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Impacts of sex differences on optogenetic, chemogenetic, and calcium-imaging tools

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Abstract

Technical innovation in neuroscience introduced powerful tools for measuring and manipulating neuronal activity via optical, chemogenetic, and calcium-imaging tools. These tools were initially tested primarily in male animals but are now increasingly being used in females as well. In this review, we consider how these tools may work differently in males and females. For example, we review sex differences in the metabolism of chemogenetic ligands and their downstream signaling effects. Optical tools more directly alter depolarization or hyperpolarization of neurons, but biological sex and gonadal hormones modulate synaptic inputs and intrinsic excitability. We review studies demonstrating that optogenetic manipulations are sometimes consistent across the rodent estrous cycle but within certain circuits; manipulations can vary across the ovarian cycle. Finally, calcium-imaging methods utilize genetically encoded calcium indicators to measure neuronal activity. Testosterone and estradiol can directly modulate calcium influx, and we consider these implications for interpreting the results of calcium-imaging studies. Together, our findings suggest that these neuroscientific tools may sometimes work differently in males and females and that users should be aware of these differences when applying these methods.

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Introduction

The introduction of *in vivo* calcium imaging, optogenetics, and chemogenetics [1−3] brought circuit- and cell-type-specific approaches to behavioral neuroscience [4,5]. These tools were primarily developed in male rodents. With increasing awareness of the importance of including both males and females in neuroscience studies [6], these tools are increasingly being used to study neural circuits in both sexes. These tools tap into the fundamental properties of neural function, so it is usually assumed that they will operate similarly in both sexes. However, this may not always be a valid assumption. Sex differences in pharmacokinetics and signal transduction can impact the performance of chemogenetic tools. Steroid hormones such as estradiol, progesterone, and testosterone can modulate synaptic inputs and neuronal excitability that could affect optogenetic tools. Finally, steroid hormones can alter the function of calcium channels that could influence the performance of genetically encoded calcium indicators. We review the extent to which these methods are impacted by gonadal hormones and discuss recommendations for assessing the significance of these effects.

Chemogenetics

Chemogenetic methods modulate neural activity using genetically modified G-protein coupled receptors and a ligand to activate them [7]. Engineered human muscarinic receptors hM3Dq (excitatory) and hM4Di (inhibitory) are activated by clozapine N-oxide (CNO) or compound 21 (C21). A second class of receptors was constructed based on kappa opioid receptors (KORD) that are activated by salvinorin A [8]. Combining hM3Dq and KORD allows for activating and inhibiting different populations of cells in the same animal. Both systems rely on a ligand and G-protein signal transduction to alter neuronal activity. Sex differences in pharmacokinetics and G-protein signal transduction could impact the performance of these tools.

Sex differences in pharmacokinetics

It is now more widely appreciated that CNO is metabolized to clozapine, which has psychoactive properties.

There is strong evidence for sex differences in clozapine pharmacokinetics [9]. Women generally have higher blood levels of clozapine than men when given the same dose, and women require lower doses for therapeutic effects. These differences may be driven by CYP1A2, an enzyme with higher activity in men than in women [10] and female rodents [11]. In both rats and mice, CNO is rapidly converted to clozapine [12], which is further back metabolized to CNO or the pharmacologically active metabolite N-desmethylclozapine. Some evidence suggests that clozapine is the key ligand for hM3Dq or hM3Di activation [7]. Together, these results suggest that the same dose of CNO could have different efficacies in males and females and that investigators should consider using lower doses of CNO for females than for males (Figure 1).

A second generation of ligands for hM3Dq/hM4Di, C21 has been put forth as an alternative [13]. C21 has better brain penetrance than CNO [14], but initial characterizations were performed primarily in male rodents [15]. There may be important sex differences in C21 efficacy. While C21 inactivated dopamine neurons expressing hM4Di at 0.5 mg/kg in both male and female mice, this same dose inactivated dopamine neurons in females that did not express hM4Di [16]. In males, 1 mg/kg of C21 inactivated dopamine neurons in mice that did not express hM4Di. Off-target effects of C21 could be mediated by serotoninergic 5-HT$_2$ receptors [17] that have higher efficacy in female rodents [18]. Little is known about the metabolism of C21 and whether off target effects occur via direct C21 action or metabolites. Together, off-target effects of C21 may be more prevalent in females, so investigators should use the lowest dose possible.
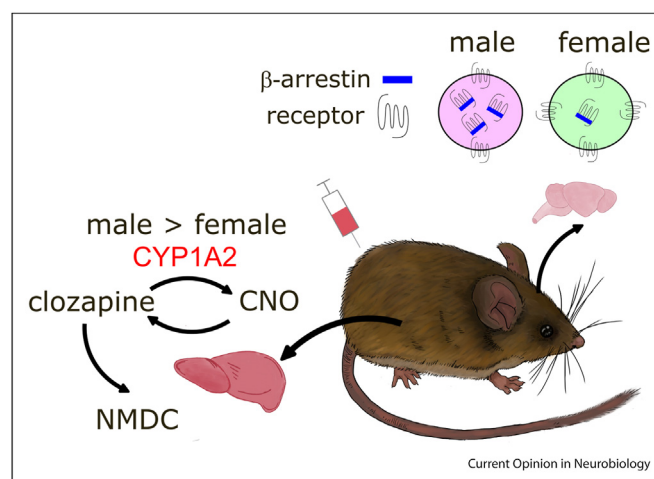
Sex differences in signal transduction
There is little direct evidence for sex differences in muscarinic receptor signal transduction, but sex differences in β-arrestin regulation of G-protein-coupled receptors have been identified. In the locus corelleus (LC), stress enhanced the expression of β-arrestin in males but not in females, and this was associated with increased internalization of corticotropin-releasing hormone (CRH) receptor 1 in males [19]. Intriguingly, in CRH overexpressing mice, discharge rates of LC neurons were lower in males than in females [20], suggesting that CRH receptor internalization reduces the sensitivity of male neurons to CRH. Muscarinic receptors can be internalized by β-arrestin [21], and the impact of internalization after repeated stimulation by CNO has been considered [22]. However, it is unknown whether chemogenetic receptors are more rapidly internalized in males than in females, possibly resulting in stronger effects of chemogenetic stimulation in females (Figure 1).

Most studies using KORD have used only male rodents [23,24]. However, recent studies observed sex differences in effects of kappa opioid receptor ligands [25,26], even though their pharmacokinetics in males and females are similar [27,28]. In males, a key mechanism for the kappa antagonist norBNI to inhibit the action of kappa opioid receptors is the activation of c-Jun N-terminal kinase [29]. In females, norBNI fails to activate c-Jun N-terminal kinase in the brain [27]. While kappa

Figure 1

Sex difference in pharmacokinetics and signal transduction: implications for chemogenetics. Metabolism of ligands within the liver is different in males compared to females. CYP1A2, a key enzyme in the metabolism of clozapine N-oxide (CNO), has higher activity in males versus females, and thus lower dosages may be more optimal for females. Sex differences have also been identified in β-arrestin regulation of corticotrophin-releasing hormone (CRH) receptors, which, like most chemogenetic receptors, are G-protein-coupled receptors. After stress exposure, males showed more receptor internalization than females. If this effect generalized to females, chemogenetic stimulation would be expected to be weaker in females. Mouse drawing by Pei Luo.

antagonists phosphorylate extracellular regulated kinase (ERK) in males, this effect was absent in females [30]. In females, high estradiol levels enhance activation of the inhibitory G-protein-coupled receptor kinase 2. When G-protein-coupled receptor kinase 2 was experimentally inhibited in female mice, kappa opioid receptor activation of ERK was restored. Thus, there are important sex differences in signal transduction of kappa opioid receptor. It is unknown if these mechanisms impact the performance of KORD. One study used KORD to examine cognitive function in ovariectomized female mice [31], which would reduce the influence of estrogens on G-protein-coupled receptor kinase 2 signaling. A recent report used KORD to target prelimbic somatostatin neurons in an alcohol self-administration paradigm [32], and the authors found that Salvinorin B had similar effects on both neural activity and alcohol self-administration in male and female mice. Thus, while sex differences in signal transduction could affect the performance of KORD, at least some circuits appear to be unaffected. Future studies should consider the possibility that KORD-based inhibition of neural circuits could be less efficacious in females than in males.

Summary
Chemogenetic tools are useful for studying neural circuits, but investigators should be cautious when interpreting sex differences in chemogenetic studies. Although sex differences observed with this technique could reflect real mechanistic differences in the regulation of behavior, they could also be driven by sex differences in pharmacokinetics or signal transduction. Ligand dosages should be optimized separately for males and females, and investigators should assess potential off-target effects in both sexes rather than assuming an effective dose in males will behave similarly in females. Reporting male and female data separately (rather than pooling) will also help in the evaluation of the effectiveness of chemogenetic manipulations. If sex differences in chemogenetic experiment outcomes are observed, *in vivo* or *ex vivo* assessments of neural activity in both sexes could help determine whether sex differences are driven by signal transduction or broader biological function. Overall, verifying sex differences with other methods and performing dose response curves with ligands will add rigor to the interpretation of chemogenetic data from males and females.

Optogenetics
Optogenetic approaches differ from chemogenetic methods in the way that these tools directly depolarize or hyperpolarize neurons to modulate their activity. Steroid hormones can modulate neuronal excitability and synaptic inputs, suggesting that optogenetic approaches could function differently in males and females.

Estrogens
One of the first discoveries of hormone regulation of neuronal function was the regulation of dendritic spines. Increased spine density increases sensitivity to glutamatergic input, thus potentially exaggerating the impact of excitatory optogenetic stimulation and impeding inhibitory stimulation. Spine density in the CA1 increases during proestrus (hen estradiol levels increase) and decreases during diestrus (when estradiol levels decrease) [33]. These changes are estrogen-dependent in the hippocampus [34] and other brain areas [35,36]. Estradiol also enhances excitatory postsynaptic potentials in CA1 pyramidal neurons in male [37] and female [38] rats. Some of these effects may be mediated by changes in spine morphology. Immature spines usually have fewer α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors, and these "silent synapses" are associated with weak or no postsynaptic depolarization [39]. Mushroom spines have wider spine heads that anchor AMPA receptors and facilitate depolarization. In the CA1, thin spines are more abundant during estrus, while mushroom spines were more abundant during diestrus and proestrus [40]. Estrogens can also alter intrinsic membrane excitability by increasing the sensitivity of CA1 pyramidal neurons to a depolarizing current [41,42]. In the dorsal striatum, medium spiny neurons in dorsal striatum also had decreased excitability in male rats compared to females [43]. Furthermore, evidence from other studies suggests that estradiol may influence inhibitory synaptic transmission. *In vitro* and *in vivo* studies with young and adult rats demonstrate that estradiol works to suppress GABAergic inhibition through sex-specific systems [44–46]. This suppression occurs through estrogen receptor α (ERα) that triggers activation of inositol triphosphate (IP3), which exists at higher levels in females than in males. This activates the IP3 receptor and ultimately leads to endocannabinoid release. Interestingly, this system is regulated via estradiol inf females but not males [44]. When it comes to learning in the hippocampus, estradiol also suppresses inhibitory transmission onto CA1 pyramidal cells [45], which allows for estradiol to potentiate excitatory transmission. Interestingly, only females require cAMP-activated protein kinase. In the same study, it was found that females but not males required not only a release of internal calcium stores but also L-type channel activation, which facilitates extracellular calcium influx [46].

Androgens
Androgens, similar to estrogens, modulate both synaptic function and intrinsic neuronal excitability. However, androgens have more variable effects on neurophysiology, and the basis for this variability is still unclear. The nonaromatizable androgen dihydrotestosterone (DHT, which binds androgen receptors) increases spine density in *ex vivo* hippocampal slices [47] and *in vivo*

[48] with similar effects observed in medial prefrontal cortex (PFC) [49]. However, chronic DHT treatment reduced the efficacy of high concentrations of NMDA on irreversible depolarization [50]. The complexity of androgens on synaptic function extends to their effects on intrinsic excitability. In the dorsal hippocampus, male gonadectomy reduced excitatory postsynaptic potential amplitudes [51] and action potentials generated from injected current-spike induction [52]. These effects were reversed by testosterone treatment. Systemic administration of the androgen-receptor antagonist flutamide also reduced measures of intrinsic excitability in the dorsal hippocampus. Androgens can also have an organizational role in synaptic transmission. Although postnatal androgen treatment had no effect on hippocampal spine density in female rats, it sensitized females to stress in adulthood. Androgenized females exposed to restraint stress as adults had more apical and basal dendrites in CA1 than females that were treated with postnatal androgen [53]. Postnatal androgen treatment also alters effects of stress in an eyeblink-conditioning task, reducing conditioned responses compared to normally cycling females [54]. Androgens also modulate neuronal function in ventral hippocampus.

A population of ventral hippocampal CA1 neurons that project to nucleus accumbens (vHPC-NAc) was less excitable in male mice than in females [55]. Male gonadectomy increased the excitability of vHPC-NAc neurons, and testosterone implants reversed this effect. Excitability also increased when vHPC-NAc neurons from males were treated with bath application of flutamide. Currently, it is unknown why androgens enhance neuronal excitability in the dorsal hippocampus and decrease excitability in the ventral hippocampus. One factor that could be involved is homeostatic scaling, which refers to the extent to which neurons adjust their intrinsic signaling properties to avoid hyperactivity or hypoactivity/excitation [56]. Homeostatic scaling can be achieved by altering excitatory inputs or through changing intrinsic membrane excitability. Thus, testosterone may increase the excitatory drive onto CA1 neurons while simultaneously reducing intrinsic membrane excitability as part of a homeostatic mechanism that regulates neuronal networks. While these mechanisms are not fully understood, androgens have important effects on neuronal activity that vary by region, circuit, or cell type.

Progesterone

Similar to androgens and estrogens, progestins modulate neuronal activity but do so through different mechanisms. Rather than targeting dendritic spines or intrinsic membrane excitability, progestins rapidly inhibit neuronal activity by allosteric binding to $GABA_A$ receptors, particularly receptors containing the δ subunit
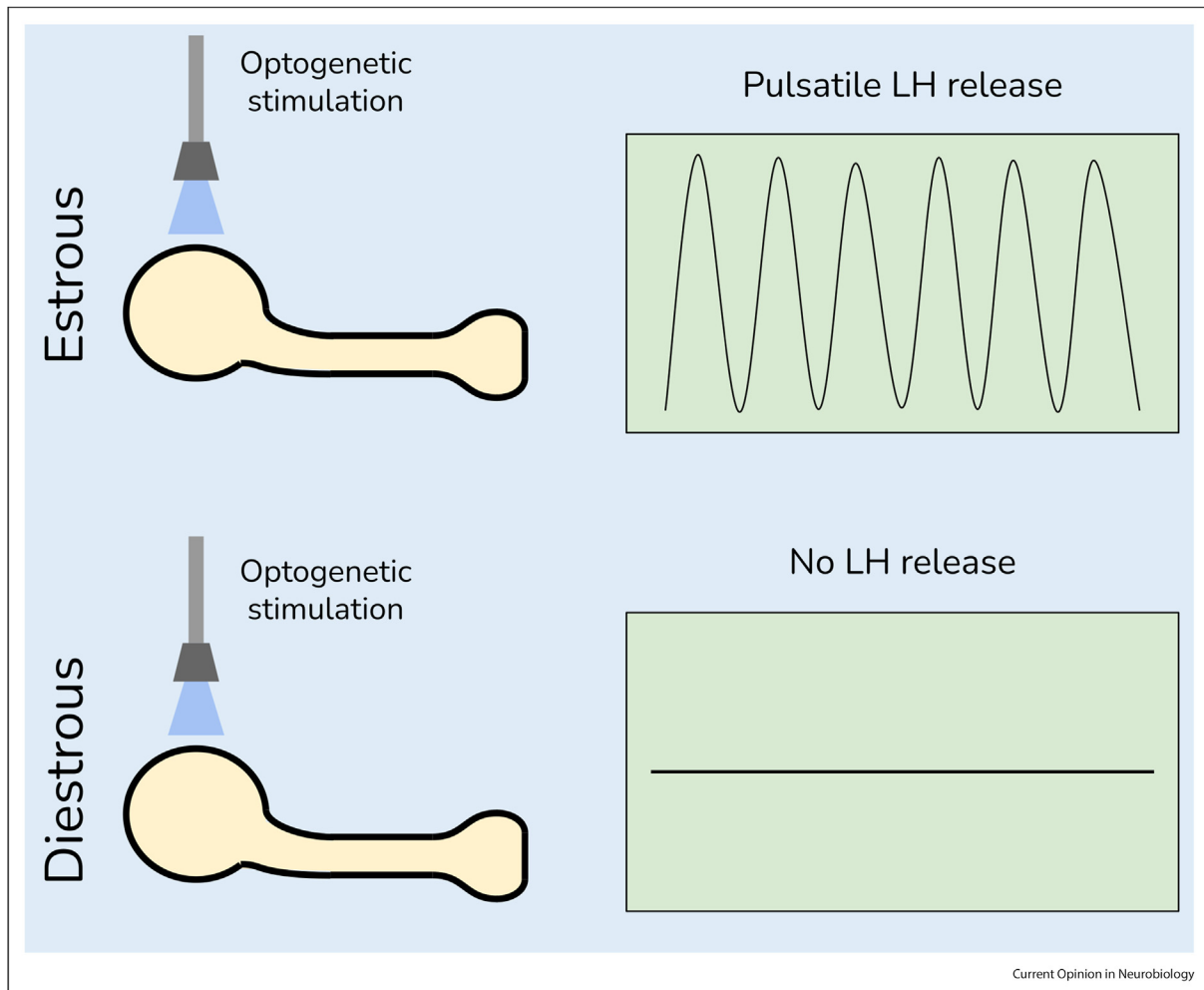
in place of the γ subunit [57]. Intriguingly, receptors expressing the δ subunit are primarily located outside of the synapse where they mediate persistent, or tonic, inhibition [58]. Thus, increased tonic inhibition of neural activity would be expected to be present under conditions of high endogenous progesterone such as late diestrus or pregnancy. This is best described in rodent seizure models. For example, in late diestrus, there is increased tonic inhibition and reduced neuronal excitability in dentate gyrus granule cells [59]. Experimental knockdown of the δ $GABA_A$ subunit blocks this effect and increases seizure susceptibility under high progesterone conditions. These effects are absent in CA1 pyramidal cells where the expression of δ $GABA_A$ subunit is low [60]. Unlike estrogens or androgens, there is little evidence for progestin modulation of intrinsic excitability. Nevertheless, progesterone modulation of tonic inhibition could impact the outcomes of optogenetic stimulation experiments.

Summary

Gonadal steroids modulate several aspects of neuronal function, but the key question is whether these effects alter the performance of optogenetic tools. A recent paper suggests that at least some optogenetic manipulations are robust to fluctuations in hormone levels [61]. Excitatory optical stimulation of the motor cortex was used to induce spreading depolarization, a slow wave of neuronal and glial excitation that is used as a preclinical model for migraines. Optogenetic induction of spreading depolarization increased sensitivity to pain to a greater extent in females than in males. However, the estrous stage did not affect spreading depolarizations, even though pain sensitivity was greater during proestrus or estrus compared to diestrus. Similarly, although spreading depolarization increased sensitivity to light, no differences were observed across the estrous cycle. Thus, in this model, while there are robust sex differences in susceptibility to spreading depolarization, the behavioral effects of optogenetic stimulation do not appear to be modulated by estrous cycle state. This may not be true for all systems.

Kisspeptin neurons in the hypothalamus drive pulsatile release of gonadotropin-releasing hormone, which in turn controls gonadal function [62]. Channelrhodopsin was expressed in kisspeptin neurons, and blue-light stimulation was applied at different stages of the estrous cycle [63]. During estrus, blue-light stimulation increased the number of luteinizing hormone pulses. In contrast, when blue light was applied during diestrus, the number of luteinizing hormones pulses was reduced (Figure 2). These differences could be driven by progesterone as optogenetic excitation of kisspeptin neurons induced surges of luteinizing hormone in the presence but not in the absence of progesterone [64]. While these data show that progestins can modulate the

Figure 2

Estrous cycle influences the effects of optogenetic stimulation of kisspeptin neurons. During estrus, circulating estrogens are elevated while progestin levels are decreasing, and optogenetic stimulation of kisspeptin neurons stimulates luteinizing hormone (LH) release. During diestrus, estrogen levels are lower while progestin levels are higher, and optogenetic stimulation of kisspeptin neurons does not stimulate GnRH release.

effects of optogenetic manipulations, the extent of these effects outside of the hypothalamus is unclear. Although there is little direct evidence for androgen effects on optogenetic stimulation, this has not been assessed directly.

Calcium imaging

Calcium imaging is a key method for monitoring neuronal activity [65]. Genetically encoded calcium indicators (such as GCaMP) allow for the targeting of specific cell populations *in vivo* using fiber photometry, 2-photon imaging, or miniature microscopes [66]. All methods assume that fluorescent signals track changes in intracellular calcium that tracks action potentials. Intracellular calcium levels can be modulated by gonadal hormones, which could influence the amplitude and fidelity of activity-dependent calcium signals.
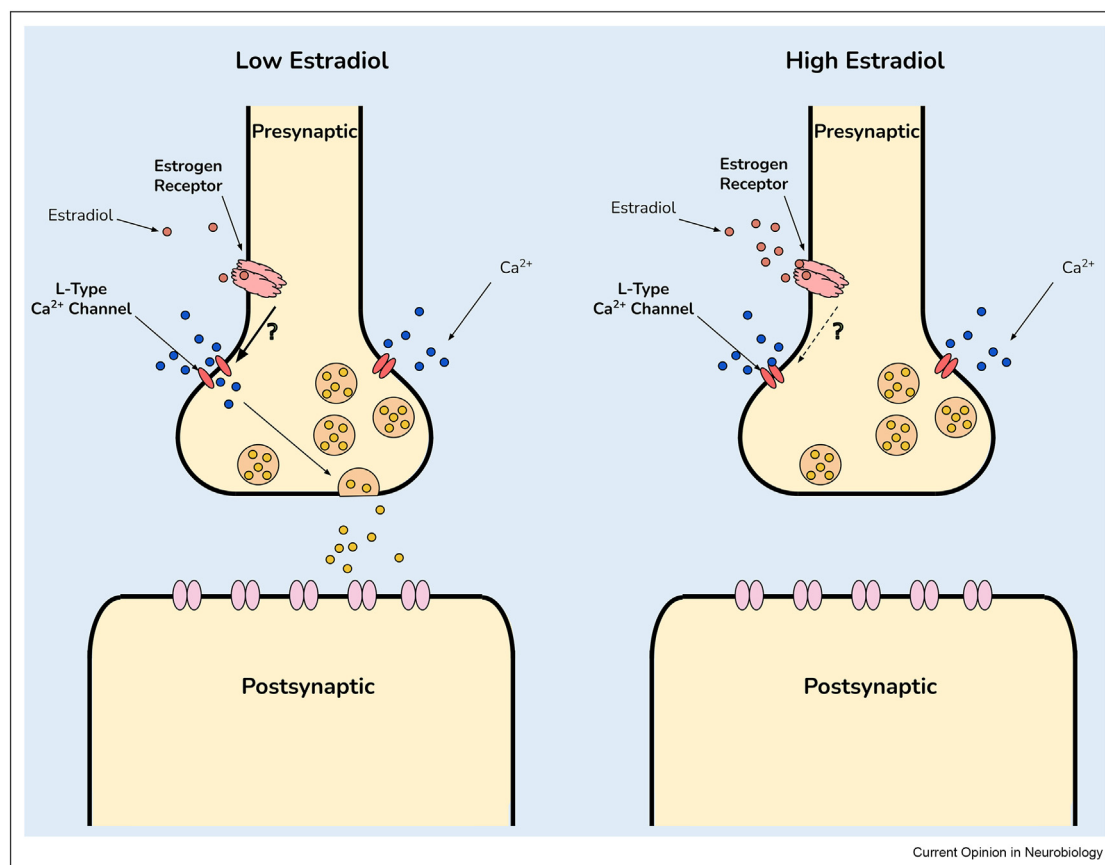
Effects of gonadal hormones on calcium influx

In cultured hippocampus neurons, DHT treatment increased baseline calcium levels, and this effect was blocked by flutamide [67]. Differences in baseline levels might not impact calcium-imaging applications that assess changes relative to a baseline (e.g. $\Delta F/F$). More relevant were findings that DHT modulated glutamate-induced calcium responses. In neurons cultured from newborn rats, DHT enhanced glutamate-induced calcium influx [68] but decreased calcium responses in cells harvested from older animals [69]. The latter outcome suggests that neurons exposed to androgens would produce blunted $\Delta F/F$ signals compared to neurons in the absence of androgens. A recent fiber photometry study also observed that male prepubertal gonadectomy enhanced calcium transients within bed nucleus of the stria terminalis (BNST) [70]. Male

California mice were randomly assigned to prepubertal castration or sham surgery and then were exposed to social defeat as adults. Calcium imaging in the BNST showed that both castrated and intact males had increased $\Delta F/F$ to a threatening male target mouse. In contrast, only castrated males exhibited increased $\Delta F/F$ to a nonthreatening male target mouse. These results suggest that the ventral BNST responds to threatening social contexts and that androgen exposure reduces neuronal excitability in less threatening contexts. A key question is whether the reduced calcium transients observed in castrated males were due to reduced neural activity or due to direct effects of androgens on calcium influx. A separate dataset found that prepubertal castration increased c-fos expression within the BNST, suggesting that increased calcium transients in castrated males were driven by increase in neural activity. Even if androgens reduced calcium influx without altering the firing rates of neurons, the observation of reduced calcium transients would imply that neurotransmitter release would be reduced [71].

Estrogens also rapidly modulate intracellular calcium concentrations (Figure 3), but these effects appear to be dose dependent [72]. At lower picomolar concentrations, estradiol induced a rapid influx of intracellular calcium in cultured hippocampal cells [73]. This effect was blocked by preincubation with nifedipine, an L-type voltage-gated calcium channel antagonist. Similar effects of estradiol (at picomolar concentrations) were observed in cultured mouse midbrain neurons [74]. However, at higher nanomolar concentrations, estradiol reduced intracellular calcium concentrations in cultured corticals, and these effects were reversed by L-type calcium channel activation [75]. These findings raise the difficult question of whether experimental concentrations of estradiol match physiological levels *in vivo*. Steroid concentrations in the brain can be much higher than in blood [76]. A recent analysis of estradiol concentrations in the BNST reported concentrations in the 1-pg/10-µL range [77]. Although these measurements provide a useful estimate of brain estradiol content; they likely reflect a lower limit on estradiol concentrations at

Figure 3

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Effects of estradiol on calcium influx: Cell culture studies find that low concentrations of estradiol facilitate calcium influx and that this effect is prevented by L-type calcium channel blockers. In contrast, the opposite effect is observed at high estradiol concentrations. The mechanism for these concentration-dependent effects of estradiol is unknown.

the synapse as neurons, and astrocytes-that expressar-omatase are capable of synthesizing estradiol in close proximity to synapses [78].

Conclusions

Sex differences can influence the function of widely used neuroscientific tools. For chemogenetics, sex differences in ligand pharmacokinetics, signal transduction, and off-target effects of chemogenetic ligands are well established. For optogenetic tools, steroids can modulate synaptic plasticity and intrinsic excitability, but these tools may be less susceptible to sex differences in efficacy. There is also evidence that hormonal modulation of calcium channels could affect measures of neural activity via calcium imaging. However, since hormone-dependent effects on calcium influx would have functional consequences for neuronal signaling, these effects would be physiologically relevant and likely not an artifact of the technique. An additional dimension not addressed in this review is the possibility that expression of genes on sex chromosomes (X and Y) could differentially impact neuronal signaling and thus the function of neuroscience tools [79,80]. Together, these findings suggest that chemogenetic, optogenetic, and calcium-imaging tools may need separate optimization for use in males and females and that caution should be taken to consider the role that sex hormones might play in the processes targeted by these tools.

Declaration of competing interest

There are no competing interests to disclose.

Data availability

No data was used for the research described in the article.

Acknowledgements

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* of special interest
** of outstanding interest

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