SUPPLEMENTARY MATERIAL

Cognitive Impacts of Estrogen Treatment in Androgen-Deprived Males: What Needs to be Resolved

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Supplementary Table 1. Studies on the effects of E2 treatment on cognitive performance of castrated male rats.

Studies	E2 Treatment Paradigm	Interval Between Castration and E2 Treatment	Behavioural Testing and Key Findings
[71]	▪ E2 was administered *via* slow-release pellets, releasing 25 pg of E2 per mL of blood per day. ▪ Plasma E2 levels were not measured but the dose was based on another study [62] with a resultant E2 levels of 26.5 pg/mL. ▪ Animals were tested 4 weeks after treatment onset.	Immediately	Animals had bilateral injection cannulae implanted in the prefrontal cortex 5 days before behavioural testing. The animals received either saline or an NMDA receptor antagonists. Below are the results of the saline-treated animals only. Barnes maze was used to study spatial ability. On the first testing day, compared to E2-treated castrated rats, castrated rats without hormone replacement had: ▪ a longer path to reach the goal hole. ▪ more number of errors. ▪ a longer time to reach the goal hole. Performance on the second day of behavioural testing was similar between both groups.
[25]	▪ E2 was administered *via* slow-release pellets, releasing 25 pg of E2 per mL of blood per day. ▪ Plasma E2 levels were not measured but the dose was based on another study [62] with a resultant E2 levels of 26.5 pg/mL. ▪ Animals were tested 4 weeks after treatment onset.	Immediately	Barnes maze was used to study spatial ability. Compared to E2-treated castrated rats, castrated rats without hormone replacement had: ▪ slower time to acquire the behavioural task on Barnes maze (shown by a higher latency to investigate the first hole in a testing, to find the goal box, and longer path length). ▪ increased errors in task performances. ▪ impaired retention of the task a week later.
[63]	▪ E2 was administered *via* Silastic tube. ▪ Plasma E2 levels were not measured but the dose was based on another study [119] in female rats with a resultant E2 levels of 90 pg/mL. ▪ Animals were habituated in the testing arena 3-5 days after surgery for 4 days, followed by 2 testing 4-7 days apart.	Immediately	Object location memory test was used to test spatial working memory. Two identical objects were placed in adjacent corners, and the rats were allowed to explore them. After a delay, one of the objects were moved to a different corner. Time exploring moved and unmoved objects were measured. Specifically: ▪ castrated rats spent similar time exploring moved and unmoved objects. ▪ castrated rats with E2 supplementation explored moved object more than unmoved object. ▪ the exploring time on the moved object was higher in rats treated with E2 than rats with no hormone replacement.

(Supplementary Table 1) contd….

Studies	E2 Treatment Paradigm	Interval Between Castration and E2 Treatment	Behavioural Testing and Key Findings
[26]	▪ E2 was administered by in the form of EB (intramuscular injection of 200 mg/kg every 4 days) or ED (intramuscular injection of 50 mg/kg daily). ▪ Both treatments resulted in supraphysiological E2 levels, but higher E2 levels was reached with ED. ▪ Hormones were administered within a 21-day period. Behavioral testing started 15 days after the treatment onset. All animals had 4 days of acquisition phases, and on day 5 the rats were tested for behavioural performance.	2 weeks	Morris water maze was used to test spatial ability. The escape latency (time to find a hidden platform, as a measure of reference memory) decreased over time, but E2 did not affect this parameter. E2 treatment also did not affect the time spent in the platform quadrant (another measure of reference memory). Castrated rats with EB (but not ED) had worse spatial working memory (assessed by measuring the difference in escape latency between the final and first day of testing) than intact rats. However, using this parameter, castrated rats without hormone treatment did not have significantly different spatial working memory than intact rats.
[23]	▪ Estrogenic compounds were administered by daily intraperitoneal injection: EB (0.32 μg), ERβ agonist (2 mg/kg), or ERα agonist (2mg/kg), raloxifene (0.5, 1 or 2 mg/kg), or tamoxifen (0.5, 1 or 2 mg/kg). ▪ Animals were tested 1 weeks after treatment onset.	1 week	Cross-maze test was used to assess spatial reference memory (measured by the numbers of correct arm choice on cross-maze over 4 days trials). Main findings are: ▪ Intact animals had increasing number of correct arm choices over 4 days period, but castrated animals had similar number of correct arm choices over time. ▪ Castrated rats with EB, ERβ agonist, raloxifene (1mg/kg dose), or tamoxifen (1mg/kg dose) similarly had increasing number of correct arm choices over time. Other doses of tamoxifen or raloxifene did not improve spatial ability.
[21]	▪ E2 was administered *via* slow-release pellets, releasing 25 pg of E2 per mL of blood per day. ▪ Plasma E2 levels were not measured but the authors used the same dose as that in another study [62], with resultant E2 levels of 26.5 pg/mL. ▪ Animals were tested 3 weeks after treatment onset.	Immediately	Spontaneous novel object recognition task was used to assess recognition memory. Two identical objects were placed in one end of an open field and the time when the rats explored the objects were measured. After a delay, one of the objects were replaced with a new object and the time when the rats explored both objects were measured. Intact rats explored the novel object more than castrated rats with or without E2 treatment.
[15]	▪ E2 was administered *via* slow-release E2 pellets, releasing 25 pg of E2 per ml of blood per day. ▪ Plasma E2 levels were not measured but the dose was the same as that in an older study [62] with resultant E2 levels of 26.5 pg/mL. ▪ Animals were tested 4 weeks after treatment onset.	Immediately	Operant conditioning chambers were used to assess dopamine-dependent prefrontal functions, including: ▪ medial prefrontal functions (spatial working memory, withholding response, extradimensional set shifting). ▪ orbital prefrontal functions (reversal learning/preservation and motivation). All tasks included water reward. Below are tasks in which E2 treatment helped improve performances in castrated rats: Withholding response—after getting a reward, rats needed to withhold from pressing a bar before getting the next reward) task. Castrated rats required a longer time than intact and castrated rats with E2 to acquire this task. Motivation—measured by "Progressive Ratio 1 Schedule of Reinforcement" paradigm (rats needed to do increasing number of bar-pressing in order to get a reward). Castrated rats had lower number of rewards (stopped bar-pressing for a reward sooner) than intact and castrated rats with E2.

(Supplementary Table 1) contd….

Studies	E2 Treatment Paradigm	Interval Between Castration and E2 Treatment	Behavioural Testing and Key Findings
[61]	▪ E2 was administered *via* a Silastic capsule, with a resultant E2 levels of 25.2 pg/mL. [Intact rats have E2 levels of 1.6 pg/mL] ▪ Behaviour training started at 2 weeks after treatment.	Immediately	T-maze was used to assess spatial learning, spatial reference memory, and spatial working memory by using a "Delayed matching-to-position" paradigm. Rats needed to find a reward in one open arm (the other arm is closed). After a delay, the rats needed to find a reward in the same baited arm, while the previously closed arm was now open and baited too. Castrated rats with E2 treatments learned to acquire the "Delayed matching-to-position" task faster than intact or castrated rats, but there was no time difference in learning this task between intact and castrated rats. With increased delay (≥30 s), castrated rats with and without E2 treatments had less correct choices (worse spatial working memory) than intact rats. Configural association negative patterning was measured in an operant conditioning chamber. In order to get a reward, rats needed to enter a food cup in response to a tone or light, but not when both stimuli were presented simultaneously. Across trials, there was no group difference between castrated rats with and without E2 in the number of response and time to respond after tone, light, or both stimuli.
[13]	▪ E2 was administered *via* slow-release pellets, releasing 25 pg of E2 per mL of blood per day. ▪ Plasma E2 levels were not measured but the dose was based on an older study [62] with a resultant E2 levels of 26.5 pg/mL. ▪ Animals were tested 4 weeks after treatment onset.	Immediately	T-maze was used to assess spatial task learning. Rats needed to get a water reward in alternating arms. Castrated rats with or without E2 had a slower time than intact rats to acquire T-maze alternation paradigm task.
[60]	▪ E2 was administered *via* a Silastic tube. ▪ Plasma E2 levels were not measured but the dose was the same as that in another study in female rats [119], with a resultant E2 levels of 90 pg/mL. ▪ Behaviour testing started 3 days after treatment onset.	10 days	Radial arm maze was used to assess spatial learning, spatial reference memory and spatial working memory. Rats needed to find a food reward in one of the 8 arms; this was repeated with no delay or 1 hour delay. Under no-delay trials, no significant difference was found between castrated rats with or without E2. When 1-hour delay was introduced between the 4^{th} and 5^{th} arm visits, castrated rats with E2 treatment achieved more correct choices than those without E2.

E2 = Estradiol. EB = Estradiol benzoate. ED = Estradiol dipropionate. ER = Estrogen receptor.

Supplementary Table 2. Studies on the effects of E2 treatment on cognitive performance of androgen-deprived genetic male populations (PCa patients on ADT and MtFs on HT).

Studies	Treatment Regime for E2-Treated Groups	Testing Paradigm and Key Findings
PCa Patients		
[76]	▪ 10 PCa patients (mean age = 71.6) were on combined therapy of LHRH agonist and non-steroidal antiandrogen. After 12 weeks on combined ADT, the patients received 1 mg/day micronized E2 in addition to the combined therapy. ▪ Serum E2 levels were 171.7 pmol/L and serum testosterone levels were 1.64 nmol/L after 12 weeks of E2 treatment.	PCa patients with or without E2 therapy were tested for: ▪ Spatial ability (mental rotation, paper folding, block design) ▪ Verbal ability [verbal memory (paragraph recall and PAL) and fluency] ▪ Visuomotor scanning and attention (digit symbol test) ▪ Working memory (letter-number sequencing test) 12 weeks of combined ADT did not affect any of the cognitive measures. However, after 12 weeks of E2 treatment, PCa patients performed worse than the placebo-treated PCa patients in immediate and delayed recall. No significant difference was observed between groups for the other cognitive measures.
[75]	▪ 18 patients (mean age = 70.3) with androgen-independent PCa received 0.6 mg/day transdermal E2 as a second line HT. ▪ 13 had received LHRH agonists alone (stopped when starting E2) and 5 were orchiectomized. ▪ Serum E2 levels were 450 pg/mL and serum testosterone levels were 9.8 ng/dl after 4 weeks of transdermal E2 treatment.	PCa patients on E2, PCa patients on ADT, and healthy male controls were tested for the following tests: ▪ Immediate and delayed paragraph recall ▪ Subject ordered pointing ▪ Trails tests At baseline, both groups of PCa patients performed worse on Trail tests, immediate and delayed paragraph recall tests than healthy male controls. At follow-up, performance on immediate and delayed paragraph recall tests improved in E2-treated men, but not on the other 2 groups which did not receive E2. A reversed pattern was found with subject ordered pointing and Trail tests; *i.e*., the E2-treated group performed worse than the other 2 groups.
[77]	▪ 13 PCa patients (mean age = 70) received 1 mg/day micronized E2 taken orally for 9 weeks. ▪ Serum E2 levels were 55.3 pg/mL and serum testosterone levels were 0.48 ng/mL. ▪ Some men had been on LHRH agonist therapy. They were tested at baseline and at 9 weeks after E2 therapy. ▪ Other men were about to start neoadjuvant therapy before external beam radiation. They were tested before starting LHRH agonist, 3 weeks later and 9 weeks after E2 therapy.	PCa patients were tested for: ▪ Rey auditory verbal learning test ▪ Benton visual retention test ▪ Trail making test ▪ Stroop test ▪ Controlled oral word association test Compared to the placebo group, PCa patients who received E2 treatment had better performances on trail making test and the Stroop test.
MtFs		
[86]	▪ 11 MtFs (mean age = 38.0) were on HT for at least 6 months with average E2 levels of 115 pmol/L and testosterone levels of 1.4 ng/mL. ▪ All received oral E2; 6 individuals also received CA. ▪ HT duration was 6-12 months for 4 individuals, 1-2 years for 5 individuals, and >2 years for 2 individuals. ▪ None was surgically castrated.	Men, MtFs on HT, MtFs not on HT were tested for mental rotation ability, but no significant difference was found between groups. [Their pattern of brain activation was also measured by using fMRI during a mental rotation task. There were some difference in the activation pattern between the 3 groups.]
[87]	▪ 8 MtFs (mean age = 22) were on CA 100 mg/day and either oral ethinyl E2 (100 μg/day) or transdermal E2 (100 μg twice a week). ▪ Their serum testosterone levels were 0.2 nmol/L and serum E2 levels were 44 pmol/L after 3 months of HT. ▪ Testosterone levels declined significantly but E2 levels did not show a significant increase.	Participants were tested for language tasks and mental rotation before starting HT and at 3 months later. No significant difference in these tasks was found between before and after HT. [Their pattern of brain activation was also measured by using fMRI during language and mental rotation tasks but there was no significant difference in the activation of the areas of interests before and after HT.]

(Supplementary Table 2) contd….

Studies	Treatment Regime for E2-Treated Groups	Testing Paradigm and Key Findings
MtFs		
[88]	▪ 74 MtFs were divided into 3 groups: AB group – MtFs (mean age = 37.1) were tested shortly pre-HT, and again 3-12 months later. BA group – MtFs (mean age = 39.6) who had been on HT for ≥28 months were tested first, and tested again after stopping HT for ≥8 weeks. Control group – MtFs (mean age = 40.3) who had been on HT for ≥3 months were tested first, and tested again at 3-12 months later. ▪ MtFs were on different daily HT regimes: AB group – 25 MtFs were on 10-100 µg ethinyl E2 for 3-14 months; 2 MtFs were on 50-100 µg ethinyl E2 and 50-100 mg CA for 4-6 months. BA group – 28 MtFs were on 5-7.5 mg Premarin for 28-120 months, 4 MtFs were on 100-150 ethinyl E2 for 28-120 months, 4 MtFs were on 5-7.5 mg Premarin and 50-120 mg CA for 50-150 months, 1 MtF was on 7.5 mg Premarin and 15 mg CA for 156 months. Control group – 5 MtFs were on 5-7.5 mg Premarin for 26-48 months, 1 MtF was on 7.5 mg Premarin and 15 mg CA for 71 months, 12 MtFs were on 50-150 µg ethinyl E2 for 6-94 months, 2 MtFs were on 50-100 µg ethinyl E2 and 100-150 mg CA for 20-60 months. ▪ Plasma E2 levels were not measured.	MtFs were tested for cognitive tasks which are known to have: ▪ sex difference favouring males (mental rotation, judgement of line angle and position). ▪ sex difference favouring females (verbal fluency, controlled associations) MtFs were tested for various memory tests which are known to have: ▪ sex difference favouring males (visual reproduction, figural memory, visual memory span). ▪ sex difference favouring females [verbal memory tests (logical memory, verbal PAL), visual memory tests (visual PAL, object memory, location memory)]. ▪ no sex difference (DS) In the AB group, DS was better after HT. In the BA group, visual paired associate memory was better after HT is stopped. In the control group, logical memory (delayed and immediate recall) and figural memory were better when measured at the second time-point. No difference in cognitive measures was found at time 1 between AB and BA group. Those on continuous HT had better cognitive performances: verbal memory [immediate recall & delayed recall] and figural memory.
[90]	▪ 22 MtFs (mean age = 31.4, range = 20-51) on CA (50 mg twice a day) in combination with either oral ethinyl E2 (0.05 mg twice a day, n = 11) or transdermal E2 patches (0.10 mg once a day, n = 11). ▪ Plasma E2 levels were not measured. ▪ MtFs were tested for cognitive tasks at 1 week before HT and at 14 weeks after HT.	MtFs, FtMs, women without gender dysphoria, and men without gender dysphoria were tested for the following tests: ▪ Verbal reasoning test ▪ Line orientation test ▪ Rotated figures (2-dimensional, 3-dimensional, 3-dimensional: same-different) ▪ Targeted throwing Visuospatial tasks (all three rotated figure tasks) of MtFs were better at the second testing (after HT) but could be due to learning because the other groups had similar improvements at the second testing.
[91]	▪ 20 MtFs (mean age = 29.1, range = 19-45) were on CA (50 mg, twice a day) and ethinyl-E2 (50 mg, twice a day). ▪ All were tested shortly before starting HT and at 3 months after HT. ▪ 18 MtFs were retested at 12 months. ▪ 14 were retested at 18 months (post-sex reassignment surgery and when they had been off HT for 5 weeks). ▪ Plasma E2 levels were not measured.	MtFs and FtMs were tested for tasks which are known to have: ▪ sex difference favouring males [rotated figures (2-dimensional, 3-dimensional), hidden figure test]. ▪ sex difference favouring females [verbal fluency (words, sentences), fine motor movements, perceptual speed task (TD2)] ▪ no sex difference (verbal reasoning test) Fine motor movement, TD2, and hidden figure test were only administered at the first and second time points. At 3 months, performances of MtFs on rotated figures (3D) did not change, on rotated figures (2D) decreased, and on hidden figure test increased. Performances on female-favouring tasks did not change significantly after 3 months of HT. At 12 months and 18 months (after 5 weeks of hormone deprivation), performances of MtFs on any of the tested tasks did not change significantly.

(Supplementary Table 2) contd….

Studies	Treatment Regime for E2-Treated Groups	Testing Paradigm and Key Findings
MtFs		
[92]	▪ 29 MtFs were on daily HT (mean age = 36.7). 19 MtFs were only on 2.5-7.5 mg Premarin for 4-72 months, 3 MtFs were on 2.5 mg Premarin plus 5.0 mg Provera for 36-60 months, 5 were on 2.5-7.5 mg Premarin plus 50-100 mg CA for 3-36 months, 2 were on 50 μg ethinyl E2 for 36-40 months. ▪ Plasma E2 levels were not measured.	MtFs on HT and MtFs not on HT were tested for DS, PAL, mental rotation, controlled associations, vocabulary. MtFs on HT performed better on PAL than MtFs who are not on HT. There was no significant difference between groups on the other tests.
[89]	▪ 15 MtFs (mean age = 32.4) were on CA (50 mg, twice a day) and ethinyl E2 (50 μg, twice a day). ▪ MtFs were tested shortly before HT and after 3 months of HT. ▪ Plasma E2 levels were not measured.	MtFs were tested for the following cognitive tasks: ▪ Card rotations test ▪ Verbal reasoning test ▪ Verbal fluency tests (word and sentence production) After 3 months of HT, MtFs performed worse on card rotation test and verbal fluency (word production), but better on verbal fluency (sentence production) and no change on verbal reasoning test.

ADT = androgen deprivation therapy; CA = cyproterone acetate; DS = digit span; E2 = estradiol; fMRI = functional magnetic resonance imaging. FtMs = female-to-male transsexuals; HT = hormone therapy; LHRH = luteinizing hormone-releasing hormone; MtFs = male-to-female transsexuals; NMDA = N-methyl-d-aspartate; PAL = paired associate learning; PCa = prostate cancer.