Cannabinoid receptor type 1 (CB1) has been extensively implicated in a variety of psychological and psychiatric disorders, including drug addiction (1, 2). Recent studies suggest that CB1 within the nucleus accumbens (NAc), a key component of the brain reward circuit, plays a particularly important role in the development and maintenance of cocaine-induced behavioral alterations (3). Compared with the extensive expression of CB1 in the striatum, the mRNA and protein levels of CB1 within the NAc are sparse, leading to the notion that CB1 at afferent terminals projecting to the NAc are largely responsible for intranAc, CB1-dependent, cocaine-induced behaviors (4–6). However, a recent study primarily targeting CB1-expressing neurons demonstrates that inhibiting the expression of CB1 within the NAc antagonizes cocaine-induced reward responses (7). This and other results (8) suggest that CB1-expressing neurons in the NAc, although sparse, are critical for cellular and behavioral alterations induced by cocaine and other drugs of abuse.

To examine these putative CB1-expressing neurons within the NAc, we generated a knock-in mouse line in which CB1-expressing neurons expressed the fluorescent protein td-Tomato (tdT). Our results show that tdT-positive neurons within the NAc were exclusively fast-spiking interneurons (FSIs). These FSIs were not only electrically connected with each other but exerted extensive inhibitory control on nearby medium spiny neurons (MSNs), the principal neurons in the NAc, via monosynaptic connections. Furthermore, the membrane excitability of these neurons became significantly up-regulated throughout short- and long-term withdrawal from repeated exposure to cocaine. These results suggest that CB1-expressing FSIs within the NAc are neural substrates targeted by cocaine exposure and influence the overall functional output of the NAc.
These findings are consistent with previous studies using different experimental approaches in cortices (4, 5, 10–14). The medial prefrontal cortex (mPFC) exhibited higher expression of tdT than adjacent cortical regions (Fig. 1B), consistent with the expression pattern of previous results (4). In addition, we observed expression of tdT in both cell bodies and fibers in layer 1 of cortex. This observation is different from some reports (14, 15) but consistent with others (10) about CB1 expression in the cortex.

The expression pattern of tdT in the basal ganglia was similar to previous reports of CB1 expression (4, 5, 10–14). In the caudate/putamen, there was a clear dorsolateral-to-ventromedial decrease in expression, with virtually all medium-sized neurons in the dorsolateral region exhibiting somatic expression (Fig. 1B, C).
C, and E). In contrast, the NAc exhibited relatively low expression of tdT, consistent with the expression pattern of CB1 reported previously (10–12). These NAc tdT-positive neurons were medium-sized and sparsely distributed, with clear tdT-positive neuronal fibers (Fig. 1B). In addition, these tdT-positive neurons were not seemingly anatomically clustered (11) but, instead, evenly distributed in both the NAc core and shell (Fig. 1B). In the midbrain, expression of tdT was high in portions of the substantia nigra (Fig. 1C and D) and limited to axonal fibers, with no somatic expression observed. Consistent with previous results of CB1 expression (4, 10, 11), no somatic expression of tdT and minimal fiber expression of tdT were observed in the ventral tegmental area (VTA) (Fig. 1D and Fig. S1).

The habenula exhibited very low expression of tdT (Fig. 1C). In the hippocampus, sparse high somatic expression of tdT was observed in the regions outside the pyramidal cell layers of the hippocampus, and large numbers of fluorescent fibers were present surrounding the pyramidal cells (Fig. 1C–E). These observations are consistent with previous results of CB1 expression (4, 5, 11, 13). In the cerebellum, where CB1 is extensively expressed (4, 10, 16), high expression of tdT was detected (Fig. 1F).

Collectively, our results suggest that CB1-expressing neurons were faithfully labeled with tdT in this mouse line.

**tdT-Positive Cells in the NAc Are FSIs.** To characterize the electrophysiological properties of CB1-expressing neurons in the NAc, we targeted these neurons based on their tdT signals (Fig. 2A) and performed whole-cell current-clamp recordings. Compared with principal MSNs, these tdT-positive neurons (n = 57) exhibited a high maximal rate of evoked action potential (AP) firing, short AP half-width, large afterhyperpolarization potential, and little inward rectification (Fig. 2B–G and Table S1). These membrane properties are consistent with those of FSIs previously reported in the striatum and NAc (17–19), and they are distinctively different from those of MSNs or other types of interneurons that have been characterized in the striatum and NAc (18, 20–24). Indeed, all tdT-positive neurons (n = 327) recorded throughout the study exhibited the fast-spiking properties, suggesting that (i) CB1-expressing NAc neurons were exclusively FSIs and, as such, (ii) NAc MSNs did not express CB1.

Using immunostaining approaches, we next attempted to verify these conclusions and to explore the biochemical properties of NAc tdT-expressing neurons. Thus far, three major types of medium-sized GABAergic interneurons have been identified in the striatum based on the differential expression of their signature proteins: (i) parvalbumin (PV), (ii) calretinin (CR), and (iii) somatostatin or neuropeptide Y or nitric oxide synthase (SOM/NPY/NOS) (21). Expression of PV can serve as a reliable marker for FSIs, because all striatal PV-expressing interneurons that have been recorded thus far are FSIs (18, 19, 22). We thus examined whether tdT-expressing FSIs in the NAc were indeed PV-expressing FSIs by dual immunostaining for tdT (using dsRed antibody) and PV in coronal slices from CB1-tdT mice (n = 5). Within the NAc shell and core, 52% of tdT-positive cells (total of 541 cells) were also labeled with PV, whereas 65% of PV-positive cells (total of 437 cells) were colabeled with tdT (Fig. 3A–D and SI Results). The partial overlap of tdT- and PV-expressing neurons suggests that at least three types of FSIs are present in the NAc: (i) FSIs expressing CB1 only, (ii) FSIs expressing PV only, and (iii) FSIs expressing both CB1 and PV. In addition, both tdT- and PV-expressing neurons within the NAc exhibited a rostral-to-caudal decrease in the density, a distribution pattern in concert with previous mRNA-based results of CB1 expression (25). It is also worth noting that the colocalization of PV and CB1 is not observed in the somatosensory cortex (14) or hippocampus (26). Nonetheless, a remaining question is whether CB1-positive, PV-negative FSIs fall into any known neuronal subtypes. SOM/NPY/NOS-expressing interneurons can be largely excluded due to their distinct electrophysiological and morphological properties (18, 22, 27–30). CR-expressing neurons, however, remained unchecked for their electrophysiological properties. Using triple immunostaining in coronal slices from CB1-tdT mice (n = 3), we observed strong and extensive CR signals in neuronal processes in the NAc shell, with very few CR-positive somas, as reported previously (31). Furthermore, very few CR-positive cells are tdT-positive (4 of
242, 2.0 ± 0.8%; Fig. 3 I, K, and L) or PV-positive (1 of 242, 0.5 ± 0.5%; Fig. 3 J–L). As such, the tdT-positive, PV-negative neurons may represent a distinct population of FSIs in the NAc. For NAc/striatal MSNs, COUP TF1-interacting protein 2 (Ctip2) has been identified as a reliable molecular marker (32). In coronal slices from CB1-tdT mice (n = 3), our immunostaining results show minimal overlap of tdT and Ctip2 in NAc neurons (22 of 788, 2.8 ± 0.4%; Fig. 3 E–H). These results confirm our electrophysiological conclusion that little CB1 is expressed in NAc MSNs.

**Synaptic Connections of tdT-Positive FSIs.** Dual recordings in the NAc shell from our CB1-tdT mice (6–8 wk old) revealed that tdT-positive FSIs were electrically coupled. In this experiment, two adjacent (75–250 μm apart) tdT-positive neurons were simultaneously recorded, one (tdT-a) in current-clamp configuration and the other (tdT-b) in voltage-clamp configuration (Fig. 4A). Current injections into tdT-a induced temporally correlated changes in the membrane potential of tdT-b, and APs elicited in tdT-a were always accompanied by temporally correlated electrical current spikes [excitatory postsynaptic current (EPSC)] in tdT-b (Fig. 4 B–D). These simultaneous events suggest that tdT-positive FSIs are electrically connected, similar to FSIs in the striatum (19). In our recordings, the majority (20 of 32) of pairs exhibited such an electrical coupling.

The tdT-positive FSIs were also connected with each other by chemical synapses; an AP in tdT-a often triggered a postsynaptic chemical current [inhibitory postsynaptic current (IPSC)] in tdT-b in the majority (23 of 32) of recorded pairs, with a delay of 0.71 ± 0.05 ms (AP peak to initiation of the synaptic current, n = 23; Fig. 4B), suggesting a monosynaptic transmission via chemical synapses. Furthermore, these synaptic currents were completely inhibited by perfusion of picrotoxin (0.1 mM, n = 4), confirming they were IPSCs.

In these paired recordings, both tdT-positive FSIs presumably expressed CB1, which may regulate the chemical synapses between them. An important and sensitive CB1-dependent short-term synaptic regulation is depolarization-induced suppression of inhibition (DSI). However, DSI was not induced at tdT-to-tdT inhibitory synapses (10 s at 0 mV, inhibition: 7.36 ± 4.50%; P = 0.86; n = 17/8; Fig. 4E). This result cannot be explained by a lack of expression of CB1 at these synapses because these synapses were sensitive to CB1-selective agonist WIN 55212-2 [(R)-(+)2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenemethanone mesylate; 5 μM; n = 6/4], the perfusion of which substantially inhibited this unitary synaptic transmission by 60.9 ± 8.0% (Fig. 4F). Furthermore, this inhibition was accompanied by an increase in the paired-pulse ratio (PPR; P < 0.04, t test; Fig. 4 G and H) and an increase in the coefficient of variance (CV) of IPSC amplitudes (P < 0.03, t test; Fig. 4 F and J), suggesting presynaptic expression. Thus, functional CB1 is expressed at presynaptic terminals of tdT-to-tdT unitary synapses but is not regulated by depolarization of postsynaptic FSIs, likely due to the lack of endocannabinoid release.
Beyond tdT-to-tdT unitary inhibitory synapses, tdT-positive FSIs received extensive excitatory inputs. These excitatory synapses exhibited different properties from excitatory synapses on MSNs. In tdT-positive neurons from the NAc shell, AMPA [2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid] receptor (AMPA)-mediated EPSCs were mostly composed of a NASPM (1-Naphthyl acetyl spermine trihydrochloride)-sensitive component, presumably mediated by GluA2-lacking AMPARs (area: 71.7 ± 3.3%, n = 9/4; Fig. 4 K and L and SI Results), which was not typically observed at excitatory synapses in MSNs (33, 34). Moreover, a clear D-(-)-2-Amino-5-phosphonopentanoic acid (APV)-sensitive, N-Methyl-D-aspartate receptor (NMDAR)-mediated component was also present (area: 58.0 ± 3.7%; Fig. 4 K and L and SI Results). These properties of excitatory synapses in NAc tdT-positive FSIs are partially consistent with those in striatal FSIs (20).

**Synaptic Transmission from tdT-Positive FSIs to MSNs: Effect of Cocaine Exposure.** Paired recording (Fig. 5A) in the NAc shell showed that tdT-positive FSIs directly innervated adjacent MSNs in the NAc shell. As shown in Fig. 5B, a single AP in tdT-positive neurons elicited a postsynaptic current, with a short delay (0.5 ± 0.06 ms, n = 24) and picrotoxin sensitivity (0.1 mM, n = 4), suggesting that they were unitary IPSCs (uIPSCs).

The NAc is a critical brain region, where exposure to cocaine triggers addiction-related neural adaptations (35, 36). Although the effects of cocaine exposure on NAc principal neurons have been extensively characterized, little is known about how cocaine exposure regulates FSIs, which impose extensive regulation on the principal neurons. Our results show that unitary synaptic transmission from tdT-positive FSIs to MSNs in the NAc did not appear to be altered by repeated exposure to cocaine (15 mg kg⁻¹ d⁻¹, 5 d, at 1 or 40 d of withdrawal). First, the PPR was...
not significantly altered in recorded uIPSCs at 1 or 40 d of withdrawal (day 1, \( P = 0.28 \); day 40, \( P = 0.42 \), \( t \) test; Fig. 5 B and C). Second, the peak amplitude of uIPSCs in MSNs was not significantly altered (day 1, \( P = 0.40 \); day 40, \( P = 0.32 \), \( t \) test; Fig. 5F). Third, the amplitude of uIPSCs fluctuated over a trial, and this fluctuation, which could be experimentally assessed by the

CV, corresponds to the number of functionally active synapses being activated during transmission. The CV of uIPSCs was not significantly altered either (day 1, \( P = 0.59 \); day 40, \( P = 0.82 \), \( t \) test; Fig. 5 D and E). Thus, potential cocaine-induced alterations in the number of active synapses per FSI-MSN pair were not detected. Fourth, the unitary synaptic transmission from tdT-positive FSIs to MSNs exhibited robust CB1-dependent DSI (induced by a 5-s depolarization of the postsynaptic MSN to 0 mV; 26.7 ± 3.7%), which was blocked by AM 251 (2 μM), an inverse agonist of CB1 (\( P = 0.01 \), \( t \) test; Fig. 5G). This DSI was not different between saline- and cocaine-administered animals on withdrawal day 1 (\( P = 0.46 \), \( t \) test; Fig. 5H) or withdrawal day 40 (\( P = 0.30 \), \( t \) test; Fig. 5I). In addition, other kinetic parameters (e.g., decay time constant, rise time, half-width of uIPSCs) were not altered (Table S2).

### Cocaine-Induced Adaptations in CB1-Expressing FSIs

We next examined the impact of cocaine exposure on the intrinsic membrane excitability and synaptic properties of tdT-positive FSIs in the NAc shell. The membrane excitability was assessed by the frequency of evoked APs (24, 37, 38). After 1 d of withdrawal from repeated cocaine administration, the membrane excitability of tdT-positive FSIs was significantly increased \([F(1,160) = 28.77, P < 0.001, \text{two-factor ANOVA}; \text{Fig. 6 A and B}]\), and it remained high on withdrawal day 40 \([F(1,160) = 64.99, P < 0.001, \text{two-factor ANOVA}; \text{Fig. 6 C and D}]\). This cocaine-induced increase was accompanied by alterations of either the threshold of APs (\( P = 0.03, \text{t test}; \text{Table S3} \)) or membrane resistance (\( P = 0.05, \text{t test}; \text{Table S3} \)). Note that the membrane excitability of MSNs in the NAc is decreased during withdrawal from repeated exposure to cocaine (24, 37, 38). Thus, exposure to cocaine differentially regulates principal MSNs and FSIs. Also note that the kinetics of APs in CB1/tdT-expressing FSIs were not altered following exposure to cocaine (Table S3).

In addition to membrane excitability, synaptic input affects the output of FSIs, which, in turn, may regulate the overall output of the NAc. We next examined the potential effects of cocaine on synaptic transmission to tdT-positive FSIs by measuring miniature EPSCs (mEPSCs) and miniature IPSCs (mIPSCs). Neither the mean frequency (cocaine vs. saline: mEPSCs: d 1, \( P = 0.21 \); d 40, \( P = 0.35 \); mIPSCs: d 1, \( P = 0.49 \); d 40, \( P = 0.67 ; t \) test) nor the mean amplitude (cocaine vs. saline: mEPSCs: d 1, \( P = 0.61; d 40, P = 0.24; \) mIPSCs: d 1, \( P = 0.35; d 40, P = 0.63; t \) test) of mEPSCs or mIPSCs in tdT-positive FSIs was significantly altered on withdrawal day 1 or 40 (Fig. 6 E–L). Thus, potential cocaine-induced alterations in synaptic inputs to tdT-positive FSIs were not detected. These results, taken together with our earlier results, depict a general picture of the output of the NAc following exposure to cocaine. Because the basal synaptic inputs to tdT-positive FSIs (Fig. 6) and the unitary synaptic transmission from tdT-positive FSIs to MSNs were not altered (Fig. 5), the increased membrane excitability of FSIs (Fig. 6) may readily result in increased inhibitory control of MSNs. This enhanced inhibitory control, together with the “direct” inhibitory effect of cocaine on the membrane excitability of MSNs (24, 37, 38), may substantially suppress the output of NAc MSNs following cocaine exposure.

### Discussion

**CB1-Expressing Neurons in the NAc.** CB1 is one of the most abundant G protein-coupled receptors in the brain (4). In the striatum, the distribution of CB1-expressing neurons exhibits a progressive dorsolateral-to-ventromedial reduction (4) (Fig. 1), with much fewer CB1-positive neurons in the NAc (4, 10, 11, 25). Based on such a low density of expression and certain behavioral patterns induced by intra-NAc manipulation of CB1 signaling, it had been hypothesized that CB1-expressing neurons in the NAc are interneurons (8, 25). Using a genetic marker combined with
Electrophysiological recording and immunohistostaining, our current study provides unambiguous evidence that unlike the dorsal striatum, where CB1 is expressed highly in both MSNs and interneurons, NAc CB1 reporter tdT-expressing neurons are exclusively FSIs (Figs. 2 and 3). This result, together with other related observations, provides several important theoretical deductions.

First, given that the primary function of CB1 is to regulate presynaptic release of neurotransmitters (39), the lack of expression in NAc MSNs suggests that synaptic projections from the NAc are not influenced by CB1 signaling. This is important in understanding NAc-originated projections, particularly the projection to the VTA, a key feedback pathway in the brain reward circuit. Our results suggest that this feedback pathway is free of CB1-mediated regulation, because neither the VTA nor the NAc projection neurons express CB1. Thus, although some inhibitory presynaptic terminals in the VTA carry CB1 (6) (Fig. 1D and Fig. S1), which may underlie CB1-mediated modulation of these synapses (40-42), these inhibitory synapses on VTA neurons are not likely projected from the NAc based on our present results. In addition, although CB1-mediated regulation of VTA-to-NAc dopamine release has been observed (43), because VTA dopamine neurons do not express CB1 (12) (Fig. 1D and Fig. S1 D and E), the VTA-to-NAc presynaptic dopaminergic terminals are also not likely regulated by CB1 signaling directly. As such, there seems no within-circuit presynaptic CB1-mediated regulation through the entire VTA (dopamine)-NAc-VTA loop. If so, CB1-mediated regulation of dopamine release to the NAc (43, 44) must be mediated by other CB1-expressing projections.

Second, NAc neurons are thought to be organized as separate functional ensembles (45). Our results suggest that adjacent CB1-expressing FSIs were often connected to each other with
both electrical and chemical synapses (Fig. 4). Illuminated primarily by the studies of striatal FSIs (19, 20, 46–51), these CB1-expressing FSIs may provide feed-forward inhibition of NAc MSNs, with high timing and anatomical specificities. Thus, the electrical coupling between CB1-expressing FSIs may serve as one type of organizer to synchronize the functional output of a small group of MSNs (ensemble).

Third, approximately half of PV-positive neurons did not express CB1 (Fig. 3 A–D). Assuming that most PV-positive neurons in the NAc are FSIs and exhibit electrical coupling as demonstrated in the striatum, only approximately half of FSIs and their controlled ensembles would be sensitive to CB1-mediated modulation. This potential dichotomy raises several intriguing questions for future studies: (i) Are these CB1 ensembles those that mediate cannabinoid-elicted emotional and motivational alterations? (ii) If so, how do these CB1 ensembles interact with other non-CB1 ensembles? (iii) Can these CB1 ensembles be selectively targeted to achieve therapeutic specificity?

Fourth, because CB1/FSIs only partially overlap with PV-expressing neurons, previous studies using PV as a marker to assess the number of FSIs in the NAc/striatum may underestimate the total number of FSIs by ~50%. This is anecdotally supported by the unexpectedly high frequency of encountering FSIs during random sampling of striatal neurons in brain slices (19) and in vivo (52). Furthermore, using both CB1 and PV as markers for FSIs, our estimated spacing between FSIs was ~122 μm (SI Results), a distance that is within the axonal arbor of FSIs (18). This estimated anatomical setup suggests extensive control of the MSN population by FSIs.

Effects of Cocaine. An important finding of the present study is that following cocaine exposure, the intrinsic membrane excitability of CB1-expressing FSIs was increased, whereas the synaptic efficacy of FSI-MSN transmission appeared to remain unaltered (Figs. 5 and 6). In the striatum, and also likely in the NAc, FSIs exert strong inhibitory control on the output MSNs. Our previous results show that the membrane excitability of MSNs is decreased following contingent or noncontingent exposure to cocaine (24, 37, 38). Thus, the effects of cocaine on CB1-expressing FSIs may increase the inhibitory influence over MSNs, further decreasing the membrane responsiveness of the NAc to excitatory input during cocaine withdrawal. As such, the increased membrane excitability of CB1-expressing FSIs during cocaine withdrawal (Fig. 6) provides a circuitry mechanism to dampen further the responsiveness of the NAc to excitatory input. Beyond the level of individual MSNs, cocaine’s effect on CB1-expressing FSIs can also exert an impact at the functional ensemble level. Because one CB1-expressing FSI may simultaneously influence an ensemble of NAc MSNs, the cellular behaviors of such a putative functional ensemble can be, to some extent, synchronized toward the same functional mode shaped by the effect of cocaine on FSIs. Collectively, our results suggest that the effects of cocaine on CB1-expressing FSIs and MSNs coordinate to decrease the functional responsiveness of NAc MSNs to excitatory input.

However, the excitatory synaptic input to MSNs in the NAc is also, per se, subject to cocaine-induced adaptation. During cocaine withdrawal, the postsynaptic responsiveness (number of postsynaptic AMPARs) of excitatory synaptic input to NAc MSNs is increased (36), functionally opposing the effects of cocaine on the membrane excitability of MSNs and FSIs. These opposing effects do not simply cancel out each other or follow a linear integrator; rather, they may reshape the input-output equation of NAc MSNs, as implied by a previous computational model (38). Specifically, the decreased membrane responsiveness during cocaine withdrawal may effectively prevent AP firing of NAc MSNs in response to low-intensity excitatory synaptic inputs that otherwise elicit APs in naive animals. These low-intensity excitatory inputs may represent basal or background stimulation, such as stimuli associated with the home cages where animals dwell. On high-intensity excitatory inputs, which can be produced by reexposure to cocaine or cocaine-associated cues during withdrawal, the effect of cocaine on excitatory synaptic strength would predominate, resulting in a higher level of AP firing in NAc MSNs than in naive animals (38). This speculation is supported by observations of hypoactivity of the NAc during cocaine withdrawal and hyperactivity of NAc on reexposure to cocaine after withdrawal (53, 54).

In summary, the present study characterized the biochemical and biophysical properties of CB1-expressing neurons in the NAc and demonstrated cocaine-induced adaptations in these neurons. These results suggest that NAc CB1-expressing FSIs may serve as critical neuronal targets for cocaine to induce cellular and behavioral alterations related to addiction.

Experimental Procedures

Experimental procedures are provided in SI Experimental Procedures. These procedures include generation of the CB1-tdT mouse line. Other sections included are experimental animals and cocaine administration; immunohistochemistry, imaging, and biochemistry; slice preparation and electrophysiology; and data analysis.

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