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Effects of gonadectomy and dihydrotestosterone on neuronal plasticity in motivation and reward related brain regions in the male rat

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Abstract

Gonadal hormones affect neuronal morphology to ultimately regulate behaviour. In female rats, oestradiol mediates spine plasticity in hypothalamic and limbic brain structures, contributing to long-lasting effects on motivated behaviour. Parallel effects of androgens in male rats have not been extensively studied. Here, we investigated the effect of both castration and androgen replacement on spine plasticity in the nucleus accumbens shell and core (NAcSh and NAcC), caudate putamen (CPu), medial amygdala (MeA) and medial preoptic nucleus (MPN). Intact and castrated (gonadectomy [GDX]) male rats were treated with dihydrotestosterone (DHT, 1.5 mg) or vehicle (oil) in three experimental groups: intact-oil, GDX-oil and GDX-DHT. Spine density and morphology, measured 24 hours after injection, were determined through three-dimensional reconstruction of confocal z-stacks of DiI-labelled dendritic segments. We found that GDX decreased spine density in the MPN, which was rescued by DHT treatment. DHT also increased spine density in the MeA in GDX animals compared to intact oil-treated animals. By contrast, DHT decreased spine density in the NAcSh compared to GDX males. No effect on spine density was observed in the NAcC or CPu. Spine length and spine head diameter were unaffected by GDX and DHT in the investigated brain regions. In addition, immunohistochemistry revealed that DHT treatment of GDX animals rapidly increased the number of cell bodies in the NAcSh positive for phosphorylated cAMP response-element binding protein, a downstream messenger of the androgen receptor. These findings indicate that androgen signalling plays a role in the regulation of spine plasticity within neurocircuits involved in motivated behaviours.

KEYWORDS

androgen, gonadectomy, pCREB, plasticity, spine density

1 | INTRODUCTION

Gonadal hormones are key regulators of rewarding behaviour.[1,2] Oestrogen, progesterone and androgen signalling in the brain is involved in the display of motivated behaviours such as copulation, aggression and physical activity.[3] Moreover, gonadal hormones have been shown to influence the susceptibility to addiction-like behaviour.[4] To understand how hormones affect behaviour, it is important to investigate the underlying neurobiological mechanisms.

One mechanism through which gonadal hormones exert their influence on motivated behaviours is by affecting the structural plasticity of neurones. Previous research has shown that spine density, spine morphology and dendrite length can be impacted by gonadal hormones in multiple brain regions involved in motivation.[5-8] These hormone-induced structural reorganisations are both sexually dimorphic and strikingly different between brain regions, and have been linked to motivated behaviour, learning, memory and addiction.[4,9-11]

Copulation is a naturally occurring motivated behaviour reliant on gonadal hormones. Earlier research has shown that structural neuronal plasticity could be at the basis of hormonal effects on sexual behaviour.[12] For example, within the hypothalamus, oestradiol appears to enhance neuronal connectivity, essential for lordosis.[13-16] Oestrogens impact additional structures in the female limbic system. For example, spine density in the hippocampus fluctuates during the oestrous cycle and oestradiol increases spine density in ovariectomised animals.[17,18] By contrast, oestradiol administration to ovariectomised hamsters or rats produces a decrease in spine density within the nucleus accumbens core (NAcC).[8,19]

Castration gradually ceases all sexual behaviour in male rats and hormonal replacement fully restores copulation.[20] Yet, in males, it remains grossly unknown what neurobiological mechanisms underlie the loss of sexual behaviour following loss of gonadal hormones, and whether hormone effects on structural plasticity could be involved. Although some studies have shown spine plasticity in response to testosterone in males, it remains unclear to what extent this is mediated by oestrogen formed through aromatisation of testosterone.[21-23] It is, however, evident that oestrogens do not simply have the same effects on spine plasticity in males as in females. For example, as mentioned, the hippocampal CA1 region exhibits increased spine density upon oestrogen treatment in females, but is unresponsive to oestrogens in males.[24,25] Instead, CA1 spine density in males is induced by dihydrotestosterone (DHT), a high-affinity ligand of the androgen receptor that is not aromatised into oestradiol.[24] Our laboratory also recently reported similar effects in the nucleus accumbens, where spine plasticity is affected by oestrogens in females and by DHT in males, again indicating that these effects in males are caused by androgens rather than oestrogens.[8,26]

Although the effects of gonadal hormones on spine plasticity are sexually dimorphic, there are indications that the underlying mechanisms through which these effects arise are homologous. Specifically, hormone-induced spine plasticity in the nucleus accumbens is mediated by activation of metabotropic glutamate receptor (mGluR) signalling, via oestradiol in females and DHT in males.[8,26] In females, the oestrogen-induced spine plasticity is reliant on membrane-bound oestrogen receptors that are coupled to mGluRs, which are activated upon oestrogen binding to the oestrogen receptor. The activation of mGluRs can induce a downstream phosphorylation pathway leading to increased phosphorylation of cAMP response-element binding protein (CREB).[27,28] CREB is a transcription factor involved in numerous behavioural outputs and implicated in spine plasticity.[29,30] Because androgen-induced spine plasticity in the nucleus accumbens in males is also mediated by mGluRs, it could be expected that androgen signalling in males has similar effects on CREB phosphorylation as oestradiol in females, perhaps mediated by membrane-associated androgen receptors.[31-33]

In the present study, we investigated the effects of gonadectomy (GDX) and androgen replacement on neuronal plasticity in putatively important brain regions for sexual motivation in male rats. We hypothesised that GDX could lead to alteration of structural plasticity in the medial preoptic nucleus (MPN), medial amygdala (MeA), NAcC and nucleus accumbens shell (NAcSh), possibly indicating a mechanism for GDX-induced loss of sexual behaviour. In addition, we investigated how androgen signalling in GDX males impacts structural plasticity. Finally, we built on the hypothesis that the observed effects could be membrane-bound androgen receptor mediated by looking at rapid induction of phosphorylated CREB (pCREB) in the striatum following DHT treatment in GDX males.

2 | MATERIALS AND METHODS

2.1 | Animals

Intact and castrated Sprague-Dawley rats (200-225 g, 8 weeks old) were purchased from Envigo Laboratories (Indianapolis, IN, USA). Castration took place 48-72 hours before the animals arrived in our facility. Animals were housed two per cage (DiI labelling) or three per cage (pCREB immunohistochemistry) and maintained under a 12:12 hour light/dark photocycle with food and water ad lib. Animals were allowed to habituate to the research facility for at least 1 week prior to the start of any experiment. All animal procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the University of Minnesota.

2.2 | Treatment, tissue processing and DiI labelling

5α-androstan-17β-ol-3-one (DHT; Steraloids Inc; Newport, RI, USA) was dissolved in cottonseed oil. Ten to 30 days after arrival, the rats were injected s.c. with 1.5 mg DHT or vehicle in a volume of 0.2 mL. The used DHT dose in the present study is based on a recent study from our laboratory showing significant effect on spine density in the NAcSh of castrated males.[26] The experiment was run in batches of two animals (cage mates) at a time. The two animals in each batch were in the same group, and treatment groups were randomised

according to a latin square design, so that average castration duration did not differ between castrated groups. Animals were killed 24 hours after hormone or vehicle treatment.

The tissue was prepared and DiOlistically labelled as described previously.[34] DiOlistic labelling involves the ballistic delivery of tungsten microparticles coated with a lipophilic fluorescent dye (here: DiI) to tissue sections. DiI labels membranes of all neurones in which a tungsten bead is embedded, providing a Golgi-like labelling of neurones, with higher throughput and without bias. Briefly, animals were killed by an Euthasol overdose (0.35 mL i.p; 390 mg mL^{-1} pentobarbital sodium, 50 mg mL^{-1} phenytoin sodium; Virbac AH Inc., Nice, France), injected with 0.25 mL heparin into the left ventricle, and transcardially perfused with 50 mL of 25 mmol L^{-1} phosphate-buffered saline (PBS, pH 7.2) followed by 500 mL of 1.5% paraformaldehyde in PBS. Brains were removed and post-fixed in 1.5% paraformaldehyde for 1 hour. Then, brains were sliced coronally into 300-μm thick sections using a VT1000 S Vibratome (Leica, Buffalo Grove, IL, USA). Sections containing the brain regions of interest (ie the caudate putamen [CPu], NAcC and NAcSh, MPN and MeA) were collected and stored in PBS until ballistic labelling.

DiI bullets were prepared from Tefzel tubing (Bio-Rad, Hercules, CA, USA) pretreated with 15 mg mL^{-1} polyvinylpyrrolidone (PVP) in deionised water. Two milligrams of DiI (Molecular Probes, Carlsbad, CA, USA) was dissolved in 100 μL of dichloromethane and applied to 90 mg of 1.3 μm tungsten microcarrier particles (Bio-Rad) spread out evenly on a glass slide. The coated tungsten particles were suspended in 10 mL PVP solution, and disaggregated by sonication and intermittent vortexing for 12 minutes. The pretreated Tefzel tubing was subsequently coated with the DiI-tungsten particles by allowing the suspension to settle in the tubing for 3 minutes, after which the suspension was quickly expelled. The tubing was dried under 0.4 LPM nitrogen gas flow using a tubing prep station (Bio-Rad) for 30 minutes, after which the tubing was cut into 1.3 cm long 'bullets'. Bullets were loaded into a Helios Gene Gun (modified barrel, 40 mm spacer, 70 μm mesh filter; Bio-Rad) and PBS surrounding brain sections was removed. DiI-tungsten particles were shot into the tissue by shooting one bullet on each section using helium gas expulsion (100 PSI). To allow DiI spreading throughout the labelled neurones, sections were kept overnight in PBS in the dark. The next day, sections were post-fixed in 4% paraformaldehyde for 1 hour, rinsed in PBS, mounted on slides and coverslipped with FluorGlo mounting media for lipophilic dyes (Spectra Services, Ontario, NY, USA) (note that the FluorGlo mounting medium is no longer available).

2.3 | DiI confocal imaging, reconstruction and quantitation

Using a Leica TCS SPE confocal microscope, brain regions of interest were identified and delineated using low magnification brightfield in conjunction with the rat brain atlas of Paxinos & Watson (6th edition) for reference.[35] For each brain region, two or three dendritic segments (70-200 μm away from soma, and more than 10 μm away from dendritic end points and bifurcations) per neurone (completely
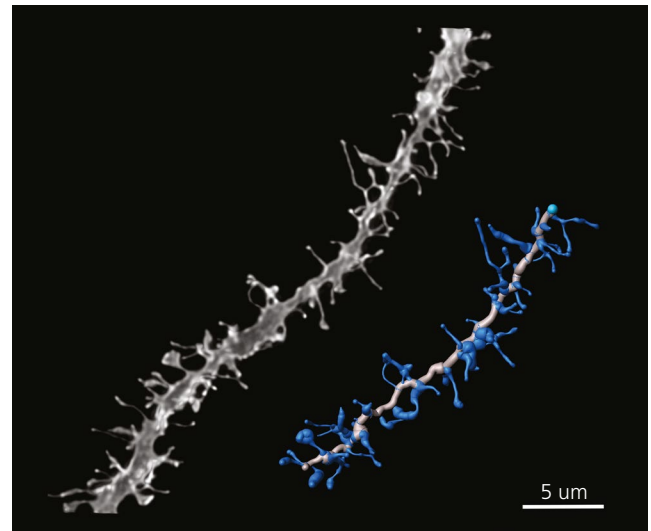

FIGURE 1 Dendritic segment reconstruction. Representative maximum projection image and three-dimensional reconstruction process of a striatal medium spiny neurone dendritic segment labelled with DiI. Image acquired at 63×. Scale bar = 5 μm

and brightly labelled, isolated from other labelled neurones), in two to six neurones (three neurones for the far majority of data points), were imaged and analysed (Figure 1). Our assessment and statistical analyses of dendritic spine densities is based upon a rigorous approach that we and others have previously used.[8,19,34,36-38] With nine or ten animals/group, this equates to approximately 80-90 dendritic segments (and thousands of spines) per group per brain region.

Dendritic segments were imaged using a Leica PLAN APO 63×, 1.4 NA oil immersion objective (11506187; Leica, Mannheim, Germany) and Type LDF immersion oil (Cargille, Cedar Grove, NJ, USA). All images were taken at an xy pixel distribution of 512 × 512, a frequency of 400 Hz, a step size of 0.12 μm and optical zoom of 5.6, with the laser power and photomultiplier being adjusted to capture the dendrite in its full dynamic range. Data from nine or ten animals were collected for each treatment group. In case there were less than two neurones in a brain region feasible for imaging, the animal was excluded from further analysis for that region. This predominantly occurred in the MPN and MeA, and explains the smaller sample sizes for these regions.

After imaging, optical sections were processed through three-dimensional (3D) deconvolution using AutoQuant X3 AutoDeblur software (Media Cybernetics, Rockville, MD, USA). Deconvoluted z-stacks were then reconstructed in the Surpass module of Imaris software (Bitplane Inc., Concord, MA, USA), through manual tracing of dendrites and spines using the Filament tool and Autodepth function. A 3D reconstruction of 15-20 μm of dendritic shaft and spines was rendered using the diameter function with a contrast threshold of 0.7, and data on spine density, spine length and head diameter were collected for each segment. Spine densities for each segment (collected as average spine density per 10 μm) were averaged across each neurone and then within each brain region for each animal, providing a region-specific spine density average for each animal that

was then used for statistical analysis. Measurements of spine length and head diameter were pooled for each treatment condition and then plotted in violin plots, as well as binned cumulative probability distributions (bin sizes: spine length, 0.5 μm; head diameter, 0.1 μm).

2.4 | pCREB immunohistochemistry

2-hydroxypropyl-β-cyclodextrin (cyclodextrin; Sigma-Aldrich, St Louis, MO, USA) was dissolved in sterile water to obtain a 45% vehicle solution. DHT was dissolved in cyclodextrin solution and 1.5 mg (high dose) or 0.15 mg (low dose) was injected i.p. in a volume of 0.2 mL. DHT was administered i.p. in this experiment instead of s.c. given the more rapid time course of CREB phosphorylation compared to spine changes measured over 24 hours. After injection, animals were put alone in a cage until lethal i.p. injection with Euthasol (0.35 mL i.p; 390 mg mL^{-1} pentobarbital sodium, 50 mg mL^{-1} phenytoin sodium, Virbac AH Inc.) 15 or 30 minutes later. Then, animals were injected with 0.25 mL of heparin into the left ventricle, and transcardially perfused with 50 mL PBS followed by 500 mL of 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde for 2 hours, and stored in 10% sucrose in PBS overnight at 4°C. The next day, brains were cut on a freezing microtome into 40-μm sections and every third section throughout the striatum was collected into 0.1% bovine serum albumin (BSA) in 25 mmol L^{-1} PBS (BSA/PBS) for immediate free-floating immunohistochemical processing. After rinsing in BSA/PBS, sections were incubated with a rabbit polyclonal primary antibody directed against serine 133 phosphorylated CREB (dilution 1:2000; cat. 06-519, Merck Millipore, Burlington, MA, USA[39,40]) in 0.3% Triton-X-100 in BSA/PBS for 48 hours at 4°C. Subsequently, sections were incubated in biotinylated goat anti-rabbit (dilution 1:200; VECTASTAIN Elite ABC-HRP rabbit-IgG Kit; Vector Laboratories, Inc., Burlingame, CA, USA) in BSA/PBS for 1 hour, avidin-biotin-peroxidase complex (dilution 1:100; VECTASTAIN Elite Kit) in PBS for 1 hour, and 3,3′-diaminobenzidine (0.8 mg mL^{-1}; Sigma-Aldrich) with 0.3% H$_2$O$_2$ in 50 mmol L^{-1} Tris buffer (pH 7.6) for 8 minutes, with repeated buffer washing in between all steps. Sections were then mounted on slides, and coverslipped using DPX mounting medium (Sigma-Aldrich). The experiment was run in batches of three animals at a time, with the same treatment injection timing (15 or 30 minutes) for each animal in the batch, and one animal per treatment group per batch. Administration of different treatments was randomised according to a latin square design so that the order of injection and perfusion would not be a factor.

2.5 | pCREB imaging and quantitation

For each animal, three sections within the central striatum were identified and imaged using a Leica DM 4000 B LED microscope and 10× objective. At the level of the nucleus accumbens, the anterior commissure has a lateral monotonic migration. Consequently, sections were matched on the anterior-posterior axis by selecting those sections in which the distance from the tip of the lateral ventricle to the medial

edge of the anterior commissure was 300-350 μm. Images were always taken on the right side of the section, without avoidance of artefacts. The same exposure and white balance settings were used across all images. The images were subsequently loaded in PHOTOSHOP (Adobe Systems Inc., San Jose, CA, USA) and a red box (300 × 500 μm) placed within the brain regions of interest. For the NAc, the box was placed medial to the ventricle for the shell and lateral to the ventricle for the core, and the distance between the boxes was kept at 100 μm for each section imaged. For the medial and lateral CPu, the top corner of the boxes touched the corpus callosum.

For pCREB + cell counting, the cell counter plugin was used in IMAGEJ (NIH, Bethesda, MD, USA). To increase intra-observer reliability, we converted the images to greyscale and used the automated threshold algorithm 'Otsu' to acquire a binary image that separated positive cells from background to use as a counting guide. Otsu's method finds a threshold value where foreground and background pixel value variance is at a minimum. Because some batch-to-batch immunohistochemistry variance is to be expected, Otsu's method works well for thresholding here because it uses information from within the image to separate background from staining. Cells were counted if they appeared with at least one pixel in the thresholded image, and standard stereology rules were applied when counting on the box borders. Intra-observer agreement of positive cell count was within the range 97.4%-99.5% for a sample size of 10 duplicate images. Cell counts of three sections were averaged across each brain region within each animal. Out of 384 total boxes, eight images contained very large artefacts in the tissue within the box, and were therefore excluded from analysis.

2.6 | Statistical analysis

All data analysis was conducted in PRISM, version 8 (GraphPad Software Inc., San Diego, CA, USA). For spine density and pCREB expression, groups were compared using a one-way ANOVA, followed by Tukey's multiple comparisons test in case of significant effect, or a Kruskal-Wallis test followed by Dunn's multiple comparisons test when the data did not pass assumptions for parametric analysis. The binned spine morphology probability distributions were compared to each other group using a Kolmogorov-Smirnov test.

3 | RESULTS

3.1 | Dendritic spine plasticity

To investigate the effects of both GDX and androgen replacement on dendritic spine plasticity, we compared intact males treated with oil (vehicle) to GDX males treated with oil, as well as to GDX males treated with DHT. We found that GDX affected spine plasticity in the MPN ($H = 12.16$, $P = 0.0002$, $\eta^2 = 0.59$) (Figure 2A) by decreasing spine density (mean difference vs intact = 2.03, $P = 0.0073$, $g = 2.35$). GDX did not affect spine density in the MeA, NAcSh, NAcC and CPu (Figure 2A). DHT administration to GDX animals rescued the GDX-induced spine loss in the MPN (mean difference vs GDX-oil = 2.15,
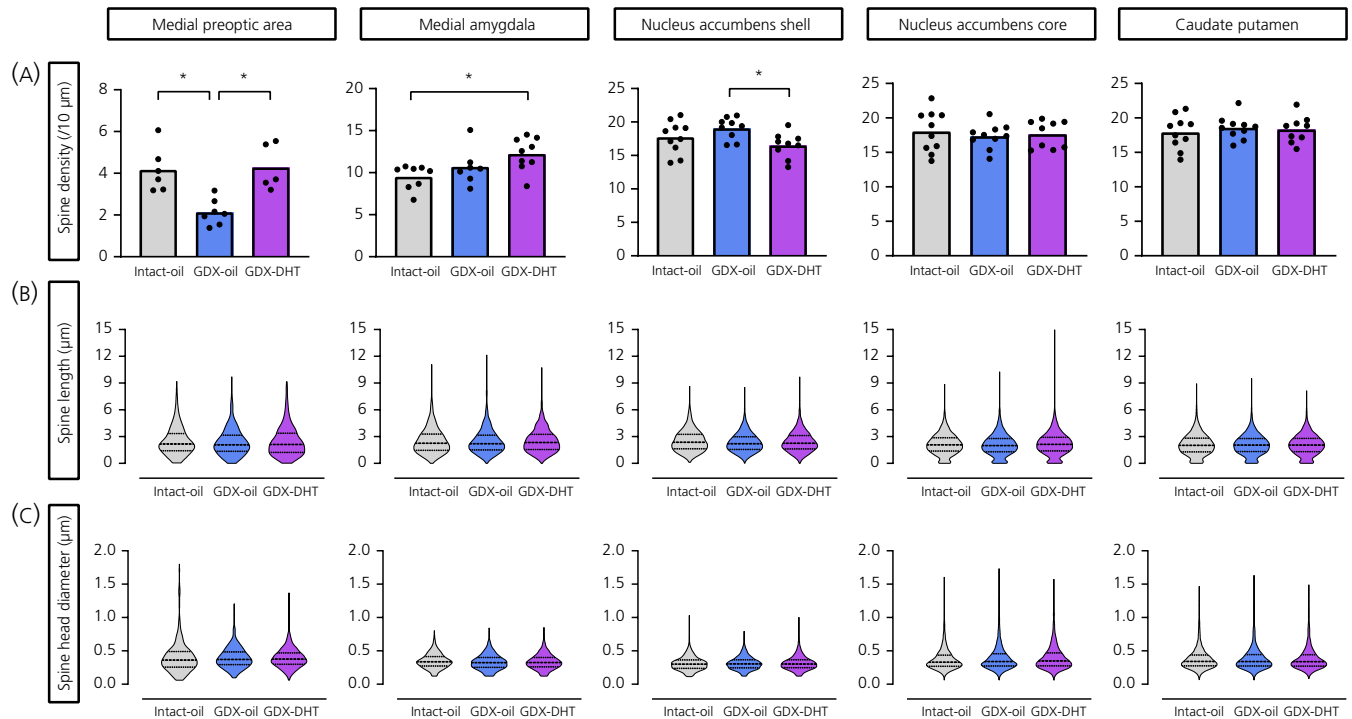
FIGURE 2 Gonadectomy (GDX) and dihydrotestosterone (DHT) affect spine plasticity differentially across several brain regions regulating motivated behaviour. A, Spine density 24 h after treatment with oil or DHT in intact and GDX males in the medial preoptic nucleus (MPN), medial amygdala (MeA), nucleus accumbens shell and core (NAcSh and NAcC), and caudate putamen (CPu). Individual values represent neurone spine density average per animal (= unit of analysis), which is comprised of the average spine density across two or three neurones per animal, calculated from the average spine density from two or three segments per neurone. n = 6, 7, 5 (MPN); 8, 7, 9 (MeA); 10, 10, 9 (NAcC); 10, 9, 9 (NAcSh); and 10, 10, 9 (CPu) animals per group. *$P < 0.05$. B, Violin plot representation of spine length distribution. For violin plots, all spine data points from all animals within the same group were pooled into one plot. Dashed line, median; dotted lines, quartiles. C, Violin plots of spine head diameter distribution

$P = 0.012$, $g = 2.54$). By contrast, DHT decreased spine density in the NAcSh in GDX animals compared to oil-treated GDX animals ($F_{2,25} = 3.56$, $P = 0.04369$, $\eta^2 = 0.22$) (Figure 2A) (mean difference vs GDX-oil = 2.54, $P = 0.0341$, $g = 1.44$). This effect, however, was not different compared to the intact-oil group. We found effects on spine density in the MeA as well ($F_{2,21} = 4.45$, $P = 0.0245$, $\eta^2 = 0.30$) (Figure 2A). Specifically, although GDX itself did not affect spine density, DHT treated GDX males had a higher spine density than oil-treated intact males (mean difference vs intact = 2.71, $P = 0.0193$, $g = 1.56$). We saw no effects of DHT on spine density in NAcC and CPu (Figure 2A) compared to either the intact-oil or the GDX-oil group.

Spine morphology gives information about spine maturation and function.[41] Because spine morphology is determined by spine length and spine head diameter, we compared the distributions of these two parameters between groups. No effects of castration or DHT treatment were observed on spine length or spine head diameter in any of the brain regions (Figure 2B,C).

3.2 | pCREB expression

To investigate the potential rapid effects of DHT we focused on the striatum, a brain region in which rapid phosphorylation of CREB has been documented.[27] We determined the number of cells expressing

pCREB by means of immunohistochemistry (Figure 3A), 15 and 30 minutes after hormone or vehicle treatment of GDX males. DHT treatment had no effect on the amount of pCREB + cells within 15 minutes of hormone administration in any of the investigated subregions (Figure 3B). After 30 minutes, however, a high dose of DHT significantly increased the number of pCREB + cells in the NAcSh ($F_{2,12} = 5.039$, $P = 0.0258$, $\eta^2 = 0.46$) (Figure 3C) compared to vehicle (mean difference 90, $P = 0.0358$, $g = 1.79$). A low dose of DHT also increased the number of pCREB + cells in the medial CPu ($F_{2,12} = 4.350$, $P = 0.038$, $\eta^2 = 0.42$]) (Figure 3C) compared to vehicle (mean difference 82, $P = 0.0321$, $g = 1.77$). No effects of DHT treatment were found in the NAcC and lateral part of the CPu.

4 | DISCUSSION

Gonadal hormones are known to regulate synaptic plasticity.[42,43] Although the literature has so far mostly characterised the effects of oestrogens in females, some evidence exists for effects of gonadal steroids in males as well.[24,31,44] In the present study, we aimed to examine the effects of loss and subsequent replacement of gonadal hormones on spine plasticity in males. We focused on brain regions that are involved in neural circuits of (sexual) motivation: the NAcC, NAcSh and CPu, which are part of dopaminergic reward processing,
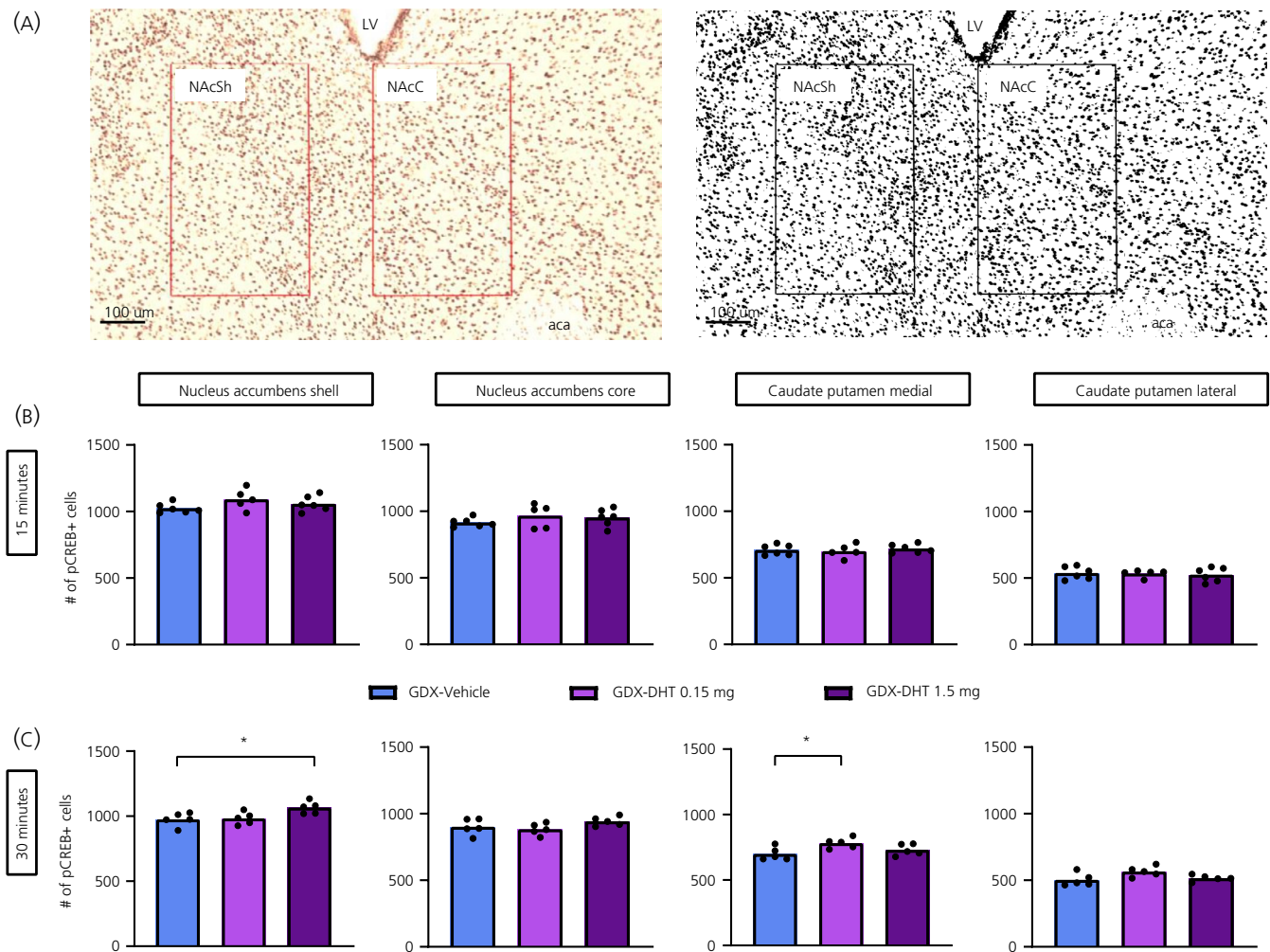
FIGURE 3 Dihydrotestosterone (DHT) rapidly induces cAMP response-element binding protein (CREB) phosphorylation. A, Representative image of phosphorylated CREB (pCREB) staining and counting box (300 μm × 500 μm) delineating in the nucleus accumbens core and shell, and corresponding Otsu thresholded image. Scale bar = 100 μm. LV, lateral ventricle; aca, anterior commissure; NacSh, nucleus accumbens shell; NacC, nucleus accumbens core. B, Number of pCREB positive cells in each brain region 15 min after i.p. treatment with oil or low or high dose DHT in gonadectomised (GDX) males. C, Number of pCREB positive cells 30 min after treatment. *$P < 0.05$

and the MPN and MeA, two regions critically involved in the display of sexual behaviour. The investigated brain regions have all been shown to be androgen target areas in rats.[45] We showed that GDX decreased spine density in the MPN, which was rescued by DHT treatment. In addition, DHT decreased spine density in the NAcSh in GDX animals, whereas it increased spine density in the MeA of GDX animals compared to intact animals. Thus, spine plasticity is differentially affected by gonadal hormones across the studied brain regions.

GDX gradually ceases copulation in male rats as a result of the loss of gonadal hormones. Oestrogen as well as androgen signalling through oestrogen and androgen receptors in the brain is necessary for the full display of male sexual behaviour.[20] Considering the high expression of androgen and oestrogen receptors in the MPN, it is therefore not surprising that the MPN is the most important brain region for regulation of sexual behaviour in males.[20] Disruption of the MPN through lesions causes gonadally intact male rats to stop copulating.[20] Local infusion of an aromatase inhibitor (preventing the formation of oestradiol and thus oestrogen receptor signalling)

or an androgen antagonist (preventing androgen receptor signalling) into the MPN suppresses copulation in gonadally intact male rats, showing a vital interaction of gonadal hormones and the MPN in male sexual behaviour.[46,47] Yet, it remains unclear what mechanism underlies the importance of the activity of gonadal hormones in the MPN for copulation in male rats. What has been shown earlier is that GDX reduces dopamine release and c-Fos expression in the male MPN upon exposure to an oestrous female.[48-50] This suggests that a lack of gonadal hormones may reduce afferent sensory information to the MPN. In the present study, we demonstrate a novel effect of GDX in the MPN of male rats. GDX drastically reduces spine density of MPN neurones, an indication of an overall decrease in synapses within the MPN. In line with reduced dopamine release and c-Fos expression in the MPN, this suggests a model in which gonadal hormones act as facilitators contributing to MPN connectivity. This connectivity may then be necessary for sexual behaviour to arise in response to the stimulus of an oestrous female. Our study shows that the GDX-induced spine loss is present in males gonadectomised

for longer than 10 days, a time point at which most male rats would have stopped copulating.[20] Future research should focus on spine plasticity at different time points after GDX aiming to reveal whether loss of spines in the MPN also coincides with the gradual loss of sexual behaviour after GDX in males. That should provide more insight in whether GDX-induced loss of spines in the MPN indeed contributes to loss of sexual behaviour.

Treatment with testosterone given systemically or locally into the MPN facilitates copulation in GDX males.[51-54] In addition, testosterone rescues GDX-induced spine loss in the MPN of male hamsters.[23] Furthermore, functional aromatisation of testosterone into oestrogen is necessary for the display of the full range of sexual behaviour in male rats. Treatment of GDX males with DHT, a high-affinity ligand of the androgen receptor that cannot be aromatised into oestrogen, is ineffective with respect to reinstating sexual behaviour.[52] Furthermore, at the same time that DHT has affinity for oestrogen receptor β (ERβ), our prior work showed that an ERβ agonist did not affect dendritic spine density in the nucleus accumbens.[26] Therefore, we expected to find that treatment with DHT would not be sufficient to rescue the GDX-induced spine loss in the MPN. Nevertheless, in the present study, we show that DHT treatment of GDX males does fully restore spine density on MPN neurones. Even though DHT-induced spinogenesis in the MPN of GDX males does not coincide with restoration of copulation,[55] androgen signalling still contributes to copulatory behaviour. For example, local infusion of an androgen receptor antagonist into the MPN of GDX males prevents the reinstatement of sexual behaviour by systemic testosterone treatment.[56] In line with this, androgen signalling in addition to oestrogen signalling is necessary for the motivational aspects of sexual behaviour, such as preference for an oestrous female, and DHT alone has mild effects on sexual incentive motivation.[57,58] Furthermore, testosterone and oestradiol both show rapid effects on firing rate in MPN neurones, but they rarely affect the same neurones.[59] Therefore, androgenic signalling may perhaps primarily influence and maintain sexual motivation through a distinct neuronal population in the MPN, mediated by spine plasticity. An important research focus in the future will be to unravel the effects of oestrogens on spine plasticity in the MPN of GDX males.

By contrast to our findings in the MPN, we found that GDX did not decrease spine density in the MeA, another important region for copulation.[20] Other studies have reported a decrease in spine density in the posterodorsal MeA, 3 months after GDX, measured on dendrites very proximal to the soma,[59,61] and in males castrated before puberty.[62] Our measures, on the other hand, were taken at least 70 μm away from the soma, not on primary dendrites and within a shorter time frame after GDX. We did find, however, that DHT has spinogenic properties in the MeA, even though we only saw this in comparison with intact males. Possibly, gonadal hormones are not necessary to maintain spine density in the MeA, but do have the ability to affect spine plasticity such as in the MPN. Another study conducted in intact pubertal males showed that a chronic high dose testosterone is transiently spinogenic in the antero- and posterodorsal MeA.[6] Thus, gonadal hormones may affect MeA spine plasticity

differentially depending on the distance of a dendritic segment from the soma, the timing of castration within life, the castration duration, and the amount of time that has passed subsequent to hormone treatment.

The present study replicated earlier results obtained from our laboratory, where we showed that, in contrast to its spinogenic effects in the MPN and MeA, DHT decreases spine density after GDX in NAcSh, but not in NAcC and CPu in GDX males.[26] Here, we used an additional control group of intact males to also establish that gonadal hormones are not necessary for maintaining spine density and morphology in the striatum in males because GDX left these variables unaffected. Another group found that, in intact males, a chronic high dose of testosterone decreases spine density in the NAcSh, and has no effect on the NAcC.[63] This suggests that androgens induce loss of spines in the NAcSh regardless of whether the male is gonadectomised or not. The rapid changes in NAcSh dendritic spines following DHT do not appear to underlie the expression of copulation in males because the effects of DHT on spines require mGluR5 signalling[26] and accumbens antagonism of mGluR5 receptors does not disrupt copulation.[64] The medial preoptic area and medial amygdala are better candidates for sites of action of DHT on copulation, and DHT modulated dendritic spines within 24 hours in these regions as well. One study has demonstrated interactions between oestrogen receptor and mGluR signalling in the medial preoptic area[65] with nothing known about similar interactions in the medial amygdala. Because both oestradiol and DHT induce rapid ERK phosphorylation in the medial preoptic area,[66] cooperative signalling through mGluR receptors could be the basis for rapid effects on copulation in males. We propose parallels with the mechanisms through which oestradiol acts to regulate female sexual behaviour. Oestrogens induce rapid membrane-mediated signalling cascades, which are followed by longer lasting transcriptional activation via nuclear receptors.[67] We envision a similar set of actions for male sexual behaviour in which androgens provide both rapid and long-term plasticity.

The small numbers of dendritic spines measured in these studies sometimes raise questions about the functional significance of these spine changes. For striatal medium spiny neurones, Golgi studies suggest that the cumulative dendritic length may be on the order of 2100 μm,[68] whereas cell fills put the number closer to about 3000 μm.[69] With an increase of three spines per 10 μm, as we see in the nucleus accumbens shell, this translates to upwards of 1000 excitatory synapses per medium spiny neurone, producing a substantial impact on the electrotonic potential of these neurones.[69] A limitation of the DiOlistic labelling approach taken in this experiment is that it is not possible to differentiate between specific neuronal cell-types such as D1 vs D2 medium spiny striatal neurones. In the MeA and MPN, neurones were selected based on similar gross morphology, which only partially addresses neuronal heterogeneity. Future research will aim to refine and combine methods in order to distinguish different neuronal populations.

Androgens can exert their action on neurones through multiple signalling pathways.[70] Although the 24 hours after hormone

treatment in the present study comprises sufficient time for genomic effects to occur, previous results from our laboratory show that DHT-induced spine plasticity in the NAcSh is mediated by mGluR5, a G protein-coupled receptor associated with the Gαq protein, suggesting that membrane-initiated signalling pathways are involved.[26] This mechanism is homologous to the mGluR5 mediated oestrogen-induced decrease in spine density in the NAcC of ovariectomised females.[8] The coupling to and regulation of mGluRs by membrane-bound oestrogen receptors has been well characterised and has been shown to mediate spine plasticity and behaviour in females.[15,71,72] Oestrogens rapidly increase phosphorylation of the transcription factor CREB through its membrane interaction with mGluRs in hippocampal and striatal neurones exclusively in female cultures.[27,28] It is important to note that, although oestrogen receptor/mGluR signalling leads to pCREB across many brain regions, only a subset of these exhibit changes in dendritic spines. Therefore, this signalling pathway may be necessary for structural changes, but it is not sufficient. In male cultures, oestradiol does not induce pCREB, whereas mGluR activation does. In addition, activation of mGluR5 mediates spine plasticity in male NAcC and NAcSh,[73] suggesting that mGluRs possibly couple to membrane-bound androgen receptor in males. The androgen receptor has indeed also been shown to migrate to the membrane,[74] using the same intracellular processes as oestrogen receptors.[75] Here, we show that DHT is capable of inducing striatal pCREB in vivo within 30 minutes of injection. Although the immunohistochemistry method that we used for assessing pCREB expression only allowed for counting of number of positive cells, and not the level of pCREB within a cell, it is possible that DHT *also* induces higher phosphorylation levels of pCREB in each individual positive cell. Still, our results point towards a pathway in which androgen binds to membrane-bound androgen receptors, which activates mGluR5 through coupling in the NAcSh but not in the NAcC. This leads to activation of a downstream signalling cascade culminating into phosphorylation of CREB, thereby enhancing its gene transcription properties. There is a large body of literature on the function of CREB, which, amongst others, is involved in learning and memory and synaptic plasticity.[29] Whether this proposed mechanism of DHT-induced plasticity, through mGluR5 or other mGluRs, can also be applied to the effects we found in the MPN and MeA will be part of future research.

5 | CONCLUSIONS

We conclude that both GDX and androgen differentially affect spine plasticity in the MPN, MeA and NAcSh, whereas NAcC and CPu remain unaffected. In the NAcSh, DHT may exert its effects through pCREB induction mediated by androgen receptor activation of mGluR5.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Patty T. Huijgens: Conceptualisation; data curation; formal analysis; investigation; visualisation; writing – original draft. **Eelke M. S. Snoeren:** Formal analysis; funding acquisition; supervision; writing – review and editing. **Robert L. Meisel:** Conceptualisation; methodology; resources; supervision; writing – review and editing. **Paul G. Mermelstein:** Conceptualisation; funding acquisition; methodology; resources; writing – review and editing.

DATA AVAILABILITY

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

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