A specific circuit in the midbrain detects stress and induces restorative sleep

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In mice, social defeat stress (SDS), an ethological model for psychosocial stress, induces sleep. Such sleep could enable resilience, but how stress promotes sleep is unclear. Activity-dependent tagging revealed a subset of ventral tegmental area γ-aminobutyric acid (GABA)–somatostatin (VTA) cells that sense stress and drive non–rapid eye movement (NREM) and REM sleep through the lateral hypothalamus and also inhibit corticotropin-releasing factor (CRF) release in the paraventricular hypothalamus. Transient stress enhances the activity of VTA cells for several hours, allowing them to exert their sleep effects persistently. Lesioning of VTA cells abolished SDS-induced sleep; without it, anxiety and corticosterone concentrations remained increased after stress. Thus, a specific circuit allows animals to restore mental and body functions by sleeping, potentially providing a refined route for treating anxiety disorders.

Acute stress activates the hypothalamic–pituitary–adrenal axis, and the resulting fast increase in blood glucocorticoid concentrations aids immediate survival (1–3). But chronically increased concentrations of glucocorticoids are harmful (4–6), as can be memories of stressful experiences (7). Although stress can cause insomnia and raise stress hormones (3, 5–6), the opposite is also true: Chronic stress increases rapid eye movement (REM) sleep (9); and sleep in rodents is induced by specific types of stress, such as social defeat stress (SDS). Although the function and benefits of sleep remain unclear, sleep is certainly restorative (10). Thus, sleep has been suggested to be one of the mechanisms for alleviating the malignant effects of stress (4, 9, 11, 12). Whether a specific circuit links stress and sleep is, however, unknown. We reasoned that the ventral tegmental area (VTA) in the midbrain could provide a link.

The VTA regulates reward, aversion, goal-directed behaviors, and social contact (13–15).

It also influences responses to stress and threats (16, 17), and strongly affects sleep and wake: VTA neurons promote wakefulness (18, 19), whereas VTA neurons induce sleep (18, 20, 21). Because some GABAergic cells in the substantia nigra (SN) and VTA are activated by stressful and aversive stimuli (16, 22–24), we hypothesized that this route allows stress to induce sleep.

Social defeat stress induces sleep

We first assessed the sleep-wake architecture of mice after they had experienced either SDS from aggressors with consecutive episodes of SDS for 1 hour, or a control procedure, in which the experimental mouse (intruder) was separated from the resident aggressor by a clear partition (fig. S1A). As a further control, instead of an aggressor mouse, we introduced a juvenile male mouse as the resident for 1 hour (fig. S1B). During this nonstress procedure, the experimental mice experienced social interaction. As a control for whether physical activity induces sleep, the mice experienced voluntary wheel running or forced treadmill running continuously for 1 hour (fig. S1C and D); alternatively, as another control, the mice were placed in an unfamiliar environment with a novel object (fig. S1E). Corticosterone (CORT) concentrations in mice increased after the SDS session (Fig. 1A), but not after exposures to juvenile mice, physical exercise, or a novel environment or objects (fig. S2, A, C, E, and G). After SDS, NREM sleep latency was shortened, and both NREM and REM sleep were continuously elevated for 5 hours (fig. 1B and C, and fig. S21), consistent with previous observations (11, 25). Mice that experienced a nonstressful procedure, voluntary wheel running, forced treadmill running or that were deprived of 1 hour of sleep by placing them in a novel environment, however, did not have induced sleep above baseline (fig. S2, A to H and J to M), suggesting that social interaction or physical exercise did not induce sleep and SDS procedures did not cause a sleep rebound while the mice were awake.

Sleep relieves SDS-induced anxiety and CORT concentrations independently

We explored potential functions of sleep after SDS. For mice allowed sufficient sleep (home cage sleep) after SDS, anxiety-like behaviors caused by SDS were rapidly reduced, as seen in the elevated plus maze and open-field assays (Fig. 1, D to F). If mild sleep deprivation over 4 hours took place immediately after SDS, the mice remained in an anxious state (Fig. 1, D to F). For mice allowed sufficient home cage sleep after SDS, raised CORT concentrations returned to baseline over 60 min (Fig. 1G). If mild sleep deprivation occurred immediately after stress, however, CORT concentrations remained elevated (Fig. 1G). But pharmacologically reducing the CORT concentrations induced by SDS during sleep deprivation, by using a corticosterone synthesis inhibitor, mepyrramine (fig. S3A), did not reduce anxiety after sleep deprivation (fig. S3, B and C).

Identification of neurons activated by stress

To identify the circuitry that induces restorative sleep, we mapped cFOS expression throughout the brain. After the SDS protocol (fig. S1A), cFOS was strongly increased in brain areas involved in stress responses (figs. S4, A and B), including the VTA (fig. 1H and fig. S4). In the VTA, cells activated (cFOS-positive) by stress predominantly expressed the GABAergic marker Vglut2 (60%) or GABA (57%) (Fig. 1I and fig. S5, A and B), whereas relatively fewer cells expressed the glutamatergic marker Vgat2 (20%) or the dopaminergic marker tyrosine hydroxylase (TH) (10%) (fig. S5, C and D). However, physical exercise did not induce cFOS in the VTA as a whole, and particularly not in VTA cells, but forced treadmill running slightly increased cFOS expression in TH-positive cells (fig. S5, E to G). For the subsequent studies, we focused on the VTA neurons, as these only induce sleep (18).

VTA neurons have persistently increased activity in response to SDS

VTA neurons rapidly and strongly responded when mice experienced an attack during SDS (Fig. 1, J and K), as assessed by GCaMP6 fiber photometry. The cells did not respond when the mice were presented with novel objects or placed in an unfamiliar environment (fig. S6, A and B). During SDS, the calcium signal in VTA neurons increased and stayed enhanced for about 5 hours (fig. 1L), correlating with the behavioral result of prolonged sleep after SDS (Fig. 1, B and C). By contrast, voluntary wheel
Figure 1 continued on next page
Fig. 1. Stress increases sleep whereas sleep reduces SDS-induced anxiety and stress activates VTA

Subsets of VTA neurons mediate SDS-induced sleep

Because only a subset of VTA neurons (20%) were excited by SDS (Fig. 1I), we undertook cFOS-dependent activity tagging linked to expression of DREADD hM3Dq-mCherry to test whether this VTA subset could induce sleep (Fig. 2A). Mice experienced either SDS or a nonstressful procedure, voluntary wheel running or forced treadmill running, while the VTA neurons were selectively activity-tagged with Cre-recombinase-dependent tagging vectors (AAV-cFOS-IRATA and AAV-TRE-DIO-hM3Dq-mCherry; Fig. 2A and fig. S7, S7, A, D, and G). Compared with pan-VTA neurons expressing mCherry, only 15% of the VTA neurons were captured by activity tagging during SDS (Fig. 2B). We then reactivated these SDS-tagged VTA neurons with clazapine N-oxide (CNO). Chemogenetic reaction decreased sleep latencies and increased sleep times (Fig. 2, C and D). Thus, reactivation of SDS-activated VTA neurons recapitulated sleep architectures induced by SDS (Fig. 2E). Of note, a few cells (2.6%) were tagged during the nonstressed procedures (Fig. 2B). However, chemogenetic reactivation of these particular tagged VTA subset cells did not elicit sleep (fig. S7, B and C). Moreover, only rare cells were tagged when mice experienced physical exercise (Fig. 2B), and therefore chemogenetic reactivation did not induce sleep (fig. S7, D to I).

To examine the necessity of VTA subset subclasses for SDS-induced sleep, we chemogenetically inhibited SDS-tagged VTA neurons using cFOS-dependent expression of DREADD hM4Di-mCherry. AAV-cFOS-tTA and AAV-TRE-DIO-hM4Di-mCherry were injected into the VTA of Gagires-Cre mice (fig. S10A). VTA neurons became selectively ChR2-tagged during SDS (fig. S10A). Mapping of VTA neurons by injecting AAV-DIO-ChR2-GFP into Vtagires-Cre mice showed broad projections (fig. S10B) (18). However, those SDS-tagged VTA neurons detected with ChR2 activity mapping primarily innervated the LH (fig. S10C). When SDS-ChR2-tagged terminals in the LH of the VTA neurons were reactivated by optogenetic stimulation, this elicited NREM sleep from waking (fig. S10, D and E).

Circuits linking stress and sleep

We next investigated the circuitry linking SDS and VTA neurons. We expressed GCaMP6 selectively in VTA neurons and used fiber photometry to measure how the VTA terminals in different locations responded to stress. Only the terminals of the VTA neurons projecting to the lateral hypothalamus (LH) had increased Ca2+ signals after SDS (Fig. 3, A and B), whereas the VTA neurons projections in the CeA, LHb, and hippocampal dentate granule (DG) cells showed no responses (fig. S8). To determine the function of the VTA-LH pathway activated by stress on sleep, we injected retro-AAA-VRE-DIO-Flpo into the LH, together with the injection of AAV-cFOS-IRATA and AAV-fDIO-hM3Dq-mCherry into the VTA of Vtagires-Cre mice (Fig. 3C). Following intersectional activity tagging during SDS and chemogenetic reactivation, the VTA-LH pathway promoted sleep (Fig. 3, D and E). The hM3Dq-mCherry labeling produced in the VTA neurons of this experiment mainly traced out axons to the LH (fig. S9).

We used optogenetics to confirm the above result. The behavioral experiments were repeated by using cFOS-based activity tagging with ChR2 delivered into the VTA of Vtagires-Cre mice (fig. S10A). VTA neurons became selectively ChR2-tagged during SDS (fig. S10A). Mapping of VTA neurons by injecting AAV-DIO-ChR2-EYFP into Vtagires-Cre mice showed broad projections (fig. S10B) (18). However, those SDS-tagged VTA neurons detected with ChR2 activity mapping primarily innervated the LH (fig. S10C). When SDS-ChR2-tagged terminals in the LH of the VTA neurons were reactivated by optogenetic stimulation, this elicited NREM sleep from waking (fig. S10, D and E).

Stress-driven input-output organizations

We investigated the identity and activity of VTA neurons relevant for stress using a rabies system, combined with activity mapping. VTA neurons were seeded with rabies coat protein and its receptor by injecting AAV-DIO-N2cG and AAV-DIO-VA-nGFP, followed by injection of RABV-N2cAG-EnvAmCherry into the VTA (Fig. 3F). The animals were then given control experiences or SDS, respectively. Then we conducted brain-wide mapping of rabies-labeled presynaptic inputs and stress-activated cFOS expression (Fig. 3F). cFOS was induced by stress in many brain regions (fig. S4), and from the rabies tracing, VTA neurons inputs originated in many locations (fig. S11) (26). However, only the lateral preoptic (LPO), paraventricular hypothalamus (PVH), and periaqueductal grey (PAG) areas had overlap with cFOS-positive cells and rabies-labeled VTA neurons inputs (Fig. 3, G, H, and I, and figs. S12, A to C, and S13A).

We determined the inputs of VTA neurons that project to the LH. AAV-DIO-N2cG and AAV-DIO-VA-nGFP were seeded as before in VTA neurons, and RABV-N2cAG-EnvAmCherry was injected into the terminal fields of the VTA neurons in the LH. As before, the mice were given control experiences or SDS. Then we mapped cFOS expression (Fig. 3F), and we obtained an identical result as above: LH-projecting VTA neurons received stress-activated inputs from the LPO, PVH, and PAG (Fig. 3, J to L, and figs. S12, D to F, and S13B).

We further determined if these stress-activated inputs were specific to the stress-activated VTA subset. The SDS-activated VTA cells were specifically ablated with Casp3 by using activity tagging (AAV-cFOS-tTA and AAV-TRE-DIO-Casp3). Then we conducted rabies tracing and activity mapping (fig. S14A). Ablation of SDS-activated VTA cells largely reduced the stress-driven inputs (cFOS/rabies) (fig. S14, B and C, and Fig. 3L).

VTA somatostatin neurons are necessary for SDS-induced sleep

Given that GABAergic VTA neurons are heterogeneous (27, 28), and only a subset of VTA neurons responded to SDS (Fig. 2B), we looked for subtypes of VTA cells responsible for SDS-induced sleep. First, we examined by single-cell quantitative polymerase chain reaction the molecular identities of SDS-tagged
Fig. 2. Sufficiency and necessity of the stress-activated VTA^Vgat neurons for SDS-induced sleep. (A) Activity-tagging protocol for testing the sufficiency of SDS-activated VTA^Vgat cells for sleep. (B) Expression and quantification of pan or activity-tagged hM3Dq-mCherry transgene in VTA^Vgat neurons (n = 4 mice per group). Scale bar, 100 μm. (C and D) Chemogenetic reactivation of tagged VTA^Vgat neurons for sleep (n = 8 mice per group). Graphs show sleep latency, percentage and time of NREM (C) or REM (D) sleep. Unpaired t test, *p < 0.05, **p < 0.01. (E) Matrix bubble summary shows fold change (Fc) of sleep parameters after SDS, nonstressed, voluntary wheel running, forced treadmill running or the chemogenetic reactivation of tagged VTA^Vgat neurons. (F) Activity-tagging protocol for testing the necessity of SDS-activated VTA^Vgat cells for sleep. (G and H) Percentage and time of NREM (G) or REM sleep (H) in mice given second SDS after chemogenetic inhibition of first SDS-tagged VTA^Vgat cells (n = 6 to 8 mice per group). Two-way ANOVA with bonferroni post hoc test. *p < 0.05, ***p < 0.001, n.s.: not significant.
Figure 3 continued on next page
cells (fig. S15A): A large proportion (42%) expressed vglut/somatostatin (sst), and others were characterized by vglut/parvalbumin (pv) (10%) or vglut expression alone (32%), the remaining cells being 2% vglut/vglut2, 2% vglut/vglut2/sst, and 2% vglut/cyp (fig. S15B). We further characterized the SDS-activated cells using reporter mice (Fig. 4, A B and fig. S15, E and F). Nearly 40% of the VTA cells using reporter mice (Fig. 4, A B and fig. S15, E and F), whereas there was no induction of SDS following SDS in VTApv cells (fig. S4B). We next determined the activity of individual subtypes responding to stress using fiber photometry (Fig. 4, C and D). Both the VTAst and VTApv populations responded transiently to SDS, but the collective calcium signal for VTAst cells was larger (Fig. 4, C and D), and only VTAst neurons had persistent activity after SDS, with enhanced activity for a few hours (Fig. 4E). By contrast, the transient activity of VTApv neurons after SDS was not sustained (Fig. 4F).

We next used activity tagging with hM3Dq to capture SDS-tagged VTAst neurons (fig. S16, A and B). Because VTAst cells are heterogeneous (28), we examined the molecular identities of SDS-tagged cells (fig. S16C). These tagged cells predominantly expressed vglut/gad1 (90%) (fig. S16D).

We tested whether VTAst cells could respond to two types of insomnia-inducing stress, restraint and cage change (7, 29). However, these procedures did not affect the acute or long-term calcium activity in VTAst neurons (fig. S17, A to D). In addition, we did not observe any VTAst neurons becoming tagged by restraint stress or cage-change stress (figs. S17E).

Next, we measured spontaneous activities of VTAst neurons across brain states. From calcium photometry, VTAst neurons were primarily active during spontaneous NREM and REM sleep (Fig. 4, G and H), whereas VTApv neurons were wake-active (Fig. 4, I and J). Chemogenetic stimulation of VTAst neurons directly increased sleep (fig. S18). We further defined how VTAst neurons link stress and sleep. We recorded the spontaneous activity of stress-tagged VTAst populations across brain states (fig. S19A). The tagged cells were also primarily active during NREM and REM sleep (fig. S19B). Chemogenetic reactivation of SDS-tagged VTAst cells was sufficient to promote NREM and REM sleep (fig. S19, C to E).

To explore whether the VTAst→LH pathway links stress and sleep, we conducted fiber photometry to measure terminal activity in the LH that responded to stress by expressing GCaMP6 in VTAst neurons (fig. S20A). The VTAst→LH projection responded to SDS (fig. S20B). Following intersectional activity tagging during SDS (fig. S20C), chemogenetic reactivation of the VTAst→LH pathway promoted sleep (fig. S20, D and E).

Finally, we examined directly whether VTAst neurons are necessary for SDS-induced sleep. Genetic ablation specifically depleted VTAst neurons (fig. S21). Lesioning of VTAst neurons decreased baseline sleep (Fig. 5, A to C). When VTAst-caspase mice were challenged with SDS, SDS-induced sleep was abolished (Fig. 5, B and C). This was also confirmed by chemogenetic manipulation; inhibition of VTAst neurons also decreased SDS-induced sleep (fig. S22). By contrast, ablation of VTApv neurons decreased baseline NREM sleep, but SDS-induced sleep could still be elicited (Fig. 5, D to F).

SDS-induced sleep by VTAst neurons reduces stress-induced anxiety

Given the proposed restorative function of sleep after SDS (Fig. 1, D and G), we explored whether this function was linked to VTAst neurons. Ablation of VTAst neurons or chemogenetic inhibition of SDS-tagged VTAst neurons had no effect on baseline anxiety-like behaviors (fig. S23). However, after SDS, mice lacking SDS-induced sleep (because of selective lesioning or inhibition of VTAst neurons or inhibition of SDS-tagged VTAst neurons (Figs. 2, F to H, and 5, A to C, and fig. S22) remained in an anxious state (Fig. 6, A to C and fig. S24), similar to the effects of sleep deprivation after SDS (Fig. 1, E and F, and fig. S25). When VTApvst neurons were unplugged, while the mice had sufficient SDS-induced sleep, the SDS-induced anxiety-like behaviors were reduced to baseline (Fig. 6, B and C, and fig. S24). We found that sleep deprivation after SDS suppressed anxiety in VTAst neurons induced by SDS (fig. S26).

However, during the sleep deprivation procedure after SDS while mice were awake, VTAst cell stimulation did not reduce anxiety even if VTAst neurons were activated (fig. S27), suggesting that the anxiolytic effects require SDS-induced sleep.

Activation of VTAst neurons suppresses corticotrophin-releasing factor levels induced by SDS

How do VTAst neurons regulate CORT production? VTAst neurons expressing hM3Dq-mCherry sent numerous mCherry-positive axons into the paraventricular nucleus (PVN) area (fig. S28, A and B), a major site of corticotrophin-releasing factor (CRF) production. After SDS, cells in the PVN are excited, as inferred from their strong expression of cFOS; however, stimulation of VTAst neurons inhibited SDS-activated cells in the PVN (fig. S28C).

We next used a genetically encoded CRF sensor (AAV-hSyn-GRABCRF1.0) (30) to determine the dynamics of CRF release around the PVN (fig. S28D and Fig. 6D). CRF sensor signals were indistinguishable between control and chemogenetic activation of VTAst neurons (fig. S28E), consistent with CORT concentrations also not changing with VTAst stimulation (fig. S28F). But after SDS, there were large increases in CRF (fig. S28G). However, chemogenetic activation of VTAst neurons prevented this increase (fig. S6E), consistent with correspondingly decreased CORT concentrations (fig. S6F). By contrast, chemogenetic inhibition of VTAst neurons further increased SDS-induced CRF concentrations (Fig. 6G), thereby increasing CORT levels after SDS (Fig. 6H).
Fig. 4. Activity of VTA\textsuperscript{Sst/\textit{Pv}} neurons responding to stress and across brain states. (A and B) cFOS expression and quantification in genetically labeled VTA\textsuperscript{Sst} (A) or VTA\textsuperscript{Pv} (B) neurons after control experience or SDS (n = 8 mice per group). Scale bar, 100 μm. (C and D) Fiber photometry measuring calcium signals in VTA\textsuperscript{Sst} (n = 10 mice per group, 45 trials) (C) or VTA\textsuperscript{Pv} (n = 6 mice per group, 15 trials) (D) neurons responding to SDS. Raw calcium signal traces, color matrix of signals for all trials, ΔF/Φ ratios across the experimental period and average ΔF/Φ ratios before and during SDS. Paired t test, ***p < 0.001. 

(E and F) Fiber photometry measuring long-term calcium signals in VTA\textsuperscript{Sst} (E) or VTA\textsuperscript{Pv} (F) neurons. Raw traces and average ΔF/Φ ratios before and after the procedures (n = 6 mice per group). Paired t test, **p < 0.01. (G to J) Fiber photometry with electroencephalography and electromyography measuring spontaneous activity across brain states. ΔF/Φ ratios in VTA\textsuperscript{Sst} (G) or VTA\textsuperscript{Pv} (I) neurons during wakefulness, NREM and REM sleep, and at transitions of vigilance states (n = 6 mice per group) (H and J). One-way repeated ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
their home cage sleep after SDS (Fig. 6J and fig. S29B), similar to the effects of sleep deprivation after SDS (Fig. 1G). However, when VTA<sup>Vgat-Sst</sup> neurons were unimpeded, the SDS-induced sleep correlated with CORT concentrations returning to baseline (Fig. 6J and fig. S29B). In addition, activation of VTA<sup>Sst</sup> cells during sleep deprivation after SDS, (i.e., activation of these cells while mice were awake) partially reduced CORT concentrations (fig. S29, C and D), but the overall CORT levels still remained elevated (fig. S29D), suggesting that sleep after SDS is also needed to reduce CORT concentrations.

Discussion
Our proposed circuit model for how SDS translates to sleep and reduction of anxiety, with VTA<sup>Vgat-Sst</sup> cells playing a central role, is shown in Fig. 6K. Once activated by SDS, VTA<sup>Vgat-Sst</sup> cells drive sleep through the LH, a brain region containing a diverse population of cells implicated in regulating stress, anxiety, and sleep-wake behaviors (31, 32). VTA<sup>Vgat-Sst</sup> cell activity is maintained for some hours beyond the stress episode, suggesting a form of plasticity that enables them to keep promoting NREM and REM sleep episodes for a sustained period. In parallel to their sleep-inducing and anxiety-reducing effects, VTA<sup>Vgat-Sst</sup> cells inhibit CRF-producing neurons in the PVN hypothalamus, thereby reducing CORT concentrations after SDS. We found that SDS-induced anxiety persisted even in the presence of CORT inhibitors. These results suggest that physiological activation of VTA<sup>Vgat-Sst</sup> neurons during and after SDS represses CRF and therefore CORT production, guarding against overproduction of CORT.

Persistently increased CORT concentrations have deleterious effects on body organs (1). We propose that the reduced anxiety comes from the sleep component. After SDS, the restorative sleep by VTA<sup>Vgat-Sst</sup> cells also aids CORT concentrations returning to baseline, so there seem to be parallel routes to reducing CORT levels, but with VTA<sup>Sst</sup> cells coordinating both mechanisms.

The output pathways regulated by VTA<sup>Vgat-Sst</sup> cells in the LH to induce sleep and reduce anxiety are unclear. VTA<sup>Gad67</sup> neurons inhibit orexin/Hcrt neurons in the LH (20). However, chemogenetic inhibition of LH<sup>Hcrt</sup> cells did not reduce anxiety after SDS (fig. S30), and orexin receptor antagonists did not restore the anxiolytic effects that were missing in VTA<sup>Sst</sup>-lesioned mice that had undergone SDS (fig. S31), suggesting that orexin/Hcrt cell inhibition is not required for the anxiolytic actions of VTA<sup>Vgat-Sst</sup> cells. Thus, identifying the targets of VTA<sup>Vgat-Sst</sup> cells requires further study. Local action within the VTA of the VTA<sup>Sst</sup> neurons is also possible.

We have shown here that GABA<sup>Sst</sup> neurons in the VTA respond to SDS, an ethological model for psychosocial stress, by inducing restorative sleep and decreasing CRF production. Targeting these neurons could potentially provide a new route for treating anxiety disorders.
Fig. 6. Activity and SDS-induced sleep by VTA\textsuperscript{Sst} neurons reduces anxiety and corticosterone concentrations. (A to C) Plan of the experimental procedure (A), tracing of locomotion for representative animals (B), time spent in the open arms of the elevated plus maze and in the center zone during the open-field test (n = 9 mice per group) (C). (D) Delivering genetically encoded CRF sensor in the PVN hypothalamus alongside chemogenetic manipulation of VTA\textsuperscript{Sst} neurons. (E and G) Raw PVN CRF sensor traces and ΔF/F ratios during SDS after chemogenetic activation (E) or inhibition (G) of VTA\textsuperscript{Sst} neurons (n = 8 mice per group). (F and H) Corticosterone concentrations following SDS after chemogenetic activation (F) or inhibition (H) of VTA\textsuperscript{Sst} neurons (n = 6 mice per group). (I and J) Plan of the procedure (I) and corticosterone concentrations (n = 4 to 9 mice per group) (J). (K) Conceptual summary diagram. (C and J) Two-way ANOVA with bonferroni post hoc test, *p < 0.05, **p < 0.01, ****p < 0.0001. (E to H) Unpaired t test, *p < 0.05, **p < 0.01, ***p < 0.001.
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