. However, the targets for the neurons in this study are unknown and an important next step will be to directly link the neuronal populations recorded here to these different projection systems. We believe that previous studies have predominantly characterized dorsal VTA dopamine neurons, which are selectively activated by rewards (1) and inhibited by noxious stimuli (6). In contrast, we now show that ventral VTA dopamine neurons are excited by noxious stimuli, which suggests the possibility that they may encode saliency. A key test of this proposal will be to investigate how these ventral dopamine neurons respond to rewards.

Our findings can help explain a number of observations that have been particularly problematic for single-system reward theories. For example, aversive stimuli evoke dopamine release at projection targets (7, 8), particularly those that receive strong innervation from the PN (i.e., the medial prefrontal cortex and the shell of the nucleus accumbens). This point remains somewhat speculative as the projection targets of our excited neurons are currently unknown. Another troublesome finding

has been that dopamine receptor antagonists interfere with the acquisition and expression of aversive conditioning (9). Our results suggest that in both of these cases, activation of ventral VTA dopamine neurons may be involved. Moreover, recent studies have highlighted functional differences between rostral and caudal VTA (34-38). For example, overexpression of glutamate receptor subunit 1 or cAMP response element-binding protein in the rostral parts of the VTA causes conditioned place preference to morphine, but in caudal VTA this leads to conditioned place aversion (34, 38). In addition, overexpression of phospholipase Cγ in caudal VTA enhances responsiveness to nociceptive stimuli (35). It is possible that these studies differentially targeted the inhibited and excited dopamine neuron populations described here. The caudal VTA in these studies refers to a region that comprises both the PBP and the compact PN (Fig. 1*G*) (11). In contrast, the rostral VTA comprises the rostral part of the PBP and the parafasciculus retroflexus (Fig. 1*G*) (11).

Lastly, it will be important to directly compare dorsal and ventral VTA dopamine neuron activity in freely-moving, awake animals. Although it is not currently feasible to use the juxtacellular labeling technique in freely-moving animals, careful single-neuron recordings which systematically explore the entire VTA may still be informative. In any case, it is likely that our findings can be extrapolated to awake animals because dopamine neuron properties and responses to noxious events appear to be relatively unaffected by anesthesia (39-42).

In conclusion, we show here that dopamine neurons located in the ventral VTA are excited by noxious footshocks, in contrast to dorsal VTA dopamine neurons which are inhibited. We suggest that these two anatomically-discrete populations represent two functionally-distinct dopamine systems within the VTA.

MATERIALS AND METHODS

Rats were treated in accordance with the Animals (Scientific Procedures) Act 1986 (UK).

Surgery. Sprague-Dawley rats (250-400 g; Charles River, UK) were anesthetized with urethane (1.3 g/kg, i.p.; Sigma, UK), plus supplemental doses of ketamine (20 mg/kg, i.p.; Ketaset, Willows Francis, UK) and xylazine (2 mg/kg, i.p.; Rompun, Bayer, Germany) as required. Body temperature was maintained using a homeothermic heating device (Harvard Apparatus, UK). The depth of anesthesia was assessed by testing reflexes to a hind-paw pinch. Corneal dehydration was prevented with application of Lacri-lube eye ointment (Allergan Pharmaceuticals, Ireland). A wide craniotomy was performed centered above the ventral tegmental area (VTA) (rostral-caudal: -5.3 mm from bregma) on either side of the sagittal suture. The prominent blood vessel on the sagittal sinus was heat cauterized at the two ends (rostral and caudal) of the opening without damaging the underlying cortex and removed along with the dura from the exposed brain area. Saline solution (0.9% w/v NaCl) was applied to the exposed cortex to prevent dehydration during recording.

Electrophysiology. Glass microelectrodes were lowered into the VTA using a micromanipulator (LSS-8000 Inchworm Microdrive System, Burleigh, Canada) to a depth of 7.8–9.0 mm (rostral-caudal: 5.0–6.0 mm; medial-lateral: 0.3–1.0 mm). Extracellular neuronal activity was monitored using the glass microelectrode (filled with 1.5 % w/v Neurobiotin (Vector Laboratories, UK) in 0.5M NaCl), which was broken back to give a final tip diameter of 1-2 μm and a resistance of 6-15 MΩ (*in situ*). Extracellular recordings were AC coupled, amplified (x1000), band-pass filtered

between 0.3 or 0.05 and 5 kHz (NeuroLog System, Digitimer, UK) and acquired with Spike2 software (version 5.08, Cambridge Electronic Design, UK) on a PC. Electrical interference from analogue signals was minimized by using HumBug (Quest Scientific, Canada). The signals were then displayed on a digital oscilloscope (TDS 2002B, Tektronics, USA) and captured using a 1401plus A-D converter (Cambridge Electronic Design, U.K.). Data were collected from neurons exhibiting broad action potentials with an initial positive deflection and a spontaneous firing rate < 10 Hz. Spike2 software was used to analyze data off-line. Neuronal activity was typically measured for 2 minutes each at two different filter settings (0.05–5 kHz and 0.3–5 kHz) before the onset of the noxious stimuli. Recordings from 0.3-5 kHz filter settings provided the baseline firing profile of individual neurons and their response to the experimental paradigm. Noxious stimuli were delivered via two silver wires (0.37 mm diameter), one attached to the plantar surface of the heel and the other to the ball of the little toe on the lateral side of the hind-paws of the rats. Noxious electrical stimulations (three trains in each series: 5 mA, 20 Hz, 4 s duration, 60 s inter-train interval) were administered using CED 1401 computer interface and a constant current isolated stimulator (DS3, Digitimer, UK) to hind-paws. Initially they were delivered on ipsi- or contra-lateral sides of the VTA recording site. Because there were no significant differences in recordings between the two sides, for subsequent experiments, three trains of multiple electrical stimulations were given only on the side contra-lateral to the VTA recording site and repeated after an interval of 60-120 s.

Juxtacellular labeling. Following recording, neurons were selectively labeled using the juxtacellular technique (14). Briefly, positive current pulses were applied through

the microelectrode (200 ms duration, 50% duty cycle, 2.5 Hz, 1-20 nA). The amount of current applied was continuously monitored and adjusted in order to obtain modulation of action potential activity in the neuron (i.e., increase in firing to passage of positive currents only). Modulation of firing was required to obtain detectable Neurobiotin labeling of the soma and dendrites of the recorded neuron.

Immunohistochemistry. At the end of the experimental session, animals were given a lethal dose of anesthetic then transcardially perfused with 200 ml of 0.1M phosphate-buffered saline (PBS) solution at pH 7.4 followed by 400 ml of 4 % w/v paraformaldehyde (PFA) solution. The brain was subsequently removed and post-fixed overnight in 4 % PFA. Initially, coronal sections (60 μm) were made on a vibrating microtome (Leica VT1000S, Leica Microsystem, Germany) using PBS in the bath. Sections were incubated in a blocking solution (PBS with 10% normal goat serum (Jackson Laboratories, USA), 0.5 % Triton, and 1 % bovine serum albumin (BSA)) for 1 h at room temperature, and then incubated for 72 h (4°C) in mouse monoclonal antibody against tyrosine hydroxylase (TH; 1:1000; Sigma, USA) in PBS with 2 % normal goat serum, 0.5 % Triton, and 1 % BSA to determine whether the neurons were dopaminergic. Following this, sections were rinsed several times in PBS and then incubated for a further 18-24 h (4°C) in either Cy3-conjugated Streptavidin and Cy5-conjugated donkey anti-mouse antibodies or Cy2-conjugated Streptavidin and Cy3-conjugated donkey anti-mouse antibodies (1:1000, Jackson Laboratories, USA) in PBS with 2 % normal goat serum, 0.5 % Triton, and 1 % BSA. Sections were then rinsed in PBS and mounted on slides in Vectashield (Vector Laboratories, USA) for viewing under a fluorescence microscope (Leica DM4000B, Leica Microsystem, Germany). After the initial experiments we modified our protocol (as

detailed below) to improve the penetration of the antibody which allowed inclusion of labeled neurons deep in the tissue which under the previous protocol would have been rejected from our analysis. After perfusion and fixation as described above, the whole rat brain was cryoprotected in 30 % sucrose in PBS, embedded in OCT medium, frozen in isopentane at -50^{o}C and sectioned at 30 µm on a cryostat (Leica CM1800, Leica Microsystems, Germany). The floating sections were rinsed in PBS and then in 0.2 % Triton-PBS solution or only in PBS. In the next stage, the sections were incubated in blocking solution (PBS with 6 % normal donkey serum, 0.2 % Triton) for 1 h at room temperature and transferred into primary antibody solution of polyclonal rabbit antibody against TH (1:1000 or 1:2000; Calbiochem, USA) in PBS with 2 % normal donkey serum and 0.2 % Triton. Following overnight incubation at room temperature, sections were rinsed several times in PBS with 0.2 % Triton and incubated for another 2-4 h at room temperature in secondary antibody solution consisting of CY3- or CY2-conjugated Streptavidin (1:1000; Jackson Laboratories, USA) and Alexa 488-conjugated goat anti-rabbit antibodies (Invitrogen, USA) or CY3-conjugated to donkey anti-rabbit antibodies (Jackson Laboratories, USA; both 1:1000, in PBS with 2 % normal donkey serum, 0.2 % Triton). Sections were rinsed in PBS with 0.2 % Triton and then PBS solution only and mounted on slides and dried. Coverslips were placed on the slides after applying Vectashield or Gel Mount (Sigma, USA) for visualization and identification. Images were stored digitally using Leica FireCam software (version 1.7.1) on an Apple Mac G3.

Anatomical localisation of labeled neurons. Cytoarchitectonic features of the VTA and the surrounding areas and more specifically of TH-positive neurons including their distribution (density) and their morphology (size, orientation, dendritic

arborisation), as described by Ikemoto (11), were used to precisely determine the anatomical localization of neurobiotin-labeled cells. These neurons were then plotted onto coronal figures from the rat brain atlas of Paxinos and Watson (16). This plotting was done blind to the physiology of the individual neurons. To more clearly depict dorsal and ventral differences, we made a single schematic sagittal view of the VTA (lateral 0.6 mm) on which we plotted all left- and right-side labeled neurons.

Data analysis. Each trace was visually inspected to ensure that the spikes identified were distinct from the noise and other artifacts. The baseline firing rate of each neuron was quantified by averaging a two-minute recording session (with band-pass filter settings of 0.3-5 kHz) prior to the application of the noxious stimuli. For each neuron, an average spike waveform width was calculated from the recordings under the two filter settings described earlier. Width was determined as the time from the onset of the action potential to the negative trough (6).

Statistical analysis. Peristimulus time histograms using 500 ms bins were created from the average of firing rate obtained during all the noxious stimulations. A neuron was considered responsive (excited or inhibited) if the mean firing rate during the first 500 ms following onset of the stimulus showed a change of 1.96 standard deviations from the mean baseline firing rate measured from the 10 s window prior to the onset of stimulation. Neurons that failed this criterion were considered to be unresponsive. Excitation at the termination of the noxious stimulation was determined for the first 500 ms following stimulation offset using the same criterion (i.e., 1.96 standard deviations above the mean baseline firing rate). For each neuron, coefficient of variation of inter-spike-interval was calculated as the ratio of the standard deviation to

the mean of the inter-spike-interval (ISI) for the two-minute baseline recording session. Statistical comparisons of percentage of spikes in a burst and coefficient of variation of ISIs of excited versus inhibited dopamine neurons were made using Mann-Whitney U-test with a significance level of $P < 0.05$. Statistical comparison of action potential waveform widths and firing rates of different classes of recorded neurons were done by using a two-tailed t-test with a significance level of $P < 0.05$.

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AUTHOR CONTRIBUTIONS

The electrophysiological data were collected and analyzed by F.B. and S.C. The anatomical data were collected and analyzed by F.B., S.C., and D.B. The manuscript was prepared by F.B., S.C., D.B. and M.U.

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FIGURE LEGENDS

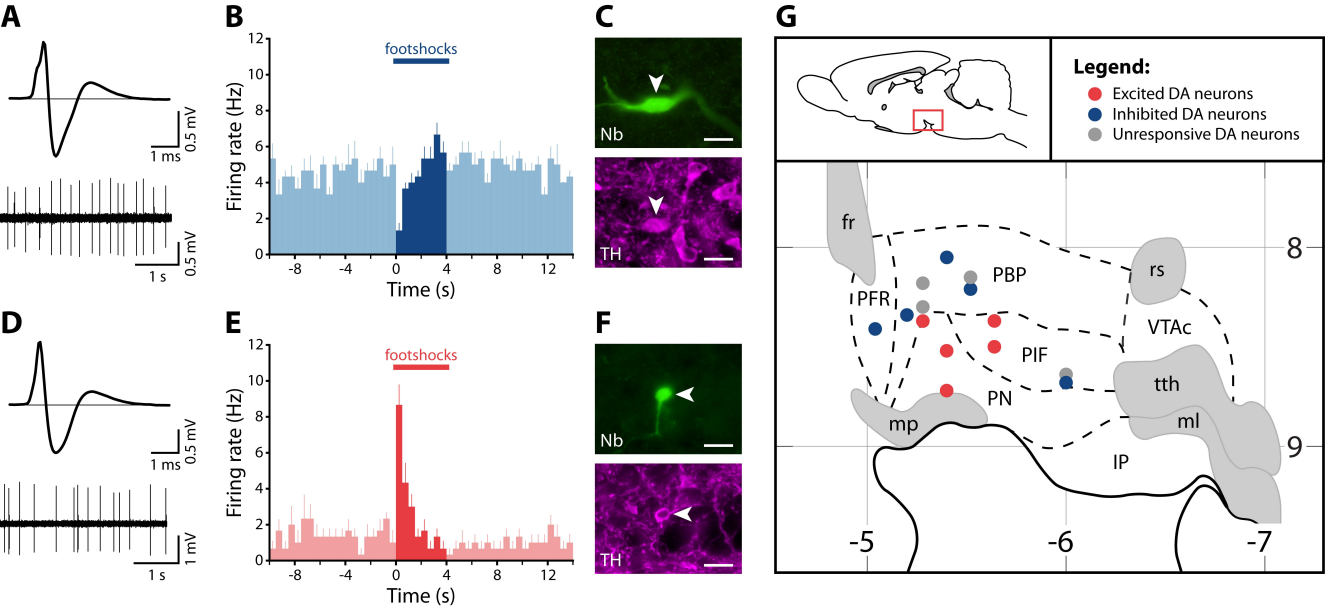
Fig. 1. Dorsal VTA (ventral tegmental area) dopamine neurons are inhibited by noxious stimuli, whereas ventral VTA dopamine neurons are excited. (*A*) Averaged extracellular waveform and baseline firing activity from a recorded neuron. This neuron showed an inhibitory response to footshocks (peri-stimulus time histogram averaged across six footshocks; mean + SEM; 500 ms bins) (*B*) and was immunohistochemically-identified as dopaminergic (TH, tyrosine hydroxylase; Nb, neurobiotin) (*C*). In contrast, a second neuron, with a similar averaged extracellular waveform and baseline firing rate (*D*) showed an excitatory response to footshocks (*E*), but was also immunohistochemically-identified as dopaminergic (*F*). Scale bars represent 20 μm. (*G*) A parasagittal schematic view of the VTA (lateral 0.6 mm) showing the distribution of individual dopamine neurons and their responses to footshocks, showing a clear anatomical segregation of functional subgroups (horizontal numbers are distance from bregma in mm; vertical numbers are depth in mm) (fr, fasciculus retroflexus; IP, interpeduncular nucleus; ml, medial lemniscus; mp, mammillary peduncle; PBP, parabrachial pigmented nucleus; PFR, parafasciculus retroflexus area; PIF, parainterfascicular nucleus; PN, paranigral nucleus; rs, rubrospinal tract; tth, trigeminothalamic tract; VTAc, ventral tegmental area caudal).

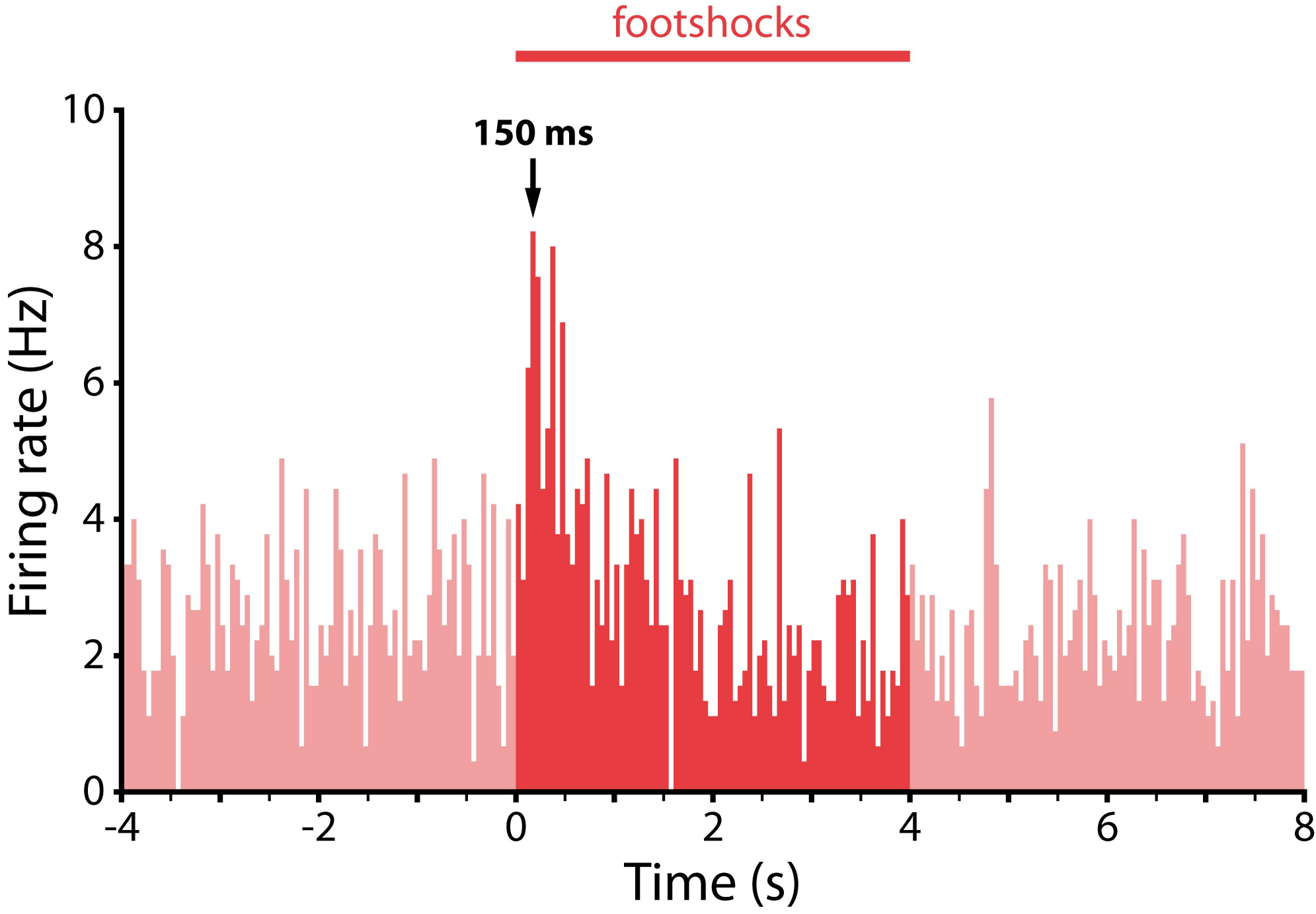
Fig. 2. Footshock-evoked excitations in ventral VTA dopamine neurons have a rapid onset, similar to that seen for reward-related excitations in dopamine neurons in previous studies. Population peri-stimulus time histogram for the 5 identified dopamine neurons that exhibited an excitatory response to footshocks (50 ms bins).

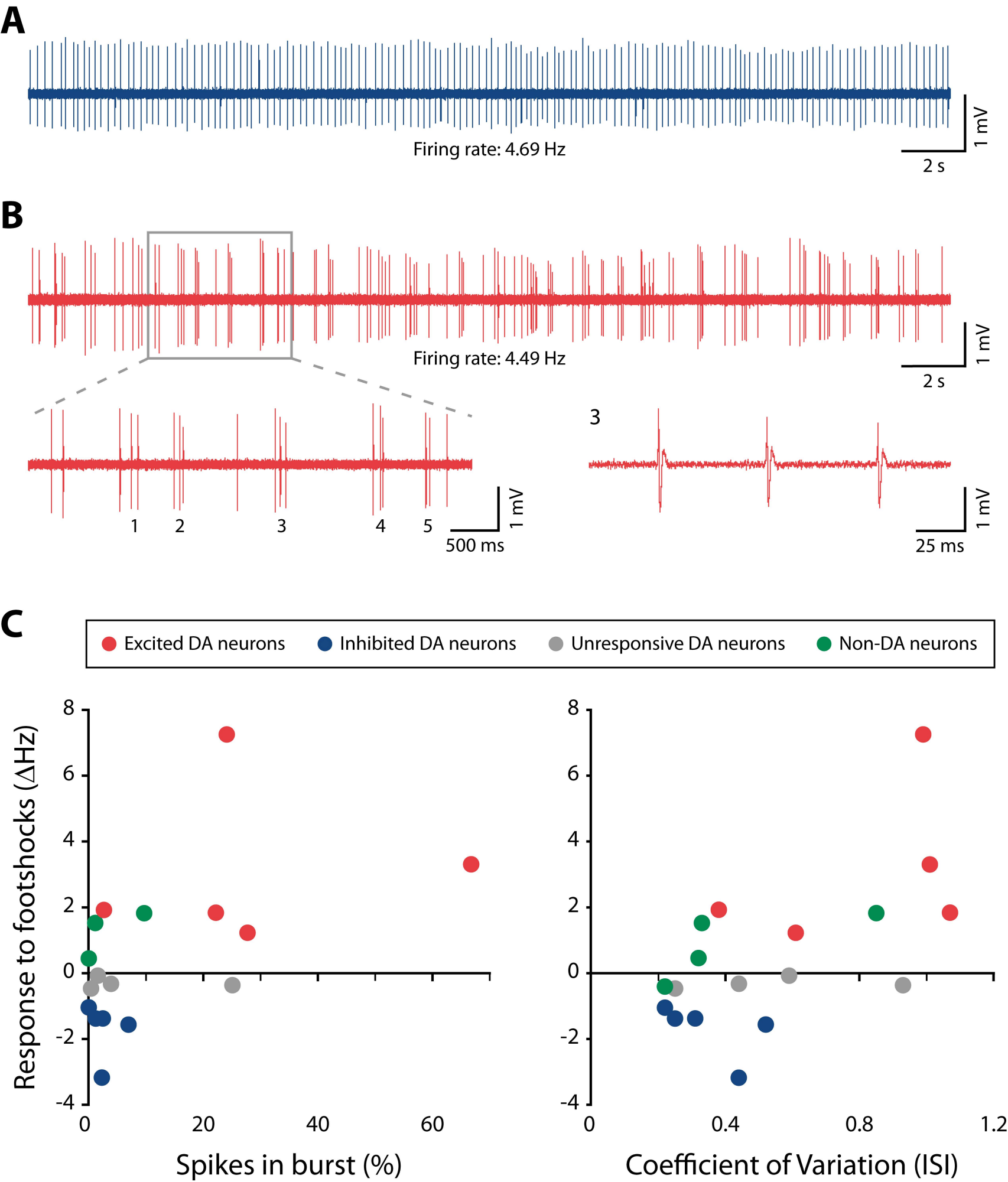
Fig. 3. Dopamine neurons that are inhibited by the noxious stimulus show tonic low-bursting single-spike activity, whereas dopamine neurons that are excited by the noxious stimulus show high levels of bursting. (*A*) Tonic firing activity in an identified dopamine neuron that was inhibited by the footshocks. (*B*) Bursting activity in an identified dopamine neuron that was excited by the footshocks (upper trace). Lower traces show an expanded view of five bursts (left) and of the third burst (right). (*C*) Scatter plots showing change in response to footshock as a function of the percentage of spikes in burst (left panel) or coefficient of variation (CV) of the inter-spike-interval (ISI; right panel). Dopamine neurons that were excited by the footshocks had higher levels of bursting and higher ISI CVs than either dopamine neurons that were inhibited by the footshocks or non-dopamine neurons (one non-dopamine neuron could not be analyzed for bursts because its high baseline firing rate (9.14 Hz) meant that its mean ISI fell within the burst criteria).

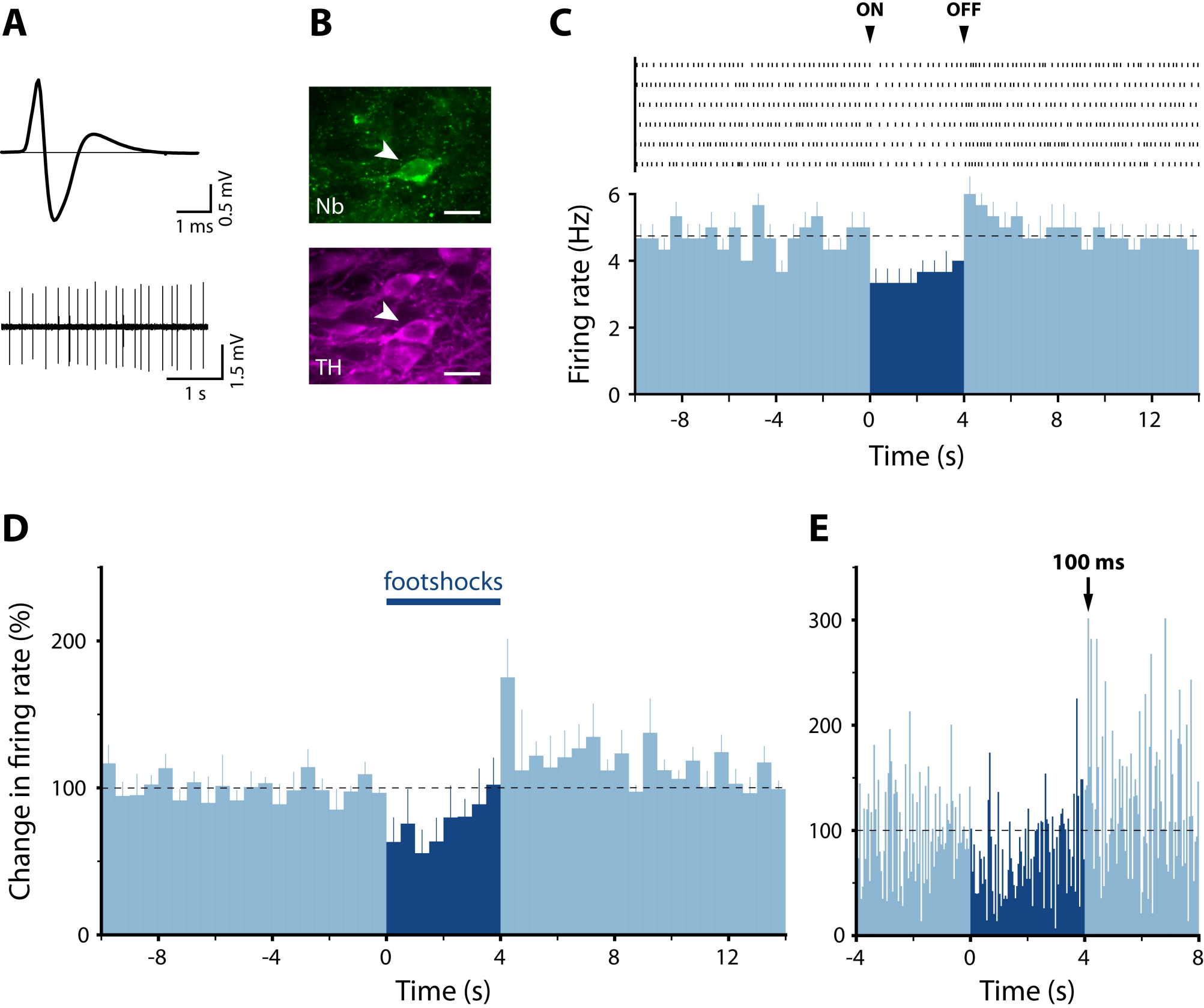
Fig. 4. Many dopamine neurons that are inhibited by (or unresponsive to) the footshocks show a significant excitation at the termination of the stimulus. (*A*) Averaged extracellular waveform and baseline firing activity from a recorded neuron. (*B*) The same neuron was immunohistochemically-identified as dopaminergic (TH, tyrosine hydroxylase; Nb, neurobiotin). Scale bars represent 20 μm. (*C*) A cumulative raster plot of this neuron (upper panel) and the resulting peri-stimulus time histogram (lower panel) averaged across six footshocks (mean + SEM; 500 ms bins) showed an inhibitory response following the onset (ON) of the stimulation and an excitation following its offset (OFF). Black, dashed line indicates mean baseline firing rate. (*D*) Population peri-stimulus time histograms (500 ms bins; mean + SEM) for the 5 identified dopamine neurons that exhibited an excitation at the offset of the

footshocks. (*E*) Higher resolution population peri-stimulus time histograms (50 ms bins; mean) showing latency of the peak response at offset of footshocks.


A
1 ms
0.5 mV
1 s
0.5 mV
B
footshocks
Firing rate (Hz)
Time (s)
C
Nb
TH
D
1 ms
0.5 mV
1 s
1 mV
E
footshocks
Firing rate (Hz)
Time (s)
F
Nb
TH
G
Legend:
Excited DA neurons
Inhibited DA neurons
Unresponsive DA neurons
fr
PFR
PBP
rs
VTAc
PIF
tth
PN
mp
ml
IP
8
9
-5
-6
-7


footshocks
150 ms
Firing rate (Hz)
10
8
6
4
2
0
-4
-2
0
2
4
6
8
Time (s)


A
Firing rate: 4.69 Hz
1 mV
2 s
B
Firing rate: 4.49 Hz
1 mV
2 s
1
2
3
4
5
500 ms
1 mV
3
25 ms
1 mV
C
Excited DA neurons
Inhibited DA neurons
Unresponsive DA neurons
Non-DA neurons
Response to footshocks (ΔHz)
8
6
4
2
0
-2
-4
0
20
40
60
Spikes in burst (%)
0
0.4
0.8
1.2
Coefficient of Variation (ISI)


A
1 ms
0.5 mV
1 s
1.5 mV
B
Nb
TH
C
ON
OFF
Firing rate (Hz)
0
2
4
6
-8
-4
0
4
8
12
Time (s)
D
footshocks
Change in firing rate (%)
0
100
200
-8
-4
0
4
8
12
Time (s)
E
100 ms
0
100
200
300
-4
0
4
8
Time (s)