Behavioral Models of Neural Pleasure Circuitry: Effects of Sex Differences

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Abstract

The mechanisms underlying the sensation of pleasant touch is currently unknown and is a challenging topic in the field of neuroscience. In addition, diverging brain structure and sensitivities to pleasure and pain sensations among males and females suggest potential sexual dimorphisms in the pleasant touch neural circuitry, which this paper aims to investigate. In this study, we used intersectional genetic manipulations to selectively activate a subset of primary sensory neurons in the dorsal root ganglia (DRG) which expresses vesicle glutamate transporter type 3 (VT3+). Following behavioral tests showed that activation of VT3+ sensory neurons in freely behaving mice promoted conditioned place preference. Moreover, we found no significant differences among male and female subjects. These results indicated the involvement of VT3+ DRG neurons in the pleasant touch neural circuitry as well as an absence of sexual dimorphisms. VT3+ neurons include two subpopulations: Tyrosine hydroxylase+ (TH+) C-low threshold mechanoreceptors (LTMRs) and TH- SA1 Aβ -LTMRs. For further specificity, a different mouse-line was used to activate TH+ DRG via intersectional genetic tools. Results were similar to the initial experiment, suggesting the specific involvement of small diameter, VT3+/TH+ C-LTMRs in the pleasant touch neural circuitry and an absence of sexual dimorphisms. These findings suggest that males and females may have the same neural circuitry underlying pleasant touch sensations. Overall, our results lead to further understanding of the mechanisms underlying pleasant touch.

Keywords: pleasant touch; sexual dimorphism; Vglut3 (VT3); dorsal root ganglia (DRG); Clozapine N-oxide (CNO); place preference assay

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Touch, the detection of mechanical stimuli on the skin, is critical for the survival and development of humans and other mammals. It allows for the sensation of pressure, temperature, proprioception, and, most importantly, nociception (DiGuiseppi & Tadi, 2019). The coding of such somatosensory information involves complex neural circuitry, beginning with various specialized cutaneous mechanoreceptors that retrieve external stimuli and feed this information to the brain. These mechanoreceptors are categorized into low-threshold mechanoreceptors (LTMRs) and high-threshold mechanoreceptors (HTMRs), each associated with Aβ -, Aδ -, or C-fibers with varying propagation speeds of the action potentials (Roudaut et al., 2012). Aβ fibers are the most myelinated nerves with the largest diameter. They carry non-discriminative touch and pressure stimuli information through the spinothalamic tract. Aδ -fibers are moderately myelinated nerves, compared to more heavily myelinated Aβ - fibers, that signal for a rapid nociceptive response and trigger muscular reflexes to remove the body away from the stimuli. This is termed the "first" pain response. C-fibers are unmyelinated, slow conducting nerves that respond to mechanical and temperature stimuli and signal for the drawn out nociceptive pain sensations commonly associated with injuries. This is termed the "second" pain response. These fibers synapse with thalamic nerve cells, which further send signals through the corona radiata to the relevant regions of the somatosensory cortex (DiGuiseppi & Tadi, 2019).

The mechanisms involved in the sensation of nociceptive stimuli such as pain and itch, especially C-fibers, may be able to help shed light on the mechanisms involved in another type of touch that is currently less investigated: pleasurable touch. Pleasurable touch, or pleasant touch, is a type of afferent touch that can increase well-being and is a significant factor in

affiliative behaviors (Lloyd, McGlone, & Yosipovitch, 2015). Pleasant touch plays an important role in homeostasis, social and affiliative interactions, and communication. In a study done with individuals from different countries, subjects from the US and Spain were able to distinguish different emotions based on how another person touched them (Hertenstein, Keltner, App, Bulleit, & Jaskolka, 2006). In a study done on rodents, slow stroking over the skin promoted hormonal responses, specifically in endorphins and oxytocin (Olausson, Wessberg, Morrison, McGlone, & Vallbo, 2008). Recent evidence supports the belief that pleasant touch promotes nurturing as well as inter-individual bonding in social species.

Types of pleasurable touch include interpersonal touch, grooming, massage, and may also potentially include itch relief, although there is still a degree of uncertainty regarding this (Lloyd, McGlone, & Yosipovitch, 2015). In an fMRI study conducted by Mochizuki and colleagues (2013) which aimed to measure the degree of pleasure experienced when scratching, a broad neural network was shown to be activated during scratching. It was found that in addition to the reward system of the brain, regions such as the primary somatosensory cortex and the insular cortex were activated, suggesting consciousness of subjective feelings. This provides a potential link between scratching and pleasure as well as a possible explanation for the subjectivity of pleasure sensations.

In another fMRI study imaging neural responses to self-scratching vs other-scratching behaviors, self-scratching activated many regions associated with reward processing, correlating with the responses observed during pleasure while scratching or itch relief. However, only some parts of these neural circuits were activated by other-scratching, indicating that pleasure of scratching and itch relief may be independent (Lloyd, McGlone, & Yosipovitch, 2015). Given these findings, it is possible that there is an overlap in neural circuitry involved in pleasant touch

sensations and nociception. In addition, a study done by bin Saif et al. (2012) discovered topographical variations in itch intensity, itch relief, and pleasurability at the forearm, ankle, and back. This was attributed to possible differences in nerve density, which agrees with recent evidence suggesting that cutaneous C-afferent fiber density impacts perceived pleasant touch (bin Saif et al., 2012). This further suggests a potential overlap in neural circuitry between nociception and pleasant touch. It is also important to mention that itch attenuation by scratching was found to act through the inhibition of spinothalamic tract neurons' transmission of itch information under the specific conditions of histamine induction (Davidson, Zhang, Khasabov, Simone, & Giesler, 2009). While scratching activates regions of the brain involved in itch processing, it also inactivates areas such as the anterior cingulate cortex which is involved in the unpleasant sensation of itch. Most importantly, neuroimaging studies have shown that the itch sensation itself leads to strong activation of the putamen in the dorsal striatum, which is involved in the anticipation of pleasure (bin Saif et al., 2012). These mechanistic indicators imply a possible link between itch sensation, relief, and pleasure circuitry.

However, while itch sensations are more commonly associated with Aβ - fibers, recent studies have placed a focus on the relevance of unmyelinated C afferent neurons, specifically low threshold mechanoreceptor C tactile (CT) afferents (C-LTMR), in pleasant touch sensations. These afferents respond to slow and light stroking and are found in non glabrous skin (Loken, Wessberg, Morrison, McGlone, & Olausson, 2009). In a study conducted by Loken et al. (2009), they demonstrated a relationship between positive hedonic sensation and neural firing of CT afferent nerve fibers. When conducting soft brush stroking on hairy skin, it was found that stroking was perceived as most pleasant when delivered at velocities that were most effective at activating CT nerve stimulation (1-10 cm s^{-1}). Furthermore, C-tactile fibers project to the insular

cortex, which processes emotions and interoceptive awareness (Olausson et al., 2002). This indicates that CT afferents may be part of a system that underlies emotional, hormonal, and affiliative responses to light, skin-to-skin touch between individuals.

Additionally, the CT affective touch hypothesis, based on recent evidence, suggests that when CTs are activated, they may have analgesic and anxiolytic effects. Liljencrantz and Olausson (2014) investigated this through tactile allodynia, a symptom in which typically innocuous moving stimuli result in pain. They studied both healthy subjects and two patients with selective denervation of A β - fibers. Typically, it is difficult to acquire data that supports a specific role of C-afferents only as it is not possible to activate C-afferents without also activating A β - fibers. However, the two patient subjects allowed for the investigation of C-afferents only. Interestingly, after being administered CT stimulation, both patients independently reported that they felt a pleasant touch experience with no hint of pain, tickle, or itch. In addition to this, this study also proposed that the significance of CT afferents in tactile allodynia was their role in loss of pain inhibition, suggesting that activation of CT afferents may lead to pleasant touch sensations through the inhibition of pain.

Recent evidence also further suggests that CT afferents are significant in hedonic or rewarding touch in the context of social interaction and relationships. This social touch can include huddling, cuddling, and other thermoregulatory touch behaviors. This overlap in thermosensation and somatosensation occurs due to CT afferents' role in both pleasant touch circuitry and thermoreceptivity (Morrison, 2016). Furthermore, the activation of these C afferent nerves is believed to influence behavior. Evolutionarily, social touch behaviors, such as huddling, can lead to the reduction of risk associated with exposure to predation in addition to thermoregulatory benefits. Researchers believe that social touch may have anxiolytic effects and

that moments of high stress or anxiety may trigger the drive to seek social proximity, just as cold temperatures may trigger the drive to seek warmth through huddling behaviors.

On another note, sexual dimorphism is an influential factor to consider when mapping neural circuitry. Due to differences in brain structure, males and females exhibit diverging behaviors, such as mating behaviors. This is caused by a lack of masculinization of the female brain, or feminization, due to the absence of testicular hormones during development (Yang & Shah, 2014). In a study done by Girard-Tremblay et al. (2014), sex differences in pain perception were investigated using electrical stimulation of the sural nerve to evoke experimental pain. It was found that induced subjective, unpleasant pain correlated with significantly increased activity of the perigenual anterior cingulate cortex in women and decreased activity of the ventromedial frontal cortex in men. The findings of this study suggest distinct neural coping mechanisms in males and females in response to unpleasant pain. In males, deactivation of an inhibitory region allows them to mobilize threat control circuits, which was unobserved in females. Females instead experienced activation of regions in the limbic system which is involved in emotion, indicating an advantage in processing, articulating, and identifying the emotional aspect of pain (Girard-Tremblay et al., 2014). Furthermore, in another study on drug use and the brain's reward system, found that females had a larger release of dopamine and higher sensitivity to inhibition of dopamine uptake during the initial exposure to stimulants (Becker, Perry, & Westenbroek, 2012). In regards to chronic drug use, it was found that females experienced an exacerbated negative affective state during withdrawal which was attributed to higher noradrenergic and corticotropin releasing factor activity.

Additionally, in the insular cortex, a region of the brain to which C-tactile fibers project to, was discovered to have significantly more cannabinoid 1 receptor (CB_1R)-expressing cells in

males than in females (Liu, Li, Zhao, Wang, & Wang, 2020). CB1R is one of the most abundant G-protein coupled receptors in the brain and modulates and mediates synaptic transmission and plasticity, impacting both emotion and behavior.

In another study, sexual dimorphisms were found in the multi-body-part receptive fields of the genital somatosensory cortex, a key region of perception (Lenschow et al., 2015). To investigate the cortical representation of the genitals, in vivo cell recordings were recorded during applied air puffs to regions of the genitals confirmed to correspond to the genital cortex through mapping experiments. They found that genital receptive field patterns in males colocalized significantly more with the forearm than those in females. Females also had significantly more colocalization with the trunk. There were rarely pure genital receptive fields. These studies provide evidence of behavioral and neural sexual dimorphisms, both of which may impact pleasant touch responses and perception. These studies also suggest a potential sexual dimorphism in neural circuitry involved in pleasant touch.

Body temperature is another sexually dimorphic characteristic that influences thermal and pain sensitivities. It is known that core body temperature in mice changes with age, however, in a study done by Sanchez-Alavez, Alboni, and Conti (2010), young female mice (3 months) had an overall higher core temperature than male mice. This temperature difference became more pronounced in older mice (24 months), due to the elimination of estrous as a confounding factor. This study also suggests gonadal dependent modulation of core temperature as a contributing factor in gender-specific longevity, which they supported with evidence of their male mice subjects outliving their female mice subjects. Moreover, recent studies showed that prenatally stressed male infant (7-day-old) Long-Evans hooded rats showed a higher pain sensitivity in response to the formalin test than their female counterparts (Butkevich, Barr, & Vershinina,

2007). This shows that there are sexual dimorphisms present in nociception and suggests potential sexual dimorphisms in the pleasant touch circuitry as well.

One transporter worth mentioning is the Vesicular Glutamate Transporter Type 3 (VGLUT3, VT3). It has been found that VT3 has a role in enhancing and inhibiting 5-hydroxytryptamine (5-HT) neural transmission in different regions of the brain, influencing serotonergic circuitry or mood regulation (Amilhon et al., 2010). Furthermore, VT3 are prevalent in excitatory glutamatergic neurons and were recently found to have a significant role in the development and regulation of innate fear in mice (Balázsfi et al., 2018). VT3 is shown to be significant in the expression of emotions and may contribute to the pleasure aspect of pleasant touch.

Vglut3-expressing C-LTMRs have been implicated in the mediation of pleasant touch and/or pain. In a study which performed genetic fate mapping, Vglut3 lineage sensory neurons were divided into two groups based on transient or persistent Vglut3 expression (Lou, Duan, Vong, Lowell, & Ma, 2013). Vglut3-transient neurons are large- and medium-diameter, myelinated mechanoreceptors that form the Merkel cell-neurite complex, commonly associated with slow adapting nerve fibers and deep static touch. Vglut3-persistent neurons are small diameter, unmyelinated nerves that are further categorized into tyrosine hydroxylase (TH)-positive C-LTMRs of non glabrous skin and TH-negative neurons that form epidermal-free nerve endings. Based on analyses of mice subjects, this study concluded that Runx1 is required to establish mechanosensitivity in C-LTMRs and is necessary for the development of Vglut3-persistent neurons. These conclusions call for further investigation into the role of Vglut3-persistent neurons in the pleasant touch neural circuitry.

Pleasant touch is a highly complex somatosensory modality and much of its underlying circuitry and mechanism is still mostly unknown. Based on previous research, there is evidence that C-LTMRs play a significant role in pleasant touch circuitry, specifically Vglut3-expression C-LTMRs. We hypothesize that unmyelinated C-LTMR afferents, specifically those which are located in the DRG, will lead to changes in behavior that indicate the activation of the pleasant touch circuitry. Furthermore, we plan to investigate the presence of sexual dimorphisms in the pleasant touch circuitry, through the investigation of DRG.

Method

1. Animals

For the purposes of this study, we interbred Vglut3::Cre (VT3::Cre) mice and Advillin^{Flpo} (Avil^{Flpo}) mice to make VT3^{Cre}/Avil^{Flpo} mice (Figure 1). These mice were then bred with the Cre and Flpo dependent Rosa-Dq mice to get our final Vglut3::Cre/Advillin^{Flpo}/Rosa26$^{CAG\text{-}ds\text{-}hM3Dq}$ (VT3/Avil/Dq) mouse line. A hM3Dq receptor, a modified version of the human M3 muscarinic (hM3) receptor was genetically inserted into our mouse line. hM3Dq can be activated by the inert clozapine metabolite clozapine-N-oxide (CNO), engaging the Gq signaling pathway. Gq signaling releases intracellular calcium stores and enhances neuronal excitation. This allows us to target dorsal root ganglia (DRG) that express the Vesicular Glutamate Transporter Type 3 (VGLUT3, VT3).

VT3/Avil/Dq mice subjects and control mice subjects were used, for a total of at least 24 subjects. This sample size was concluded using a G power analysis program with a significance level required for regression analysis of 0.05, a large effect size of 0.72, a power of 0.95 according to Cohen's Law, and the number of groups set to 4 (Faul, Erdfelder, Buchner, & Lang, 2009). Based on our power analysis, having at least 6 subjects in both the control and treatment

group would allow us to detect any relevant effects. Subjects underwent a place preference assay with supplemental behavioral tests.

Moreover, an additional THcreER/Avil/Dq mouse-line was used (Figure 2). We interbred TH::CreER (TH::CreER) mice and Advillin^{Flpo} (Avil^{Flpo}) mice to make TH^{CreER}/Avil^{Flpo} mice. These mice were then breeded with the Cre and Flpo dependent Rosa-Dq mice to get our final TH::CreER/Advillin^{Flpo}/Rosa26$^{CAG\text{-}ds\text{-}hM3Dq}$ (THcreER/Avil/Dq) mouse line. A hM3Dq receptor, a modified version of the human M3 muscarinic (hM3) receptor was genetically inserted into our THcreER mouse line, allowing us to excite dorsal root ganglia (DRG) expressing tyrosine hydroxylase (TH). Additionally, after the mice were bred, they were injected with Tamoxifen (IP. 500 ug, 8 mg/3 ml for 3 days) 5 days post-DOB to activate cre and allow mRNA to migrate and translate TH. These subjects also underwent a place preference assay with supplemental behavioral tests. The necessary sample size for the THcreER/Avil/Dq mouse-line subjects was consistent with the VT3/Avil/Dq mouse-line subjects. All experimental methods were approved by the Experimental Animal Ethics Committee of University of Michigan (IACUC Protocol #PRO00009325).

2. Place Preference Assay

The place preference assay consisted of a 9 day protocol (Figure 3). On the first day, the mice were freely habituated in a dark/bright chamber, with complete access to the entire chamber, for 30 minutes. On the second day, the mice were freely habituated again, entering at the bright chamber, and this was recorded for 10 minutes. From the 3rd to the 8th day, mice underwent training in 30 minute sessions, being restricted to either the bright or dark chamber. Training alternated between bright and dark habituation until 3 days of each had been done. The bright chamber habituation was performed for 30 minutes starting 30 minutes post-CNO

injection (intraperitoneal; i.p, 5mg/kg), and the dark chamber habituation was performed for 30 minutes starting 30 minutes post-saline injection (i.p). CNO-injections were used for the chemogenetic activation of Vglut3 + and TH+ sensory neurons. Training and habituation are necessary to eliminate possible confounding variables during data collection. On the 9th day, after the training concluded, data collection began. At 30 minutes post-CNO injection, the mice were placed in the bright/dark chamber with complete access to the both sides of the chamber and recorded for 30 minutes. The time spent on each side of the bright/dark chamber was observed to determine chamber preference. The data was collected in 10 minute intervals.

3. Von Frey

As previously mentioned, the place preference assay was supplemented with additional behavior tests. For the Von Frey test, we acclimated mice for 30 minutes per day for 3 days in an elevated chamber with a mesh floor. Von Frey filaments were held to the hindpaw from below for 2 seconds until the filament bowed, then removed. Stimuli were presented at intervals of 5 s and positive response was noted if the paw was quickly withdrawn. Flinching or licking upon removal of the filament was also considered a positive response. The cut-off of a 1.40 g hair (~ 10% of the body weight of the smaller mice at P30) was selected as the upper limit for testing and testing was initiated with the 0.16 g filament. Stimuli were always presented in a consecutive fashion, whether ascending or descending. In the absence of a paw withdrawal to the initially selected filament (O), a stronger stimulus was presented; in the event of paw withdrawal (X), the next weaker stimulus was chosen. Every filament was used in 3 trials. If mice got positive responses in 2 trials, we considered that it was a real positive response. Optimal threshold calculation by this method requires 6 responses.

4. Mechanical Itch

To test mechanical itch, the hair behind the ears or the nape of the neck was shaved 3 days before the examination. Mice were habituated for 15 minutes for 3 consecutive days in a behavioral testing device. When testing, mice were placed in a plastic enclosure (10 cm x 10 cm x 12 cm) and allowed to habituate for 15 minutes. Mice received 5 separate mechanical stimuli for ~1 s at intervals of 3-5 s at randomly selected areas on the skin behind the ear. Mechanical stimulation was delivered to a 0.07 mN von Frey filament. The scratching response of the hindpaw to the poking site was considered a positive response. We measured the percentage of positive responses across five stimuli, resulting in an acute mechanical itching score.

5. Hargreaves Hyperalgesia Test

For the Hargreaves Hyperalgesia test, mice were placed in a clear plastic chamber with a glass floor and habituated 30 min per day for 3 days. On the third day, after a 30-min habituation, radiant heat (laser light: 23% intensity) was applied under the glass floor directly beneath the hindpaw. The heat stimuli was stopped when mice engaged in lifting the hindpaw and/or flinching/licking. The response latency was measured as paw withdrawal latency. This was repeated 4 times with 10 min intervals (same hindpaw, for acute thermal test) or 5 min intervals (different hindpaw, testing thermal hyperalgesia after inflammation).

6. RotaRod Test

For the RotaRod performance test, mice were placed on the RotaRod apparatus (IITC Life Science, CA, USA) for 15 minutes for habituation, with the RotaRod initially being static when the mouse is placed, then starting at 4 rotations per minute (rpm) and continuously accelerating to 40 rpm. After habituation, mice were placed on the RotaRod apparatus and their latency to falling off the rod was measured. Mice underwent three trials with 15 minute intervals of rest between each trial.

7. Light Brush Test

To assess dynamic allodynia, light stroking of the external lateral side of the hind paw in the direction from the heel to the toe with a paintbrush (5/0, cut the tip and made it blunt. The total length of brush is about 5 mm. And then remove the outer layer of hairs). The typical response of naive mice to the stimulation is fast movement or lifting of the stimulated paw aside (score 0). However, after the development of the neuropathy, several pain-like responses can be observed, such as: sustained lifting (more than 2 seconds) of the stimulated paw towards the body (score 1), strong lateral lifting above the level of the body [what they do is like kicking to the lateral side, it is not a flinching, it is more like a super paw withdrawal] (score 2), and flinching or licking of the affected paw (score 3). Stimulation was repeated 3 times at intervals of at least 3 minutes, to obtain the average score for each individual mouse.

8. Pinprick Test

For the pin prick test, mice were habituated to a Von Frey chamber for 20 minutes for two days before testing. When testing, we poked the hind paw of the mice, alternating between left and right with a 5 second rest period in between. The hindpaw was poked for a total of 10 times (5 times per paw). Positive responses consist of lifting, shaking, and lifting hindpaw and control mice were used to confirm positive responses.

9. Gradient Temperature Test

For the gradient temperature test, mice were habituated on the temperature gradient for 30 minutes one day before testing. On test day, mice were habituated for 15 minutes then injected with CNO (i.p, 5mg/kg, 100 ul). They were then placed in the middle of a temperature gradient ranging from 5^{o}C on one end to 54^{o}C on the other end and recorded for 30 minutes for pre-CNO injection responses. After the initial recording, mice were immediately recorded for 30

minutes more, for post-CNO injection responses. Data was collected as the percentage of the observation time spent at that temperature and analyzed using MATLAB and Prism.

10. Statistical Analysis

Due to multiple independent variables, place preference data was analyzed through an ANOVA test. A threshold of $p < .05$ was accepted as statistically different and $p > .05$ considered non-significant. Supplementary tests were subjected to Student's unpaired, two-tailed t-tests and were accepted as statistically significant if $p < .05$. All results are expressed as mean ± SEM. Statistical analysis was performed in Prism 7 (GraphPad).

Results

Activation of VT3+ or TH+ DRG Contributes to Pleasant Touch Circuitry

Mice are considered nocturnal and should have a preference for darkness. However, our data shows that VT3/Avil/Dq mice have a preference for light post-CNO injection, making it reasonable to believe that the Vglut3 positive DRG neuron is involved in the neural circuitry involved in pleasure. By using this VT3/Avil/Dq mouse line, we can ensure that VT3 positive (VT3+) DRG, which is mostly differentiated from the brain and spinal nerves by the additional expression of Advillin, will be activated by inert CNO due to the presence of a modified human M3 muscarinic receptor (termed as Dq). This allows us to specifically target small diameter DRG. The place preference assay was conducted to confirm the activation of pleasant touch neural circuitry, as mice should feel pleasant in and prefer the light chamber against their natural behavior post-CNO injection. The place preference assay was also conducted to investigate any potential sex differences in the synaptic connection of the Vglut3 positive DRG neuron.

In females, there was no significant difference, $F(3, 18) = 1.228$, $p = .885$, in the preferences of the control (-213.00s±32.80, n=6) and VT3/Avil/Dq (-157.60s±73.76, n=7) mice

before the CNO was administered (Figure 4). Post-CNO injection resulted in a significant change, $F(3, 18) = 14.48$, $p = .001$, in preference for the bright chamber in control (-174.80s±51.88, n=6) and VT3/Avil/Dq (270.60s±65.78, n=7) female mice (Figure 5). This same pattern was observed among male control and VT3/Avil/Dq mice. There was no significant difference, $F(3, 18) = 1.228$, $p = .466$, in the preferences of the control (-301.60s±53.18, n=5) and VT3/Avil/Dq (-165.30s±40.78, n=4) male mice pre-CNO injection (Figure 4), but there was a significant difference, $F(3, 18) = 14.48$, $p = .001$, in place preference between control (-207.60s±66.14, n=5) and VT3/Avil/Dq (327.00s±123.00, n=4) male mice post-CNO injection (Figure 5). Both female and male VT3/Avil/Dq mice had a significant change in preference towards the bright chamber, indicating the activation of the pleasant touch circuitry through the activation of Vglut3+ DRG neurons.

The same place preference assay was done for the THcreER/Avil/Dq mouse-line subjects, in order to further investigate the significance of Vglut3+ DRG in the pleasant touch neural circuitry. We hypothesized that there is a subset of Vglut3+ DRG neurons that also expresses tyrosine hydroxylase (TH), as there is evidence that among VGLUT3-persistent neurons, a subset of TH+ C-LTMRs innervate hairy skin (Lou et al., 2013). The THcreER/Avil/Dq mouse-line should allow us to increase the specificity of our study by targeting a distinct population of Vglut3+ DRG.

In females, there was no significant difference, $F(3, 8) = 0.691$, $p = .811$, in the preferences of the control mice (-255.50s±62.94, n=4) and THcreER/Avil/Dq mice (-144.00s±44.00, n=2) before the CNO was administered (Figure 6). Post-CNO injection resulted in a significant change, $F(3, 8) = 22.07$, $p = .002$, in preference for the bright chamber between control (-161.50s±17.95, n=4) and THcreER/Avil/Dq (283.00s±111.00, n=2) female

mice (Figure 7). This same pattern was observed among male control and THcreER/Avil/Dq mice. Before the CNO injection, there was no significant difference, $F(3, 8) = 0.691$, $p = .682$, between control (-289.30s±133.60, n=3) and THcreER/Avil/Dq (-155.30s±40.04, n=3) male mice (Figure 6). Post-CNO injection, there was a significant difference, $F(3, 8) = 22.07$, $p = .003$, between the place preference of the control (-129.30s±33.65, n=3) and THcreER/Avil/Dq (264.00s±66.97, n=3) male mice (Figure 7). Both female and male THcreER/Avil/Dq mice had a significant change in preference towards the bright chamber, indicating the activation of the pleasant touch circuitry through the activation of TH+ neurons.

No Sexual Dimorphisms Revealed by Activation of VT3+ or TH+ DRG

ANOVA tests were also done to check for sexual dimorphisms in the preferences of male and female VT3/Avil/Dq mice. Between the control male (-301.60s±53.18, n=5) and female (-213.00s±32.80, n=6) mice, there was no significant difference, $F(3, 18) = 1.228$, $p = .712$, in the results, pre-CNO injection. There was also no significant difference, $F(3, 18) = 14.48$, $p = .989$, between control males (-207.60s±66.14, n=5) and control females (-174.80s±51.88, n=6) post-CNO injection (Figure 4 and 5). When comparing the VT3/Avil/Dq male (-165.30s±40.78, n=4) and female (-157.60s±73.76, n=7) mice, there was also no significant difference, $F(3, 18) = 1.228$, $p = .999$, observed pre-CNO injection. Post-CNO injection, there was also no significant difference, $F(3, 18) = 14.48$, $p = .952$, in the place preferences between VT3/Avil/Dq males (327.00s±123.00, n=4) and VT3/Avil/Dq females (270.60s±65.78, n=7) (Figure 4 and 5). Based on this data, it is reasonable to conclude that there is no sexual dimorphism present in Vglut3 positive DRG neuron's portion of the pleasant touch neural circuitry.

In regards to the THcreER/Avil/Dq mouse line, there was no significant difference, $F(3, 8) = 0.691$, $p = .989$, in the pre-CNO injection place preferences between the control males

(-289.30s±133.60, n=3) and control females (-255.50s±62.94, n=4) (Figure 6). There was also no significant difference, $F(3, 8) = 22.07$, $p = .962$, between the control males (-129.30s±33.65, n=3) and control females (-161.50s±17.95, n=4) post-CNO injection (Figure 7). When comparing the THcreER/Avil/Dq male (-155.30s±40.04, n=3) and female (-144.00s±44.00, n=2) mice, there was also no significant difference, $F(3, 8) = 0.691$, $p = .999$, observed pre-CNO injection (Figure 6). Post-CNO injection, there was also no significant difference, $F(3, 8) = 22.07$, $p = .995$, between male (264.00s±66.97, n=3) and female (283.00s±111.00, n=2) THcreER/Avil/Dq mice (Figure 7). Based on this data, it is reasonable to conclude that there is no sexual dimorphism present in Vglut3+ or TH+ DRG neuron's portion of the pleasant touch neural circuitry.

Supplementary Tests Ensure No Subject Abnormalities

Supplemental tests were also performed to check for potential subject exclusions. The Von Frey Test and light poking behind the ears was conducted to assess neuropathic pain and mechanical itch sensitivity, respectively. The Hargreaves Hyperalgesia Test was performed to assess thermal nociception as well as the RotaRod performance test to assess locomotion. Subjects also underwent light touch (brush, 3 trials) to assess dynamic pain and pin prick tests to assess threshold sensitivity, as well as a gradient temperature test.

Among male mice, there was no significant difference ($p = .471$) in terms of the Hargreaves test between the control (13.90±1.208, n=5) and VT3/Avil/Dq (15.53±1.690, n=6) mice. In terms of mechanical itching responses, there was no significant difference ($p = .199$) between the control (56.00±7.483, n=5) and VT3/Avil/Dq (70.00±5.774, n=4) mice. In terms of the Von Frey test, there were no significant differences ($p = .330$) between the control (0.356±0.039, n=5) and VT3/Avil/Dq (0.306±0.021, n=4) mice. The RotaRod test also showed

no significant difference (p = .315) between the control (131.00±11.11, n=5) and VT3/Avil/Dq (147.80±10.30, n=4) mice. There was also no significant difference in light touch responses (p = .519) between the control (2.333±0.333, n=3) and VT3/Avil/Dq (2.667±0.333, n=3) mice. Lastly, our data displayed no significant difference in pinprick responses (p = .374) between the control (90.00±5.774, n=3) and VT3/Avil/Dq (96.67±3.333, n=3) mice. (Figure 8) This indicates that among the male mice, the treatment group had no changes in transient or chronic sensitivity and, like the control group, there was normal functioning of the nervous system.

Among female mice, there was no significant difference (p = .100) in terms of the Hargreaves test between the control (15.53±1.690, n=6) and VT3/Avil/Dq (18.77±0.850, n=7) mice. In terms of mechanical itching responses, there was no significant difference (p = .813) between the control (56.67±6.146, n=6) and VT3/Avil/Dq (60.00±11.55, n=7) mice. In terms of the Von Frey test, there were no significant differences (p = .087) between the control (0.360±0.036, n=6) and VT3/Avil/Dq (0.466±0.042, n=7) mice. The RotaRod test also showed no significant difference (p = .280) between the control (93.79±8.963, n=6) and VT3/Avil/Dq (104.50±4.268, n=7) mice. There was also no significant difference in light touch responses (p = .423) between the control (3.000±0.000, n=2) and VT3/Avil/Dq (2.000±1.000, n=2) mice. Lastly, our data displayed no significant difference in pinprick responses (p > .999) between the control (95.00±5.000, n=2) and VT3/Avil/Dq (95.00±5.000, n=2) mice. (Figure 9) This indicates that among the female mice, the treatment group had no changes in transient or chronic sensitivity and also had normal functioning of the nervous system.

In regards to the gradient temperature assay, this test was used to assess any changes in thermal sensitivity due to the CNO injection. We used VT3/Avil/Dq mice subjects before and after CNO injection, in order to activate VT3+ DRG. Mice generally favor temperatures around

30-32ºC. However, after Vglut3+ neurons were activated in VT3/Avil/Dq mice by CNO, there was a shift in temperature preference towards slightly higher temperatures. This shift was seen in male VT3/Avil/Dq mice post-CNO injection (Figure 10) as well as female VT3/Avil/Dq mice post-CNO injection (Figure 11).

The THcreER/Avil/Dq mouse line was also subject to the same supplementary tests to check for potential subject exclusions. Among male mice, there were no significant differences (p = .571) between the control (11.01±0.683, n=4) and THcreER/Avil/Dq (12.84±2.971, n=4) mice in terms of the Hargreaves test. There was no significant difference (p = .955) between the control (77.50±10.31, n=4) and THcreER/Avil/Dq (78.36±10.41, n=4) mice regarding mechanical itching responses. The results of the Von Frey test also showed no significant difference (p = .353) between the control (0.305±0.069, n=4) and THcreER/Avil/Dq (0.413±0.081, n=4) mice. There was also no significant difference in the RotaRod test (p = .835) between the control (135.60±19.20, n=4) and THcreER/Avil/Dq (140.50±12.52, n=4) mice. Furthermore, there was no significant difference in light touch responses (p = .670) between the control (2.500±0.500, n=4) and THcreER/Avil/Dq (2.750±0.250, n=4) mice. (Figure 12) Interestingly, there was a significant difference in the results of the pinprick test (p = .049) between the control (100.00±0.000, n=4) and THcreER/Avil/Dq (90.00±4.082, n=4) mice, however, this could have been due to human error or a limited number of mice subjects (Figure 12). Overall, among the male mice, the THcreER/Avil/Dq treatment group had no changes in transient or chronic sensitivity and, like the control group, there was normal functioning of the nervous system.

Among female mice, there were no significant differences (p = .332) between the control (11.06±1.080, n=6) and THcreER/Avil/Dq (13.43±2.353, n=4) mice in terms of the Hargreaves

test. There was no significant difference (p = .648) between the control (68.31±7.082, n=6) and THcreER/Avil/Dq (63.00±8.699, n=4) mice regarding mechanical itching responses. The results of the Von Frey test also showed no significant difference (p = .826) between the control (0.395±0.057, n=6) and THcreER/Avil/Dq (0.413±0.038, n=4) mice. There was also no significant difference in the RotaRod test (p = .961) between the control (123.10±13.78, n=6) and THcreER/Avil/Dq (124.20±14.65, n=4) mice. Furthermore, there was no significant difference in light touch responses (p > .999) between the control (2.500±0.224, n=6) and THcreER/Avil/Dq (2.500±0.289, n=4) mice. Lastly, our data displayed no significant difference in pinprick responses (p > .999) between the control (88.33±3.073, n=6) and THcreER/Avil/Dq (95.00±5.000, n=4) mice. (Figure 13) This indicates that among the female mice, the treatment group had no changes in transient or chronic sensitivity and also had normal functioning of the nervous system.

In regards to the gradient temperature assay, we used THcreER/Avil/Dq mice subjects in order to activate TH+ DRG through CNO injections. After TH+ neurons were activated in THcreER/Avil/Dq mice by CNO, a shift in temperature preference towards slightly higher temperatures was observed. This shift was seen in male THcreER/Avil/Dq mice post-CNO injection (Figure 14) as well as female THcreER/Avil/Dq mice post-CNO injection (Figure 15).

Discussion

In both male and female VT3/Avil/Dq mice, there was a significant preference for the light chamber after the activation of VT3+ DRG. This indicates that VT3+ DRG is significant in the neural circuitry underlying pleasant touch. Activation of these neurons appears to have led to the activation of a pleasant sensation, as the mice changed their preference for the dark chamber to the light chamber, going against their natural preference.

Additionally, these same place preference results were consistent with that of the THcreER/Avil/Dq mouse-line. A significant change in preference for the dark chamber to the light chamber was observed post-CNO injection in male and female THcreER/Avil/Dq mice, suggesting that it is specifically VT3+ DRG that also express tyrosine hydroxylase which are involved in the activation of the pleasant touch neural circuitry. Furthermore, this indicates a sufficiency of this subpopulation of VT3+/TH+ C-LTMRs in the DRG in activating pleasant touch in both males and females.

On the other hand, no significant differences in chamber preference between male and female mice before and after the CNO-injection was observed overall in the control, VT3/Avil/Dq, or THcreER/Avil/Dq mouse line. Based on these findings, we cannot conclude that there are any sexual dimorphisms present in the pleasant touch neural circuitry. Although our data does not indicate any sexual dimorphisms, more research is needed to investigate the complex mechanisms underlying these neural processes. Despite no significant differences between males and females in behavioral responses to the activation of VT3+/TH+ DRG neurons, underlying mechanisms may be more complicated.

Interestingly, regarding the tests supplementing the place preference assays, we did observe a change in the thermal preferences of VT3/Avil/Dq male and female mice post-CNO injection. As commonly known, mice generally favor temperatures around 28-32 degrees celsius, however, we observed an increased preference for warmer temperatures in VT3/Avil/Dq mice after CNO injection, which stimulates the VT3+ DRG. This change in thermal preference was also observed in THcreER/Avil/Dq male and female mice, post-CNO injection. These results could be explained through social behavior.

One form of pleasant touch is social touch, which includes thermoregulatory processes like cuddling and hugging. Previous studies have revealed that social touch relies on tactile thermosensory pathways and suggests that social touch is neurally related to temperature through cutaneous C afferents. More specifically, according to the "social touch hypothesis," CT afferents respond to a range of tactile stimuli mostly occurring during social interaction and can impact behavior (Morrison, 2016). It is possible that the activation of the pleasant touch circuitry via CNO injection induced C or CT afferents through the pleasant touch circuitry and influenced our mice subjects to prefer higher temperatures, as pleasure and warmth can stem from social touch.

Due to the current Covid-19 pandemic, there was a shortage of mice subjects during the course of this experiment, however, this experiment is still ongoing. A larger population of subjects is needed to further ensure the accuracy of our results. Furthermore, our data does not investigate specific mechanisms, including on a molecular level, underlying the generation of pleasant touch sensations. Previously, a new class of proteins, piezo proteins, were identified as ion channels which are believed to mediate mechanosensory transduction in mammalian cells (Bagriantsev, Gracheva, & Gallagher, 2014). In situ hybridizations also revealed the presence of piezo 2 mRNA in 20% of adult mice DRG, which are classified as both mechanoreceptive and nociceptive (Delmas, Hao, & Rodat-Despoix, 2011). The potential and definitive role of specific mechanoreceptors in the pleasant touch circuitry still need to be investigated. Pleasant touch is a complex somatosensory modality and much of this complicated process has yet to be mapped and is potentially sexually dimorphic.

Our study only begins the investigation of the synaptic connections involved in pleasant touch circuitry. Much of this circuitry has yet to be discovered and may overlap or be

antagonistically related with circuitry underlying other modalities such as itch and pain. In addition to pleasant touch, C-fibers respond to general mechanical and temperature stimuli and are commonly associated with nociceptive pain sensations. According to a recent study done by Vrontou, Wong, Rau, Koerber, and Anderson (2013), there are two classes of C-fibers that respond to mechanical stimuli in vivo and are categorized by their expression of MrgprB4 and MrgprD. Unsurprisingly, MrgprD+ C-fiber neurons were activated by pinching. However, MrgprB4+ C-fibers were not activated by pinching but by the massage-like stroking of hairy skin. It is unclear why MrgprB4+ C-fibers are not also activated by pinching however, based on this information, C-fibers may be the connection between pleasure and pain. There is still so much we do not know, but our current study has provided a means to a deeper understanding of pleasant touch.

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Figures

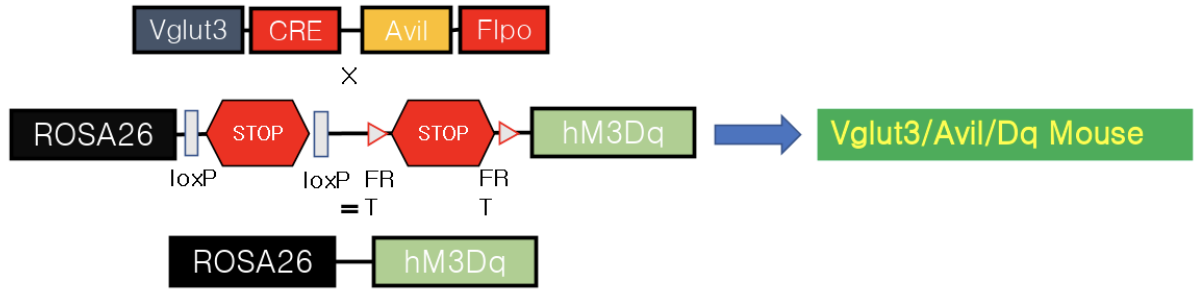

Figure 1. Chemogenetic activating of Vglut3+ sensory neurons

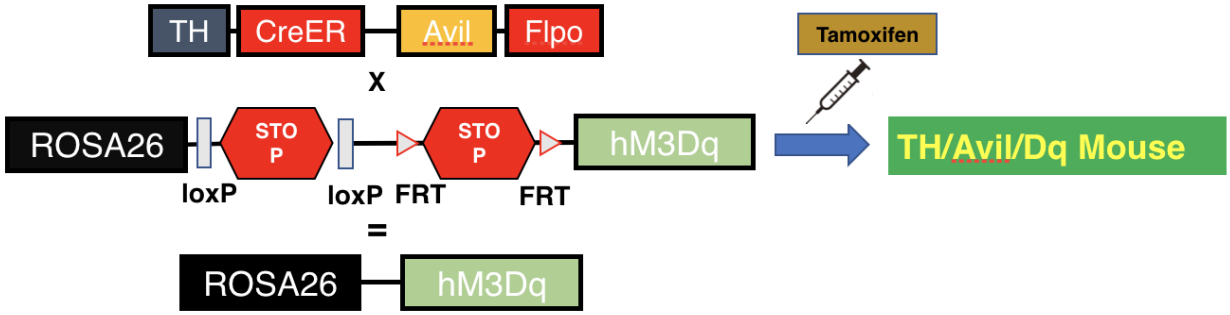

Figure 2. Chemogenetic activating of TH+ sensory neurons

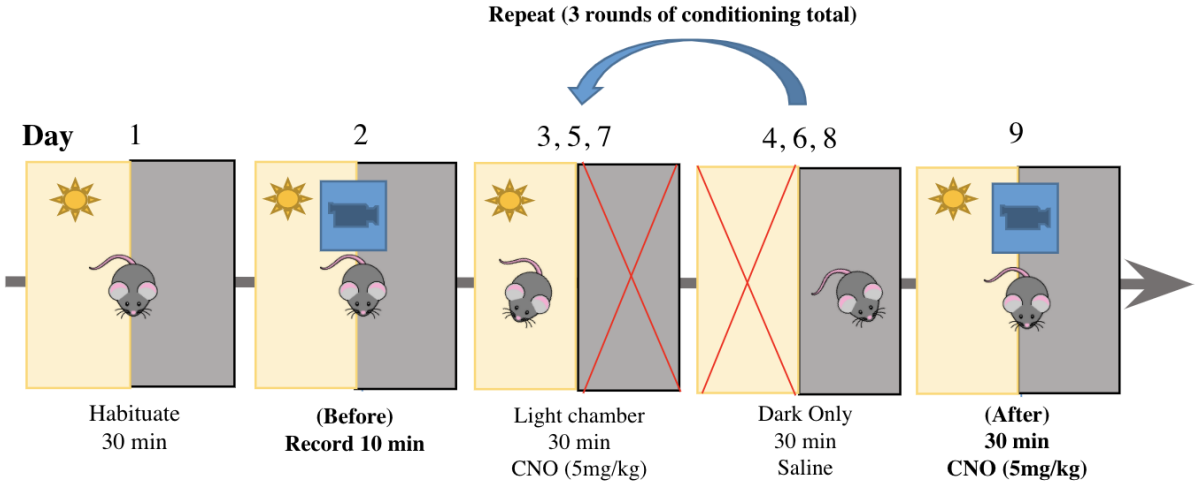

Figure 3. Place Preference Behavioral Assay

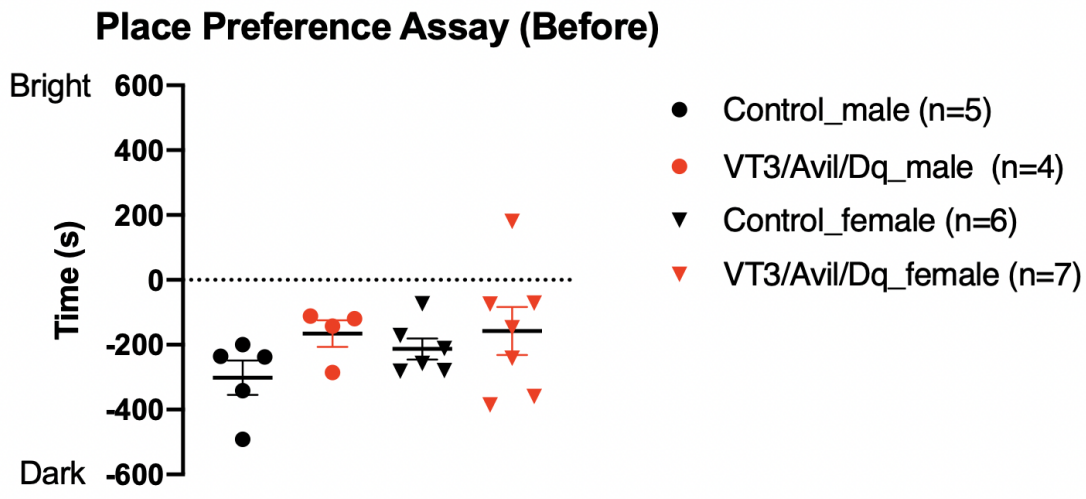

Figure 4. Control vs VT3/Avil/Dq Place Preference Assay (Before results)

Note. One-way ANOVA, error bar shows mean ± standard error of the mean (s.e.m.).

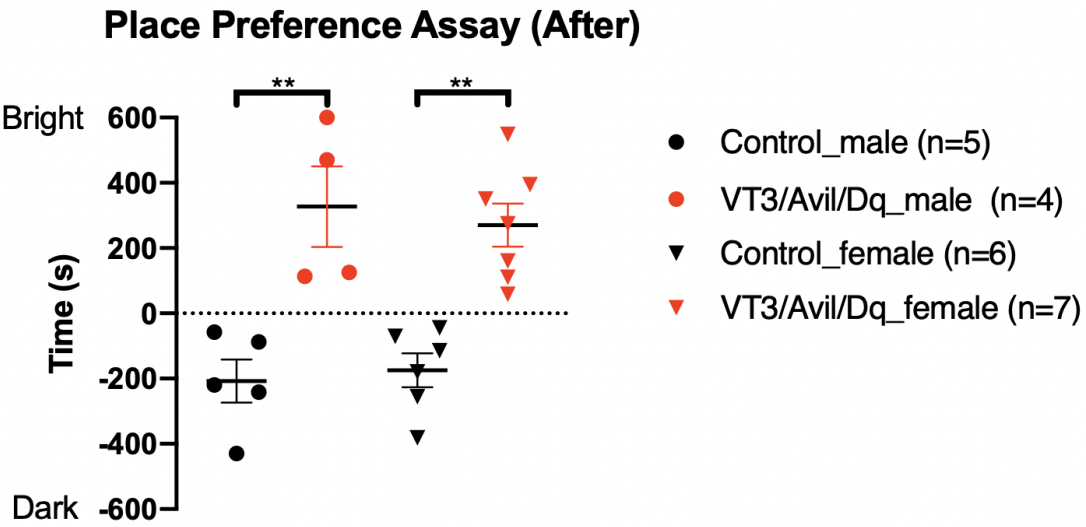

Figure 5. Control vs VT3/Avil/Dq Place Preference Assay (After results)

Note. One-way ANOVA, ** $p<0.01$, error bar shows mean ± standard error of the mean (s.e.m.).

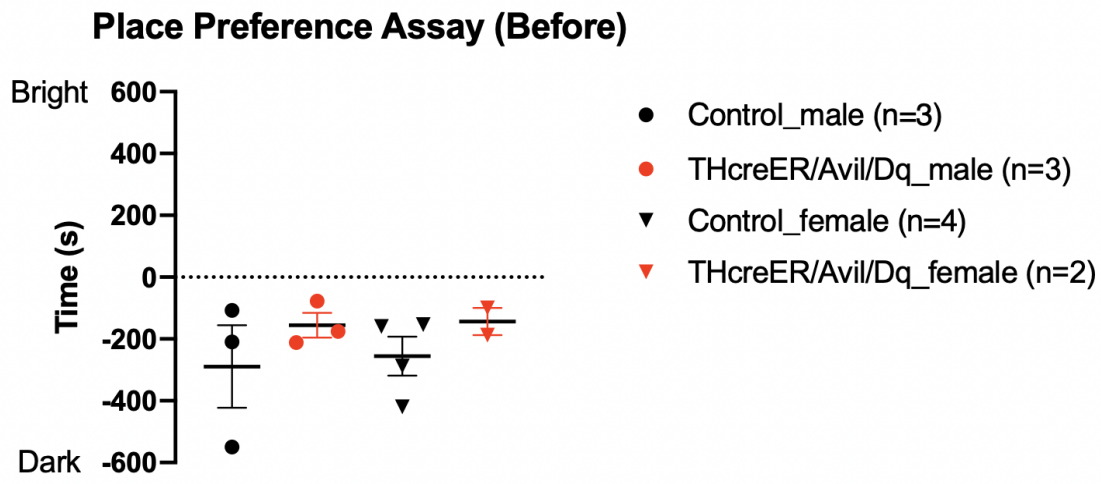

Figure 6. Control vs THcreER/Avil/Dq Place Preference Assay (Before results)

Note. One-way ANOVA, error bar shows mean ± standard error of the mean (s.e.m.).

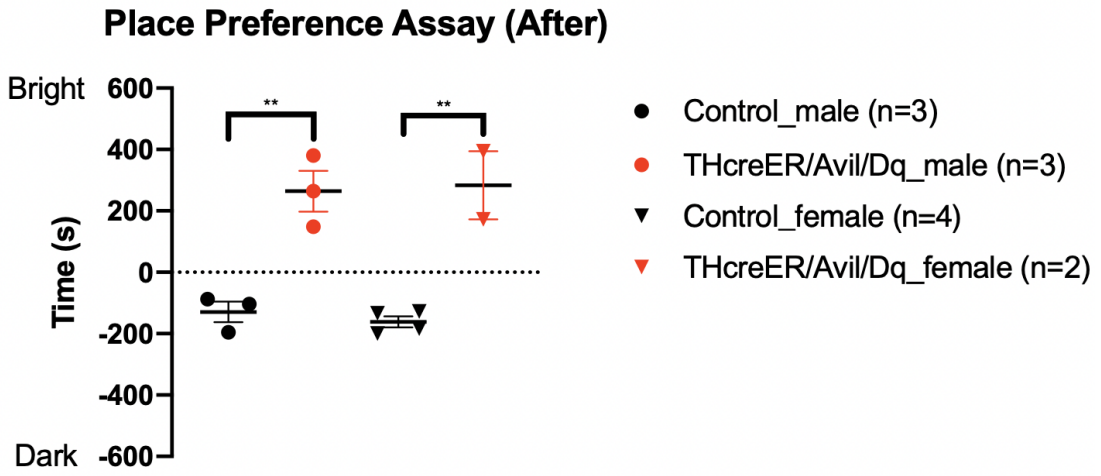

Figure 7. Control vs THcreER/Avil/Dq Place Preference Assay (After results)

Note. One-way ANOVA, ** $p<0.01$, error bar shows mean ± standard error of the mean (s.e.m.).

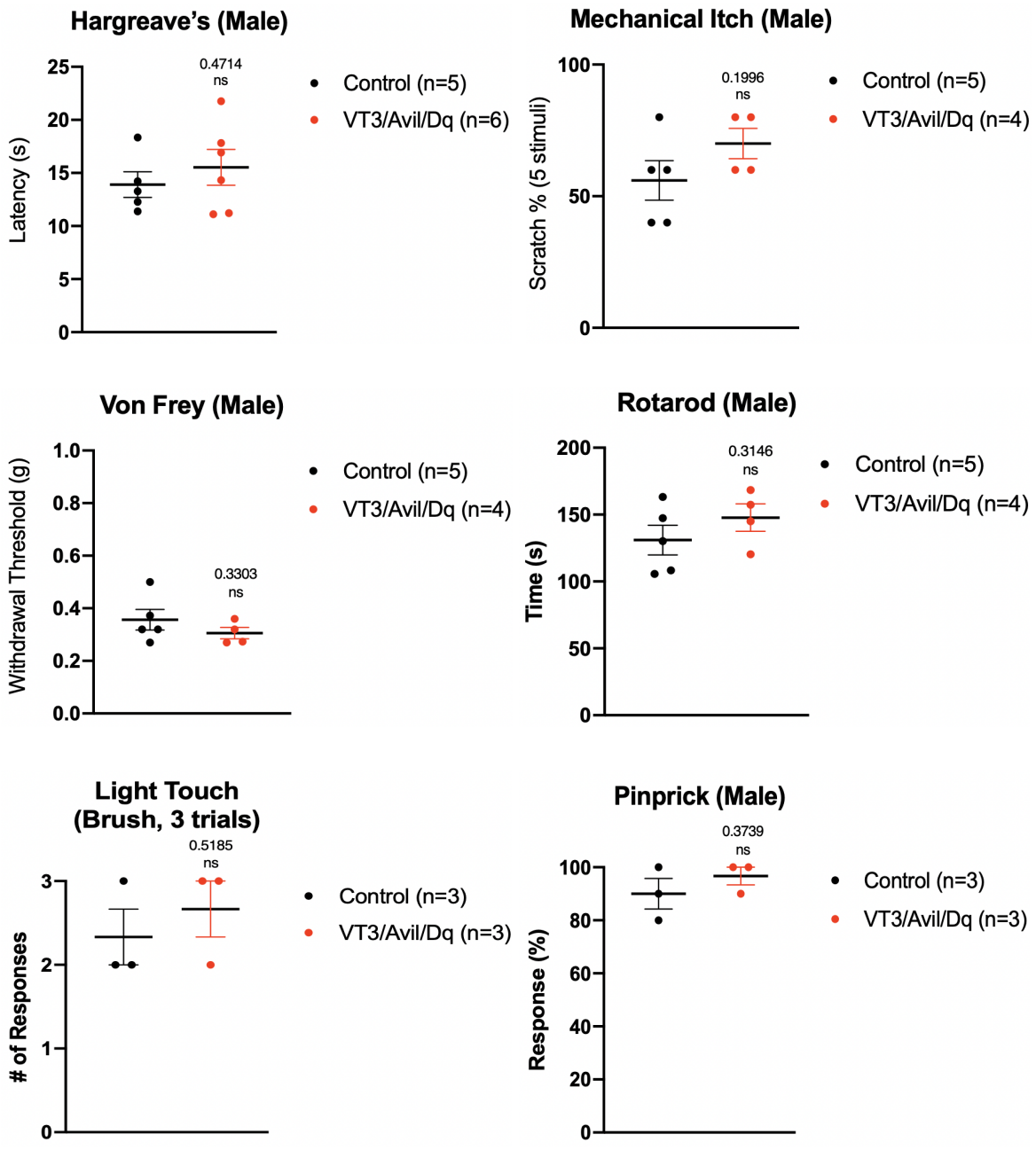

Figure 8. Control vs VT3/Avil/Dq Male Behavioral Assays

Note. Unpaired, two-tailed Student's t-test, error bar shows mean ± standard error of the mean (s.e.m.). The corresponding P-values are listed above the treatment group data points on the graph ($p > .05$).

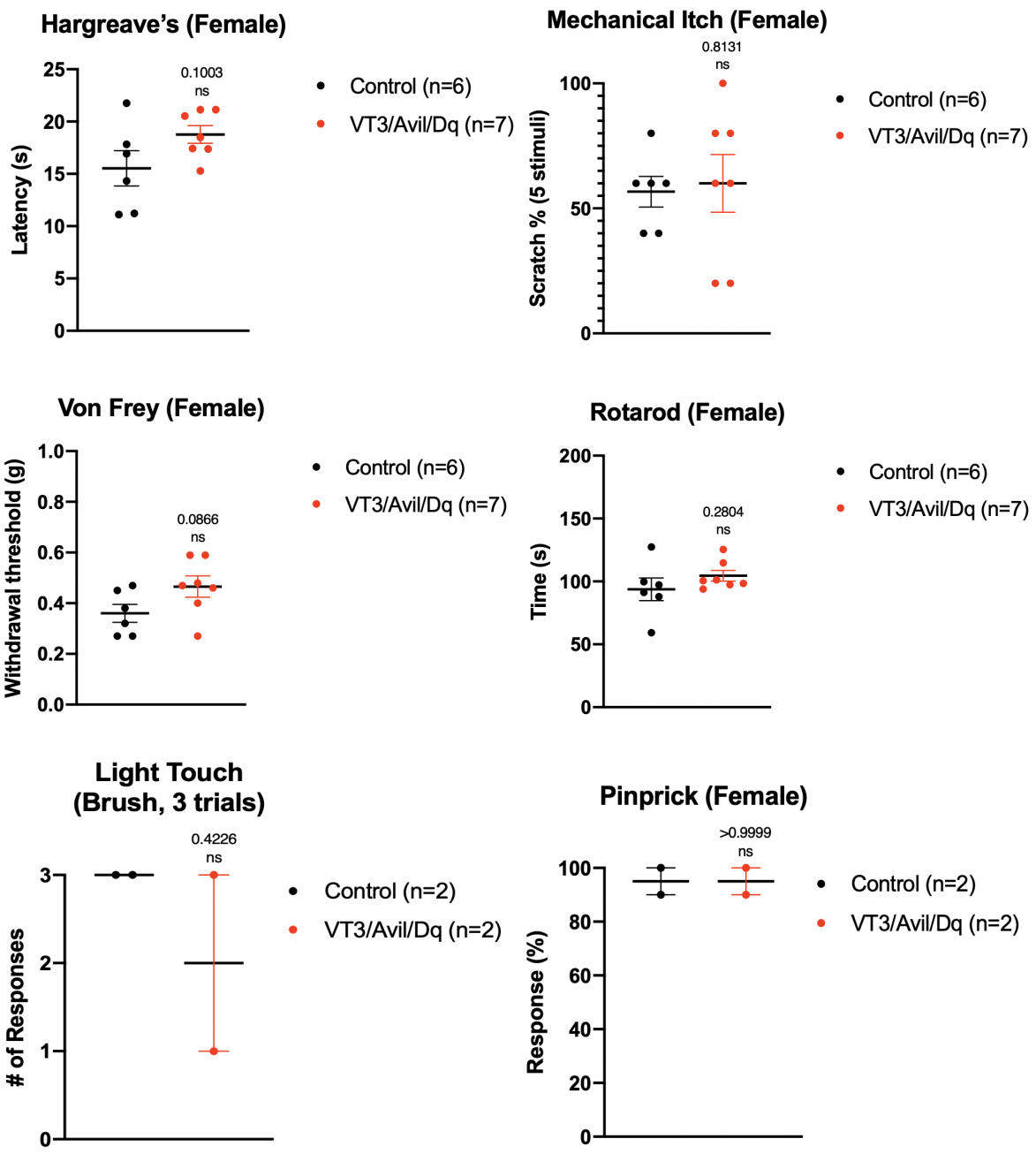

Figure 9. Control vs VT3/Avil/Dq Female Behavioral Assays

Note. Unpaired, two-tailed Student's t-test, error bar shows mean ± standard error of the mean (s.e.m.). The corresponding P-values are listed above the treatment group data points on the graph ($p > .05$).

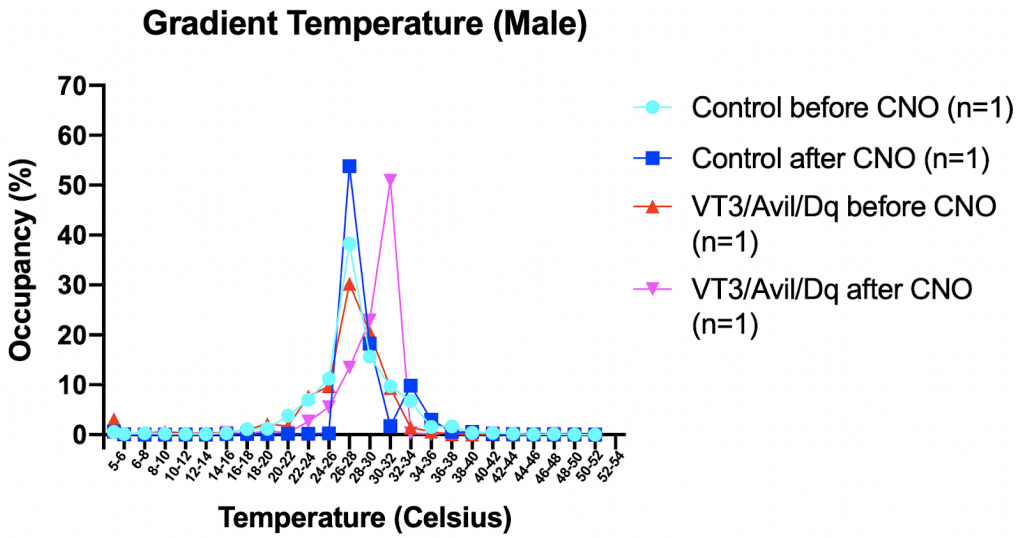

Figure 10. Control vs VT3/Avil/Dq Males Gradient Temperature Assay

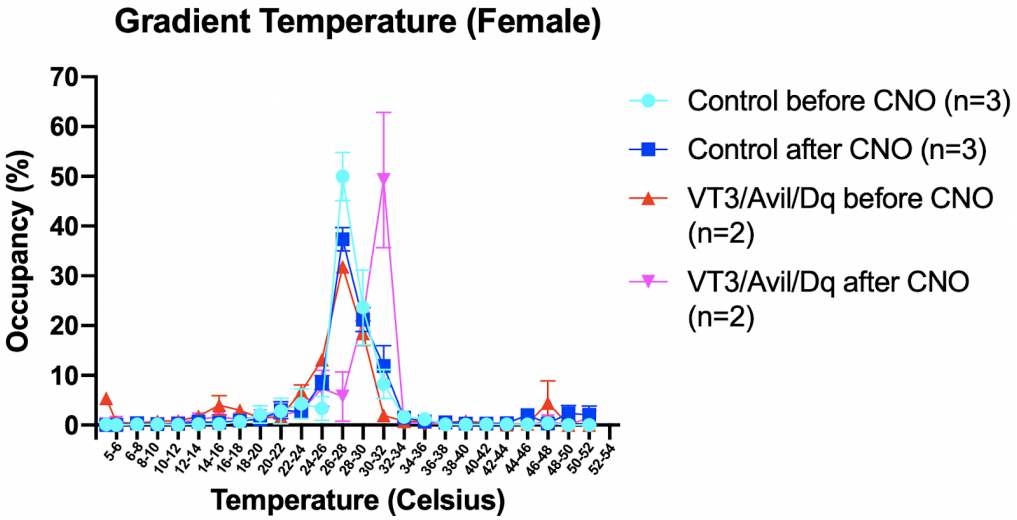

Figure 11. Control vs VT3/Avil/Dq Females Gradient Temperature Assay

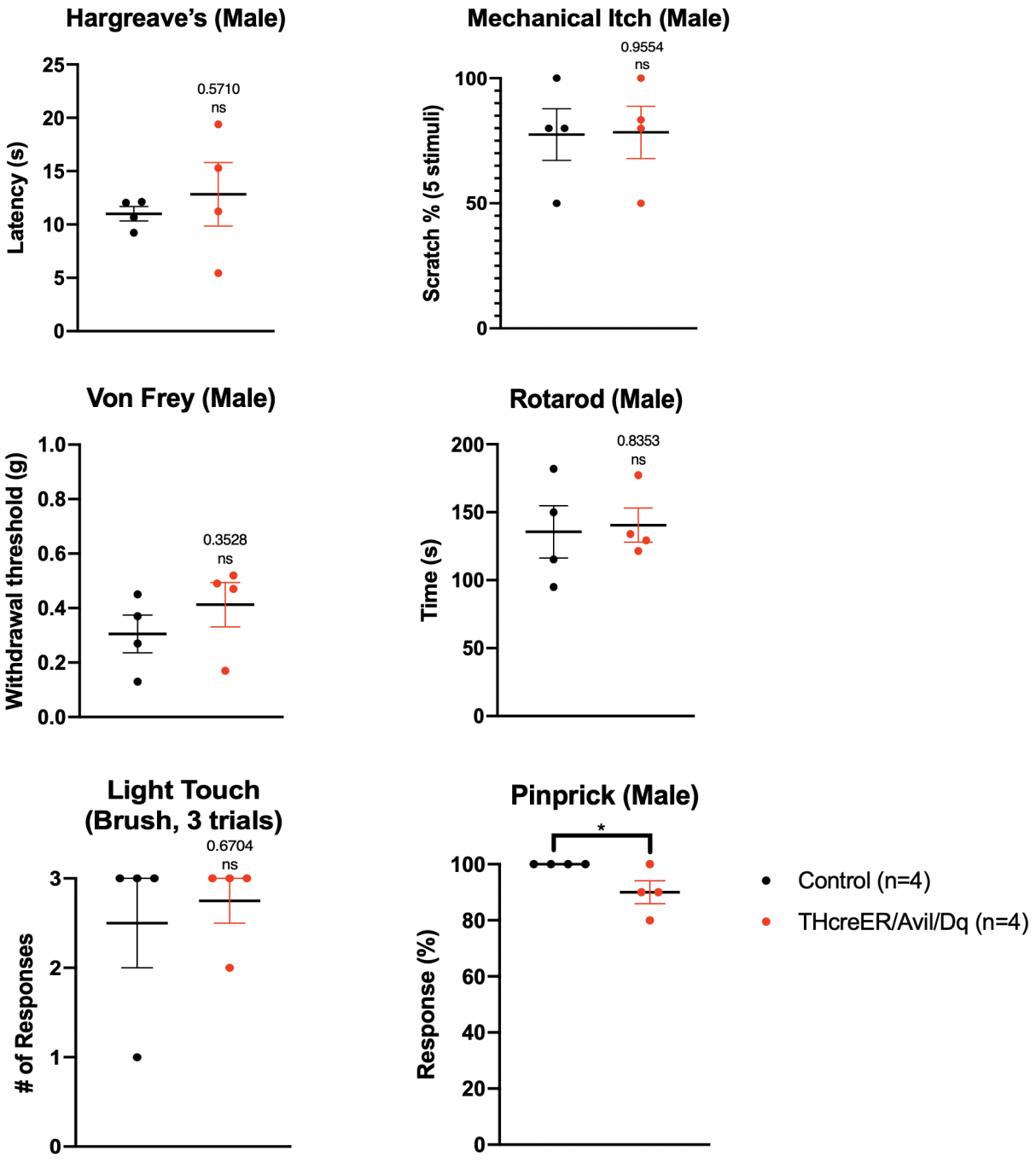

Figure 12. Control vs THcreER/Avil/Dq Male Behavioral Assays

Note. Unpaired, two-tailed Student's t-test, error bar shows mean ± standard error of the mean (s.e.m.), * $p < .05$. The corresponding P-values are listed above the treatment group data points on the graph ($p > .05$).

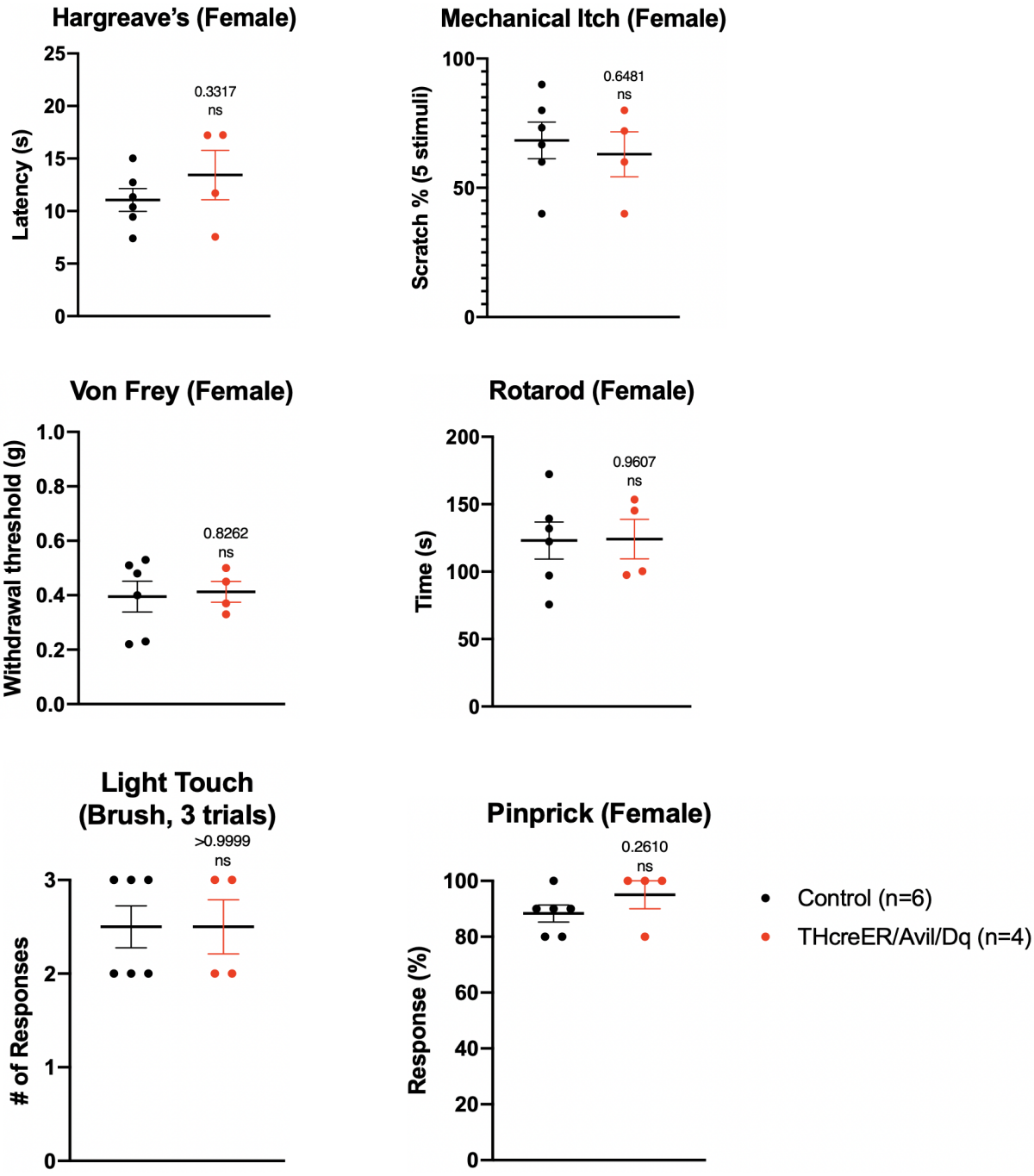

Figure 13. Control vs THcreER/Avil/Dq Female Behavioral Assays

Note. Unpaired, two-tailed Student's t-test, error bar shows mean ± standard error of the mean (s.e.m.). The corresponding P-values are listed above the treatment group data points on the graph ($p > .05$).

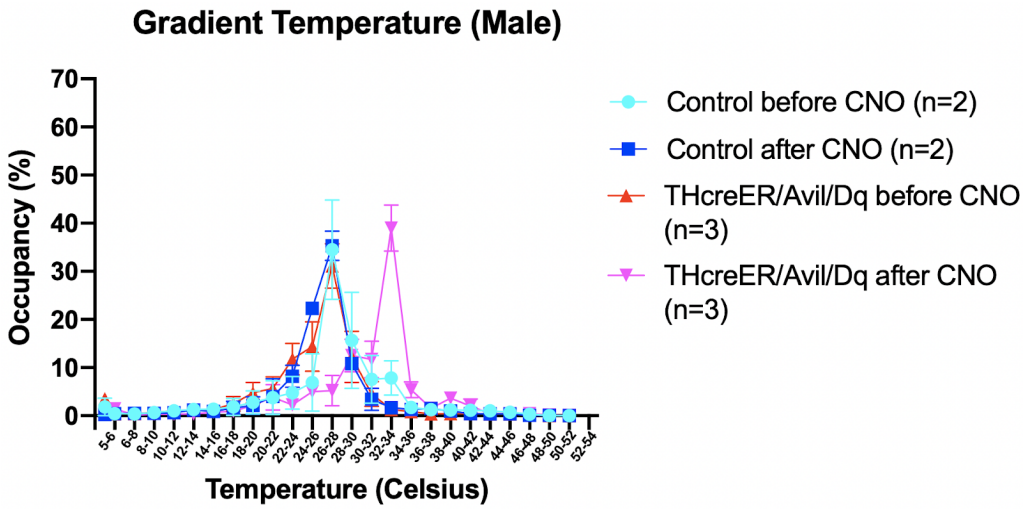

Figure 14. Control vs THcreER/Avil/Dq Males Gradient Temperature Assay

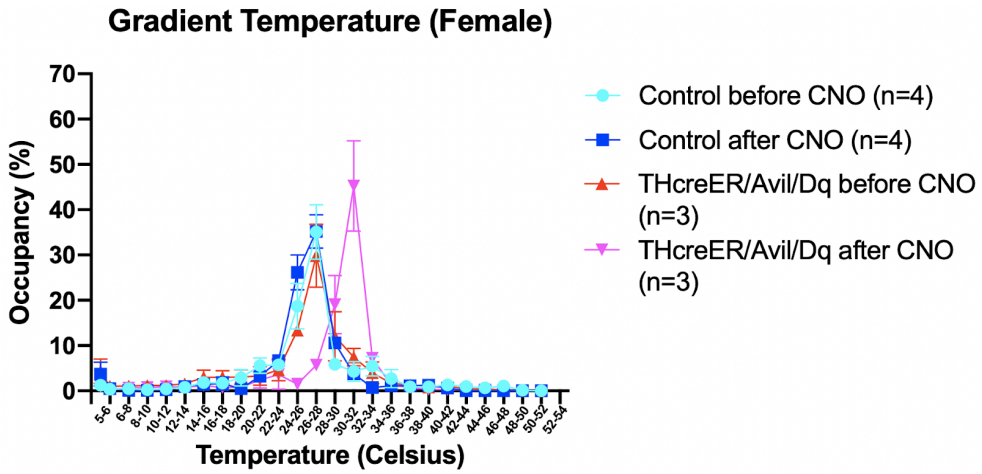

Figure 15. Control vs THcreER/Avil/Dq Females Gradient Temperature Assay