Voluntary urination control by brainstem neurons that relax the urethral sphincter

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Voluntary urination ensures that waste is eliminated when safe and socially appropriate, even without a pressing urge. Uncontrolled urination, or incontinence, is a common problem with few treatment options. Normal urine release requires a small region in the brainstem known as Barrington’s nucleus (Bar), but specific neurons that relax the urethral sphincter and enable urine flow are unknown. Here we identify a small subset of Bar neurons that control the urethral sphincter in mice. These excitatory neurons express estrogen receptor 1 (BarESR1), project to sphincter-relaxing interneurons in the spinal cord and are active during natural urination. Optogenetic stimulation of BarESR1 neurons rapidly initiates sphincter bursting and efficient voiding in anesthetized and behaving animals. Conversely, optogenetic and chemogenetic inhibition reveals their necessity in motivated urination behavior. The identification of these cells provides an expanded model for the control of urination and its dysfunction.

Urination (also known as micturition) is a fundamental behavior that requires coordination of the bladder and urethral sphincter1–3. Humans urinate involuntarily and reflexively at birth but acquire voluntary control with learning and development. Unfortunately, this motivated control is ultimately disrupted in one in three adults worldwide4. The neurons in the brain that control urination remain obscure, partly because most studies have focused on reflex urinary bladder filling and voiding can be easily controlled and monitored in anesthetized animals. However, voluntary urination occurs before the bladder reaches capacity and must be studied in awake, behaving animals. Because of this experimental complication, there is little understanding of the neural substrates underlying natural, voluntary urination behavior and continence.

House pets commonly demonstrate that many animals, in addition to humans, can learn to control urination behavior. Moreover, territorial males of many wild animals, including fish5, rodents6–9 and primates8, deliberately urinate in their domain to transmit social scents such as pheromones. Male mice in particular scent-mark prolifically6,7 to attract female mating partners. However, exuberant urination behavior is metabolically wasteful and may attract other aggressive males10 or predators11. Mice offset these risks by limiting voluntary scent marks to critical social environments such as those most likely to contain females4. Therefore, the use of female odor to promote rapid and robust scent marking behavior in the male mouse serves as an experimental platform to identify neurons controlling voluntary urination.

The switch from urine storage to deliberate elimination is known to depend on brain input, as spinal cord injury acutely prevents voluntary urination. Bar (also known as the pontine micturition center or M-region), is a well-conserved and heterogeneous population of neurons in the dorsal pons that was identified as the major brain center regulating urination almost a century ago12,13. Bar contains at least three different cell types defined by physiology14, gene expression15,16 and histology17. The best-studied among these express corticotropin releasing hormone (CRH, also known as corticotropin releasing factor)15–20. BarESR1 neurons increase their firing rate under anesthetized bladder and colon distension, as well as during awake, diuretic-induced urination15,21. Moreover, optogenetic stimulation of these neurons generates an increase in bladder pressure15. However, the smooth muscle of the bladder wall contracts slowly via autonomic, involuntary control, which alone is not sufficient for voiding. Urine release is ultimately gated by the external urethral sphincter (EUS), which is normally constricted but relaxes to allow urine flow (Fig. 1a). In humans, this relaxation precedes bladder contraction and initiates voluntary urination22,23. The EUS is composed of striated muscle to permit fast control via somatic, voluntary motor neurons, which are monosynaptically inhibited by interneurons in the dorsal gray commissure (DGC) in the spinal cord24–26. Broad electrical or chemical stimulation of Bar drives urination27,28, and current models assume that this occurs through a single, divergent Bar projection to the spinal cord to control both bladder and EUS11,29. However, Bar neurons that relax the urethral sphincter have not been identified.

Here we establish a voluntary urination assay in male mice by quantifying their rapid generation of scent marks following detection of female odor. We find that this behavior depends on a previously uncharacterized subpopulation of spatially clustered neurons in Bar that express high levels of estrogen receptor 1 (BarESR1 neurons). These neurons project heavily to the DGC and increase their activity in freely behaving mice just before voluntary scent marking urination. BarESR1 neurons drive efficient voiding when photostimulated in awake animals, and urinary muscle recordings in anesthetized animals indicate a distinct mechanistic role in urethral sphincter relaxation. Chemogenetic inhibition of BarESR1 but not BarESR1 neurons abolishes natural scent-marking urination, and acute BarESR1 photoinhibition abruptly terminates ongoing EUS relaxation. Thus, BarESR1 neurons are indispensable for driving urethral
Articles of the Bar neurons projecting to the spinal cord lack CRH expression, are unlikely to facilitate voluntary urination. Approximately half modest effects on urination in awake animals, suggesting that they have small Bar subpopulations. Immunostaining for ESR1 protein in CRH-Cre mice confirmed a small Bar subpopulation (~200 cells) expressing high amounts of ESR1 (BarESR1 neurons; Fig. 1b–f). The majority of BarESR1 neurons (about three-quarters of the BarESR1 population; Fig. 1f) did not overlap with CRH-tdT, and the overlapping minority are likely to represent an upper bound on coexpression because tdT integrates Crh promoters activity over the lifetime of the animal. BarESR1 neurons are found in a dorsal cluster within the Nissl-defined ovoid Bar nucleus, whereas BarCRH neurons are more numerous (~500 cells), ventrally biased, and extend further along the rostrocaudal axis beyond traditional, Nissl-defined Bar borders (Fig. 1d,e). Moreover, in CRH-Cre mice, 96.8% of BarESR1 neurons (n = 3 mice) overlap with reporter expression (Supplementary Fig. 1a), confirming that the Crh and Esr1 promoters are active in largely independent Bar populations.

To investigate the potential for BarESR1 neurons to relax the urethral sphincter, we evaluated their neurotransmitter identity and anatomical connections to the lower urinary tract. Immunostaining with anti-ESR1 in Vgat-Cre and Vglut2-Cre mouse lines crossed to fluorescent reporter lines (marking GABAergic and glutamatergic neurons, respectively), as well as in situ hybridization, revealed that the majority of BarESR1 neurons express Vglut2 (93.6% reporter overlap, n = 3 mice) and not Vgat (2.2% reporter overlap, n = 4 mice).

**Results**

A cell type in Barrington’s nucleus with a role in urination. Our initial tests and a previous study of BarCRH neural function showed modest effects on urination in awake animals, suggesting that they are likely to facilitate voluntary urination. Approximately half of the Bar neurons projecting to the spinal cord lack CRH expression, and their molecular identity and function is undetermined. We took a candidate approach to identifying molecular markers for Bar neurons that may function to promote urinary sphincter relaxation and voluntary urination in male mice and provide a promising tool for the future study of continence and incontinence.

Fig. 1 | A novel cell type in Barrington’s nucleus with projections biased to sphincter-inhibiting interneurons. a, Urination requires sphincter relaxation. b, ESR1-immunostaining in Bar (dotted oval) in CRH-tdT mouse. LC, locus coeruleus; 4V, fourth ventricle. c, Larger view of CRH-tdT (top) and anti-ESR1 (bottom) channels from Bar (mean ± s.e.m. for BarESR1 neurons; Fig. 1b–f) and cell percentages (bottom) in Bar (mean ± s.e.m. for BarCRH neurons; Fig. 1b–f). The majority of Bar ESR1 neurons (about three-quarters of the BarESR1 population; Fig. 1f) did not overlap with CRH-tdT, and the overlapping minority are likely to represent an upper bound on coexpression because tdT integrates Crh promoters activity over the lifetime of the animal. BarESR1 neurons are found in a dorsal cluster within the Nissl-defined ovoid Bar nucleus, whereas BarCRH neurons are more numerous (~500 cells), ventrally biased, and extend further along the rostrocaudal axis beyond traditional, Nissl-defined Bar borders (Fig. 1d,e). Moreover, in CRH-Cre mice, 96.8% of BarESR1 neurons (n = 3 mice) overlap with reporter expression (Supplementary Fig. 1a), confirming that the Crh and Esr1 promoters are active in largely independent Bar populations.

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Supplementary Fig. 1b–k). Injection of the retrograde tracer cholera toxin B (CTB) into the lumbosacral spinal cord resulted in colabeling with BarESR1 cells, indicating their direct projections to urinary targets (Supplementary Fig. 1l–n). To further investigate BarESR1 axonal projections, we unilaterally injected adeno-associated virus (AAV) expressing Cre-dependent GFP into Bar of ESR1-Cre or CRH-Cre animals and imaged the lower thoracic to sacral spinal cord (Fig. 1g,h,i). The lumbosacral mediolateral column (ML) contains preganglionic autonomic neurons that excite the bladder (along with intermingled interneurons)26, and the lumbosacral DGC contains interneurons that directly inhibit (relax) sphincter motor neurons of the dorsolateral nucleus via Bar input15–28 (Fig. 1k). Consistent with the known role in bladder pressure regulation, BarCRH-GFP axons showed a dense focal projection to the ML (Fig. 1g,h) with only sparse fibers arcing further medially or to thoracolumbar levels T13–L2 (Supplementary Fig. 2a,b). BarESR1-GFP axons projected similarly across the lumbosacral ML, with additional lighter fibers seen in the thoracolumbar ML (Supplementary Fig. 2c). However, they also provided much denser innervation of the sphincter-inhibiting DGC, extending rostrally from the proposed L3–L4 burst generator29 to midsacral levels (Fig. 1g,h bottom and Supplementary Fig. 2c). Bilateral labeling of BarESR1 or BarCR1 neurons with a second Cre-dependent virus (AAV-FLEX-ChR2) confirmed the same projection patterns (Fig. 1i and Supplementary Fig. 2b,c). Thus, the cell body distribution, molecular expression and efferents of BarESR1 neurons indicate that they constitute an uncharacterized cell type within Bar, distinct from BarESR1 neurons.

On detecting the odor of a female, male mice promptly urinate to show their command of the territory and advertise their availability to mate30. We promoted this voluntary urination by adding female odor (female urine) to an arena lined with absorbent paper and recorded the male’s position from above and their urine output from below. This enabled quantification of both the timing and abundance of voluntary urination events during freely moving behavior (Supplementary Fig. 3). To determine the temporal activation of BarESR1 cells in relation to this natural urination behavior, we unilaterally injected Bar with AAV-FLEX-GCaMP6s, encoding a fluorescent calcium indicator, in ESR1-Cre animals and imaged population calcium activity with fiber photometry (Fig. 2a,b). We observed robust, discrete increases in fluorescence that were highly correlated with detected urination events, unlike randomly chosen intervals (Fig. 2c–e). The lags for maximal cross-correlation between urine detection and GCaMP fluorescence transients revealed no significant difference between the timing of BarESR1 population activity and urine marks (GCaMP preceded by 0.37±0.16 s, mean±s.e.m., n=76 urination events across 7 mice, P=0.18, Wilcoxon signed rank test). Altogether, we find that ESR1 defines a novel cell type in Bar with anatomical and physiological features consistent with a direct role in urination.

**Fig. 2 | BarESR1 activity increases during urination events.** a. Schematic of fiber photometry experiment and example urine quantification with control odor (gray shading) and female odor (yellow shading) on bottom camera view. b. Example GCaMP6s expression in Bar (gray dotted oval) of ESR1-Cre mouse. Dotted orange rectangle shows approximate fiber location. c. Example BarESR1-GCaMP6s fluorescence (top, green) and derivative of urine detection (Δurine, bottom, yellow). d. GCaMP6s fluorescence synchronized to Δurine peaks (green) or at shuffled times (black) for all mice (thick line and shading are mean±s.e.m., respectively; n=76 urination events from 7 mice). e. Correlation coefficient between GCaMP6s and Δurine traces at zero lag (green) and random lag (gray) for all mice (mean±s.e.m., same events as d). Scale bar, 100 µm. ***P=1.1×10−45 (Mann–Whitney U test).

**Artificial activation of BarESR1 neurons promotes urination in awake and anesthetized animals.** BarCRH-ChR2 photostimulation was previously shown to drive bladder pressure increases during urethane-anesthetized cystometry31, but the sufficiency of these cells in awake urination has not been characterized. To determine whether either of these distinct Bar populations promote urination in behaving animals, we first bilaterally infected BarESR1 or BarCR1 neurons with AAV-FLEX-ChR2, encoding the excitatory optogenetic protein channelrhodopsin2, or with AAV-FLEX-GFP (BarESR1-ChR2, BarESR1-GFP or BarCR1-ChR2; Fig. 3a–c), and performed slice recordings to confirm that both BarESR1-ChR2 and BarCR1-ChR2 neurons reliably responded to photostimulation at frequencies previously used in electrical stimulation (Supplementary Fig. 4). We then quantified and compared the latency and amount of urine induced by photostimulation in awake, freely moving mice without urine-promoting odor cues. While photostimulation of BarESR1-GFP neurons produced no effect on urine excretion, photostimulation of BarESR1-ChR2 neurons led to robust, frequency-dependent urine volume release, following light onset with a mean latency of 2.1 s (Fig. 3d–h and Supplementary Video 1). Over 96% of BarESR1-ChR2 stimulation trials at 10–50 Hz resulted in urination (Fig. 3d,f). In comparison, photostimulation of BarCR1-ChR2 neurons during freely moving behavior had a much smaller effect on urination despite generally higher ChR2 viral infection levels (Fig. 3c–h and Supplementary Video 2). Less than 37% of BarCR1-ChR2 stimulation trials at 10–50 Hz resulted in the voiding of urine (Fig. 3f). Of this subset, the latency and amount of urine produced differed from that of BarESR1-ChR2 at all frequencies tested (Fig. 3d–h). We additionally investigated the extent to which BarESR1 and BarCR1 neural activity could initiate voiding without conscious sensory input. Photostimulation under isoflurane anesthesia, known to depress reflex urination2,21, resulted in urine voiding in 43% of the BarESR1-ChR2 trials, but only 6% of the
BarESR1 trials, with none of the BarCRH-ChR2 voids occurring during the photostimulus window (Fig. 3f,i and Supplementary Video 3). This indicates that BarESR1 neuronal activity induces rapid and efficient urination and hints at a distinct mechanism from neighboring BarCRH activity, which is known to increase bladder pressure. BarESR1 neurons drive urination by controlling the urethral sphincter.

To directly test the effect of BarESR1 and BarCRH neurons on urinary muscle targets, we performed EUS electromyography (EMG) and cystometry (bladder filling and pressure recording) under isoflurane anesthesia (Fig. 4a). We perfused saline at a constant rate into the bladder to stimulate reflex voiding and observed natural cycles of bladder pressure increase and associated EUS bursting muscle patterns, which correlated with voiding and subsequent bladder pressure decrease (Fig. 4b). These bursting contractions interspersed with periods of muscle relaxation are believed to enable efficient urine flow through the narrow rodent urethra. Following observation of regular cystometry cycles, we stopped the saline pump when the bladder was “filled” or “empty” (75% or 10% of the volume observed to trigger reflex urination, respectively) and initiated 5 s of photostimulation (Fig. 4b). We found that both BarESR1-ChR2 and BarCRH-ChR2 photostimulation...
produced reliable, time-locked bladder pressure increases at similar latencies (Fig. 4c,d). The initial latency and slope of the bladder pressure increase by stimulation of each cell type were indistinguishable by our analysis; however, the peak pressure and end pressure (25 s after stimulus onset) were significantly less for BarESR1-ChR2 photostimulation. This difference occurs because only with the BarESR1-ChR2 photostimulation did we observe abundant urine release, which results in a sharp pressure decrease below the starting value (Fig. 4c–f and Supplementary Video 4). When BarESR1-ChR2 photostimulation ceased, the bladder usually returned to the same pressure level observed before BarESR1-ChR2 stimulation (Fig. 3c,d,f), independently confirming our observations that significant urine release does not normally occur through activation of this cell population (Supplementary Video 5).

The reason for the observed differences in photostimulated urine release become clear only when examining the EUS EMG responses. The photostimulated urination in BarESR1-ChR2 mice coincided with a reliable bursting pattern of sphincter activity, the extent of which was dependent on bladder fill level (Fig. 5a,b and Supplementary Fig. 5a,b). Pulsatile urination occurred during the bursting periods (Supplementary Video 4), consistent with previous observations of urine flow during the relaxation phases between bursts14,15 and our calculations of relaxation time between burstlets (Fig. 5c,d). Frequency analysis of the sphincter EMG signal also shows that 85% of the BarESR1-ChR2 stimulations with a filled bladder resulted in sphincter relaxation and bursting, and associated voiding (Fig. 5e,f and Supplementary Fig. 5a,b). Additionally, we observed burst-like EMG responses in the absence of bladder contractions on a subset of empty-bladder trials (Supplementary Fig. 6), such that the effect of BarESR1 neurons on the sphincter cannot be solely due to reflex activity from bladder afferents. In contrast, photostimulation of BarCRH-ChR2 neurons produced either no detectible change in sphincter activity, tonic sphincter discharge (constriction) or rare irregular bursting (13% of trials), which was always preceded by tonic (constricting) activity and accompanied by bladder pressure increase (Fig. 5b–c). This tonic activity increase was characteristic of the spinal guarding reflex, a compensatory tonic contraction of the EUS mediated through bladder afferents to prevent urination during bladder distension.

The extent to which urethral sphincter bursting occurs during natural, awake rodent behavior varies across sex and species16–20 and remains controversial. Thus, to investigate natural sphincter activity, we surgically implanted a wireless pressure recorder into the corpus spongiosum, which surrounds the urethra and can serve as a proxy for the urethral activity, and in which bursting duration corresponds to the amount of urine release38. Upon recovery, we analyzed urination behavior in response to odor cues and found urethral sphincter bursting patterns to occur during the awake behavior (Supplementary Fig. 5c–f). Notably, the duration and slope
of the spectral power seen during the BarESR1-ChR2 photostimulation bursts mimicked wirelessly recorded pressure during awake, natural scent-marking urination (Supplementary Fig. 5b,e,f). Overall, these awake and anesthetized urinary recordings indicate that stimulation of both Bar populations increase bladder pressure equally, but only BarESR1 neurons relax the EUS via bursting to enable efficient urine flow as in natural, awake urination in male mice.

**BarESR1 but not BarCRH neurons are indispensable for voluntary scent marking urination.** No single cell type in Bar has been shown to be necessary for voluntary urination. To investigate the extent to which Bar neurons participate in this motivated behavior, we established a rapid behavioral assay that compares the voluntary baseline urination rate (2 min in the presence of a control odor) to the rate during the subsequent 2 min in the presence of motivating female urine odor (Fig. 6 and Supplementary Video 6). The reliable and rapid change in the amount of urine marks in response to female urine indicates that olfactory cues access circuits that relax the EUS and generate voluntary urination.

To test whether Bar neurons are necessary for this response, we bilaterally infected them with AAV-FLEX-hM4Di, encoding an inhibitory chemogenetic receptor, in ESR1-Cre or CRH-Cre mice (BarESR1-hM4Di or BarCRH-hM4Di; Fig. 7a,b). Individuals were then injected with either its ligand, clozapine-N-oxide (CNO), or saline on alternate days and assayed for their urination rate in the presence of female urine. Female-odor-evoked urination was reversibly diminished following CNO injections in BarESR1-hM4Di but not BarCRH-hM4Di or wild-type control mice (Fig. 7d,e), despite higher viral infection levels in CRH-Cre mice (Fig. 7c), and without affecting locomotion or odor sampling (Supplementary Fig. 7a,b). A previous study found a subtle effect on urination from BarCRH-hM4Di inhibition at a much longer 2-h timescale, which we replicated here (Supplementary Fig. 7c) and which is consistent with a modulatory role for either BarCRH or the third population of BarCRH-ESR1 neurons that would be expected to be inhibited with both drivers.

We additionally examined the necessity of BarESR1 neurons at faster timescales by bilaterally injecting them with AAV-FLEX-ArchT, encoding an optogenetic inhibitor (BarESR1-ArchT; mice; Fig. 7f and Supplementary Fig. 8a). We compared urination during 2 min of photoinhibition with female odor present to an additional 2 min immediately after photoinhibition ceased (Fig. 7g). Sniffing of the female odor did not differ during and after photoinhibition,
urination is difficult to trigger and study in model organisms. Here we leveraged the natural behavior of male mice, which are highly motivated to scent mark their territory in environments likely to contain females, irrespective of bladder pressure. Our quantitative behavioral assay allowed us to identify and study a subpopulation of Bar neurons that are critical to achieve voluntary urination. We further show that optogenetic stimulation of these BarESR1 neurons in male mice under isoflurane anesthesia can be used as a powerful model of controlled urination. The surprising ability of this minority subset of Bar neurons to drive robust urination comes not from the induced bladder pressure increase, but their ability to relax the EUS and gate urine release. It will be of great interest to determine whether the function of the BarESR1 neurons is conserved across evolution since ESR1 expression has also been described in Bar of primates and human urination similarly depends on relaxing the urethral sphincter11,25. Our findings in mice suggest an updated model of supraspinal urinary control in which Bar sends molecularly and functionally distinct parallel projections to downstream urinary targets (Supplementary Fig. 9a), with BarESR1 neurons serving as a key subset to enable the study of neural mechanisms underlying voluntary urination.

The majority of neurons in Bar express CRH, yet their anatomy and optogenetic activation indicates a role in focal bladder contraction without relaxing the urethral sphincter. Moreover, chemogenetic inhibition of BarESR1 neurons does not show this subset of neurons to be essential in generating odor-evoked voluntary urination. However, BarESR1 cells have been reported to play a role in urination patterns regulated by long-term social status changes, while CRH itself has a negative effect on urination at longer timescales33,34. In agreement with these findings, we replicated an earlier finding that inhibition of BarESR1 neurons leads to a modest decrease in urine marks over a much longer 2-h assay (Supplementary Fig. 7c)3. Additionally, BarESR1 neurons have been proposed to map to the 'direct' subset of Bar neurons whose firing rates correlate directly with reflexive bladder contraction1 and which some have suggested function to prolong the contraction and maintain appropriate pressure rather than initiate urination14. The activity of about one-fourth of Bar neurons does not correlate with reflexive bladder activity at all14, and our results suggest that urethral inhibition provides another meaningful dimension to Bar categorization. However, a more complete and dynamic catalog of gene expression and spiking activity in Bar neurons during a variety of conditions will likely be needed to fully classify their roles.

Liquid waste elimination is a fundamental animal need, but it must compete with many other potential priorities in a complicated world. These behavioral interactions could explain the need for heterogeneous urination control at the brainstem level, as demonstrated by the actions of BarESR1 and BarESR3 neurons described here. For example, scent marking to conspecific odor cues is highly sexually dimorphic, but all adult mice also urinate under extreme stress2,43. Most trials with female odor, but not with control odor, resulted in urination within seconds of light termination. This suggests that the immediate urine release resulted from priming by odor cues rather than trivial rebound activity upon the cessation of photoinhibition (Fig. 7g,h and Supplementary Fig. 8c). Finally, photoinhibition during cystometry revealed that ongoing BarESR1 activity is necessary to maintain sphincter bursting, since initiating brief photoinhibition during a reflexive urination event terminated EUS bursting activity and urine release within milliseconds (Supplementary Fig. 8d,e). Together, our experiments indicate that BarESR1 neurons are essential for urethral inhibition (relaxation) and voluntary urination promoted by olfactory cues in male mice.

**Discussion**

Bar is well established to be the key conserved brainstem node that coordinates the switch from urine storage to elimination. It is currently modeled as a single projection to the spinal cord that diverges to coordinate both the smooth, involuntary, slow muscle of the bladder wall and the voluntary, striated, fast muscle of the EUS. However, neurons that relax the EUS have not to our knowledge previously been identified, at least in part because voluntary but urination was largely inhibited during the photoinhibition window (Fig. 7f–j, Supplementary Fig. 8b and Supplementary Video 7). Urine marks during habituation with control odor only (gray) or with female odor (yellow shading) (thick line and shading are mean ± s.e.m., respectively; n = 12 mice). ***P = 0.00049 (Wilcoxon signed rank) for number of urine marks at 4 min compared to that at 2 min.

**Fig. 6 |** Naive male mice rapidly and robustly scent mark to female odor cues. a, Scent marking behavior in wild-type mice. Left: after 2 min exposure to control odor (black shading), right: after another 2 min with female odor (yellow shading). b, Raster plot of urine marks detected. c, Urine marks during habituation with control odor only (gray) or with female odor (yellow) (thick line and shading are mean ± s.e.m., respectively; n = 12 mice). ***P = 0.00049 (Wilcoxon signed rank) for number of urine marks at 4 min compared to that at 2 min.
Fig. 7 | Chemogenetic and optogenetic inhibition of BarESR1 neurons impairs voluntary scent marking urination. a, Schematic of chemogenetic inhibition of Bar during scent marking urination. b, Example hM4Di expression in Bar of ESR1-Cre (top) and CRH-Cre (bottom) mice; larger views minus Nissl on the right. c, Number of Bar cells infected with hM4Di virus versus CNO urine inhibition index (see Methods) for all mice (green for ESR1-Cre, magenta for CRH-Cre). d, Raster plots of urine marks on consecutive days with either CNO or saline (BarESR1-hM4Di, top; BarCRH-hM4Di, middle; CNO-only control, bottom). e, Fraction of maximum urine marks across all CNO or saline days for BarESR1-hM4Di (top; n = 8, P = 0.00013 Friedman’s test, day 2 saline **P = 0.0058, day 4 saline **P = 0.011 Dunn-Sidak post hoc differences from CNO days 1 and 3), BarCRH-hM4Di (middle; n = 10, P = 0.29 Friedman’s test) and CNO control (bottom; n = 7, P = 0.86 Friedman’s test) mice (thin lines, individual mice; thick lines, mean ± s.e.m.). f, Schematic of optogenetic inhibition of BarESR1 during scent marking urination. g, Δurine amount around the 2-min photoinhibition period. Female odor presented within 15 s of light on, and subsequent sniff periods shown in blue. n = 9 trials from 3 mice. h, Δurine amount ± s.e.m. from end of photoinhibition for control odor and female odor (thick line and shading are mean ± s.e.m., respectively; n = 9 total trials from 3 mice). i, j, Urine amount (i, **P = 0.0039) and female odor sniff time (j, P = 0.20) during the 2-min photoinhibition period and 2 min immediately following (mean ± s.e.m., same trials as h, Wilcoxon signed rank tests). Green shading denotes photoinhibition periods; n.s., not significant. Scale bars, 100 μm.

stimulating center” by anatomical and correlational evidence for roles in behaviors such as defecation, sexual behavior and childbirth, which require varying levels of somatic (for example, BarESR1) and autonomic (for example, BarCRH) coordination of the pelvic ganglia, but for which little is known in the CNS47, include perineal, muscular and physiological heterogeneity in this relatively small nucleus. For example, ejaculation requires striated urethral muscles for semen expulsion, but simultaneous bladder inhibition and backflow prevention44, and likely uses the same pattern generator in the L3–L4 DGC proposed to drive bursting urination in rodents46. The current data shows that BarESR1 neurons provide input to the DGC at these rostral lumbar levels (Supplementary Fig. 2c) that could drive the expulsion phase of ejaculation, while bladder pressure could be independently inhibited. Furthermore, the idea of regulation at different timescales could extend to other pelvic autonomic functions such as pheromone release from the preputial gland, which is also known to be regulated by social status46 and needs to be synchronized with the urine stream. Other slow functions attributed to the pelvic ganglia, but for which little is known in the CNS47, include sperm production and transport, prostate gland secretions, and various reproductive secretions that differ by species48,49. Thus, while the role of BarESR1 neurons in voluntary urination is clear, our demonstration of functional heterogeneity across Bar invites further
study into potential roles for all its neurons in regulating various other pelvic functions.

Incontinence directly or indirectly affects nearly everyone at some point in their life, yet we still have relatively little understanding of how the brain functions or fails to function during this process. Common disruptions include detrusor-sphincter dyssynergia in paraplegics or after spinal cord injury, pudendal nerve damage during childbirth, stress urinary incontinence, Fowler’s syndrome (inability to voluntarily relax the EUS in women), paruresis (inability to urinate in public) and nocturnal enuresis or bedwetting. BarESR1 neurons can now serve as an important new target for greater understanding of cause and effect in these disorders. Furthermore, the ability to direct voluntary urination in behaving male mice on a timescale of seconds also opens up new avenues for recording and manipulating neural activity during natural urination that is not driven by bladder distension. BarESR1 neurons form a critical node in this relatively simple and robust social behavior that can be leveraged to rigorously ask how such behavior is modulated by age, sex, state and learning.

Methods
Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-018-0204-3.

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Author contributions
J.A.K., J.C. and L.S. designed the study, analyzed the data and wrote the manuscript. S.S. aided in the cystometry; O.G. supported S.S. E.H.-J.W and B.K.L. aided in the fiber photometry. V.L. performed slice physiology. All other experiments were performed by J.A.K. and J.C.

Competing interests
The authors declare no competing interests.

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Methods

Animals. All animal procedures were conducted in accordance with institutional guidelines and protocols approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute. Mice were group housed at weaning (5–8 per cage), single housed for at least 1 week before any testing, and maintained on a 12/12 light/dark cycle with food and water available ad libitum. All mice were males with a mean age of ~10 weeks when single housed (range 8–12 weeks) and a mean weight of ~27 g (range 25–33 g). The number of mice used for each experiment is listed below where applicable and in the figure legends. All mouse lines are available at The Jackson Laboratory: CRH (stock no. 012704), ESR1-Cre (stock no. 007990), BALB/cByJ (stock no. 000651), UPI (stock no. 016963), ROSA-LSL-tdTomato (Ai9, stock no. 007990), and Therapeutics Core (GT3) and injected bilaterally at 8 x 1012 GC/mL. We did not see efficient expression using this virus in CRH-Cre animals, so for CRH-Cre DREADD inhibition, AAV1-CAG-FLEX-hM4Di-mCherry (Addgene plasmid no. 50461, a gift from Bryan Roth) was produced by Virovek and injected bilaterally at 4 x 1012 GC/mL. For photoinhibition, AAV9-CAG-FLEX-ArchT-GFP (UNC AV2220) was injected bilaterally at 2.2 x 1012 GC/mL in ESR1-Cre mice. For CRH-Cre DREADD inhibition, AAVd1-1/EF1a-CAG-FLEX-hM4Di+mcCherry (Addgene plasmid no. 52536, a gift from Scott Stermon) was produced by the Salk Institute Gene Transfer Targeting and Therapeutics Core (GT3) and injected bilaterally at 8 x 1012 GC/mL. We did not see efficient expression using this virus in CRH-Cre animals, so for CRH-Cre DREADD inhibition, AAVd1-1/EF1a-CAG-FLEX-hM4Di+mcCherry (Addgene plasmid no. 50461, a gift by Bryan Roth) was produced by Virovek and injected bilaterally at 4 x 1012 GC/mL. For photoinhibition, AAV9-CAG-FLEX-ArchT-GFP (UNC AV2220) was injected bilaterally at 2.2 x 1012 GC/mL in ESR1-Cre animals, and the same virus and titer were used for anatomical axon tracing unilaterally in both ESR1-Cre and CRH-Cre animals. For fiber photometry, AAV-CAG-FLEX-GCaMP6s (UPenn AV-9-PV2818) was unilaterally injected at 3.2 x 1012 GC/mL in ESR1-Cre animals.

Viral injection and fiber optic implantation. Injections were made using pulled glass pipettes (tips broken for an inner diameter of 10–20 μm) and a Picospritzer at 25–75 μL/s. For Bar injections, the overlaying muscle was removed and a medial–lateral angle of 33° was used to avoid the fourth ventricle. The pipette entry coordinate relative to bregma was 5.3 mm caudal, 2.5 mm lateral and a medial–lateral angle of 33° was used to avoid the fourth ventricle. The pipette was left in place for 5 min after injection before slowly retracting. Fiber optic implants (4 mm length, PleXon 230 μm diameter for ChR2 or ArchT) were attached over frontal cortex for animals with implants. After injection and implantation, the skull was covered with superglue and dental cement to seal the craniotomy and hold the implants in place.

Spinal cord CTB injection. A 1–2 cm incision was made over lumbar segments, and the connective tissue and muscle overlying the vertebrae were minimally dissected to expose L1 and L2 vertebrae. Vertebrae and underlying spinal segments were located by spinal process tendon attachments and spinal process shape, and confirmed by pilot injections of Diod Dye. A spinal adipator was used for the stereotaxic frame (Stoelting S1690) was used to clamp L2 transverse processes, and a beveled glass pipette was lowered into the space between L1 and L2 vertebrae, 400 μm lateral to the spinal process midline and 600 μm below dura, to target the sacral mediodorsal column bladder preganglionic neurons. After injection of 150 nL CTB-488 (ThermoFisher, 0.5% in PBS), the pipette was left in place for 5 min before slowly retracting, and then the injection site was covered with gelfoam and the overlying skin was sutured. Survival time was 5 d.

Odor-motivated urination assay. Sexually naive male mice were briefly prescreened for urination responses to 100 μL male urine (>1 s odor sampling period with >3 urine marks within 1 min) before any further testing or manipulation, which excluded 21% of all mice tested. The remaining 79% had surgical procedures and recovery or a 2-week waiting period before starting hormone injection. Mice were habituated in the behavior room 3 days before hormone injection, for 16, 8 and 4 min durations on days 1, 2 and 3, respectively. On day 3, control stimuli (100 μL tonic water, which fluoresces under UV illumination) were pipetted from above at 0 min and 2 min and the baseline response was recorded. On subsequent test days, a 4-min assay was used, with 100 μL tonic water delivered at 0 min and 100 μL female urine delivered at 2 min. All behavior was conducted during light hours under dim red light, and 70% ethanol was used to clean equipment between trials. The recording box consisted of a UV-opaque acrylic homecage with the bottom cut out, placed on top of 0.35-mm chromatography paper (Fisher Scientific 05-714-4) resting on clear glass (Supplementary Fig. 4a). Two wide-angle cameras (Logitech C930e), one above on a modified cake top and one below the bottom glass stream videoed to a laptop computer at 15 frames per second at 360 pixel resolution. An analog pulse controlled LEDs in each camera field of view to synchronize cameras. Two UV tube lights (American DJ Black-24BLB) surrounded by foil walls were used to evenly illuminate the chromatography paper from below. Videos were cut using Adobe After Effects and subsequently analyzed for urine marks using custom MATLAB software. The red and green channels of the RGB camera frames were used for urine detection and the blue channel for mouse tracking. An output video with urine detection overlay was generated to manually verify automatic spot detection. Noldus Ethovision XT was used to automatically track mice and determine distance traveled and odor sniffing periods, defined as when the nose point occluded the female urine stimulus.

Female urine collection. Adult (~8–16 weeks) C57BL/6J male mice were housed 5 per cage; soiled male bedding was introduced into the cage 24 h before the first collection night to induce estrus, and urine was pooled from 4 cages (20 mice total) over 4 d such that the stimulus consisted of a mix from all stages of the estrous cycle. Male mice were placed in a metabolic cage for 12 h at ambient temperature, and urine was collected directly into a sterile tube on dry ice and temporarily stored at ~20°C in the morning. After 4 consecutive nights of collection, urine was thawed on ice and rapidly passed through a 0.22-μm filter (Millipore Steriflip SCG005255) before aliquoting and storing at ~80°C. Two different batches of urine were collected for all experiments, and each was used with both control and experimental groups.

Chemogenetic inhibition. After hM4Di viral injection, mice were allowed at least 21 d for recovery and expression, and then intraperitoneally injected 45–55 min before testing either with control saline plus 0.5% DMSO or with clonazepam N-oxide (CNO, 5 mg/kg, Enzo Life Sciences BML-NSI05-0025) in saline plus 0.5% DMSO. Control saline injections were performed on the 3 habituation days before female urine was given. Then, on days 4, 5, 6 and 7, mice received CNO, saline, CNO and saline, respectively, before the female urine countermarking assay described above. CRH-Cre mice were tested for 2 more days (CNO then saline) using the same assay but with 2-h duration. Mice with fewer than three urine marks on 2 min after stimulus were eliminated from analysis (8 of 33 mice), as were mice that did not have bilateral hM4Di expression that spanned at least ±100 μm from the Bar rostral-caudal center, defined by ovoid Nissl clustering medial to locus coeruleus (an additional 7 of 33 mice, after behavioral exclusions). The CNO urine inhibition index (CII) was calculated as [(fraction max. urine marks on CNO days) – (fraction max. urine marks on CNO days)] / [(fraction max. urine marks on CNO days) + (fraction max. urine marks on CNO days)], such that CII = 2 represents complete inhibition by CNO relative to saline while CII = 0 represents no difference between saline and CNO days.

Optogenetic stimulation. For photostimulation experiments, fiber-implanted mice were briefly anesthetized with 5% isoflurane before connecting and disconnecting patch cables (Plexon 0.5 m, 230 μm diameter). An LED current source (Mightex BLS-SA02-US) driving two 465-nm PlexBright compact LED modules (Plexon) through a dual LED commutator (Plexon) provided 10 ± 1 mW exiting the fiber tips. Optical power was measured (Thorlabs PM20A) before and after each session. Mice were placed in the same recording box described above for behavior, but with thinner 0.19-mm firing field. Videos were cut using Adobe After Effects (version CS5) and subsequently analyzed for urine marks using custom MATLAB software (version 2014b). Urine amount was calculated from urine-covered paper (Plexon 0.5 m, 230 μm diameter). An LED current source (Mightex BLS-SA02-US) driving two 465-nm PlexBright compact LED modules (Plexon) through a dual LED commutator (Plexon) provided 10 ± 1 mW exiting the fiber tips. Optical power was measured (Thorlabs PM20A) before and after each session. Mice were placed in the same recording box described above for behavior, but with thinner 0.19-mm firing field. Videos were cut using Adobe After Effects (version CS5) and subsequently analyzed for urine marks using custom MATLAB software (version 2014b). Urine amount was calculated from urine-covered paper (Plexon 0.5 m, 230 μm diameter).
third day under 1.5% maintenance isoflurane anesthesia. Four anesthetized 50-Hz, 15-ms, 5-s photostimulation bouts separated by 1 min, 1 min, and 5 min were conducted, and then the isoflurane was removed and the mouse was allowed to recover. Recording continued before waiting for two awake 50-Hz, 15-ms, 5-s bouts separated by 5 min to confirm that awake urination was intact. After all experiments, mice were perfused and checked for viral expression and fiber placement as described for immunohistochemistry. Mice that did not have at least unilateral ChR2 expression as described above, 2 min of constant photostimulation was applied 10 s after control odor and 10–15 s before female urine. Urine marking behavior continued for 2 min after photoinhibition ceased. Mice that did not have bilateral ArchT expression that spanned >100 μm from the Bar rostral-caudal center were excluded from analysis (7 of 10 mice).

Optogenetic inhibition. For photoinhibition, all procedures were same as for photostimulation described above except for the following changes: fiber-implanted mice were not anesthetized before connecting patch cables, but were habituated to the procedure for at least 3 d before testing. On the final habituation day, control odor and then female urine stimulants were applied (two of 30 s, one of 2 min, separated by at least 30 s) to test the baseline effects of ArchT inhibition on urine output. Plexon 550 nm PlexBright compact LED modules were used, providing 0.5 ± 1 mW exiting the fiber tips. During the odor-motivated urination assay as described above, 2 min of constant photoinhibition was applied 10 s after control odor and 10–15 s before female urine. Urine marking behavior continued for 2 min after photoinhibition ceased. Mice that did not have bilateral ArchT expression that spanned >100 μm from the Bar rostral-caudal center were excluded from analysis (9 of 29 mice).

Slice electrophysiology. Mice were deeply anesthetized with isoflurane, and acute 300-μm coronal brain sections were prepared after intracardial perfusion of ice-cold choline-based slicing solution containing (in mM) 25 NaCl, 1.25 NaH2PO4, 25.0 NaHCO3, 0.5 CaCl2, 1.25 MgCl2, 10 glucose, 2.0 sodium ascorbate, 0.4 sodium glutamate, 2.0 CaCl2, 0.3 1 M Na2-EDTA. Brains were transferred quickly and sliced in the same solution with a vibratome (LeicaVT1200). Sections were transferred to a recovery chamber and incubated for 15–20 min at 35°C in recovery solution consisting of (in mM) 118 NaCl, 2.6 NaHCO3, 11 glucose, 15 HEPS, 2.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, 0.4 sodium ascorbate, 3.1 sodium pyruvate. Brains were maintained at room temperature for at least 30 min until transfer to the bath for recording. Cutting solution, recovery solution and ACSF were constantly bubbled with 95% O2/5% CO2. Slices were transferred to a recording chamber on an upright fluorescence microscope continuously perfused with oxygenated ACSF consisting of (in mM) 125 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 11 glucose, 1.3 MgCl2, and 2.0 CaCl2. Back illumination was provided by fluorescent markers were visualized with a 40x water-immersion objective with epifluorescence and infrared differential interference contrast video microscopy. Recording pipettes with 3–5 MΩ resistance were pulled from borosilicate glass (G10TF-4; Warner Instruments). The internal solution for current-clamp recording consisted of the following (in mM): 140 NaCl, 10 HEPS, 0.5 EGTA, 20 KCl 4 MgCl2, 0.3 Na2-ATP, 10 phosphocreatine. Recordings were made using a MultiClamp700B amplifier and pClamp software (Molecular Devices). The signal was low-pass filtered at 1kHz and digitized at 10kHz with a Molecular Devices digitizer. For photostimulation of ChR2, 15-ms, 5-s duration blue light pulses were emitted from a collimated LED-emitting diode (473 nm; Thorlabs) driven by a 1-Cube LED driver (Thorlabs) under the control of a Digidata 1440 A Data Acquisition System and pClamp software. Light was delivered through the reflected-light fluorescence illuminator port and the 40x objective (light power at maximum setting measured at 13.45 mW). Analysis was performed in either Clampfit (Molecular Devices) or OriginPro 2016 (Origin Lab).

Immunostaining. Animals were perfused with cold PBS followed by 4% PFA, and the brain and spinal cord were dissected and postfixed in 4% PFA at 4°C for 24–48 h. The brain and spinal cord were then washed in PBS, embedded in 1% low melting point agarose and cut on a vibratome at 50 μm for ESR1 staining or 100 μm for Nissl-only staining. Spinal cords were cut transversely across the entire thoracolumbar and lumbo sacral region and matched to segments using Nissl landmarks. For ESR1 immunostaining, free-floating sections were blocked in 1% BSA (Sigma A3059) in 1% PBST (PBS plus 1% Triton X-100) for 3 h, followed by primary incubation with anti-ESR1 antibody17,52,65 (antigen is mouse C-terminal fragment, Santa Cruz sc-542, 100 μg/mL diluted 1:500 in 1% BSA, 0.3% PBST) overnight at room temperature. Sections were washed three times with 0.1% PBST and blocked again at room temperature for 1 h before incubating in secondary antibody (ThermoFisher Alexa-Fluor 488 or 647 anti-rabbit IgG H+L, diluted 1:2,000 in 1% BSA, 0.3% PBST) at room temperature for 3 h. Nissl stain (ThermoFisher NeuroTrace Blue or Deep Red diluted 1:200) was also included here if necessary, or incubated for 2 h in 0.3% PBST if used alone. Sections were washed twice in 0.1% PBST followed by twice in PBS, then mounted with ProLong Diamond (ThermoFisher).

Fluorescence in situ hybridization. Mice were anesthetized with isoflurane before perfusing with cold PBS and cutting at 50 μm on dry ice. Coronal sections were cut at 20 μm and stored at −80°C until processing according to the protocol provided in the RNAseq Multiplex Fluorescent v2 kit (Advanced Cell Diagnostics). Sections were fixed in 4% PFA, dehydrated and hybridized with mixed probes: Crh (Mm-cdh, cat. 316091), Er1 (Mm-Emr1-02-C2, a 162Z probe targeting 1308–2125 of NM_007956.5), Slc12a1 (Gupt, Mm-Slc12a1-C2, cat. 319191) and Slc7a6 (Vglut2, Mm-Slc7a6-C2, cat. 319171) for 2 h at 40°C, followed by amplification. Signal in each channel is developed using TSA cyanine 3, fluorescein and cyanine 5 (PerkinElmer) individually. Sections were counterstained with DAPI and mounted with ProLong Diamond.

Confocal microscopy. Images were captured with Nikon A1 confocal microscope with a 10x air, 20x air or 40x oil objective. Nikon Elements software settings were optimized for each experiment to maximize signal range, and z-stack maximum projections were used for representative images and axonal projections while single optical slices were used for quantification of cell body overlap. For RNAseq, z-stacks were collected in 1-μm increments throughout the z axis.

Anatomical quantification. The rostrocaudal center of Bar was defined as the two consecutive 50-μm sections with the greatest ESR1 and CRH-tdt labeling whenever possible, or by distinctive ovoid Nissl boundaries. Custom MATLAB scripts were used to draw ROIs around bar and semiautomatically count cells with clear cell body staining. Cells with high expression of ESR1 were distinguished from background labeling by thresholding in the ESR1 color channel just below the mean intensity level of nearby parabrachial neurons with established strong ESR1 expression17,47. Cartesian coordinates for cell locations were saved and the centroid of CRH-tdt cells was used to register different sections to generate the overlay plot in Fig. 1d and Supplementary Fig. 9. For calculation of fluorescence...
intensity ratio (Fig. 1b) in the lumbosacral mediolateral column (ML) and dorsal gray commissure (DGC), all intact L5–S2 sections with visible axons were used. A rectangular ROI was drawn using the Nissl color channel to encapsulate the MLs and area in between. This ROI was then equally divided into medio–lateral thirds and the Bar axon color channel was used to calculate the sum of pixel intensity across each third. The ratio was calculated as this total pixel intensity in the middle DGC third divided by that of the two ML thirds averaged together.

**Statistics and reproducibility.** Nonparametric tests, which do not make assumptions about data distributions or variances, were used for all experiments, as detailed in the figure legends. The Wilcoxon signed rank test (MATLAB “signrank”) was used for comparison of two paired groups, and the Mann–Whitney U test (also known as Wilcoxon rank sum test; MATLAB “ranksum”) for two unpaired groups. Friedman’s test (MATLAB “friedman”) was used to compare across CNO and saline treatments for 4-d DREADD experiments, followed by Dunn–Sidak post hoc tests (MATLAB “multcompare”). Points with error bars represent mean ± s.e.m.

For most experiments, no statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications. We used the effect size from preliminary wild-type chemogenetic experiments to calculate sample sizes for ESR1-Cre and CRH-Cre chemogenetic experiments (Fig. 7d,e), using the “sampsizespwr” function in MATLAB. For each experiment, animals were maintained under identical conditions, such that no randomization was used to assign groups. Data collection and analysis were generally not performed blind to the conditions of the experiments. However, automated data analysis in MATLAB and Ethovision was used to track animal behavior such that no blinding is necessary to ensure behavioral data integrity. Semiautomated analyses similarly assisted cell counting, where the Nissl channel was used to manually define the Bar ROI, rather than the cell-counting channel.

In Fig. 1bc, Bar sections from n = 6 mice were imaged, shown together in Fig. 1d–f. In Fig. 1g–i, injection sites and lumbosacral spinal cords from n = 5 unilateral AAV-FLEX-GFP injection animals and n = 5 bilateral AAV-FLEX-ChR2 animals were used (10 total each for ESR1-Cre and CRH-Cre lines) with similar results to calculate the total sum in Fig. 11. In Fig. 2a–c, fiber photometry with video recording and injection site imaging was recorded in n = 7 mice with similar results. In Fig. 3b, n = 10 mice were imaged with similar injection sites in both ESR1-Cre and CRH-Cre lines, as quantified in Fig. 3c. In Fig. 3b, cystometry recordings were repeated in n = 3 ESR1-Cre mice and n = 5 CRH-Cre mice, similar to examples shown. In Fig. 5c, EMG recordings were repeated in n = 6 ESR1-Cre mice and n = 5 CRH-Cre mice, similar to examples shown. In Fig. 6a, scent marking behavior was repeated in n = 12 wild-type mice with similar results. In Fig. 7a,b, scent marking behavior with chemogenetic inhibition and imaging of injection sites was repeated in n = 8 ESR1-Cre mice and n = 10 CRH-Cre mice with similar results. In Supplementary Fig. 1a–c, similar results were obtained in n = 3 ESR1-ZsGreen, n = 4 Vgat-ZsGreen, and n = 3 Vglut2-ZsGreen reporter mice. In Supplementary Fig. 1d–k, similar RNAcope in situ hybridization results were obtained in n = 5 mice for each probe combination. In Supplementary Fig. 1m,n, similar CTB tracing results were obtained in n = 2 mice. In Supplementary Fig. 2bc, injection sites and lumbosacral spinal cords from n = 5 unilateral AAV-FLEX-ArchT-GFP injection animals and n = 5 bilateral AAV-FLEX-ChR2 animals were used (10 total each for ESR1-Cre and CRH-Cre lines), as in Fig. 1g–i but with different example animals shown. For Supplementary Fig. 3b, similar video recordings were made for all optogenetic, chemogenetic and fiber photometry experiments reported here. In Supplementary Fig. 4a,b, patch clamp recordings were repeated with similar results in n = 6 Bar*ESR1-Cre* neurons from 2 mice, n = 12 Bar*ESR1-Cre* neurons from 3 mice, and n = 4 Bar*ESR1-GFP neurons from 2 mice. In Supplementary Fig. 5b, the data were repeated with similar results in 33 trials across 6 mice, as summarized in Fig. 5b, e top. In Supplementary Fig. 5c–f, wireless cortical spongiosum recordings were repeated in n = 2 mice with similar results. In Supplementary Fig. 6, Bar*ESR1-Cre* stimulation during cystometry was repeated in the empty–bladder condition for 45 trials across 6 mice, with about half showing similar weak burst responses as shown in Fig. 5b. In Supplementary Fig. 8a, Bar*ESR1-Cre* expression was similar in n = 3 bilaterally infected mice. In Supplementary Fig. 8d, Bar*ESR1-Cre* similarly inhibited ongoing reflexive bursting in n = 5 trials across 2 animals, as shown in Supplementary Fig. 8e.

**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Code availability.** Custom MATLAB scripts were used to analyze and plot all data. The main analysis code was used for automated urine detection from raw video data, as detailed in Supplementary Fig. 3. The same code was used for all behavior data and is available in the Supplementary Software file or online at http://github.com/stowerslab/smuf/.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- n/a | Confirmed
- [x] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- [x] An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [x] The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- [x] A description of all covariates tested
- [x] A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- [x] A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- [x] For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- [x] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- [x] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- [x] Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- [x] Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

- Logitech Webcam Software was used to log video data. Biopac Acknowledge (version 5) was used to log cystometry and EMG data. Mightex BioLED Controller software was used to generate LED patterns for photostimulation and photoinhibition. Nikon Elements (version 4) was used to collect all confocal imaging data. Hamamatsu HCImage was used to log photometry data. DSI Ponemah software was used to log corpus spongiosum wireless pressure recordings. Molecular Devices pClamp software was used to log patch clamp physiology data.

Data analysis

- Adobe After Effects (version CS5) was used to trim videos, and urine marks were subsequently analyzed using custom MATLAB software (version 2014b). Noldus Ethovision XT was used to automatically track mice and determine distance traveled and odor sniffing periods. Molecular Devices Clampfit and OriginLab OriginPro software was used to analyze patch clamp physiology data. MATLAB was also used to compute all statistics and plot all data.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Analysis code is available in the Supplementary Software file or online at: github.com/stowerslab/smuf. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Field-specific reporting

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☐ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | For most experiments we did not have pre-specified effect size and used sample sizes consistent with other studies in the field. We used the effect size from preliminary wild-type chemogenetic experiments to calculate sample sizes for ESR1-Cre and CRH-Cre chemogenetic experiments (Fig. 7d-e), using the 'sampsizepwr' function in MATLAB (> 6 mice). |
| Data exclusions | No individual data points are excluded |
| Replication | Certain experiments included criteria for failed replication (e.g. animals did not behave in control conditions or viral injections were incorrectly targeted), in which case the data were not analyzed further:  
(1) Preliminary behavioral data gathered before any manipulation experiments established a criterion for animals that perform voluntary urination behavior. The number of mice that did not fulfill this criterion is detailed in the Methods "Odor-motivated urination assay" and "Chemogenetic inhibition" sections.  
(2) Preliminary immunostaining data gathered before any manipulation experiments established the limits of Bar used for determination of injection hits or misses (same criteria for ESR1-Cre and CRH-Cre). The number of mice that did not fulfill this criterion is detailed in Methods "Chemogenetic inhibition" and "Optogenetic stimulation/inhibition" sections.  
(3) For cystometry and EMG, we only photostimulated mice for which bladder-distension bursting was seen, such that we have a positive control for bursting. The number of mice that did not fulfill this criterion is detailed in the Methods "Electromyography and cystometry" section. |
| Randomization | For each experiment, animals were maintained under identical conditions, such that no randomization was used to assign groups. |
| Blinding | Data collection and analysis were generally not performed blind to the conditions of the experiments. However, automated data analysis in MATLAB and Ethovision was used to track animal behavior such that no blinding is necessary to ensure behavioral data integrity. Semi-automated analyses similarly assisted cell counting, where the Nissl channel was used to manually define the Bar region-of-interest, rather than the cell-counting channel. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Unique biological materials |
|      | Antibodies |
|      | Eukaryotic cell lines |
|      | Palaeontology |
|      | Animals and other organisms |
|      | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq |
|      | Flow cytometry |
|      | MRI-based neuroimaging |
### Antibodies

| Antibodies used | For ESR1 immunostaining, we used a previously characterized primary antibody (Santa Cruz catalog# sc-542 / MC-20, lot# A0716, rabbit polyclonal, 100μg/mL diluted 1:500 in 1% BSA / 0.3% PBST, refs. 17,52, & 65). Santa Cruz went out of business during the course of the study, but the antigen for this antibody is the mouse ESR1 C-terminus fragment, which is believed to recognize a specific N-terminus truncated ESR1 isoform, as detailed in ref. 65. We used standard secondary antibodies from ThermoFisher (Alexa-Fluor 488, catalog# A11070, lot# 1812158, or 647, catalog# A21246, lot#1924449 ,anti-rabbit IgG H+L, 2mg/mL diluted 1:2000 in 1% BSA / 0.3% PBST). |
|--------------------|---------------------------------------------------------------------------------------------------------------|

### Validation

| Validation | The ESR1 primary antibody has been previously validated in several studies (refs. 17,52, & 65) as well as by the manufacturer (www.scbt.com/scbt/product/eralpha-antibody-mc-20). We performed initial testing in hypothalamic areas with established ESR1 expression. The secondary antibodies have been used successfully in our lab and many other labs with a variety of different primary antibodies and mouse tissues. We also initially used a primary-antibody-negative control to verify specificity and compared the signal-to-noise ratio against other secondary antibody options. |

### Animals and other organisms

| Policy information about | studies involving animals; ARRIVE guidelines recommended for reporting animal research |
|--------------------------|----------------------------------------------------------------------------------------|
| Laboratory animals       | All animal procedures were conducted in accordance with institutional guidelines and protocols approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute. Mice were group housed at weaning (<5 per cage), single housed for at least 1 week before any testing, and maintained on a 12/12hr light/dark cycle with food and water available ad libitum. All mice were males with a mean age of ~10 weeks when single housed (range 8-12 weeks), and a mean weight of ~27g (range 25-33g). The number of mice used for each experiment is listed below where applicable and in the figure legends. All mouse lines are available at The Jackson Laboratory: CRH-Cre (ref. S1, stock #: 012704), ESR1-Cre (ref. 52, stock #: 017911), Vgat-Cre (stock #: 016962), Vglut2-Cre (stock #: 016963), ROSA-LSL-TdTomato (Ai9, stock #: 007909), ROSA-LSL-ZsGreen (Ai6, stock #: 007906), and BALB/cByJ (stock #: 000651). CRH-Cre and ESR1-Cre mice were backcrossed into the BALB/cByJ background for 3+ generations. |
| Wild animals             | No wild animals were used. |
| Field-collected samples  | No field-collected samples were used. |