Toll-like receptor 4 deficiency alters nucleus accumbens synaptic physiology and drug reward behavior

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Behavioral manifestations of drug-seeking behavior are causally linked to alterations of synaptic strength onto nucleus accumbens (NAc) medium spiny neurons (MSN). Although neuron-driven changes in physiology and behavior are well characterized, there is a lack of knowledge of the role of the immune system in mediating such effects. Toll-like receptor 4 (TLR4) is a pattern recognition molecule of the innate immune system, and evidence suggests that it modulates drug-related behavior. Using TLR4 knockout (TLR4.KO) mice, we show that TLR4 plays a role in NAc synaptic physiology and behavior. In addition to differences in the pharmacological profile of *N*-methyl-D-aspartate receptors (NMDAR) in the NAc core, TLR4.KO animals exhibit a deficit in low-frequency stimulation-induced NMDAR-dependent long-term depression (LTD). Interestingly, the synaptic difference is region specific as no differences were found in excitatory synaptic properties in the NAc shell. Consistent with altered NAc LTD, TLR4.KO animals exhibit an attenuation in drug reward learning. Finally, we show that TLR4 in the NAc core is primarily expressed on microglia. These results suggest that TLR4 influences NAc MSN synaptic physiology and drug reward learning and behavior.

Toll-like receptor 4 | neuroimmune | cocaine | nucleus accumbens | synapse

The integration of dopaminergic and glutamatergic signals within the nucleus accumbens (NAc) is key to processing motivation, reward, and goal-directed behavior (1). Exposure to drugs of abuse leads to behavioral adaptations by recruiting molecular mechanisms of learning and memory within the reward system (2, 3). Adaptations in NAc synaptic properties following exposure to drugs of abuse have been extensively characterized in a circuit-specific manner (4–8). Although these studies revealed important insights into neuronal factors and alterations, they largely ignored the contribution of nonneuronal mechanisms to synaptic adaptations underlying drug-related behaviors. Recent studies have begun to elucidate the role of the innate immune system and, more specifically, microglia in drug reward behavior and physiology (9, 10). However, many questions remain regarding the role of the innate immune system in supporting synaptic reorganization within the reward circuitry.

Toll-like receptor 4 (TLR4) is a pattern recognition molecule of the innate immune system linked to alcohol (11), morphine (12), and cocaine (COC)-associated behaviors (13). However, the conclusions surrounding alcohol and COC have been disputed (14, 15). TLR4 recognizes gram-negative bacteria and "danger signals" released by damaged tissue (16). Beyond pathogen detection, TLR4 is associated with a wide range of behaviors including stress-induced depression (17), visceral pain (18), and opioid reward (12). Despite the growing number of studies pointing to TLR4's involvement in various motivated behaviors, there has been no examination into its role in glutamatergic synaptic physiology. Additionally, the localization of TLR4 within NAc subregions is unknown. To address these questions, we performed cell-type–specific electrophysiology in the NAc core and shell subregions, field potential recordings, drug reward behavioral assays, and fluorescent in situ hybridization. Our findings suggest that TLR4 plays a role in basal NAc core synaptic physiology, plasticity, and drug reward behavior. We also confirm microglia as the primary cells expressing *Tlr4* in the NAc core.

Results

TLR4.KO and Wild-Type Mice Exhibit Synaptic Differences in the NAc Core but Not Shell. Within the NAc core and shell subregions, 90–95% of neurons are medium spiny neurons (MSN) expressing D1 or D2 dopamine receptors (1). Although similar in morphology, these MSNs differ in biochemistry, anatomical connectivity, and function (19–22). Furthermore, experience-dependent changes of glutamatergic synapses occur in a cell-type–specific manner (5, 6, 22–25), and activation of these NAc MSN subtypes differentially regulates drug reward behavior (3, 5, 23). Therefore, we addressed whether TLR4 influenced excitatory synaptic function within the NAc in a cell-type–specific manner.

To assess cell-type–specific NAc MSN physiology, we bred wild-type (WT) and TLR4.KO mice to bacterial artificial chromosome transgenic mice expressing the tdTomato fluorophore driven by the D1 dopamine receptor promotor. Whole-cell voltage clamp recordings were made from MSNs that expressed [D1(+)] or lacked [D1(−)] tdTomato fluorescence. Presence or absence of fluorescence defines D1 and D2 MSNs as described previously (6, 26). To determine the impact of TLR4 expression on excitatory synaptic properties in the NAc core, we assessed pre- and postsynaptic properties. In NAc core D1(+) MSNs, TLR4.KO animals exhibit a significantly decreased AMPA receptor (AMPAR)/NMDA receptor [NMDAR; AMPAR/NMDAR (A/N)] ratio compared

Significance

Substance use disorders affect >8% of the US adult population. Mitigating this problem requires a better understanding of how these substances alter reward system structures such as the nucleus accumbens (NAc). Although classically studied in a neuron-centric manner, the immune system contributes to synaptic and behavioral changes associated with drug experience. We report that the pattern recognition molecule toll-like receptor 4 (TLR4) plays a role in NAc synaptic physiology and drug reward. We show NAc subregion-specific differences in basal synaptic properties consistent with alterations in induction of NMDA receptor-dependent synaptic plasticity in TLR4 knockout (TLR4.KO) animals. Furthermore, we show that TLR4.KO animals exhibit deficits in drug reward learning and confirm microglia as the primary cell type expressing TLR4.

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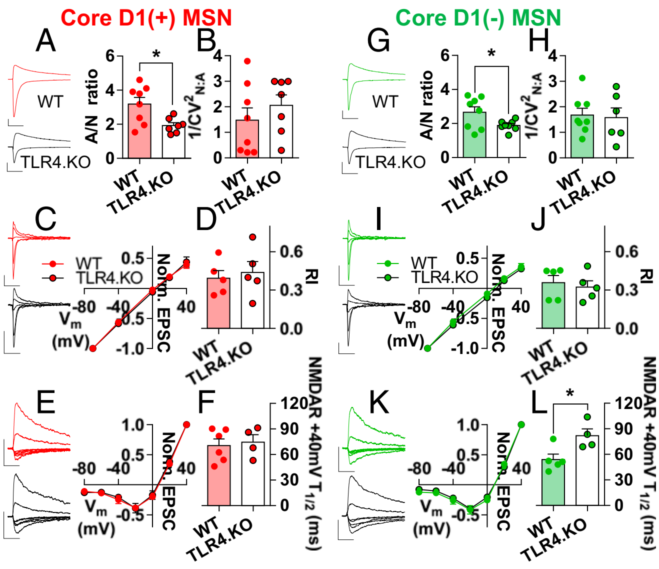

Fig. 1. Altered synaptic properties in NAc core of TLR4.KO mice. (*A*, *Left*) Representative −70 mV and +40 mV evoked current traces from D1(+) MSNs of WT (red) and TLR4.KO (black) animals. The peak current at −70 mV and the current magnitude of 50 ms following current flow at +40 mV was used to calculate the A/N ratio (WT: $n_{(cells)}/N_{(mice)}$ = 8/4; TLR4.KO: *n*/*N* = 7/4). (*Right*) Summary plot of D1(+) A/N ratio. (*B*) Summary ratio of $1/CV^2_{NMDAR}$ to $1/CV^2_{AMPAR}$ ($1/CV^2_{N:A}$) in D1(+) MSNs (WT: *n*/*N* = 8/4; TLR4.KO: *n*/*N* = 7/4). (*C*, *Left*) Representative isolated AMPAR current traces recorded between −70 to +40 mV from D1(+) MSNs. (*Right*) Mean AMPAR I–V plot from D1(+) MSNs. (*D*) RI: $I_{+40\ mV}/I_{-70\ mV}$ (50 μM D-APV; *n*/*N* = 5/3 for both WT and TLR4.KO). (*E*, *Left*) Representative isolated NMDAR current traces recorded between −80 and +40 mV from D1(+) MSNs. (*Right*) Mean NMDAR I–V plot from D1(+) MSNs. (*F*) Time to half-peak of +40 mV NMDAR currents (10 μM NBQX; WT: *n*/*N* = 6/4; TLR4.KO: *n*/*N* = 4/3). (*G*–*L*) Representative traces and summary plots of D1(−) MSNs for A/N ratio (WT: green, *n*/*N* = 8/4; TLR4.KO: black, *n*/*N* = 8/4), $1/CV^2_{N:A}$ (WT: *n*/*N* = 8/4; TLR4.KO: *n*/*N* = 5/4), AMPAR I–V and RI (*n*/*N* = 5/3 for both WT and TLR4.KO), and NMDAR I–V and NMDAR time to half-peak (WT: *n*/*N* = 5/4; TLR4.KO: *n*/*N* = 4/3). All recordings were taken in the presence of picrotoxin (50 μM). (Scale bars: 100 pA; 50 ms.) **P* < 0.05, unpaired *t* test.

with WT (Fig. 1*A*). This suggests either a decrease in postsynaptic strength through reduced AMPAR transmission or an increase in NMDAR transmission. Altered AMPAR transmission may result from differential AMPAR stoichiometry or synaptic quantity. To test for differences in AMPAR stoichiometry, we assessed AMPAR current-voltage (I–V) relationships and rectification index (RI) (Fig. 1 *C* and *D*). We found no differences in AMPAR stoichiometry in D1(+) MSNs. To test for alterations in synaptic AMPAR function, we analyzed the amplitudes of miniature excitatory postsynaptic currents (mEPSC) and found no difference between WT and TLR4.KO D1(+) MSNs (Fig. S1 *A* and *B*). Not surprisingly, we also found no differences in spontaneous EPSC amplitudes (sEPSC; Fig. S1*C*). Whereas A/N ratios represent synaptic transmission from a subset of evoked afferents, mEPSCs and sEPSCs sample indiscriminately. To assess quantal events sampled from the evoked afferents used in A/N ratios, we used a Sr^{2+}-based artificial cerebral spinal fluid (ACSF) to record and analyze electrically stimulated asynchronous EPSCs (asEPSC) (6, 27). We found no differences in asEPSC amplitudes on D1(+) MSNs (Fig. S1*D*). Together, these results suggest that AMPAR transmission is not altered in TLR4.KO D1(+) MSNs. Therefore, the decreased A/N ratios are likely caused by altered NMDAR transmission.

Differences in NMDAR transmission may stem from receptor number, function, stoichiometry, or expression of NMDAR-only synapses. The latter are known as "silent" synapses and are an important substrate for metaplasticity (24). To assess potential differences in silent synapses, we calculated the ratio of $1/CV^2_{NMDAR}$ to $1/CV^2_{AMPAR}$ ($1/CV^2_{N:A}$) as described previously (26). We found no evidence for differences in silent synapses between D1(+) MSNs from WT and TLR4.KO animals (Fig. 1*B*). Alterations in NMDAR stoichiometry are associated with experience-dependent changes in NAc MSN physiology (19). Therefore, we assessed NMDAR I–V relationships and decay kinetics for initial investigation into NMDAR stoichiometry as a potential cause of altered postsynaptic strength. We found no significant differences between WT and TLR4.KO animals in the NMDAR I–V relationship of D1(+) MSNs (Fig. 1*E*). However, we observed a trend toward increased time to half-peak of +40-mV dual component responses taken from the A/N ratios (Fig. S2; WT = 48.2 ± 8.010 ms; TLR4.KO = 64.56 ± 6.682 ms; *P* = 0.1474, unpaired *t* test); to our surprise, we found no differences in isolated NMDAR decay kinetics in TLR4.KO D1(+) MSNs (Fig. 1*F*).

To characterize presynaptic properties of TLR4.KO animals, we examined glutamate release probability using paired-pulse ratios (PPR) and mEPSC frequency. PPR is inversely proportional to the presynaptic release probability. We observed that D1(+) MSNs from TLR4.KO animals have a decreased PPR at the 20-ms but not at the 50-, 100-, 200-, or 400-ms interstimulus intervals (ISI) (Fig. S3*A*). However, this result was not corroborated by mEPSC frequency (Fig. S3 *B* and *C*; WT = 2.727 ± 0.2791 Hz; TLR4.KO = 3.367 ± 0.5189 Hz; *P* = 0.2598, unpaired *t* test). Together, these data suggest that TLR4.KO animals have altered postsynaptic properties, possibly due to altered NMDAR transmission, in D1(+) MSNs in the NAc core.

Cell-type–specific differences in NAc MSN synaptic physiology underlie behavioral differences in reward and motivation (5, 20, 22, 28). Thus, we also assessed synaptic properties in NAc core D1(−) MSNs of TLR4.KO animals. We found that, similar to the D1(+) cells, D1(−) MSNs exhibit a decreased A/N ratio (Fig. 1*G*). In this population of MSNs, no differences were found for $1/CV^2_{N:A}$, AMPAR I–V, RI, mEPSC amplitude, sEPSC amplitude, asEPSC amplitude, PPR, mEPSC frequency, and NMDAR I–V (Fig. 1 *G*–*L*, Fig. S1 *E*–*H*, and Fig. S3 *D*–*F*). However, we found that TLR4.KO D1(−) MSNs exhibit significantly slower NMDAR decay kinetics compared with WT (Fig. 1*L*). These observations suggest that TLR4.KO MSNs exhibit altered NMDAR stoichiometry without alterations in AMPAR transmission or presynaptic release properties.

The specific GluN2 subunit greatly influences NMDAR deactivation kinetics. GluN2A subunits exhibit the fastest deactivation kinetics with the widest abundance in the adult synapse whereas GluN2D has the slowest deactivation kinetics with GluN2B and 2C in the middle (29). To determine the functional NMDAR profile, we applied the GluN2B antagonist Ifenprodil (3 μM) (23) to pharmacologically isolated NMDAR currents from NAc MSNs. This was followed by D-APV (50 μM) to confirm that recorded currents were from NMDARs. If TLR4.KO MSNs exhibit increased GluN2B function, then Ifenprodil will cause a greater depression of NDMAR transmission in these cells. Ifenprodil caused a significant decrease in NMDAR currents from WT D1(+) MSNs (Fig. 2 *A* and *B*). However, we found TLR4.KO D1(+) MSNs to be insensitive to Ifenprodil (Fig. 2 *A* and *B*). Although less common, increased NMDAR decay kinetics may also be caused by GluN2C or GluN2D subunits (29). To test for this, we assessed the effect of the GluN2C/D-positive allosteric modulator CIQ (30 μM) (6) on isolated NMDAR currents from D1(+) MSNs. Whereas CIQ did not cause a significant difference from baseline in WT MSNs, it caused a significant potentiation in TLR4.KO cells (Fig. 2 *C* and *D*). The Ifenprodil and CIQ experiments were also repeated on D1(−) MSNs. Ifenprodil did not cause any significant difference from baseline in either WT or TLR4.KO D1(−) MSNs (Fig. 2 *E* and *F*). CIQ did not significantly alter NMDAR transmission from WT D1(−) MSNs; however, the compound caused a modest yet significant increase in NMDAR currents in TLR4.KO D1(−) MSNs (Fig. 2 *E*–*H*). Taken together, these Ifenprodil/CIQ experiments provide evidence for decreased GluN2B function on TLR4.KO D1(+) MSNs along with increased GluN2C/D function in both D1(+) and D1(−) cells. Our finding that TLR4.KO animals express altered NMDAR properties on both subtypes of MSNs compared with WTs suggests a shared mechanism through which TLR4 affects

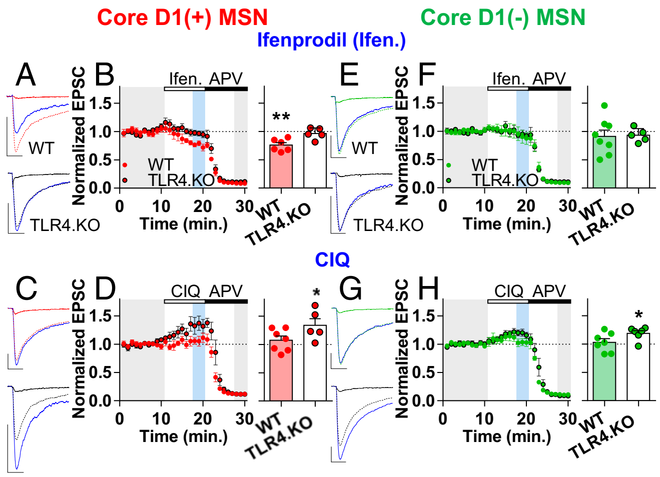

Fig. 2. Altered NAc core NMDAR pharmacological profile in TLR4.KO mice. (*A*) Representative D1(+) MSN NMDAR traces from WT (red) and TLR4.KO (black) animals overlaid with traces following Ifenprodil (3 μM; blue) and APV (50 μM; solid nonblue) application. (*B*, *Left*) Summary plot of D1(+) Ifenprodil experiments. (*Right*) Quantification of Ifenprodil response on the normalized EPSCs (WT: *n*/*N* = 6/3; TLR4.KO: *n*/*N* = 5/4). (*C*) Representative D1(+) MSN NMDAR traces from WT and TLR4.KO animals overlaid with traces following CIQ (30 μM; blue) and APV (solid nonblue) application. (*D*, *Left*) Summary plot of D1(+) CIQ experiments. (*Right*) Quantification of CIQ response on normalized EPSCs (WT: *n*/*N* = 7/4; TLR4.KO: *n*/*N* = 5/4). (*E*–*H*) Representative traces, summary plots, and quantification of D1(–) MSNs for Ifenprodil (WT: green, *n*/*N* = 8/5; TLR4.KO: black, *n*/*N* 5/4) and CIQ experiments (WT: *n*/*N* = 7/4; TLR4.KO: *n*/*N* = 6/4). All experiments were performed holding the cell at –50 mV using a low-Mg^{2+} solution with picrotoxin (50 μM) and NBQX (10 μM). (Scale bars: 100 pA; 50 ms.) *$P < 0.05$, **$P < 0.01$, one-sample *t* test vs. baseline value of 1.0.

synaptic physiology. Altered NMDARs in NAc MSNs are associated with behavioral adaptations affecting motivation, including chronic pain (23), COC experience (6), and chronic intermittent ethanol exposure (30). A basal difference in NMDAR transmission on both MSN types raises the possibility that TLR4.KO animals may exhibit altered learning mechanisms related to NAc core-dependent motivational and reward behavior.

The anatomy and physiology of the NAc shell is distinct from the NAc core with different hippocampal, prefrontal cortical, and midbrain dopaminergic inputs (1, 8, 31). Thus, it is not surprising that experience-dependent changes in MSN synaptic physiology differ between the subregions (8, 26). Unlike the core subregion, we observed no postsynaptic differences between TLR4.KO and WT D1(+) and D1(−) NAc shell MSNs as assessed through A/N ratios and $1/CV^2_{N:A}$ (Fig. 3). We did, however, observe a reduction of PPR in TLR4.KO D1(−) MSNs [Fig. S4; genotype effect $F(1,13) = 6.632$, $P = 0.0231$, two-way repeated measures ANOVA] suggesting altered presynaptic release probability. We conclude that TLR4.KO animals exhibit an alteration in postsynaptic properties in both MSN subtypes of the NAc core but not shell subregions.

TLR4.KO Mice Exhibit Long-Term Depression Deficits in the NAc Core. Synaptic plasticity is a substrate for learning and memory. Within the NAc, perturbations in plasticity mechanisms are associated with alterations in reward and motivation-related behaviors (20, 22, 23). In addition, behavioral experiences related to stress (22), pain (23), and drugs of abuse (6–8, 24, 27) alter plasticity mechanisms in the NAc. With evidence for altered NMDAR transmission on NAc core MSNs, we thought that TLR4.KO animals might exhibit changes in NMDAR-dependent synaptic long-term depression. To test this hypothesis, we performed extracellular field potential recordings from the NAc core. Using a well-established NMDAR-dependent low-frequency (LFS) stimulation protocol (3 × 3 min, 5 Hz stimulation of NAc afferents with 5 min between each LFS train) (22, 27, 32), we assessed long-term depression (LTD) in WT and TLR4.KO mice. In support of our hypothesis, this stimulation protocol induced a depression of evoked field potential responses in slices from WT animals but not TLR4.KO animals (Fig. 4 *A* and *B*). To confirm that this lack of LFS-LTD is not due to general lack of plasticity mechanisms in TLR4.KO animals, we assessed LTD dependent on group II metabotropic glutamate receptors (mGluR). Application of the group II mGluR agonist LY 379268 (200 nM, 10 min) (33) caused a significant reduction in field potentials in both WT and TLR4.KO animals (Fig. 4 *C* and *D*). Thus, the NAc core of TLR4.KO animals exhibit impairments in NMDAR-dependent LTD without deficits in group II mGluR-dependent LTD. In combination with our results showing differences in NMDAR subunit composition, these data suggest the lack of LTD in TLR4.KO is due to impairments in induction mechanisms. Loss of TLR4 function therefore hinders the ability of LFS to reduce synaptic strength in NAc core MSNs.

TLR4.KO Mice Exhibit Deficits in Drug Reward Learning. The NAc core is a nexus for drug-seeking and motivational behavior (1); therefore, the inability to regulate synaptic strength in this region has implications in associated learning. Deficits in NMDAR-dependent synaptic plasticity mechanisms are associated with altered drug reward behavior (33, 34). With evidence for a deficit in NAc core NMDAR-dependent LTD, we hypothesized that TLR4.KO animals exhibit altered drug reward learning. To test this, we performed a COC place conditioning (conditioned place preference, or CPP) assay as previously described (19) (Fig. 5*A*). In this assay, all mice were given three injections of COC and three injections of saline (one of each/context pairing day; Fig. 5*A*). We found that TLR4.KO mice have a significant attenuation in preference following conditioning with a 5-mg/kg dose (Fig. 5*B*). To test whether this result signifies an impairment or a complete loss of COC reward learning, we examined two higher doses of COC (10 mg/kg and 15 mg/kg). In support of TLR4.KO animals having decreased COC reward learning, we found no significant differences in CPP between WT and TLR4.KO animals for 10 mg/kg and 15 mg/kg COC (Fig. 5*B*).

Additionally, TLR4.KO animals did not maintain a change in preference for 10 mg/kg COC when assessed 14 d later (Fig. 5*C*), suggesting that TLR4.KO animals may have a decreased persistence of drug reward learning. Furthermore, TLR4.KO animals display less COC-induced hyperactivity than WT mice (Fig. 5*D*). The differences between genotypes are most evident at the highest COC dose tested.

Importantly, these reductions in preference are not due to differences in episodic memory as TLR4.KO animals do not significantly differ in preference change from WT mice at 15 mg/kg COC (Fig. 5*B*) and show no deficits in novel object recognition, a hippocampus-associated task (Fig. 5*E*). In addition, anhedonia is not a likely cause for impaired drug reward learning as we found no

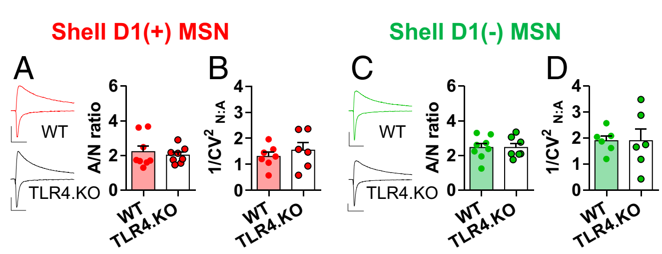

Fig. 3. Lack of postsynaptic differences NAc shell. (*A*, *Left*) Representative evoked current traces recorded from −70 mV and +40 mV from D1(+) MSNs of WT (red) and TLR4.KO (black) animals. (*Right*) Summary plot of A/N ratios (WT: *n*/*N* = 8/5; TLR4.KO: *n*/*N* = 8/4). (*B*) Summary plot of $1/CV^2_{N:A}$ in D1(+) MSNs (WT: *n*/*N* = 7/4; TLR4.KO: *n*/*N* = 6/4). (*C* and *D*) Representative traces and summary plots of D1(–) MSNs for A/N ratio (WT: green, *n*/*N* = 8/5; TLR4.KO: black, *n*/*N* = 7/4), $1/CV^2_{N:A}$ (WT: *n*/*N* = 6/5; TLR4.KO: *n*/*N* = 6/4), All recordings taken in the presence of picrotoxin (50 μM). (Scale bars: 100 pA; 50 ms.) $P > 0.05$ for all comparisons, unpaired *t* test.

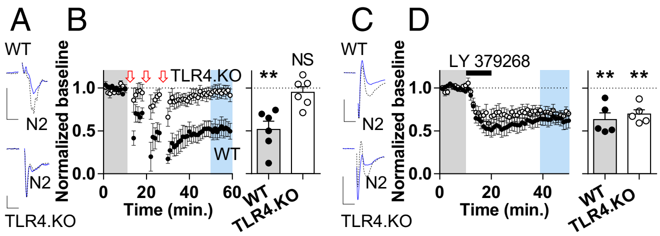

Fig. 4. Impaired NMDAR-dependent LTD and intact group II mGluR LTD in the NAc core of TLR4.KO mice. (*A*) Representative traces from baseline (dashed black) and post LFS (blue) from WT and TLR4.KO field potential experiment. (*B*, *Left*) Summary plot of NMDAR LTD (LFS); 3 × 3 min, 5 Hz separated by 5 min) from NAc core. Arrows denote LFS. (*Right*) Quantification of LFS experiments (WT: $n_{\text{experiments}}/N$ = 6/5; TLR4.KO: *n*/*N* = 6/4). (*C* and *D*) Representative traces, summary plot, and quantification of group II mGluR agonist application (LY 379268, 200 nM, 10 min) effect on N2 responses. (WT: *n*/*N* = 5/4; TLR4.KO: *n*/*N* = 5/3). All recordings taken in the presence of picrotoxin (50 μM). (Scale bars: 0.4 mV; 4 ms.) NS: not significant. $^{**}P < 0.01$, one-sample *t* test of normalized N2 from last 10 min of experiment vs. baseline value of 1.0.

differences between WT and TLR4.KO animals for the sucrose preference test (Fig. 5*F*). Finally, to control for basal behavioral states, we assessed open field locomotor activity and center time. No differences were observed for distance traveled. However, there was a trend toward decreased center time in the TLR4.KO animals (WT = 843.5 ± 98.52 s; TLR4.KO = 615.3 ± 79.72 s; $P = 0.0923$, unpaired *t* test) (Fig. S5 *A* and *B*). These results support our hypothesis that TLR4.KO animals exhibit specific alterations in drug reward learning.

TLR4 in NAc Core Expressed Primarily on Microglia. To determine where *Tlr4* was expressed within the NAc core, we performed multiplex fluorescent in situ hybridization from frozen NAc core sections taken from naive WT mice. Prior studies looking into *Tlr4* expression in the NAc used fluorescence activated cell sorting followed by qPCR but did not differentiate between the core and shell subregions (35). Consistent with results from the NAc as a whole (35), we found that the majority (~80%) of TLR4-expressing cells in the NAc core could be classified as microglia (Fig. 6; *Tlr4*+, *Iba1*+, *Gfap*−; $n_{(\text{cells})}$ = 114/143; $N_{(\text{animals})}$ = 4) [where *n* is the number of cells counted and *N* is the number of mice]. The rest included astrocytes (*Tlr4* +, *Gfap*+, *Iba1*−; *n* = 9/143), cells expressing both astrocytic and microglial markers (*Tlr4*+, *Iba1*+, *Gfap*+; *n* = 13/143), and some other cell populations (*Tlr4*+, *Iba1*−, *Gfap*−; *n* = 7/143). These results suggest the possibility of an interaction between NAc core MSNs and microglia mediating the synaptic and behavioral effects observed in TLR4.KO animals.

Discussion

In the present study, we used TLR4.KO and cell-type–specific reporter mice to investigate the interaction between the innate immune system and key elements of the reward circuit. We provide evidence that TLR4 significantly influences NAc core NMDAR synaptic transmission, synaptic plasticity, and COC-induced behavioral plasticity. Whereas we observed altered NMDAR transmission and plasticity in the NAc core, we found no postsynaptic differences between WT and TLR4.KO animals in the neighboring NAc shell. Furthermore, we found that these mice exhibit blunted behavioral responding to COC and that NAc core *Tlr4* is primarily expressed on microglia. These results suggest that TLR4, likely expressed on microglia, is a molecular regulator in the NAc associated with reward learning.

TLR4 and Drug Reward Behavior. Although numerous neuron-centric studies have revealed important insights into how drugs of abuse alter behavior and NAc physiology, far less is known about the role of the innate immune system in this sequela. One intriguing molecular target is the pattern recognition molecule TLR4. Along with its function in innate immunity, research suggests that TLR4 may play a role in reward behaviors associated with alcohol (11), morphine (12), and COC (13). Northcutt et al. (13) demonstrated that TLR4 antagonists diminish COC self-administration in rats through an effect mediated by the ventral tegmental area. These investigators also found COC reward behavior diminished in the C3H/HeJ mouse line. Although the C3H/HeJ line is deficient in TLR4, these mice are also homozygous for an inversion spanning 20% of chromosome 6 (36), making it difficult to draw specific conclusions. A later study also found that the pharmacologic reduction in COC reward in rats may be due to nonspecific effects as the same doses TLR4 antagonists also caused decreased food reward behavior (14). Additionally, TLR4's role in alcohol reward has also been disputed (15). These discrepancies prompt continued investigation of the nature of TLR4's involvement in drug reward.

TLR4 and NAc Synaptic Physiology. The NAc is a brain region that integrates information on motivation and reward to initiate goal-directed behavior (1). Virtually every drug of abuse causes changes within the NAc (2, 3), and reversal of synaptic changes leads to reversal of drug reward learning (5, 7, 8). These changes occur in a subregion (8, 37) and cell-type–specific (20, 24, 25) manner. We found that TLR4.KO mice had decreased A/N ratios in both D1(+) and D1(−) MSNs in the NAc core but not shell. With no observed differences in AMPAR function, we conclude that these differences are due to alterations in NMDARs.

Our results from TLR4.KO animals showed significantly slower isolated NMDAR decay kinetics in D1(−) MSNs and a

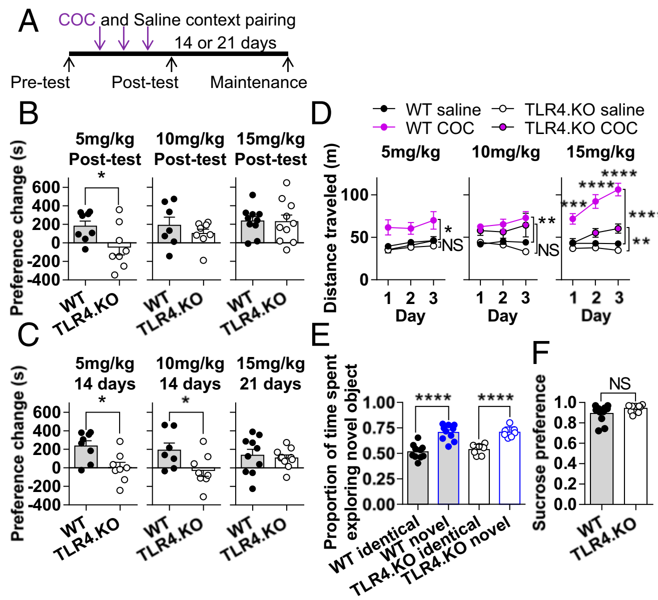

Fig. 5. TLR4.KO mice exhibit attenuated drug reward learning without deficits in episodic memory or expression of anhedonia. (*A*) Timeline of COC CPP at three doses of COC (5, 10, and 15 mg/kg). (*B*) Preference changes assessed at the posttest time point compared with pretest. $^{*}P < 0.05$, unpaired *t* test. (*C*) Preference changes at the maintenance time point compared with pretest. $^{*}P < 0.05$, unpaired *t* test. (*D*) Locomotor response to saline and COC during context pairing days: 5 mg/kg WT saline vs. COC $F(1,14) = 5.851$, $P = 0.0298$; 5 mg/kg TLR4.KO saline vs. COC $F(1,14) = 0.4099$, $P = 0.5324$; 10 mg/kg WT saline vs. COC $F(1,12) = 14.55$, $P = 0.0025$; 10 mg/kg TLR4.KO saline vs. COC $F(1,14) = 4.178$, $P = 0.0602$; 15 mg/kg WT saline vs. COC $F(1,18) = 92.46$, $P < 0.0001$; 15 mg/kg TLR4.KO saline vs. COC $F(1,18) = 13.83$, $P = 0.0016$, two-way repeated measures ANOVA. $^{***}P < 0.001$, $^{****}P < 0.0001$, Sidak post hoc test for TLR4.KO COC vs. WT COC. (*E*) Proportion of time(s) spent exploring objects in novel object recognition task. Genotype effect $F(1,15) = 0.1981$, $P = 0.6626$; object effect $F(1,15) = 87.32$, $P < 0.0001$; WT identical vs. novel, $t = 7.658$, $P < 0.0001$; TLR4.KO identical vs. novel, $t = 5.777$, $P < 0.0001$, two-way repeated measures ANOVA. $^{****}P < 0.0001$, Sidak post hoc test. (*F*) Summary of 18-h two-bottle choice sucrose preference test. NS: not significant, unpaired *t* test. n = 7–11 animals/group for all experiments.

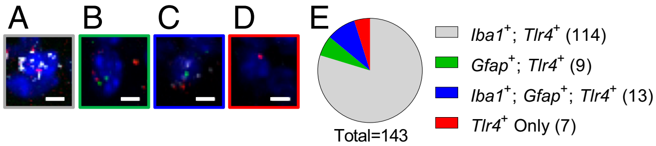

Fig. 6. NAc core *Tlr4* expression is primarily on microglia. Multiplex fluorescent in situ hybridization was performed using RNAscope to detect mRNA transcripts for *Tlr4* (red), *Iba1* (white), and *Gfap* (green) on a background of DAPI (blue). (*A*) Representative *Iba1*+, *Tlr4*+ cell. (*B*) Representative *Gfap*+, *Tlr4*+ cell. (*C*) Representative *Iba1*+, *Gfap*+, *Tlr4*+ cell. (*D*) Representative *Tlr4*+ cell. (*E*) Summary of cell counts (*n*/*N* = 143/4); 79.72% of quantified cells were *Iba1*+, *Tlr4*+. (Scale bars: 5 μm.)

trend toward slower dual component (AMPAR and NMDAR) kinetics in D1(+) MSNs. As increased NMDAR decay kinetics are commonly associated with up-regulation of GluN2B subunits (23) but could also be due to greater functional expression of GluN2C/D, we hypothesized an up-regulation of their function TLR4.KO MSNs. NMDAR pharmacology experiments showed decreased GluN2B function in D1(+) MSNs from TLR4.KOs along with increased GluN2C/D function in both MSN populations. As GluN2C and GluN2D NMDAR subunits exhibit deactivation kinetics even slower than that of GluN2B (38), their up-regulation in TLR4.KO MSNs provides an explanation for decreased A/N ratios.

Another possibility for decreased A/N ratios is an increase in NMDAR-only– containing silent synapses. In the neighboring NAc shell, silent synapses generated in the context of COC exposure are enriched with the GluN2B NMDAR subunit (24). We found no significant differences in $1/CV^2_{N:A}$, an estimation of the number of silent synapses (26), along with reduced GluN2B function on D1(+) MSNs from TLR4.KOs. Together, these results argue against increased NMDAR transmission due to increased silent synapse number. Although we did not test for synaptic NMDAR density, our results point toward altered NMDAR subunit stoichiometry contributing to the differences observed for A/N ratios. Alterations of NAc NMDAR GluN2 subunits are associated with behavioral paradigms known to affect motivation and reward. In the NAc core, this includes increased GluN2B function in D1(−) MSNs following chronic pain (23), increased GluN2C/D function of thalamic inputs onto D1(+) MSNs following COC exposure and withdrawal (6), and aversion-resistant ethanol intake requiring GluN2C function on prefrontal-cortical and insular inputs (39). In addition, studies performed examining the neighboring shell subregion or the NAc as a whole provide additional evidence for the importance of GluN2 subunits on motivation/reward behaviors (24).

With basal differences in NMDAR subunit profiles, we hypothesized that TLR4.KO animals exhibit alterations in NMDAR-dependent plasticity. This idea was supported through extracellular field potential recordings revealing a lack of NMDAR-associated LFS-LTD in TLR4.KO mice. Much controversy remains on the role of the specific NMDAR subunits in scaling synaptic plasticity from long-term potentiation (LTP) to LTD where some suggest LTD is dependent on GluN2B whereas GluN2A is important for LTP (29). An intriguing possibility is that TLR4 regulates the NMDAR-dependent threshold for inducing LTP vs. LTD. Rather than prevent plasticity, reduced GluN2B and increased GluN2C/D function in TLR4.KO mice may shift the propensity for synaptic changes. Further experiments are necessary to test this possibility. In combination, our experiments suggest TLR4.KO animals exhibit a NAc core-specific alteration of NMDAR transmission and a deficit in NMDAR-dependent synaptic plasticity. Thus, TLR4 may play a role in the developmental profile of NMDARs at NAc core synapses.

A lack of LFS-LTD in the NAc core is associated with a range of behavioral manipulations affecting motivation and reward including COC experience (33), exposure to palatable foods (32), and chronic restraint stress (22). However, many of these plasticity assays were only examined following behavioral experiments, and distinctions between LTD induction and expression were not always clarified. Psychostimulants such as COC can depress excitatory synapses in the NAc core (34), and blocking NMDAR-dependent LTD expression through inhibiting AMPAR endocytosis prevents locomotor sensitization (40). On the other hand, loss of NMDAR-dependent LTD is associated with COC-exposed mice exhibiting signs of "addiction" (drug seeking, motivation, and continued use despite negative consequences) compared with COC-exposed "nonaddicted" mice (33). Given the range of behavioral adaptations associated with NMDAR-dependent plasticity, it is difficult to make a specific prediction for the behavioral effect of a presumed change in the NMDAR-dependent LTD induction mechanism in naive TLR4.KO animals. We found that TLR4.KO mice express attenuations in COC CPP and associated locomotor sensitization, both noncontingent drug reward learning processes.

To confirm the cell type(s) expressing TLR4 in the NAc, we performed fluorescent in situ hybridization on brain sections. We found that the majority of *Tlr4*-expressing cells in the NAc core are microglia, echoing fluorescent-assorted cell sorting/qPCR results from the NAc as a whole (35). Given the many differences seen in WT mice when examining NAc synaptic properties in an input-specific manner, it will be interesting to see whether TLR4 and/or microglia influence any part of this physiology and associated behaviors.

Microglia, Immune Signaling, and Drug Reward. Throughout the brain, microglia and their associated cytokines play important roles in modulation of synapses via multiple mechanisms. These include complement-mediated pruning of spines during development of the reticulogeniculate system (41), regulation of synaptogenesis/elimination in the motor cortex through microglial BDNF (42), and the homeostatic scaling up of synapses in the hippocampus through TNFα (43). Microglia also exhibit brain-region–specific differences in cellular aging and transcriptional profiles (44), which may underlie observed synaptic differences between the NAc core and shell. Importantly, microglia have also been implicated in drug addiction. Methamphetamines induce activation of microglia in humans (9). Additionally, rodent models also support a role for microglial adaptation following COC administration (45).

In the NAc core, microglial TNFα scales down synaptic strength on D1(+) MSNs in response to COC, and lacking this cytokine exacerbates COC locomotor sensitization (10). This suggests that TNFα combats drug-induced increases in synaptic strength. TNFα is one of several known cytokines released in response to ligands binding TLR4 (46). TNFα from peripheral monocytes can also influence motor learning and cortical dendritic spine dynamics independently of microglia (47), suggesting a complex interplay between central and peripheral immune modulators. We showed that TLR4.KO animals express a basal difference in NAc NMDARs with an attenuation of COC reward learning and associated motor changes. This suggests that a mechanism independent of TNFα underlies the associated findings. Given the importance of microglia in shaping synaptic physiology and behavior along with TLR4's role in detecting factors including damaged tissue signals in addition to pathogens (16), it is tempting to think about loss of a constitutively active signaling cascade or altered gut-brain communication (48) perturbing microglia to cause a basal change in NAc physiology.

In summary, we show that TLR4 influences NAc synaptic function and COC behavior. These results expand upon the spectrum of immunologic communications with the nervous system that modulates behavior. Given TLR4's link to conditions affecting motivation and reward such as drug exposure and depression (12, 17), it is tantalizing to imagine the NAc as a nexus for neuroimmune interactions in such pathologies.

Materials and Methods

For more detailed descriptions of materials and methods, see *SI Materials and Methods*.

Mice. TLR4.KO, WT, and Drd1a-tdTomato male mice aged 6–12 wk were used in accordance with policies approved by the Institutional Animal Care and Use Committee at Vanderbilt University. All mice were on a C57BL/6 background.

Electrophysiology. Electrophysiological recordings from NAc sagittal slices (250 μm) were performed similar to previously described (6, 20, 49) (*SI Materials and Methods*).

Histology. Fresh-frozen 16-μm mouse brain sections were used. All procedures for multiplex fluorescent in situ hybridization were performed per RNAscope fluorescent multiplex assay protocol (Advanced Cell Diagnostics Inc.) (*SI Materials and Methods*). Probes used included mouse *Tlr4*, *Gfap*, and *Iba1* (*Aif1*).

Behavior. Conditioned place preference (19), novel object recognition (50), sucrose preference (51), and open field tests (19) were performed similarly to previously described protocols (*SI Materials and Methods*).

Data Analysis. All data are presented as a mean ± SEM. Individual data points represent individual cells for whole-cell physiology, slices for field potentials, and animals for behavioral assays. Sample sizes are presented as *n*/*N*, where *n* is the number of cells (whole cell) or slices (field potentials) and *N* is the number of mice. Statistical significance was tested using one-sample *t* tests, unpaired *t* tests, two-way, and repeated measures ANOVA with further comparisons made using Sidak post hoc tests. Representative traces have had stimulus artifacts removed.

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