**UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF**

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# Sex-dependent responsiveness of hippocampal neurons to sex neurosteroids: A role of Arc/Arg3.1

**Dissertation**

zur Erlangung des Grades eines Doktors der Medizin
an der Medizinischen Fakultät der Universität Hamburg.

vorgelegt von:
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aus Paderborn

Hamburg 2023

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am: 12.12.2023

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg.

Prüfungsausschuss, der/die Vorsitzende: Prof. Dr. Barbara Schmalfeldt

Prüfungsausschuss, zweite/r Gutachter/in: Prof. Dr. Gabriele M. Rune

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Received: 4 May 2021 | Revised: 23 December 2021 | Accepted: 7 January 2022

DOI: 10.1111/jne.13090

ORIGINAL ARTICLE

Journal of Neuroendocrinology WILEY

# Sex-dependent responsiveness of hippocampal neurons to sex neurosteroids: A role of Arc/Arg3.1

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**Funding information**
Deutsche Forschungsgemeinschaft, Grant/Award Number: DFG-436/4-1

## Abstract

Sex steroids, such as estradiol ($E_2$) and dihydrotestosterone (DHT), regulate hippocampal plasticity and memory in a sex-dependent manner. Because the activity-regulated cytoskeleton protein Arc/Arg3.1 is essential for long-term memory formation and synaptic plasticity, we investigated the expression of Arc/Arg3.1 with respect to its responsiveness to $E_2$ and DHT in male and female hippocampal neurons. For the first time, we show that, in hippocampal neurons, Arc/Arg3.1 expression is sex-dependently regulated by sex steroids. No difference in the expression between sexes was observed under control conditions. Using a quantitative real-time polymerase chain reaction, western blot analysis and quantitative immunoreactivity, upregulation of Arc/Arg3.1 protein expression was observed in specifically female hippocampal neurons after application of $E_2$ to the cultures. Conversely, upregulation of Arc/Arg3.1 was seen in specifically male neurons after application of DHT. A quantitative real-time PCR revealed that the sex-dependency was most pronounced on the mRNA level. Most importantly, the effects of $E_2$ in cultures of female animals were abolished when neuron-derived $E_2$ synthesis was inhibited. Our results point to a potentially important role of Arc/Arg3.1 regarding sex-dependency in sex steroid-induced synaptic plasticity in the hippocampus.

**KEYWORDS**
Arc/Arg3.1, estrogen, hippocampus, neurosteroids, sex-specificity, testosterone

## 1 | INTRODUCTION

It has been widely established that the immediate early gene Arc/Arg3.1 plays a master role in synaptic plasticity and memory formation.[1] Subsequent to its discovery in 1995 by Kink et al.[2] and Lyford et al.,[3] it has been shown that it is regulated by neuronal activity, and that its mRNA is strongly induced and transported to dendrites upon long-term potentiation (LTP) stimulation.[4] Within dendrites, it has been suggested that Arc/Arg3.1 is translated on site.[5] Changes in Arc/Arg3.1 have been directly linked to information processes in the brain.[6,7] In classical Arc/Arg3.1 knockout mice, Plath et al.[7] demonstrated strong impairment of synaptic plasticity and long-term memory, whereas short-term memory remained unaffected, as did brain morphology, neural activity and synaptic transmission. In a recent study, it was shown that Arc/Arg3.1 is transiently upregulated during the first postnatal month, and that conditional removal of Arc/Arg3.1 during the first month after birth impairs learning capacity up to adulthood. Post developmental removal, however, leaves learning capacity intact, whereas long-term memory storage is affected throughout life.[8]

A recent extensive review from Karyn Frick's laboratory on estradiol ($E_2$) as a neuromodulator of learning and memory summarizes the great number of studies on this topic carried out throughout the last three decades.[9] Meanwhile, it has been widely established that $E_2$ is a potent regulator of the neural mechanisms that are critically involved in memory formation, including cell signaling, gene expression, protein synthesis, extrinsic and intrinsic excitability, dendritic spine formation, and neurogenesis. Despite the fact that $E_2$ is a female hormone, $E_2$ was also shown to effect memory in males, even if the signaling of $E_2$ appears to be sex-dependent.[10–15] Furthermore, similar effects, as compared to $E_2$ effects, were attributed to testosterone in males. $E_2$ effects, however, appeared to be specific in females.[16–19] It was also shown that hippocampal neurons are capable of synthesizing $E_2$ and dihydrotestosterone (DHT) de novo from cholesterol.[20,21] Inhibition of $E_2$ or DHT synthesis, either pharmacologically or by genetic approaches, revealed a clear sex-dependency; inhibition of $E_2$ synthesis, via inhibition of aromatase, reduced spine and spine synapse density and impaired LTP in the female, but not male, hippocampus. Inhibition of DHT, the more potent testosterone metabolite, via inhibition of 5α-reductase, resulted in synapse loss and LTP impairment in males, but not females.[22–25] Inconsistencies between effects after inhibition of sex steroid synthesis and effects after exogenous application of sex steroids were recently reviewed by Brandt et al.[26]

To the best of our knowledge, in the context of sex steroid-induced synaptic plasticity, the role of Arc/Arg3.1 has not yet been considered in any of the numerous studies. In the present study, we show, for the first time, that Arc/Arg3.1, a protein that is directly related to memory, is responsive to sex steroids in a sex-dependent manner.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Wistar rats (Institute of Neuroanatomy, University of Hamburg, Germany) were maintained under control conditions, and water and food were available ad libitum. Experiments were conducted in accordance with the German and European Union laws on protection of experimental animals, following approval by the local authorities of the City of Hamburg (Committee for Food Safety and Veterinary Services, Hamburg Authority for Social Affairs, Family, Health and Consumer Protection, Germany, Org 996). Embryonic day 18 (E18) rats were used for culturing hippocampal cells in dispersion.

### 2.2 | Dispersed cultures

Primary hippocampal neurons were prepared from embryonic day 18 Wistar rats, as described by Banker and Gosslin.[27] The cells were cultured on poly-L-lysine-coated (0.1 mg $mL^{-1}$) glass coverslips in Neurobasal A medium, supplemented with 0.5% B27 (Invitrogen), 0.5 mM glutamine (Gibco), and the antibiotics penicillin and streptomycin (1 ×; Gibco) were used at a density of 125,000 cells per well (24-well-plate) for RNA and protein extraction, or 80,000 cells per well for immunocytochemistry. The purity of the neuronal culture was tested by immunocytochemistry according to von Schassen et al[28]

### 2.3 | Treatment

After 21 days in vitro, the differentiated neurons were stimulated with testosterone ($10^{-8}$ M), dihydrotestosterone ($10^{-8}$ M), estradiol ($10^{-8}$ M), letrozole ($10^{-8}$ M) and finasteride ($10^{-8}$ M), or they were treated with the vehicle as controls for 1 h. In addition to this stimulation, pretreated cells were stimulated with letrozole, finasteride or the vehicle every 48 h from day 14 to day 21 in vitro. Stimulation with the sex steroids was then performed on day 21 for 1 h.

### 2.4 | Quantitative real-time polymerase chain reaction (RT-qPCR)

To determine mRNA levels of Arc, 500,000 cells were pooled for one sample after stimulation, as described previously. The total RNA extraction was carried out using the RNeasy Mini Kit (Qiagen), in accordance with the manufacturer's instructions. Then, 100 ng of mRNA was converted using a cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific). A sample preparation without enzymes (NEM; non-enzyme mix) was used as a negative control for cDNA synthesis. Afterwards, samples were prepared according to the TaqMan Universal PCR MasterMix (Thermo Fisher Scientific) protocol and the following primers were added to amplify Arc and the endogenous control hypoxanthine phosphoribosyl transferase 1: Rn00571208_g1 (Arc) and Rn01527840_m1 (hprt1; Thermo Fisher Scientific). Each sample was measured as a triplet, together with the associated NEM and H2O as negative controls. The RT-qPCR was performed using the StepOne Real-time PCR System (Applied Biosystems) and analyzed with the associated StepOne software (version 2.3). For mathematical evaluation, the efficiency-corrected model according to Pfaffl[29] was used ($n = 7$ or 8 per condition).

### 2.5 | Western blotting

Hippocampal cell extracts were prepared with RIPA buffer containing 150 mM NaCl, 50 mM Tris, 1% NP40 and a protease inhibitor cocktail (Roche). The homogenates were centrifuged at 13,000 *g* at 4°C for 30 min, and the supernatant was collected and frozen until further use. Protein concentrations were determined with the Bradford protein assay (Bio-Rad). After performing sodium dodecyl sulfate-polyacryamide gel electrophoresis with 10% acrylamide under reducing conditions, a subsequent western blot was treated with 5% non-fat milk powder solution (in phosphate-buffered saline [PBS] plus 0.3% Triton X-100) and analyzed with the polyclonal rabbit antibody to Arc (SySy 156 003, dilution 1:1000) and a polyclonal rabbit antibody to GAPDH (sc-25778, dilution 1:1000) in 5% non-fat milk powder solution (in PBS plus 0.3% Triton X-100). The incubation time was 12 h at 4°C.

Proteins were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore), or an ECL-Plus kit (GE Healthcare) and quantified by densitometry using ImageJ, version 1.49n (NIH). GAPDH expression, which was not affected by either treatment, was determined as an internal standard. Experimentally treated cultures were always compared with the respective controls from the same culture and presented as "expression relative to GAPDH" (mean ± SEM; $n = 1$ refers to pooled neurons deriving from one culture, $n = 4–6$ per condition).

### 2.6 | Immunofluorescence and immunocytochemistry

Coverslips were washed with PBS, and the cells were subsequently fixed with cold MeOH for 10 min. Permeabilization was performed with PBS containing 0.4% Triton X-100. After several washes with PBS, the cells were covered with Protein Block Serum-Free (Dako) for 30 min. For immunolabeling, the following antibodies were applied in Antibody Diluent with Background Reducing Components (Dako): rabbit anti-Arc (SySy 156 003, dilution 1:500) and chicken anti-Map2 (ab 92434, dilution 1:1000).

The primary antibody was incubated overnight at 4°C and washed several times with PBS the next day. Primary antibodies were detected with Alexa Fluor 647 goat anti-rabbit IgG antibody (A-21245, dilution 1:1000; Thermo Fisher Scientific) and Alexa Fluor 488 goat anti-chicken IgY antibody (A-11039, dilution 1:1000; Thermo Fisher Scientific). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma).

### 2.7 | Image acquisition and data analysis

To analyse the Arc distribution in neurons, stained cultures were imaged using a AxioObserver (Zeiss) with integrated Apotome 2 and Plan-APO 40x/Oil Objective (NA 1.4). *Z* stacks were collected from four various regions per coverslip of approximately 20 x 1 µm spaced images ($n = 1$). To achieve blinded image collection, neurons were chosen for imaging using the Map2 staining. Treatment and sex were also blinded.

Using Imaris, version 9.6.0 (Oxford Instruments), the nuclei were reconstructed on DAPI channel and dendrites on the Map2 channel using the volume reconstruction tool. To measure Arc levels in the soma of neurons, a spherical region of interest with a diameter of 4 µm was placed in each soma using the Spot tool. The thresholds for reconstruction were maintained for all images. Staining intensity in the Arc channel was measured separately in the reconstructed areas ($n = 3$ per condition).

### 2.8 | Statistical analysis

All data were tested with a non-parametric test due to their non-normal distribution. The Mann–Whitney test was used to compare two samples and the Kruskal–Wallis test was used when more than two samples were compared. In case there was a significant difference in the groups ($p < .05$), a post-hoc test (Dunn's procedure) for multiple comparison was followed. To reduce the problem of multiple testing, a Bonferroni adjustment was also performed, and the alpha was adjusted for statistical significance. XLSTAT 2021 software (Addinsoft) was used to calculate and present the data.

## 3 | RESULTS

### 3.1 | Arc/Arg3.1 in dissociated hippocampal neurons

Dissociated embryonic hippocampal neurons, cultured for 21 days, clearly revealed Arc/Arg3.1 mRNA as evidenced by RT-qPCR and exhibited the typical protein band at 55 kDa in the western blot analyses. The Arc/Arg3.1 protein was also revealed by immunohistochemistry. Punctate staining was found in the nucleus, perikaryon and dendrites. Under control conditions, no difference was observed between cultures that originated from male animals and cultures that originated from female animals (Figure 1).

### 3.2 | Sex steroids upregulate Arc/Arg3.1 in a sex-dependent manner

For our experiments we used embryonic dissociated cultures, comprising an established cell culture system for testing the synaptic response to various stimuli. We and others have found that responsiveness to sex steroids is very well preserved in this in vitro model, even after long-term treatment.[22,24] We applied $E_2$ and DHT at doses of $10^{-8}$ M into the medium of our cultures, a dose that is commonly used to test the effects of exogenously applied sex steroids on hippocampal synaptic plasticity,[30] and this, in particular, led to a maximum increase in Arc/Arg3.1 expression in a stable cell line.[31] To determine whether the local synthesis of sex steroids, namely $E_2$ and DHT, which were shown to induce synaptic plasticity in the hippocampus in a sex-dependent manner,[25] are of any relevance, we additionally used finasteride to inhibit DHT synthesis, via blocking 5α-reductase, and letrozole to inhibit $E_2$ synthesis, via blocking of aromatase. The doses of sex steroid synthesis blockers were adopted from previous studies, in which we tested the efficacy of the blockers.[22,23,25] Because testosterone is the substrate of both enzymes, aromatase and 5α-reductase,[25,32] it has to be considered that the inhibitors increase testosterone levels as side effects.[25]

Arc/Arg3.1. is an immediate early gene that responds to stimuli in a rapid manner. We therefore tested the time-dependency of upregulation of Arc/Arg3.1 mRNA in response to brain-derived neurotrophic factor (BDNF) as a positive control of our culture system. BDNF induces somatodendritic expression of Arc/Arg3.1 mRNA in dentate granule cells and Arc/Arg3.1 protein is required for the induction and stabilization of BDNF-LTP.[33] As expected, we found a time-dependent increase in Arc/Arg3.1, with a maximum after 55 min of BDNF stimulation (Figure 2A). We also tested the time-dependent

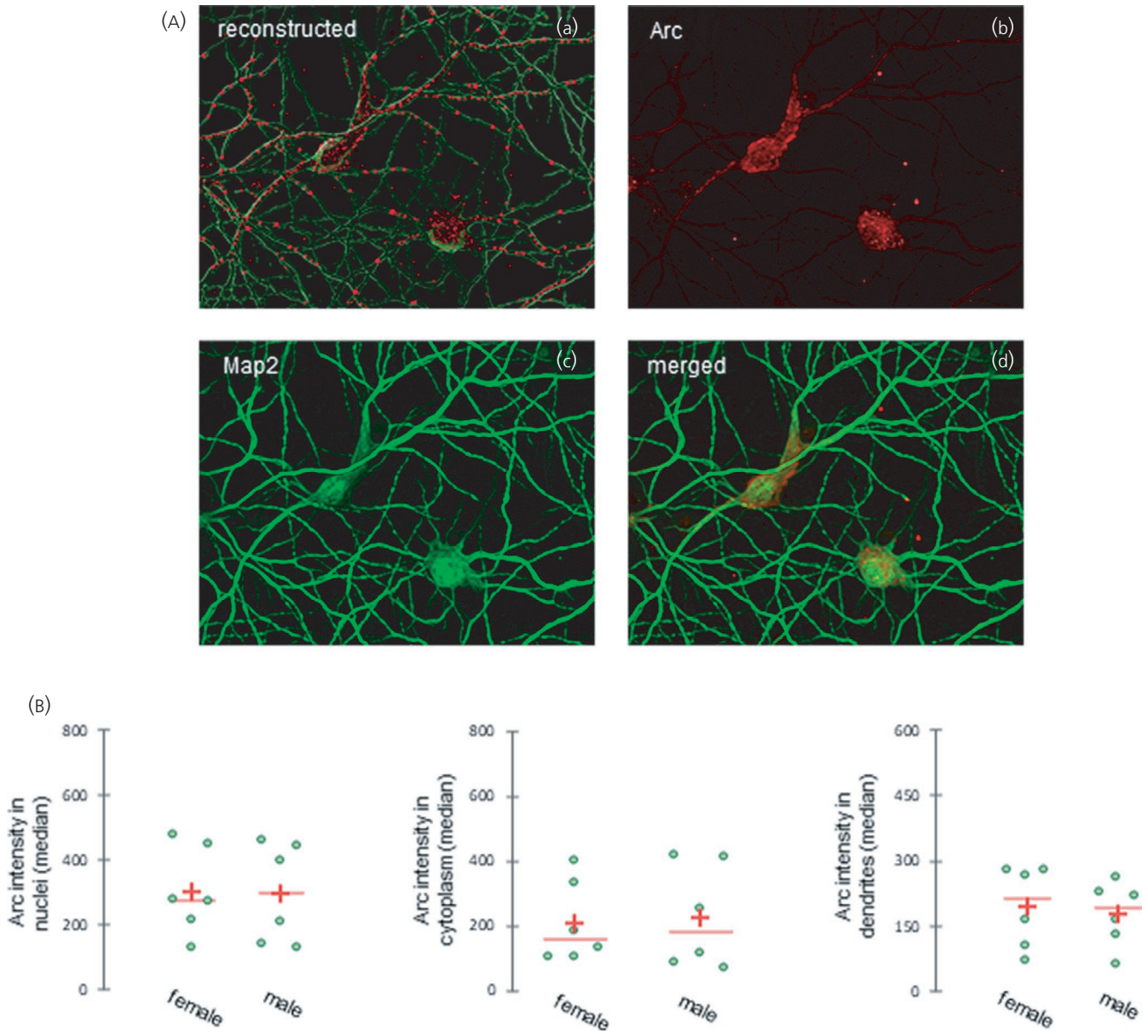


**FIGURE 1** No difference of Arc/Arg3.1 expression and distribution after 21 days in vitro (DIV) was found between female and male neurons under control conditions. (A) Reconstruction of immunocytological staining of unstimulated male neurons to illustrate Arc/Arg3.1 distribution in hippocampal neuron cultures after 21 DIV. Imaris volume reconstruction based on MAP2 staining (green) and spot reconstruction based on Arc/Arg3.1 staining (red) to visualize Arc/Arg3.1 expression and localization in primary neuronal cultures (a) 3D reconstruction. (b,c) Original Arc/Arg3.1 and Map2 staining data shown as an extended focus projection. (d) Merged channels). (B) Image analysis of Arc/Arg3.1. immunoreactivity in the nuclei, cytoplasm and dendrites. Non-parametric Mann–Whitney test ($\alpha = 0.05$) shows no significant differences in Arc/Arg3.1 distribution between females and males under control conditions. Points, single values; cross, mean; line, median ($n = 6$)

increase after application of $E_2$ and DHT. On the mRNA level, an $E_2$-induced increase in Arc/Arg3.1 expression was observed in female, but not male, neurons after 15 min of exposure, which persisted up to 120 min. A similar time-dependency was seen in male, but not female, neurons after application of DHT to the cultures. (Figure 2B,C).

RT-qPCR revealed a clear upregulation of Arc/Arg3.1 mRNA in response to DHT in cultures of male animals, but no effect was seen in cultures of female animals. Conversely, Arc/Arg3.1 mRNA was upregulated in cultures of female animals, but not in cultures of male animals after treatment of the cultures with $E_2$. Testosterone downregulated the expression of Arc/Arg3.1 in female cultures, but upregulated Arc/Arg3.1 mRNA in male cultures, which, however, did not become statistically significant (Figure 3). In general, the upregulation was more pronounced after DHT than after E2 treatment. Based upon our earlier findings that inhibition of local synthesis of $E_2$ and DHT, respectively, results in impairment of synaptic plasticity,[24,25] we also looked at the effects of sex steroid blockers. However, no effect was seen on the mRNA level when sex steroid biosynthesis was blocked for 1 h in the neurons, neither after blocking E2 synthesis, nor after blocking DHT synthesis (Figure 3).

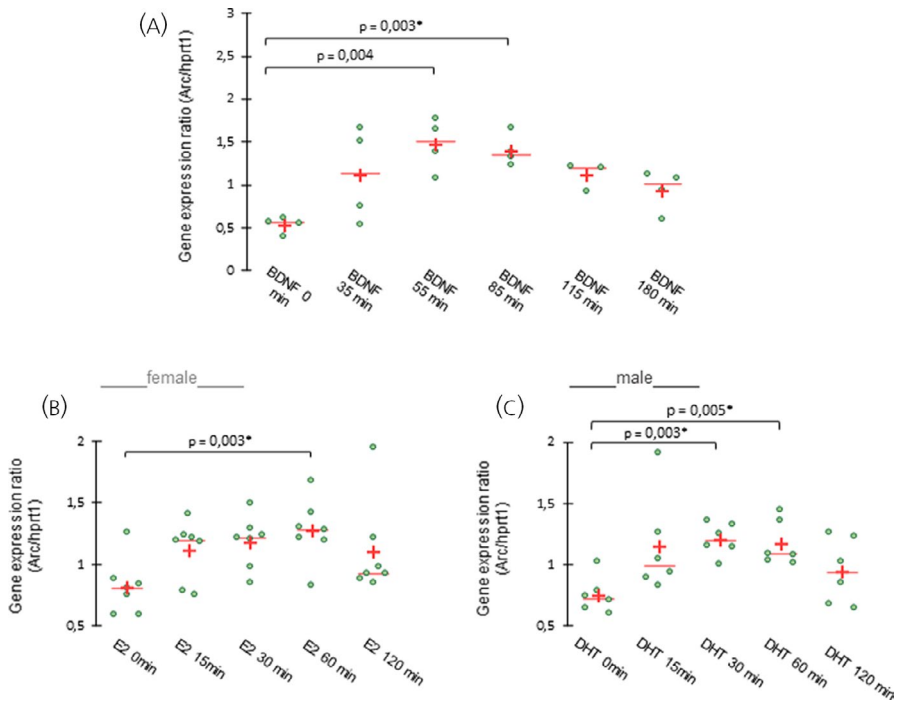


**FIGURE 2** Time-dependency of Arc/Arg3.1 expression in response to brain-derived neurotrophic factor (BDNF), estradiol ($E_2$) and dihydrotestosterone (DHT). Analysis of hippocampal Arc/Arg3.1 mRNA expression (normalized to hprt1 mRNA) in dissociated hippocampal cultures of female and male embryonic day 18 (E18) rats after 21 days in vitro. (A) Time dependency of Arc/Arg3.1. expression after BDNF ($10^{-8}$ M) treatment, to test the responsiveness of the cultures and to detect a temporal expression peak. Maximal expression was found after 85 min ($p = .003$) and 55 min ($p = .004$, Bonferroni corrected $p = .0033$, $n = 4$). (B) and (C) time dependency of Arc/Arg3.1. expression after sex steroid treatment. The females show a time-dependent increase after treatment with $E_2$ and reach their peak after 60 min (Bonferroni corrected $p = .0167$). Males peak after 30 min and remain at a significant level at 60 min (Bonferroni corrected $p = .0083$, $n = 6$ or 7). For better comparability, a 1-h treatment was chosen for both sexes. Statistical analysis was performed by a Kruskal–Wallis test followed by Dunn's procedure. Points, single values; cross, mean; line, median. $^*p < .05$

Using western blot analysis, we observed a similar pattern of regulation of Arc/Arg3.1 on the protein level. DHT was effective in male cultures, whereas $E_2$ was effective in female cultures (Figure 4). After inhibition of local $E_2$ and local DHT synthesis, no changes in Arc/Arg3.1 expression were observed, neither in male, nor in female cultures.

To further investigate the distribution of Arc/Arg3.1 within the neurons, we performed immunocytochemistry followed by image analysis to compare quantitatively staining intensities in various compartments of the neuron, namely the nucleus, cell soma and dendrites, after stimulation with sex steroids. The results are widely consistent with our results obtained by western blotting, insofar as $E_2$ upregulates Arc/Arg3.1 in female neurons mainly in the nuclei and the associated cytoplasm of the somata (Figure 5), whereas DHT predominantly upregulates Arc/Arg3.1 in the soma and dendrites of the male neurons (Figure 6).

### 3.3 | $E_2$ fails to upregulate Arc/Arg3.1 in the hippocampus if neuronal synthesis of $E_2$ is inhibited

Regarding the ineffectiveness of sex steroid blockers with respect to Arc/Arg3.1 protein and mRNA expression, we considerded that 1 h of treatment might not provide sufficient time to downregulate the synthesis of E2 or DHT, respectively. As another approach, we therefore pre-treated cultures of female animals with letrozole, our aromatase inhibitor, and cultures of male animals with finasteride, the blocker of 5α-reductase. The pre-treatment resulted in a downregulation of Arc/Arg3.1 in female cultures. A similar effect was not seen in male cultures that were pre-treated with finasteride (data not shown). Most importantly, $E_2$ failed to upregulate Arc/Arg3.1 protein expression in female cultures that were pre-treated with letrozole. Obviously, neuronal $E_2$ synthesis is a prerequisite for the upregulation of Arc/Arg3.1 in response to exogenously applied $E_2$ (Figure 7). A similar effect was not observed when we pre-treated male hippocampal cultures with finasteride. By contrast, DHT upregulated Arc/Arg3.1, as under control conditions, and was not affected by the pre-treatment (data not shown).

## 4 | DISCUSSION

In the present study, we show for the first time that transcription and translation of the immediate early gene Arc/Arg3.1 is upregulated by sex steroids in a sex-dependent manner. $E_2$ upregulates Arc/Arg3.1 in female, but not male, neurons and, vice versa, testosterone upregulates Arc/Arg3.1 in male, but not female, neurons. It appears

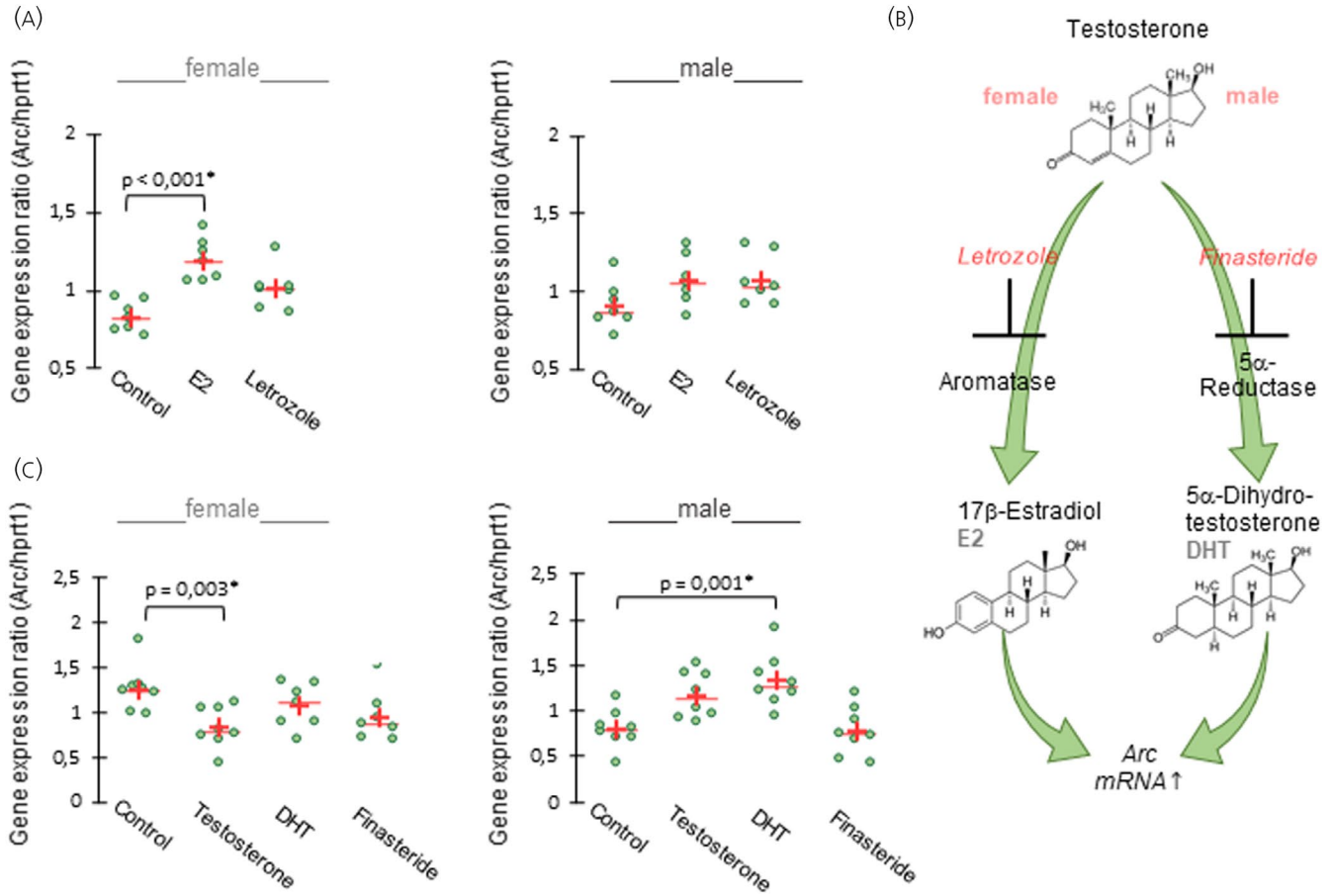


FIGURE 3 Arc/Arg3.1 mRNA transcription responds to sex steroid treatment in a sex-specific manner. (A) Arc/Arg3.1 mRNA levels increased significantly after 1 h of treatment with estradiol ($E_2$) ($10^{-8}$ M) in female, but not male, neurons. No effect was observed after application of letrozole, the inhibitor of $E_2$ synthesis, for 1 h (Bonferroni corrected $p = .0167$, $n = 7$) (B) Schematic overview of final sex steroid synthesis. Testosterone is either converted to $E_2$ via activity of aromatase or converted to dihydrotestosterone (DHT) via activity of 5α-reductase. Aromatase can be blocked by letrozole and 5α-reductase by finasteride. (C) In cultures of male, but not female neurons, a significant increase in Arc/Arg3.1 mRNA in response to 1 h of treatment with DHT was seen. In female neurons, the expression decreased in response to testosterone and increased in tendency in cultures of male neurons (Bonferroni corrected $p = .0083$, $n = 7$ or 8). No effect was seen after application of finasteride after 1 h, neither in female, nor in male cultures. Statistical analysis was performed by a Kruskal–Wallis test followed by Dunn's procedure. Points, single values; cross, mean; line, median. $^*p < .05$

that local neurosteroid synthesis is required to induce the effects because the effect of $E_2$ is abolished when neuronal $E_2$ synthesis is pharmacologically inhibited by letrozole.

### 4.1 | Arc/Arg3.1 and sex steroids in synaptic plasticity

Both Arc/Arg3.1 and sex steroids are involved in synaptic plasticity and memory. Synaptic plasticity describes the molecular basis of learning and memory, which includes modifying neuronal synapses in response to electrical activity. In the course of memory formation, two temporal phases have been defined. Short-term memory involves changes of synaptic efficacy by modifying existing proteins. Long-term memory requires new gene transcription and protein production, such as BDNF, to stabilize recent changes. LTP, long-term depression (LTD) and homeostatic plasticity represent cellular parameters for long-term memory. In LTP, specific synapses are strengthened. In LTD, specific synapses are weakened. In homeostatic plasticity, neuronal activity is balanced to maintain the maximal sensitivity of the neuron to future activity-dependent synaptic plasticity. Finally, synaptic plasticity is associated with structural changes, such as synapse formation/retraction and underlying changes of the actin cytoskeleton in postsynaptic dendritic spines.

Arc/Arg3.1 is induced under various experimental conditions, including LTP,[3,5] LTD,[34] novel environmental exploration[6,35,36] and learning.[37–39] Blocking Arc/Arg3.1 protein expression either by anti-sense oligonucleotide injection[40] or by knockout,[7] impairs LTP, whereas short-term memory is not affected. Arc/Arg3.1 protein also regulates synaptic strength and homeostatic scaling by promoting the internalization of AMPA-Rs. These findings indicate that Arc/Arg3.1 expression is regulated at multiple levels, which turns out to be altered in cognitively impaired aged rats.[41] Translation of newly induced Arc/Arg3.1 mRNA is required for cofilin phosphorylation

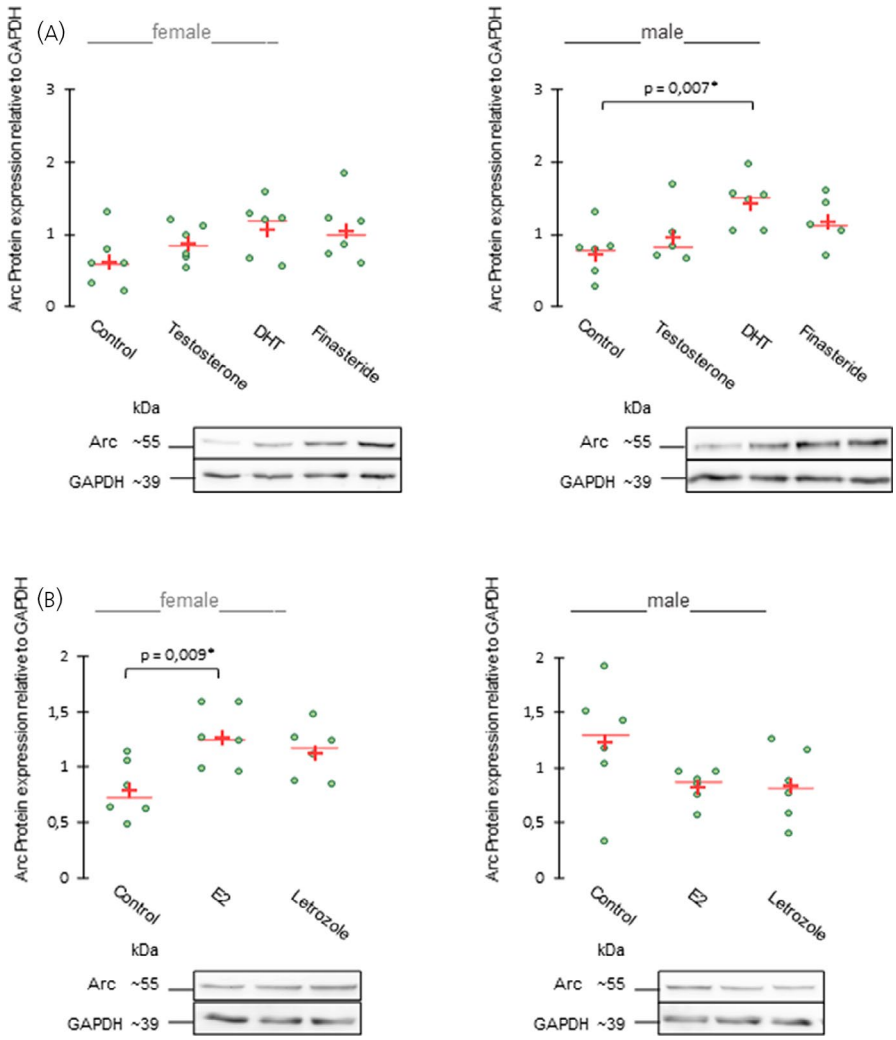


**FIGURE 4** Arc/Arg3.1 translation responds to treatment with sex steroids in a sex-specific manner. Western blot analysis of Arc/Arg3.1 protein expression in hippocampal neurons at 21 days in vitro after a 1-h treatment with sex steroids and their inhibitors. Protein levels were normalized to GAPDH and expressed relative to the control. (A) Male, but not female, neurons respond with a significant increase in protein expression to the treatment with dihydrotestosterone (DHT) ($10^{-8}$ M; Bonferroni corrected $p = .0083$, $n = 5$ or 6). (B) Female, but not male, neurons respond to stimulation with estradiol ($E_2$) (Bonferroni corrected $p = .0167$, $n = 6$). Statistical analysis was performed by a Kruskal–Wallis test followed by Dunn's procedure. Points, single values; cross, mean; line, median. $^*p < .05$

and stable expansion of the F-actin cytoskeleton underlying LTP consolidation[42] and, consistently, spine density is increased after overexpression of Arc/Arg3.1 in vitro, and disruption of Arc/Arg3.1 decreases spine density in vivo.[43] In addition, Arc/Arg3.1 synthesis is necessary for the activity of key translation factors during LTP consolidation, such as BDNF. BDNF is a member of the neurotrophin family of small, secreted peptides, and is critically involved in hippocampal plasticity and, in particular, in late LTP formation.[44] Activity-dependent synthesis and release of BDNF drives LTP, protein synthesis and structural remodelling in hippocampal synapses,[45,46] and it is required for hippocampal memory.[47–49] BDNF-induced LTP, in turn, was found to be associated with rapid Arc/Arg3.1-dependent enhancement.[50]

Among sex steroids, namely testosterone, progesterone and $E_2$, the influence of $E_2$ on learning and memory is the most extensively documented, as reviewed previously.[9] It was reported as early as in the 1990s that $E_2$ increases spine and spine synapse density in the hippocampus[51,52] and stimulates TBS-induced LTP. The magnitude of LTP,[53,54] together with that of spine density,[55] correlates with the estrous cycle, and it is highest during proestrus when $E_2$ levels in serum reach their maximum. $E_2$ has also been found to activate BDNF levels[56–60] and its receptor TrkB, at least in the context of rapid action of $E_2$ via signalling cascades.[61–64] Finally, in numerous studies, it was shown that $E_2$ enhances memory consolidation.[9] The particular role of BDNF/TrkB with respect to $E_2$-induced memory consolidation was also recently reported.[65–68]

The role of androgen has been less investigated compared to that of $E_2$.[19] Nevertheless, numerous studies provide evidence that testosterone exerts similar effects on hippocampal synaptic plasticity analogous to those of $E_2$ in females.[16,18,69-71] Similar to $E_2$, testosterone has also been shown to enhance memory, which is associated with changes in BDNF/TrkB levels.[72,73] Collectively, a notable overlap exists in the mechanisms through which sex steroids and Arc/Arg3.1 influence hippocampal plasticity and memory. It could very well be that sex steroids function upstream of LTP, Arc/Arg3.1 mobilization, F-actin assembly/disassembly, cofilin phosphorylation and spine formation.

## 4.2 | Arc/Arg3.1 is regulated by sex steroids in a sex-dependent manner

In the context of sex steroid-induced synaptic plasticity, the sex of the animals is often neglected and, regarding $E_2$ function, the focus

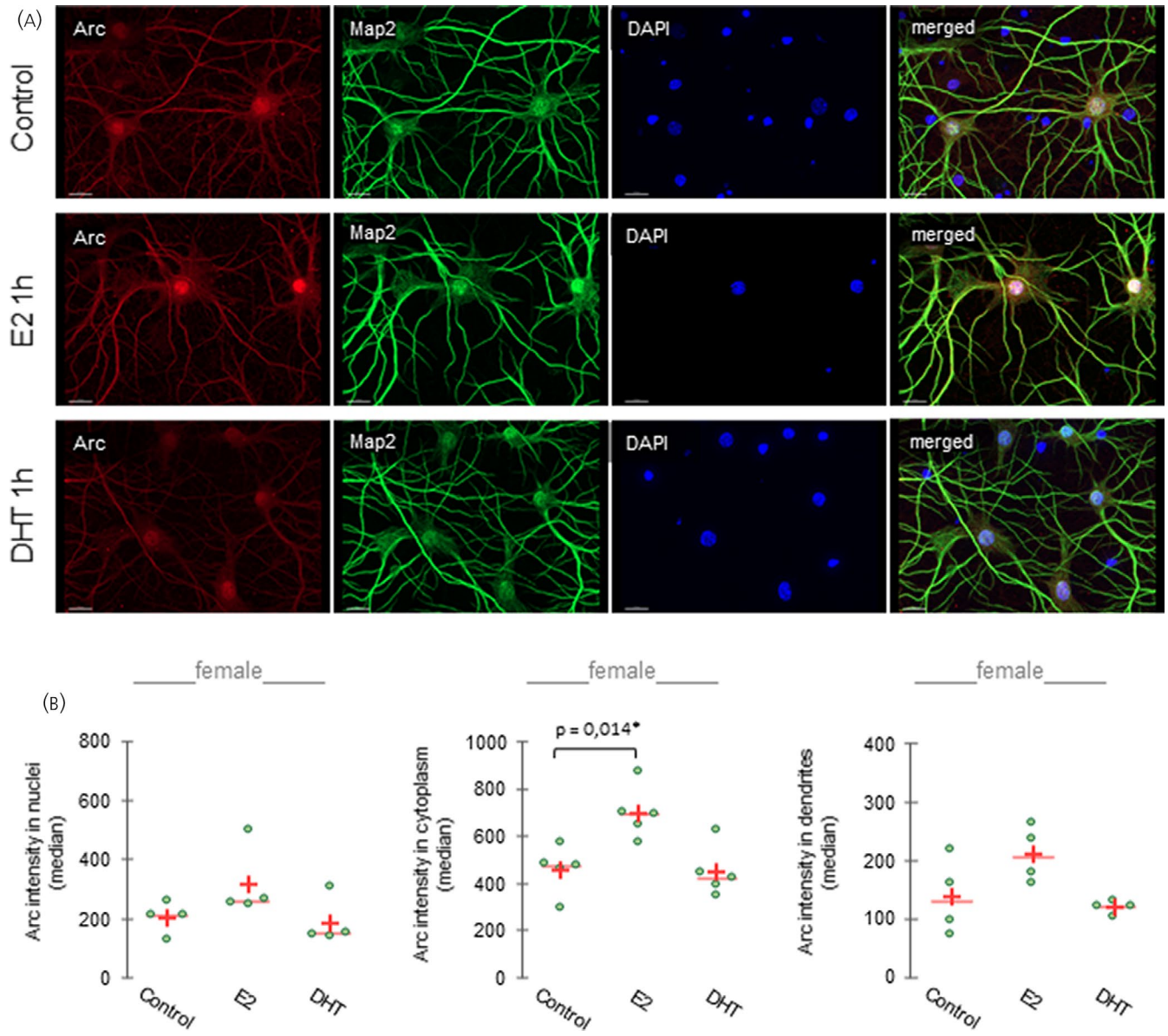


FIGURE 5 Arc/Arg3.1 protein expression is increased in female neurons after treatment with estradiol ($E_2$). (A) Example figures of Arc/Arg3.1 immunoreactivity in dissociated female hippocampal neurons at 21 days in vitro. Staining was performed after a 1-h treatment with $E_2$ ($10^{-8}$ M) and dihydrotestosterone (DHT) ($10^{-8}$ M). Co-staining with MAP2 (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue) was used to reconstruct the neurons for analysis. (B) Image analysis of Arc/Arg3.1. immunoreactivity in the nuclei, cytoplasm and dendrites. In female neurons, Arc/Arg3.1 immunoreactivity was significantly enhanced in response to $E_2$ and unaffected in response to DHT (Bonferroni corrected $p = .0167$, $n = 4$). Statistical analysis was performed by a Kruskal–Wallis test followed by Dunn's procedure. Points, single values; cross, mean; line, median. $^*p < .05$

is clearly on data from female animals. Nevertheless, it should be noted that effects regarding synaptic plasticity were also shown after application of $E_2$ to the male hippocampus.[9,30] Except for our finding that DHT decreases spine number in female dissociated cultures,[25] studies on the effects of DHT in the female hippocampus do not exist so far.

In ovariectomized animals, CA1 pyramidal spine synapse density increases after treatment with $E_2$ or testosterone.[18] The rescue of spine density in response to testosterone is likely induced by local $E_2$ production.[23] In orchiectomized males, however, $E_2$ has no significant effect,[16] whereas replacement with either testosterone or the non-aromatizable DHT both reverse orchiectomy-induced spine synapse loss. These early data already suggest a sex-dependency in that $E_2$ appears to be the relevant sex steroid in females and testosterone in males regarding synaptic plasticity in the hippocampus, for which our recent studies also provide evidence.[24,25] The clear sex-dependency became evident after inhibition of local synthesis of $E_2$ in females or DHT synthesis, respectively, in males.[24,25] In terms of physiology, this approach appeared to us more adequate compared to the commonly used exogenous application of sex steroids. Sex steroids are locally synthesized and secreted[20,21] and they exert their functions in a paracrine manner.[74]

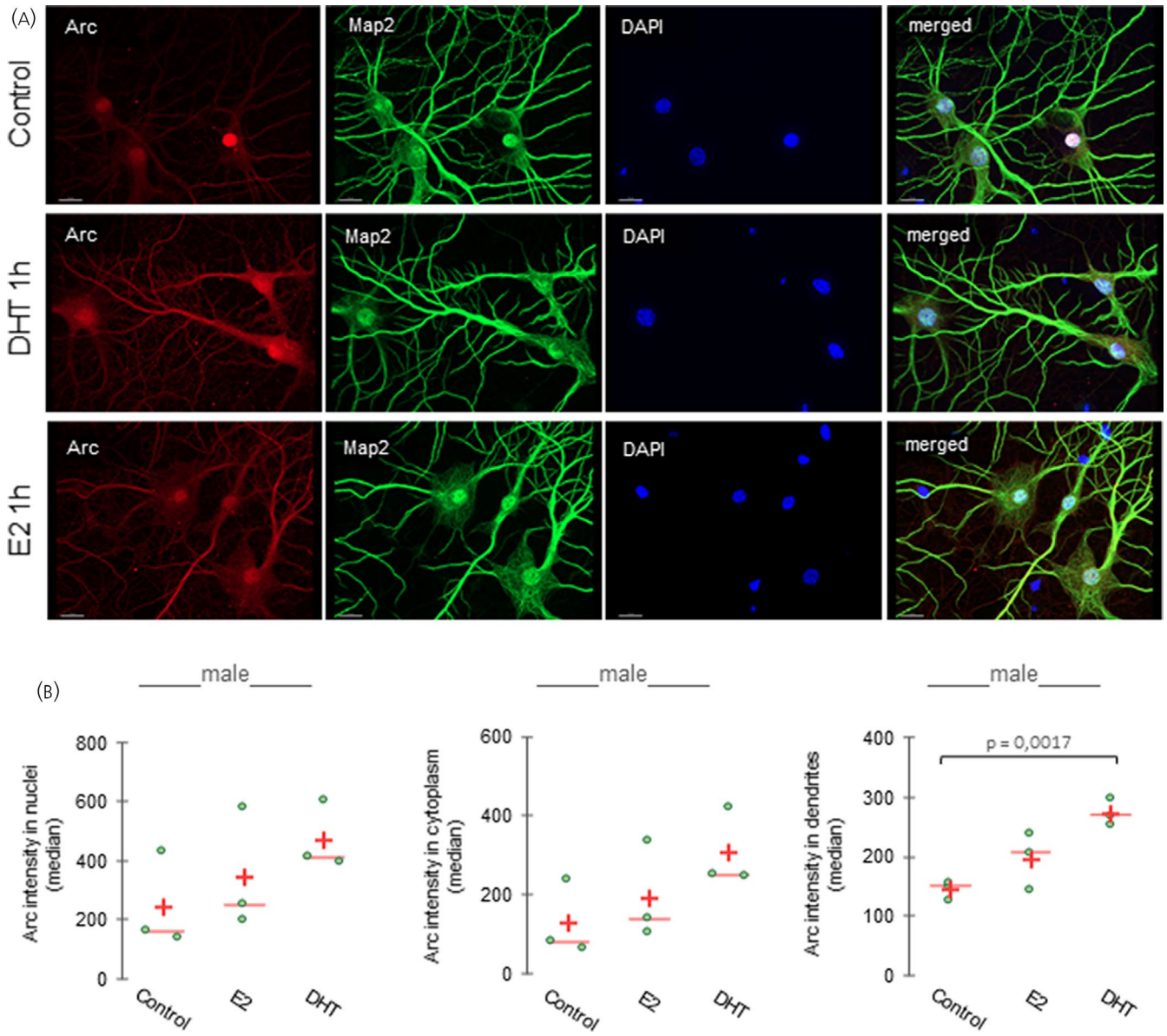


**FIGURE 6** Arc/Arg3.1 protein expression is enhanced after stimulation with dihydrotestosterone (DHT). (A) Example figures of Arc/Arg3.1 immunoreactivity in dissociated male hippocampal neurons at 21 days in vitro. Staining was performed after a 1-h treatment with estradiol ($E_2$) ($10^{-8}$ M) and dihydrotestosterone (DHT) ($10^{-8}$ M). Co-staining with MAP2 (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue) was used to reconstruct the neurons for analysis. (B) Image analysis of Arc/Arg3.1. immunoreactivity in the nuclei, cytoplasm and dendrites. In male neurons, Arc/Arg3.1 immunoreactivity was enhanced in response to DHT, in all areas of the neuron, especially in dendrites ($p = .017$). Statistical analysis was performed by a Kruskal–Wallis test followed by Dunn's procedure (Bonferroni corrected $p = .0167$, $n = 3$). Points, single values; cross, mean; line, median

Upon this background, it is even more amazing that the clear sex-dependency regarding Arc expression ($E_2$ specifically only in females and testosterone/DHT specifically only in males) was seen after exogenously administered testosterone/DHT and $E_2$ in the present study. The effects after testosterone treatment were more pronounced than the effects following $E_2$ treatment, which could be a result of the higher threshold for hippocampal LTP in females compared to males.[30] Above all, the demonstration of the $E_2$ effect required neuron-derived $E_2$ in female cultures. After long-term pre-treatment of the cultures with letrozole, an aromatase inhibitor, the upregulation of Arc/Arg3.1 in response to $E_2$ was abolished. It appears that a certain level of $E_2$ in the tissue, which, according to Hojo and Kawato[75] is considerably higher than in serum, is a prerequisite for the demonstration of $E_2$ function. This was shown in $E_2$-induced spine formation. Application of $E_2$ together with letrozole compensated the effects of application of letrozole only up to the control level, but not beyond that.[76] Moreover, estrogen receptors (ER) could play an additional role, which also has been demonstrated in E2-induced synaptic plasticity. Downregulation of E2 synthesis downregulated ERα, the ER subtype responsible for spine formation on the genomic level,[20,77,78] and spine formation, in turn, depends on Arc/Arg3.1 induction.[43] Unfortunately,

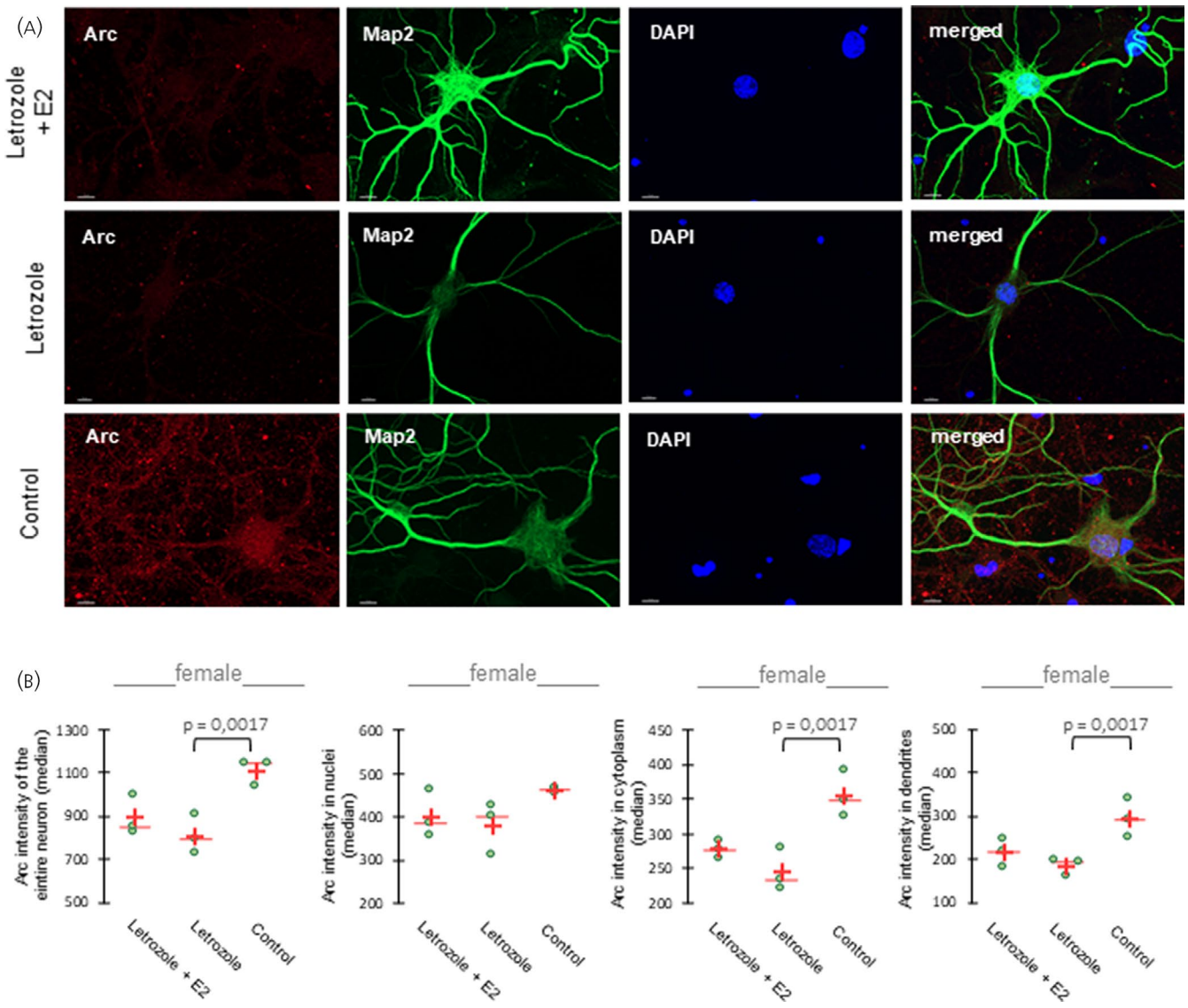


FIGURE 7 Inhibition of neuron-derived estradiol ($E_2$) synthesis reduced expression of Arc and abolished the effects of estradiol ($E_2$) in female neurons. (A) Example figures of Arc/Arg3.1 immunoreactivity in dissociated female after pre-treatment of the cultures with letrozole from 14 to 21 days in vitro (DIV) every 48 h to inhibit neuronal $E_2$ synthesis. At 21 DIV, one pre-treated group was treated with $E_2$ for 1 h (Letrozole + $E_2$), one pretreated group received no $E_2$ treatment (Letrozole) and an untreated group served as a control group. MAP2 (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue) co-staining was used to reconstruct the neurons for analysis. (B) Arc/Arg3.1 staining intensity of neurons within the three groups. Pre-treatment with letrozole abolished the enhancing effect of $E_2$ on Arc/Arg3.1 expression. This effect was similar in all compartments of the neuron, namely the nuclei, cytoplasm and dendrites. Letrozole alone induced reduced Arc/Arg3.1 expression compared to the untreated control group. This became particularly evident in the cytoplasm and in dendrites ($p = .017$). With Bonferroni corrected $p = .0167$, statistical significance is closely missed. Statistical analysis was performed by a Kruskal–Wallis test followed by Dunn's procedure ($n = 3$). Points, single values; cross, mean; line, median

in the case of testosterone, this could not be demonstrated, very likely because finasteride not only inhibits DHT synthesis, but also rapidly enhances testosterone levels[79,80] and testosterone and DHT act synergistically with respect to Arc/Arg3.1 expression.

Finally, we hypothesize that sex-dependent maintenance of LTP and synaptic stability in response to sex neurosteroids[25] goes along with, or even mediates, sex- and sex steroid-dependent induction of Arc/Arg3.1. This would explain why Arc/Arg3.1 is critical for both increases and decreases in synaptic strength.[81] Future experiments are required to decipher the role of Arc/Arg3.1 in sex-dependent responsiveness of hippocampal neurons to sex steroids.

## ACKNOWLEDGEMENTS

The study was supported by the Deutsche Forschungsgemeinschaft (DFG 436/7-1). Open access funding enabled and organized by ProjektDEAL.

## CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

## AUTHOR CONTRIBUTIONS

**Janina Brökling:** Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualization. **Bianka Brunne:** Software; Visualization. **Gabriele M. Rune:** Conceptualization; Data curation; Funding acquisition; Project administration; Supervision; Writing – original draft.

## PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/jne.13090.

## DATA AVAILABILITY STATEMENT

Data are available from the corresponding author upon reasonable request.

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**How to cite this article:** Brökling J, Brunne B, Rune GM. Sex-dependent responsiveness of hippocampal neurons to sex neurosteroids: A role of Arc/Arg3.1. *J Neuroendocrinol*. 2022;34:e13090. doi:10.1111/jne.13090

## 2. Darstellung der Publikation mit Literaturverzeichnis

### Kontext der Arbeit

Die vorgelegte Veröffentlichung (Brokling et al., 2022) beschäftigt sich mit dem geschlechtsspezifischen Einfluss von Neurosteroiden auf die Expression von Arc/Arg3.1 (engl.: Activity-regulated cytoskeleton-associated protein) in hippocampalen Neuronen. Das aktivitätsregulierte Zytoskelett-assoziierte Protein Arc/Arg3.1 gehört zu der Familie der Immediate Early Genes (IEG) und nimmt eine zentrale Rolle innerhalb der synaptischen Plastizität und der Gedächtnisbildung ein (Shepherd and Bear, 2011). 1995 wird erstmalig von Link et al. und Lyford et al. seine Regulation durch neuronale Aktivität, sowie die verstärkte Transkription von Arc/Arg3.1 mRNA durch Langzeitpotentierung (LTP) und ihren Transport in die Dendriten beschrieben (Link et al., 1995, Lyford et al., 1995). Die Übersichtsarbeit von Nikolaienko et al. (2018) veranschaulicht neben den zahlreichen Interaktionsproteinen auch die Signalkaskaden, in die Arc/Arg3.1 involviert ist. Hier zeigt sich, dass Arc/Arg3.1 über die Endozytose von α-Amino-3-hydroxy-5-methyl-4-isoxazol-propionsäure Rezeptoren (AMPAR) Langzeitdepressionen (LTD) initiiert und Einfluss auf die homöostatische Skalierung nimmt (Nikolaienko et al., 2018).
Klassische Arc/Arg3.1 Knockout-Mäuse weisen starke Beeinträchtigungen in der synaptischen Plastizität in Form von Defiziten in der Langzeitgedächtnisbildung auf, wohingegen das Kurzzeitgedächtnis unbeeinträchtigt bleibt (Plath et al., 2006). Von besonderer Relevanz scheint das Protein auch während der frühen Gehirnentwicklung zu sein, in der es zu einer transienten Hochregulation der Arc/Arg3.1 Expression kommt. Wird in diesem Zeitraum ein konditioneller Knockout initiiert, zeigen die betroffenen Tiere lebenslange Lernbeeinträchtigungen. Wird das Gen erst nach dieser kritischen Phase ausgeschaltet, bleibt die initiale Lernfähigkeit intakt, allerdings ist eine Konsolidierung ins Langzeitgedächtnis nicht möglich (Gao et al., 2018).

Die Einflussnahme von Estradiol (E2) auf Neuronen und ihre Plastizität wurde im Jahr 1990 erstmals von Woolley et al. beschrieben und gelang folglich in den Mittelpunkt vieler Forschungsarbeiten (Gould et al., 1990, Gould et al., 1991, Woolley, 1998, Woolley et al., 1990). Einen umfassenden Überblick über die Forschungsergebnisse der letzten drei Jahrzehnte zu diesem Thema gibt die Arbeitsgruppe von Karyn M. Frick (Taxier et al., 2020). E2 hat sich seither als wirksamer Regulator neuronaler Mechanismen bestätigt. Es spielt eine entscheidende Rolle in der Gedächtnisbildung, einschließlich Zellsignalübertragung, Genexpression, Proteinbiosynthese, der extrinsischen und intrinsischen Erregbarkeit, der Bildung von dendritischen Dornfortsätzen und der

Neurogenese. Obwohl es sich bei E2 um ein weibliches Hormon handelt, konnte gezeigt werden, dass es auch im männlichen Gehirn das Gedächtnis beeinflusst, jedoch seien in diesem Fall andere Signalwege involviert (Huang and Woolley, 2012, Tabatadze et al., 2015, Oberlander and Woolley, 2016, Ruiz-Palmero et al., 2016, Wang et al., 2018, Jain et al., 2019).

Geschlechtsspezifische Effekte zeigten sich auch unter dem Einfluss von Testosteron. Analog zu den Forschungsergebnissen von Woolley konnten Leranth und Kollegen den Einfluss von Androgenen auf Neuronen nachweisen. In männlichen, gonadektomierten Ratten zeigte sich durch eine Reduktion der Sexualhormone ein Verlust von dendritischen Dornfortsätzen und Synapsen im Hippocampus (Leranth et al., 2004, Leranth et al., 2003, MacLusky et al., 2006). Des Weiteren konnte gezeigt werden, dass hippocampale Neuronen eigenständig in der Lage sind, mit Hilfe der Enzyme Aromatase und 5$\alpha$-Reduktase aus Cholesterin *de novo* die Neurosteroide E2 und Dihydrotestosteron (DHT) zu synthetisieren (Hojo et al., 2004, Kretz et al., 2004, Torres and Ortega, 2006, Castelli et al., 2013).

Geschlechtsspezifische Unterschiede in Bezug auf die Neurosteroide werden insbesondere ersichtlich, wenn diese *de novo* Synthese pharmakologisch oder genetisch gehemmt wird: Die Hemmung der E2-Synthese durch die Inhibition der Aromatase reduziert die Dichte der Dendriten und Synapsen und beeinträchtigt die Entstehung von LTPs im weiblichen, nicht jedoch im männlichen Hippocampus. Durch die Hemmung der 5$\alpha$-Reduktase wird die Synthese von Testosteron zu seinem potenteren Metaboliten DHT inhibiert und führt in dem männlichen Hippocampus zu einer Synapsenreduktion und einer beeinträchtigten LTP-Bildung (Kretz et al., 2004, Brandt et al., 2020, Vierk et al., 2012, Fester et al., 2009).

Basierend auf den bisherigen Forschungsergebnissen stellt sich die Frage, ob es einen potenziellen Zusammenhang zwischen neurosteroid-induzierter synaptischer Plastizität und der Expression von Arc/Arg3 gibt. Da bereits oben genannte Studien ein klares Indiz für einen geschlechtsspezifischen Unterschied in der Gedächtnisbildung zeigen, sollen in einem ersten Schritt mögliche geschlechtsabhängige Unterschiede in der Expression von Arc/Arg3.1 untersucht werden.

**Methoden**

Um den geschlechtsspezifischen Einfluss der Neurosteroide auf hippocampale Neurone zu untersuchen, wurden primäre Dispersionskulturen genutzt. Dazu wurden die Hippocampi von Wistar Ratten im Embryonalstadium 18 (E 18) präpariert, nach ihren Geschlechtern getrennt und unter regelmäßigen Vitalitätskontrollen im Inkubierschrank kultiviert. Nach 21

DIV (engl. days in vitro, deut.: Tage in vitro) zeigten die Zellen deutliche Zellausläufer und Zellverbindungen.

Je nach Versuchsaufbau erfolgte anschließend die Stimulation mit den Neurosteroiden Testosteron, DHT und Estradiol oder mit dem Aromatasehemmer Letrozol beziehungsweise 5α-Reduktasehemmer Finasterid. Die Kontrollgruppen wurde zur besseren Vergleichbarkeit mit Dimethylsulfoxid (DMSO) als Vehikel behandelt. Die Konzentration von $10^{-8}$ M orientiert sich an der Veröffentlichung von Chamnainsawat & Chongthammakum (Chamniansawat and Chongthammakun, 2009), die den Einfluss von Estradiol auf Arc/Arg3.1 in einer SH-SY5Y Zelllinie untersuchten, sowie an internen Vorversuchen. Der Wachstumsfaktor BDNF (engl.: Brain-derived neurotrophic factor, deut.: Vom Gehirn stammender neurotropher Faktor) wurde in Vorversuchen genutzt und diente als Stimulanz zur Erstellung einer Zeitreihe, um die Zeitspanne der Arc/Arg3.1 Expression präziser einschätzen zu können. Daran orientierend wurden geschlechtsspezifische Zeitreihen erstellt, welche mit Neurosteroiden stimuliert wurden und eine gemeinsame signifikante Steigerung der Expression bei etwa 60 min zeigten. In weiteren Versuchen wurde deshalb eine Stimulationsdauer von 60 min gewählt.

Um eine Hochregulation der Genexpression zu detektieren, wurde nach RNA Aufbereitung aus den stimulierten Dispersionskulturen eine quantitative Echtzeit-Polymerase-Kettenreaktion (engl.: quantitative real-time polymerase chain reaction, RT-qPCR) durchgeführt. Als endogene Kontrolle wurde Hypoxanthin-Phosphoribosyl-Transferase 1 (hprt1) genutzt, welche durch die Stimulantien nicht beeinflusst wurde. Das Effizienz-korrigierte Model von Pfaffl (Pfaffl, 2001) wurde vor der statistischen Auswertung angewendet.

Mittels Western Blot wurde die Menge an Arc/Arg3.1 auf Proteinebene analysiert. Dazu wurden die Zelllysate nach einstündiger Stimulation aufgearbeitet. Mit Hilfe der Proteinbestimmung nach Bradford konnte photometrisch die Proteingesamtmenge in den Proben bestimmt werden und jeweils 30µg pro Probe im Western Blot nachgewiesen werden. Zur Auswertung wurde die Expression von Arc/Arg3.1 ins Verhältnis zur endogenen Kontrolle Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) gesetzt.

Die immunzytochemische Färbung ermöglichte die genauere Lokalisation der Arc/Arg3.1 Verteilung. Dazu wurde neben Arc/Arg3.1 sowohl das Mikrotubuli-assoziierte Protein 2 (Map2) zur Rekonstruktion der Neurone als auch deren Zellkern mittels 4′,6-Diamidin-2-phenylindol (DAPI) angefärbt. Mittels der Analysesoftware *Imaris* wurden die Neurone rekonstruiert und jeweils die Intensität der Arc/Arg3.1 Färbung im Nucleus, im Cytoplasma des Somas und in den Dendriten ausgewertet.

Die statistische Auswertung der nicht normalverteilten Daten wurden mit einem nichtparametrischen Test durchgeführt. Je nach Anzahl der zu vergleichenden Gruppen

wurde der Mann-Whitney Test (zwei Gruppen) oder der Kruskal-Wallis Test (> zwei Gruppen) durchgeführt. Stellte sich ein signifikanter Unterschied dar ($p<0,05$), wurde ein anschließender post-hoc Test (Dunn Prozedur) angeschlossen. Durch das multiple Testen und der einhergehenden erhöhten Irrtumswahrscheinlichkeit einen Fehler 1. Art (fälschliches Ablehnen der Nullhypothese) zu begehen, wurde eine Bonferroni-Korrektur zur Anpassung des Signifikanzniveaus durchgeführt. Die Berechnung und Darstellung der Daten erfolgte mit Hilfe der XLSTAT 2021 Software.
Für detailliertere Angaben zu der Methodik wird auf die Originalarbeit verwiesen (Brokling et al., 2022)

**Ergebnisse**

In den hippocampalen Dispersionskulturen konnte nach 21 DIV in beiden Geschlechtern unter Kontrollbedingungen sowohl Arc/Arg3.1 mRNA als auch das Protein nachgewiesen werden. Dabei zeigte sich kein signifikanter Unterschied zwischen den Geschlechtern, weder in der quantitativen Menge noch im Verteilungsmuster des Proteins.
Nach Stimulation der weiblichen und männlichen Neuronenkulturen mit jeweils E2 und DHT zeigte sich eine deutliche zeitabhängige geschlechtsspezifische Genexpression. Die weiblichen Neuronen reagierten bereits nach 15-minütiger Stimulation mit E2 mit einer Hochregulation der Arc/Arg3.1 Genexpression und blieben auf einem erhöhten Niveau. Nach einer Stunde zeigte sich ein signifikanter Unterschied im Vergleich zur Ausgangssituation, dieser Unterschied reduzierte sich nach zweistündiger Stimulation. Die Stimulation mit DHT erwirkte in den weiblichen Neuronen keine signifikante Steigerung, jedoch in den männlichen Neuronen. Auch sie zeigten bereits nach 15-minütiger DHT Stimulation eine gesteigerte Genexpression, erreichten bereits nach 30 min einen signifikanten Anstieg, der auch nach einstündiger Stimulation nachzuweisen war und fiel dann ebenfalls nach zwei Stunden wieder ab. Bei den männlichen Neuronenkulturen blieb ein signifikanter Effekt unter E2 aus.
Ebenso konnte auf Proteinebene ein identischer geschlechtsabhängiger Effekt der Arc/Arg3.1 Expression mittels Western Blot gezeigt werden. Eine einstündige Stimulation mittels den Inhibitoren Letrozol und Finasterid reichte nicht aus, um einen Effekt zu erzielen.
Zahlreiche Studien konnten bislang zeigen, dass Arc/Arg3.1 an verschiedenen Lokalisationen über unterschiedliche Signalwege an der synaptischen Plastizität beteiligt ist (Nikolaienko et al., 2018, Zhang and Bramham, 2020). Deshalb stand im Fokus des nächsten Versuchsaufbaus die Lokalisation des exprimierten Arc/Arg3.1 Proteins. Auch hier zeigten sich geschlechtsspezifische Unterschiede: Während sich Arc/Arg3.1 in den weiblichen Neuronen vor allem signifikant in dem Cytoplasma des Somas anreicherte und

nur nachrangig in den Dendriten und in den Nuclei, zeigte sich bei den männlichen Neuronen die deutlichste Anreicherung in den Dendriten.
Wie einleitend bereits beschrieben, konnten viele Arbeiten den Effekt nicht nur durch exogen hinzugefügte Neurosteroide erzielen, sondern durch Hemmung der Steroidsynthese auch einen Rückgang synaptischer und dendritischer Fortsätze provoziert werden. Um zu untersuchen, ob ein geringerer Estradiolspiegel in den Zellkulturen durch die endogene Hemmung der Estradiolsynthese mittels des Aromatasehemmers auch einen Effekt auf die Arc/Arg3.1 Expression hat, wurden die weiblichen Neuronenkulturen nach 14 DIV eine Woche lang alle 48h mit Letrozol behandelt und die Kontrollgruppe im gleichen Zeitintervall mit dem Vehikel DMSO. Am 21 DIV wurde eine vorbehandelte Gruppe für eine Stunde mit E2 stimuliert, während die anderen beiden mit dem Vehikel behandelt wurden.

Die immunhistochemische Auswertung zeigte einen deutlichen Rückgang der Arc/Arg3.1 Expression in der vorbehandelten Gruppe mit Letrozol. Als weiteres Ergebnis zeigte sich, dass durch die Vorbehandlung mit Letrozol der hochregulierende Effekt durch E2 ausblieb. Es bleibt also festzuhalten, dass Arc/Arg3.1 unter dem Einfluss von endogenen E2 stärker exprimiert wird und eine exogene Stimulation mit E2 frustran ist, sobald die endogene Steroidsynthese zuvor blockiert wurde.
Als ergänzendes Experiment wurde der identische Versuchsaufbau für die männlichen Neuronen mit der 5$\alpha$-Reduktase Finasterid und DHT durchgeführt. Hierbei zeigte sich jedoch keine verminderte Arc/Arg3.1 Expression, es zeigte sich lediglich eine wenig effizientere Stimulation durch DHT nach der Vorbehandlung mit Finasterid. Dieses Ergebnis lässt sich am ehesten dadurch begründen, dass durch die gehemmte DHT Synthese vermehrt Testosteron zur Verfügung steht. Dies ist zwar weniger potent, könnte aber durch die einwöchige Vorbehandlung einen stimulierenden Effekt erzielt haben, sodass das Grundniveau der Arc/Arg3.1 Expression bereits leicht erhöht war. Diese Hypothese müsste mit weiteren Versuchen validiert werden und ist als Ausblick zu betrachten.

**Diskussion**

Die Ergebnisse der vorgelegten Arbeit zeigen, dass sowohl die Transkription als auch die Translation von Arc/Arg3.1 durch E2 und DHT geschlechtsabhängig beeinflusst wird und somit die Neurosteroide Einfluss auf die synaptische Plastizität nehmen. Synaptische Plastizität beschreibt die molekulare Adaption auf Zellebene durch elektrische Aktivitäten während Lernprozessen und der Gedächtnisbildung. Dabei wird zwischen dem Kurzzeit- und dem Langzeitgedächtnis differenziert. Während für die kurzfristige Anpassung vor allem bereits existierende Proteine modifiziert werden, benötigt eine langfristige Anpassung

die Transkription von Genen und eine Proteinbiosynthese zur Verbesserung der neuronalen Konnektivität. Dabei sorgen LTPs für eine Verbesserung der synaptischen Verbindungen, beispielsweise in Form eines gestärkten Aktin-Zytoskeletts der Synapsen. LTDs führen hingegen zu einer Minderung synaptischer Verbindungen.
Studien zeigen, dass die Arc/Arg3.1 Expression durch verschiedene Stimuli induziert werden kann, wie zum Beispiel durch LTPs (Steward et al., 1998), durch LTDs (Wilkerson et al., 2018), durch die Entdeckung neuer Umgebungen (Vazdarjanova and Guzowski, 2004) und durch Lernprozesse (Guzowski et al., 2001, Pinaud et al., 2001). Wird die Arc/Arg3.1 Expression durch anti-Sense Oligonukleotid-Injektionen (Guzowski et al., 2000) oder mittels Knockouts (Plath et al., 2006) gehemmt, zeigt sich die Gedächtniskonsolidierung beeinträchtigt.
Wie einleitend erwähnt, konnten viele Studien den Einfluss von Neurosteroiden auf die synaptische Plastizität beweisen, jedoch nur selten unter Berücksichtigung des Geschlechts. Auch wenn der Fokus der Untersuchungen des E2-Effekts auf die verbesserte Synapsendichte bei den Weibchen lag, konnten Arbeiten auch einen Effekt auf männliche Neuronen verzeichnen (Bayer et al., 2013). *Vice versa* besteht nur eine dünne Studienlage für den Einfluss von DHT auf die Neuronen des weiblichen Hippocampus (Brandt et al., 2020).
Besonders deutlich wird die Relevanz von Neurosteroiden für die synaptische Plastizität, wenn statt einer exogenen Applikation die lokale Steroidsynthese gehemmt wird und eine verminderte Synapsendichte zu beobachten ist (Prange-Kiel et al., 2006, Brandt and Rune, 2020).
In der vorliegenden Arbeit sorgt bereits eine einstündige Stimulation mittels Neurosteroiden zu einer Hochregulation der Arc/Arg3.1 Expression. Da Arc/Arg3.1 jedoch lediglich ein Marker für synaptische Plastizität ist und eine Hochregulation nicht direkt Aufschluss über die funktionelle Bedeutung gibt, stehen die Ergebnisse in keinem Widerspruch zu den bisherigen Neurosteroiduntersuchungen.
Des Weiteren zeigte die verstärkte Arc/Arg3.1 Anreicherung geschlechtsspezifische Verteilungsmuster nach der einstündigen Stimulation mittels Neurosteroiden. Da Arc/Arg3.1 funktionell sehr vielseitig ist und auch in Abhängigkeit seiner Lokalisation verschiedene Funktionen erfüllt (Bloomer et al., 2007, Wee et al., 2014, Pastuzyn et al., 2018), bleibt zu spezifizieren, welche funktionelle Relevanz die geschlechtsspezifische Arc/Arg3.1 Verteilung in den Neuronen mit sich bringt.

Die weiblichen Neurone zeigten zudem eine verminderte Arc/Arg3.1 Expression nach einer einwöchigen Behandlung mit Letrozol. Dass weibliche Neuronen mit einer verminderten Synapsendichte auf einen reduzierten Estrogenspiegel reagieren, stimmt mit dem Ergebnis

bisheriger Studien überein (Zhou et al., 2010). Zhang und Bramham (2020) veranschaulichen in ihrer Übersichtsarbeit, wie Arc/Arg3.1 über eine Interaktion mit Drebrin A und F-Aktin die Synapse stabilisiert und das Recycling von AMPAR induziert, sodass eine schnellere Depolarisation der Neurone folgt. Auf der funktionellen Ebene konnten Vierk und Kollegen zeigen, dass nach einer einwöchigen Behandlung von weiblichen Schnittkulturen mit Letrozol die Induktion von LTPs durch theta-bursts nicht mehr möglich war (Vierk et al., 2012).
Die Ergebnisse unseres Versuchs – eine reduzierte Arc/Arg3.1 Expression durch Letrozol – könnte den molekularen Hintergrund für die neuronale Beeinträchtigung erklären. Widersprüchlich zu dieser Hypothese stehen jüngste Ergebnisse von Kryke-Smith und Kollegen (2021) die zeigten, dass auch Arc Knockout-Mäuse theta-burst induzierte LTPs bilden konnten, welche elementar für die Gedächtnisbildung sind. Konträr dazu stehen vorherige Forschungsergebnisse, in denen sich Arc/Arg3.1 als notwendig für die Induktion und Konsolidierung von LTPs ist herausstellte (Messaoudi et al., 2007).

Des Weiteren zeigte sich in diesem Versuchsaufbau, dass nach Vorbehandlung mit Letrozol eine darauffolgende Stimulation mit E2 keine Hochregulation der Arc/Arg3.1 Expression erzielen konnte. Erklärend für dieses Ergebnis könnte eine Runterregulierung der Estrogenrezeptoren (ER) durch Feedback-Mechanismen aufgrund des niedrigen E2 Spiegels in den Neuronen sein. Eine konzentrationsabhängige Regulation der ER Expression wurde bereits mehrfach in der Literatur beschrieben und könnte auch im vorliegenden Fall als Erklärungsansatz dienen (Foster, 2012, Bean et al., 2014, Kumar et al., 2015).
In einem weiteren Versuch wurden die männlichen Neuronen mit dem 5$\alpha$-Reduktase Hemmer Finasterid für eine Woche vorbehandelt. Jedoch zeigte sich keine verminderte Arc/Arg3.1 Expression zu der unbehandelten Vergleichsgruppe, welche mit DHT stimuliert wurde. Auch die vorbehandelte Gruppe ließ sich nach einer Woche nicht mehr mit DHT stimulieren. Dieses Ergebnis lässt sich ebenfalls durch einen Rückkopplungsmechanismus durch den verminderten Hormonspiegel erklären. Unter Anbetracht der Tatsache, dass Finasterid die Synthese von Testosteron zu DHT hemmt und auch Testosteron eine Affinität zu den Androgenrezeptoren besitzt, besteht die Möglichkeit, dass die Zellen auch unter Finasterid einem erhöhten Androgenspiegel ausgesetzt waren und dadurch verstärkt Arc/Arg3.1 exprimiert haben.

Die Parallelen in der Relevanz von Neurosteroiden und Arc/Arg3.1 für die synaptische Plastizität lassen auf einen potenziellen Zusammenhang schließen und sollten als Erklärungsansatz für bisher beobachtete Geschlechtsunterschiede weiterverfolgt werden.

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## 3. Zusammenfassung

Sexsteroide wie Estradiol (E2) und Dihydrotestosteron (DHT) regulieren die hippocampale Plastizität und das Gedächtnis geschlechtsspezifisch. Da das aktivitäten-regulierte Zytoskeleton–assoziierte Protein Arc/Arg3.1 essenziell für die Entstehung von Langzeitgedächtnis und synaptische Plastizität ist, haben wir die Expression von Arc unter dem Einfluss von E2 und DHT in männlichen und weiblichen hippocampalen Neuronen untersucht. Zum ersten Mal konnte gezeigt werden, dass die Expression von Arc/Arg3.1 in hippocampalen Neuronen durch Sexsteroide geschlechtsspezifisch reguliert wird. Grundlegende Unterschiede in der Arc/Arg3.1 Expression zwischen den Geschlechtern unter Kontrollbedingungen konnten nicht gezeigt werden. Mittels quantitativer real-time Polymerasen Ketten-Reaktion, Western Blot und quantitativer Immunreaktivität konnte eine Hochregulation von Arc/Arg3.1 durch die Hinzugabe von E2 in den weiblichen Neuronenkulturen beobachtet werden. Im Gegensatz dazu wurden in den Kulturen der männlichen Neuronen eine gesteigerte Arc-Expression durch DHT erzielt. Die quantitative real-time PCR zeigte, dass die Geschlechtsabhängigkeit auf der mRNA-Ebene besonders stark ausgeprägt war. Hervorzuheben ist, dass die Auswirkungen von E2 in Kulturen weiblicher Tiere aufgehoben wurden, sobald die endogene E2-Synthese in den Neuronen zuvor gehemmt wurde. Die Ergebnisse deuten auf eine möglicherweise wichtige Rolle von Arc/Arg3.1 in Bezug auf die durch Sexualsteroide induzierte geschlechtsspezifische synaptische Plastizität im Hippocampus hin.

## 4. Summary

Sex steroids, such as estradiol (E2) and dihydrotestosterone (DHT), regulate hippocampal plasticity and memory in a sex-dependent manner. Because the activity-regulated cytoskeleton protein Arc/Arg3.1 is essential for long-term memory formation and synaptic plasticity, we investigated the expression of Arc/Arg3.1 with respect to its responsiveness to E2 and DHT in male and female hippocampal neurons. For the first time, we show that, in hippocampal neurons, Arc/Arg3.1 expression is sex-dependently regulated by sex steroids. No difference in the expression between sexes was observed under control conditions. Using a quantitative real-time polymerase chain reaction, western blot analysis and quantitative immunoreactivity, upregulation of Arc/Arg3.1 protein expression was observed in specifically female hippocampal neurons after application of E2 to the cultures. Conversely, upregulation of Arc/Arg3.1 was seen in specifically male neurons after application of DHT. A quantitative real-time PCR revealed that the sex-dependency was most pronounced on the mRNA level. Most importantly, the effects of E2 in cultures of female animals were abolished when neuron-derived E2 synthesis was inhibited. Our

results point to a potentially important role of Arc/Arg3.1 regarding sex-dependency in sex steroid-induced synaptic plasticity in the hippocampus.

## 5. Eigenanteil

Das Thema der Forschungsarbeit wurde innerhalb des Forschungsschwerpunkts *Neurosteroide und ihr Einfluss auf die synaptische Plastizität* von Prof. Rune auf Grundlage vorheriger Arbeiten aus dem Institut für Neuroanatomie des Universitätsklinikums Hamburg-Eppendorf (UKE) konzeptioniert. Das methodische Vorgehen wurde mit ihr und der Co-Autorin Dr. Brunne erstellt.

Nach der Einarbeitung im Labor durch Prof. Lars Fester und den Medizinisch-technischen Assistentinnen (MTA) des Instituts erfolgte eine überwiegend selbständige Bearbeitung der Versuche meinerseits.

Dazu zählte die Erstellung der Dispersionskulturen nach einem etablierten Protokoll des Instituts, wobei das Töten der Tiere von den MTA übernommen wurde. Die Präparation der Hippocampi aus den embryonalen Gehirnen, sowie die Aufarbeitung dieser für die Kulturen erfolgte eigenständig. Ich war verantwortlich für die anschließende Stimulation der Zellen und für die Aufarbeitung der mRNA und der Proteine, die nach etablierten Protokollen erfolgte. Die Durchführung der RT-qPCRs, Western Blots und Immunzytologie erfolgte durch mich. Dr. Brunne war für die Einarbeitung und Optimierung der Aufnahmen am Zeiss Mikroskop verantwortlich, sowie die Konzeptionierung einer Auswertung in der Imaris Software. Nach erfolgter Einarbeitung übernahm ich eigenständig die Aufnahmen am Mikroskop und die Auswertung in Imaris.

Die Aufarbeitung der Daten und die Durchführung der Statistik erfolgte in Rücksprache mit Frau Prof. Rune und der statistischen Beratung des Instituts für Medizinische Biometrie und Epidemiologie des UKE. Die Visualisierung der Daten und Erstellung der Abbildungen und der dazugehörigen Legenden lag ebenfalls in meinem Verantwortungsbereich.

Die Ausformulierung des Papers erfolgte überwiegend durch Frau Prof. Rune, lediglich das Kapitel „Materials and Methods“ sowie die Legenden der Abbildungen habe ich formuliert.

Der Einreichungsprozess beim Journal sowie die Bearbeitung der Revision erfolgte in enger Zusammenarbeit mit Frau Prof. Rune.

## 6. Danksagung

An erster Stelle möchte ich meiner Doktormutter Prof. Rune danken, die mir von Beginn an das Vertrauen und die Zuversicht entgegengebracht hat, erfolgreich in ihrem Institut promovieren zu können. Dabei stand sie mir nicht nur jederzeit mit wissenschaftlichem Rat zur Seite, sondern half bei Bedarf stets mit einer gehörigen Portion Motivation und Optimismus aus.

Eine ebenso große Hilfe waren mir alle Medizinisch-technischen Assistentinnen des Instituts, die mich nicht nur mit dem Labor vertraut gemacht haben, sondern mir jederzeit mit tatkräftiger Unterstützung zur Seite standen.

Für die vielen fachlichen Diskussionen – ob im Data Club oder bei einer Tasse Kaffee – bedanke ich mich bei allen WissenschaftlerInnen und Doktoranden der Neuroanatomie.

Ein großes Dankeschön gilt meinen Eltern, die mir auch aus der Ferne immer den Rücken stärken und mich stets auf meinem Weg begleiten, sowie meinen FreundInnen, auf die ich mich während allen Höhen und Tiefen verlassen kann.

## 7. Lebenslauf

Lebenslauf aus datenschutzrechtlichen Gründen nicht enthalten

## 8. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....