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VPLIV SOCIALNEGA STRESA, SPOLNIH HORMONOV MED PUBERTETO IN PSIHOAKTIVNIH ZDRAVIL NA OBNAŠANJE ODRASLIH MIŠI

Doktorska disertacija

INFLUENCE OF SOCIAL STRESS, SEX HORMONES DURING PUBERTY AND PSYCHOACTIVE DRUGS ON THE BEHAVIOR OF ADULT MICE

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Vpliv socialnega stresa, spolnih hormonov med puberteto in psihoaktivnih zdravil na obnaģanje odraslih miģi

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IZVLEČEK

Ključne besede: Vedenje ģivali; socialna osamitev ï fiziologija; spolni hormoni; moģgani ï rast in razvoj; puberteta ï fiziologija; materinstvo; oļetovstvo; depresija ï terapija z zdravili; fluoksetin ï terapevtska raba; miģi; samice; samci

Obdobje pubertete/ mladostniģtva je ļas tako spolnega kakor tudi kognitivnega, ļustvenega in socialnega dozorevanja, kar je posledica poveļanega izloļanja in delovanja steroidnih spolnih hormonov iz spolnih ģlez. Spolni hormoni med puberteto dokonļno preoblikujejo delovanje kortikalnega in limbiļnega dela centralnega ģivļnega sistema ter s tem vplivajo na razvoj sposobnosti izraģanja razliļnih spektrov obnaģanj v odraslem obdobju. Socialna osamitev in drugi stresni dejavniki med tem razvojno obļutljivim obdobjem lahko trajno prizadenejo dozorevanje moģganov, njihovo nevroendokrino odzivnost ter pustijo dolgotrajne spremembe v obnaģanju. V doktorski disertaciji smo prouļevali socialno prepoznavanje, ģensko spolno ter starģevsko obnaģanje miģi, ki smo jih pred nastopom pubertete trajno socialno osamili, trajno nastanili v socialne skupine ali zaļasno osamili le med puberteto in jih ponovno skupinsko nastanili. Miģim smo odstranili moda in jajļnike pred puberteto ali po njej ter jim v odraslem obdobju dovajali estradiol. Preverili smo tudi uļinkovanje zdravila proti depresiji fluoksetina na depresivnemu in anksioznemu podobno obnaģanje pri miģjih samcih in samicah, nastanjenih v skupinah. Socialna osamitev med puberteto je zmanjģala sposobnost socialnega uļenja ter nepovrnljivo prizadela sposobnost socialnega prepoznavanja predvsem pri samicah. Osamitveni stres med puberteto je tudi nepovrnljivo poslabģal izraģanje spolne sprejemljivosti pri samicah ter trajno spremenil izraģanje estrogenskega receptorja Ŭ (ERŭ) v anteroventralnem delu periventrikularnega jedra (AVPV) in v ventromedialnem jedru hipotalamusa (VMH). Odsotnost spolnih hormonov med puberteto, ģe posebej estradiola pri samicah, je nepovrnljivo poslabģala sposobnost izraģanja starģevskega obnaģanja, usmerjenega k mladiļem. Dolgotrajno prejemanje fluoksetina je pri obeh spolih enako moļno zmanjģalo izraģanje obnaģanja, podobnega depresivnemu, vendar ni imelo uļinka na obnaģanje, podobno anksioznemu. Samice so bile bolj anksiozne v primerjavi s samci. Naģe ugotovitve dokazujejo, da imajo socialna osamitev in spolni hormoni med puberteto moļan in trajen uļinek tako na moģgane kot posledilno na sposobnost izraģanja razliļnih spektrov obnaģanj v odraslem obdobju.

ABSTRACT

Keywords: Behavior, animal; social isolation ï physiology; gonadal steroid hormones; brain ï growth and development; puberty ï physiology; maternal behavior; paternal behavior; depression ï drug therapy; fluoxetine ï therapeutic use; mice; female; male

Puberty/ adolescence is the period during which an individual attains reproductive as well as cognitive, emotional and social maturation due to the elevated secretion and action of gonadal steroid hormones secreted by gonads. These steroid hormones direct proper development of cortical and limbic circuits in the brain and program a variety of adult behaviors. Social isolation and other stressful events during this vulnerable period can have profound consequences on different behaviors due to alterations in developing brain and their neuroendocrine responses. Present studies explored social recognition, female sexual and parental behavior in mice of both sexes that were individually housed or housed in social groups during and after puberty, and mice that were individually housed during puberty only, followed by housing in social groups. Mice were gonadectomized either before or after puberty and received estradiol in adulthood. Furthermore, the influence of chronic fluoxetine treatment on depressive and anxiety like behavior in socially housed, gonadally intact male and female mice was also investigated. Social isolation during puberty impaired social recognition as well as irrecoverably affected the ability of social recognition in females only. Social isolation stress during puberty irrecoverably reduced receptive sexual behavior in females and permanently altered the expression of estrogen receptor Ŭ (ERŬ) in the anteroventral periventricular nucleus (AVPV) and in the ventromedial nucleus of the hypothalamus (VMH). The absence of sex hormones during puberty, especially the lack of estradiol in females, irreversibly reduced the ability of pup-directed parental behaviors. Chronic fluoxetine treatment equally reduced the expression of depressive like behavior in both sexes of mice, but fluoxetine had no effect on anxiety like behavior. These results suggest that social isolation and sex hormones during puberty have profound and long-lasting influences on brain development and subsequently on the ability to express different behaviors in adult life.

KAZALO VSEBINE

SEZNAM OKRAJŠAV

5- HTT	Prenaġalec 5-hidroksitriptamina/ Prenaġalec serotonina
5- HT	5-hidroksitriptamin/ Serotonin
ACTH	Adrenokortikotropni hormon/ Kortikotropin
AVP	Arginin vazopresin/ Vazopresin
AVPV	Anteroventralni del periventrikularnega jedra
BNST	Spodnje jedro konļne proge
CoA	Kortikalni del mandlja
CRH	Sproġļevalni hormon kortikotropina/ Kortikoliberin
EB	Estradiol benzoat
EPM	Dvignjeni labirint v obliki kriġa (*Elevated plus maze*)
ERŭ, ERƃ	Estrogenski receptor Ŭ, Estrogenski receptor ƃ
FSH	Folikle stimulirajoļi hormon
FST	Test prisilnega plavanja (*Forced swim test*)
GnRH	Sproġļevalni hormon gonadotropinov/ Gonadoliberin
HPA	Hipotalamo-hipofizno-nadledviļna os (*Hypothalamic-pituitary-adrenal axis*)
HPG	Hipotalamo-hipofizno-gonadna os (*Hypothalamic-pituitary-gonadal axis*)
LH	Luteinizirajoļi hormon
LQ	Koliļnik lordoze (*Lordosis quotient*)
LS	Stranski septum
MAOI	Zaviralci oksidaz monoaminov (*Monoamine oxidase inhibitors*)
MeA	Medialni del mandlja
MPOA	Medialni del predoptiļnega podroļja
mRNK	Informacijska RNK
OF	Test odprtega polja (*Open field*)
OT	Oksitocin
OTR	Oksotocinski receptor
POA	Predoptiļno podroļje
PVN	Paraventrikularno jedro hipotalamusa

SON	Supraoptiļno jedro
SSRI	Selektivni zaviralci ponovnega privzema serotonina (*Selective serotonin reuptake inhibitors*)
TCA	Triciklіļni antidepresivi
V1aR	Vazopresinski receptor 1a
VMH	Ventromedialno jedro hipotalamusa

1 UVOD

Ljudje in ģivali smo socialna bitja, ki ģivimo v manjģih ali veļjih socialnih zdruģbah, kar nam omogoļa, da razvijemo normalno vrstno specifiļno obnaģanje, ki je v doloļeni skupnosti priļakovano, zaģeleno in sprejemljivo. Ena od osnovnih veģin socialnega obnaģanja je socialno prepoznavanje, ki predstavlja osnovo za socialno ģivljenje v socialni zdruģbi. Obstanek socialne zdruģbe kot tudi obstoj same vrste pa je v veliki meri odvisen predvsem od uspeģnega razmnoģevanja in oskrbe ter preģivetja mladiļev.

Puberteta je obdobje, v katerem posameznik dozoreva tako fiziļno kot psihiļno, kar je posledica poveļanega izloļanja steroidnih spolnih hormonov iz spolnih ģlez. V ļasu pubertete namreļ poteka ģe zadnje preoblikovanje in reorganizacija moģganov, ki sta odvisni od delovanja spolnih hormonov, delno pa sta podvrģeni vplivom razliļnih stresorjev, kar lahko privede do (dolgo)trajnih sprememb v moģganih in poslediļno do sprememb obnaģanja v odraslem obdobju.

Razliļne razpoloģenjske oziroma ļustvene motnje so z nastopom pubertete pogosto spolno razliļne. Pojavnost depresivnih in anksioznih motenj je najmanj dvakrat pogostejģa pri ģenskah v primerjavi z moģkimi. Pri zdravljenju se najpogosteje uporabljajo selektivni zaviralci ponovnega privzema serotonina (SSRI), med katerimi je bil fluoksetin kot prvi dostopen na trģiģļu. Kljub znanemu spolnemu dimorfizmu v farmakokinetiki in farmakodinamiki nekaterih antidepresivnih zdravil, terapija ostaja enaka pri obeh spolih.

1.1 NAMEN DELA IN HIPOTEZE

Namen naģega dela je bil ugotoviti, ali socialna osamitev in spolni hormoni med puberteto ter psihoaktivna zdravila, kot je fluoksetin, vplivajo na obnaģanje odraslih samcev in samic miģi.

V prvem sklopu naģega dela smo si zastavili vpraģanje, ali socialna osamitev med puberteto vpliva na sposobnost socialnega prepoznavanja in ali izzove spremembe v oksitocinskem (OT) ter vazopresinskem (AVP) sistemu v konļnih in vmesnih moģganih. V ta namen smo miģi obeh spolov pred puberteto (od 30. dneva starosti) trajno socialno osamili ali skupinsko nastanili po 3 miģi na kletko. Da bi preverili moģnost povrnitve morebitnih sprememb zaradi

osamitvenega stresa, smo dodatno skupino miģi zaļasno osamili le med puberteto ter jih 60. dan starosti ponovno nastanili v socialne skupine.

V drugem sklopu dela smo si zastavili vpraģanje, ali socialna osamitev in odsotnost spolnih hormonov med puberteto vplivata na sposobnost izraģanja starģevskega obnaģanja pri obeh spolih ter spolnega obnaģanja pri samicah in ali pride do sprememb v izraģenosti estrogenskega receptorja Ŭ(ERŭ) v konļnih in vmesnih moģganih. V ta namen smo miģi obeh spolov pred puberteto (od 25. dneva starosti) trajno socialno osamili ali skupinsko nastanili po 3 miģi na kletko ter dodatno skupino miģi zaļasno osamili le med puberteto ter jih 60. dan starosti ponovno nastanili v socialne skupine. Miģim smo odstranili spolne ģleze pred puberteto (25. dan) ali po njej (60. dan) ter jim v odraslem obdobju dovajali estradiol z namenom, da bi preverili moģnost povrnitve morebitnih sprememb zaradi odsotnosti spolnih hormonov med puberteto.

V tretjem sklopu dela smo si zastavili vpraģanje, ali dolgotrajno prejemanje fluoksetina zmanjģa obnaģanje, podobno depresivnemu in anksioznemu, drugaļe pri samcih kot pri samicah. V ta namen smo miģi obeh spolov po odstavitvi nastanili po 3 v kletko in po puberteti polovici miģim zaļeli dodajati fluoksetin v pitno vodo, druga polovica pa je prejemala vodo brez fluoksetina.

Glede na zastavljene cilje smo postavili naslednje delovne hipoteze:

Hipoteza 1: Socialna osamitev v mladosti vpliva na socialno prepoznavanje odraslih miģi.

Hipoteza 2: Zgodnja odstranitev spolnih ģlez in socialna osamitev vplivata na starģevsko in ģensko spolno obnaģanje odraslih miģi.

Hipoteza 3: Antidepresiv fluoksetin zmanjģa depresivnemu in anksioznemu vedenju podobno obnaģanje, razliļno pri samicah in samcih.

2 PREGLED LITERATURE

2.1 PUBERTETA IN/ ALI MLADOSTNIĠTVO

Puberteta in mladostniģtvo (adolescenca) se pogosto uporabljata kot sopomenki, nanaģajoļi se na razvojni prehod iz otroģtva v odraslost. Sisk in sod. so puberteto definirali kot obdobje, med katerim posameznik postane spolno zrel in sposoben razmnoģevati se, kar je posledica poveļanega izloļanja steroidnih spolnih hormonov iz spolnih ģlez, obdobje mladostniģtva pa poleg spolnega dozorevanja zajema ģe kognitivno, ļustveno in socialno dozorevanje obnaģanja, kar je posledica izrazitega preoblikovanja strukture in delovanja moģganske skorje ter limbiļnega dela moģganov (pregledno v 1, 2). Pri miģih lahko merimo tri pokazatelje pubertete pri samicah in enega pri samcih: odpiranje noģnice (v povpreļju 28. dan), zaļetek poroģenevanja noģnice (v povpreļju 43. dan) in zaļetek spolnega ciklusa oziroma prisotnost noģniļnega ļepka po uspeģni paritvi (najpozneje do 60. dneva) pri samicah (linija C57BL/6J) ter loļitev prepucija od penisa (v povpreļju 30. dan) pri samcih (linija C57BL/6J) (3, 4).

2.1.1 Puberteta in spolni hormoni

Hipotalamus predstavlja povezavo med ģivļnim in endokrinim sistemom (5). Dozorevanje hipotalamo-hipofizno-gonadne (HPG) osi med puberteto se zaļne z aktivacijo nevronov, ki izloļajo sproģļevalni hormon gonadotropinov (GnRH) (2). GnRH usmerja sintezo in sproģļanje hipofiznih gonadotropinov, luteinizirajoļega hormona (LH) in folikle stimulirajoļega hormona (FSH), ki delujejo usklajeno in spodbujajo tvorbo tako spolnih hormonov kot tudi spolnih celic v spolnih ģlezah (5, 6).

Spolni hormoni spadajo med steroidne hormone, ki nastajajo iz holesterola in imajo za osnovo sterolno ali ciklo-pentano-perhidro-fenantrensko jedro (6). Pri samcih Leydigove celice v modih izloļajo androgene hormone, kot sta testosteron in androsteron. Pri samicah celice ovojnice jajļnega folikla v jajļnikih izloļajo estrogene hormone, kot so 17 ß-estradiol, estron in estriol, celice rumenega telesa pa gestagene hormone, kot sta progesteron in pregnenolon (7). Spolni hormoni vplivajo tako na razvoj spolnih organov (rast semenskih kanalļkov, spermatogeneza ter razvoj akcesornih spolnih ģlez pri samcih in razvoj materniļne sluznice pri samicah) kot tudi na rast in razvoj celotnega telesa (spremembe v presnovi, sekundarni

spolni znaki) (5, 6) in ne nazadnje tudi na cel spekter obnaģanj (socialno, materinsko, spolno) (8, 9, 10).

2.1.1.1 Aktivacijski in organizacijski uļinek spolnih hormonov

Steroidni hormoni spolnih ģlez vplivajo na razvoj kot tudi na delovanje moģganov in posledilno na obnaģanje. Delovanje spolnih hormonov na ģivļni sistem delimo na aktivacijsko in organizacijsko (pregledno v 2, 11, 12, 13). Pri aktivacijskem delovanju spolni hormoni zaļasno (prehodno) spremenijo aktivnost tarļnih celic in s tem spodbudijo doloļeno obnaģanje. Nasprotno pa je organizacijsko delovanje spolnih hormonov trajno (nepovrnljivo), saj se preoblikuje ģivļni sistem in posledilno se spremeni tudi aktivacijsko delovanje (1). Spolni hormoni naļeloma delujejo organizacijsko na ģivļni sistem v perinatalnem obdobju, medtem ko do aktivacijskih uļinkov prihaja pozneje, v odraslem obdobju (pregledno v 14, 15, 16). Vedno veļ raziskav pa dokazuje, da imajo spolni hormoni organizacijske uļinke na moģgane tudi pozneje v ģivljenju, med puberteto in morebiti celo v odraslem obdobju (11, 17, 18, 19, 20, 21). V moģganih glodavcev so podroļja delovanja spolnih hormonov pogosto spolno razliļna, zaradi ļesar je posledilno razliļno tudi obnaģanje. Nekateri deli hipotalamusa, ki so spolno razliļni, so predoptilno podroļje (POA), arkvatno jedro, spodnje jedro konļne proge (BNST), ventromedialno jedro (VMH) in paraventrikularno jedro (PVN) (22).

2.1.1.2 Spolni hormoni med puberteto in vpliv na obnaģanje v odraslem obdobju

Moģki spolni hormoni imajo organizacijske uļinke na ģirok spekter za moģki spol znaļilnih obnaģanj. Raziskave pri samcih podgan, zlatega hrļka in puģļavskih skakaļev razkrivajo, da kastracija pred puberteto poslabģa izraģanje socialnega (agresivno obnaģanje, oznaļevanje ozemlja) in moģkega spolnega obnaģanja v primerjavi s samci s prisotnimi spolnimi ģlezami med puberteto (pregledno v 21). Dolgotrajno dodajanje testosterona v odraslem obdobju pa ne odpravi posledic njegove odsotnosti med puberteto (23). Podobno velja tudi za samce miģi, razen moģkega spolnega obnaģanja, ne glede na to, ali so bila moda odstranjena pred puberteto ali po njej (24). Tudi obnaģanje, podobno anksioznemu, je podvrģeno

organizacijskim uļinkom testosterona, saj podganji samci, kastrirani pred puberteto, moļneje izraģajo obnaģanje, podobno anksioznemu, kot pa samci, ki med puberteto ģe imajo moda (25).

Ģenski spolni hormoni imajo prav tako organizacijske uļinke na ģirok spekter za ģenski spol znaļilnih obnaģanj (pregledno v 13), saj spodbujajo obnaģanja, znaļilna za samice v odraslem obdobju. Po drugi strani pa raziskave o vplivu ģenskih spolnih hormonov med puberteto pri samicah zlatega hrļka (pregledno v 21) in podgan (26) razkrivajo uļinke slabenja ģenskih (defeminizacija) in krepitve moģkih oblik (maskulinizacija) spolnega obnaģanja.

2.1.1.3 Spolni hormoni med puberteto ter oksitocin in vazopresin v odraslem obdobju

Oksitocin (OT) in vazopresin (AVP) sta nonapeptida, ki v glavnem nastajata v velikih ģivļnih celicah paraventrikularnega in supraoptiļnega jedra (SON), v manjģem obsegu pa v majhnih parvocelularnih nevronih v paraventrikularnem jedru in spodnjem jedru konļne proge, ģe manj pa v predoptiļnem podroļju in mandlju (6, 27). Uravnavanje sistemov OT in AVP je moļno podvrģeno vplivu ģenskih in moģkih spolnih hormonov (pregledno v 28).

Raziskave o vplivu spolnih hormonov med puberteto na sistem OT (29, 30) ne kaģejo sprememb v ģtevilu nevronov OT, le nastajanje in sproģļanje hormona OT je z odraģļanjem veļje ter sorazmerno z veļanjem koliļine spolnih hormonov. Poveļano izraģanje mRNK za OT v hipotalamusu sovpada s priļetkom pubertete in je pri samicah najveļje med pojatvijo, kar je povezano s poveļanimi vrednostmi estrogenov (29, 31), pri samcih pa s poveļanimi vrednostmi testosterona (30). Odstranitev jajļnikov pri samicah podgan pred puberteto in po njej zmanjģa izraģanje mRNK za hormon OT v paraventrikularnem in supraoptiļnem jedru, dodajanje estradiola (29, 30) in progesterona pa izraģanje zopet poviģa (32, 33, 34). Estrogeni ne spodbujajo samo prepisovanja hormona OT, ampak poveļujejo tudi vrednosti hormona OT v krvni plazmi (35), jih zniģujejo v hipofizi (31), poveļujejo ģtevilo oksitocinskih receptorjev (OTR) (36), spodbujajo sproģļanje hormona OT iz nevritskega in dendritskega dela nevronov kot tudi poveļujejo elektriļno aktivnost nevronov OT (37). Testosteron ali kastracija pri odraģļajoļih (med puberteto) in odraslih samcih podgan imata podobne uļinke na sistem OT kot estradiol ali odstranitev jajļnikov pri samicah (30, 38).

Edina raziskava o vplivu spolnih hormonov med puberteto na sistem AVP ugotavlja, da imajo kastrirani samci podgan zniģane vrednosti hormona AVP v krvni plazmi v primerjavi s kontrolnimi samci, nasprotno pa imajo samice z odstranjenimi jajļniki poviģane vrednosti v primerjavi s kontrolnimi (39). Uļinke odstranitve spolnih ģlez pri samcih odpravi dodajanje testosterona, pri samicah pa dodajanje estradiola in progesterona (39). V odraslosti je uļinek spolnih hormonov ravno obraten, in sicer kastracija zviģuje, dodajanje testosterona pa zniģuje koliļino hormona AVP v krvni plazmi pri odraslih samcih podgan (40), medtem ko jo odstranitev jajļnikov pri odraslih samicah zniģuje, dodajanje estrogenov pa zviģuje (35, 40). Samice imajo med pojatvijo viģje vrednosti AVP v plazmi (40), samci pa naļeloma viģje vrednosti v primerjavi s samicami (39). Kastracija pri odraslih podganjih samcih zmanjģa ģtevilo imunoreaktivnih celic in izraģanje mRNK za AVP v stranskem septumu (LS), spodnjem jedru konļne proge, medialnem delu mandlja (MeA) in nekaterih drugih delih, medtem ko dodajanje testosterona (41) ali dihidrotestosterona skupaj z estradiolom lahko te spremembe prepreļi (42). Pri odraslih samicah podgan nihanje vrednosti estradiola v posameznih fazah spolnega ciklusa nima vpliva na izraģanje mRNK za AVP v supraoptiļnem jedru (31), kot tudi nima vpliva na koliļino vazopresinskega receptorja v stranskem septumu (36), medtem ko imajo samice ob koncu brejosti in med laktacijo moļnejģe izraģanje mRNK za AVP v supraoptiļnem jedru (31).

2.1.2 Puberteta in stres

V ļasu pubertete potekata preoblikovanje in reorganizacija moģganov, ki sta odvisna predvsem od delovanja spolnih hormonov (13). Vedno veļ pa je raziskav, ki ugotavljajo, da izpostavljenost razliļnim stresorjem v ļasu pubertete lahko privede do (dolgo)trajnih sprememb v moģganih in posledilno v obnaģanju v odraslem obdobju (pregledno v 43), morda prav zaradi medsebojnega delovanja med spolnimi hormoni in hormoni hipotalamo-hipofizno-nadledviļne (HPA) osi (44, 45, 46).

Stres lahko definiramo kot odziv organizma na razliļne zunanje ali notranje dejavnike ali stresorje, ki ogroģajo normalno delovanje in notranje ravnovesje organizma (47). Naloga odziva osi HPA na stresorje je povrniti fiziološko ravnovesje v organizmu in prepreļiti prekomeren odziv obrambnih mehanizmov na stres, ki bi lahko privedli do poģkodbe, bolezni ali smrti (48, 49). Dolgotrajen ali zelo intenziven stres je za organizem ģkodljiv (distres),

kratkotrajen in blag pa celo koristen (evstres) (50). Uļinke stresa na organizem je v sredini dvajsetega stoletja prvi opisal Hans Selye z modelom splošnega prilagoditvenega sindroma (51). Organizem se odzove na stres z enakim vzorcem telesnih reakcij ne glede na vzrok stresa, pri dolgotrajnejši izpostavljenosti stresorju pa sledi odziv v treh fazah: alarm (boj ali beg), prilagoditev in izļrpanost (bolezen ali smrt). Pri odgovoru na stres sodelujeta dva glavna endokrina odziva, in sicer takojšnji živļni/ simpatiļni odziv (noradrenalin, adrenalin) pri akutnem stresu ter zapozneli endokrini/ glukokortikoidni odziv, znaļilen za dolgotrajnejši, kroniļni stres (52).

Stresor sproži nastajanje in izloļanje sproļevalnega hormona kortikotropina (CRH) in AVP v paraventrikularnem jedru. CRH in AVP se sprostita v hipofizni portalni žilni sistem, kjer izzoveta sproļanje adrenokortikotropnega hormona (ACTH) iz adenohipofize. ACTH spodbudi izloļanje glukokortikoidov (kortizol pri ljudeh, kortikosteron pri glodavcih) v skorji nadledviļne žleze (49, 53). Glukokortikoidi so pomembni posredniki v intermediarni presnovi, saj pospešujejo sproļanje energetskih rezerv ter s tem podprejo delovanje srļno-žilnega sistema, pospešijo izloļanje vode iz organizma, imajo protivnetni uļinek in zavirajo imunski odgovor. Vendar pa dolgotrajnejša izpostavljenost visokim vrednostim glukokortikoidov lahko poškoduje številne organske sisteme, vključno s centralnim živļnim sistemom (48, 49). Glukokortikoidni receptorji so izražени v številnih celicah po celotnih možganih, vendar jih je največ v hipokampusu, paraventrikularnem jedru in ļelnem delu možganske skorje (npr. 54, 55).

2.1.2.1 Dozorevanje hipotalamo-hipofizno-nadledviļne (HPA) osi

Za puberteto je znaļilno dokonļno dozorevanje delovanja nevroendokrinega sistema in s tem trajne spremembe v številnih nevroendokrinih oseh, najbolj izrazite v osi HPG (pregledno v 1, 13). Manj izrazite, vendar zelo pomembne spremembe v puberteti so opazne tudi v osi HPA (pregledno v 44, 45, 56). Podganam pred puberteto in odraslim podganam, izpostavljenim akutnemu fiziļnemu ali psihiļnemu stresorju, se ravni hormonov ACTH in kortikosterona poveļata primerljivo enako. Vendar pa stresni odziv s povrnitvijo stresnih hormonov na bazalno raven traja pri predpubertetnih samicah (57) in samcih (58) skoraj dvakrat dlje kot pri odraslih živalih, kar kaže na pomembnost obdobja pubertete tudi pri dozorevanju odziva (negativne povratne zanke) osi HPA in s tem zaļite organizma pred visokimi vrednostmi

glukokortikoidov (59, 60). Pri odraslih podganjih samcih se po stresnem dogodku beljakovina Fos (ki kaģe aktivnost nevronov) izraģa v ģtevilnih podroļjih moģganov, medtem ko je pri samcih v ļasu pubertete izraģenost te beljakovine omejena veļinoma na paraventrikularno jedro (61).

Testosteron pri odraslih samcih krajģa stresni odziv, medtem ko ga estradiol pri samicah daljģa (62). Odrasli samci imajo niģje bazalne in stresne vrednosti CRH, ACTH in kortikosterona kot samice (63). Pri predpubertetnih samicah in samcih spolni hormoni nimajo bistvenega vpliva na stresni odziv (57, 58).

2.1.2.2 Socialna osamitev med puberteto in vpliv na obnaģanje v odraslem obdobju

V ļasu pubertete potekajo v moģganih ģe zadnje morfoloģke in funkcionalne spremembe, zato so moģgani zelo ranljivi in obļutljivi na delovanje stresorjev, kar se lahko odraģa v spremembah obnaģanja v odraslosti (npr. 44, 45, 46, 56, 60, 64). Za socialne ģivali, kot so miģi (65), podgane (66) in prerijske voluharice (67), je lahko socialna osamitev zelo stresna. Pri podganah naj bi delovala pomirjujoļe na samce in stresno na samice (68), medtem ko ima prenaseljenost ravno obraten uļinek (merjenje kortikosterona v krvi) (69). Veļina raziskav socialne osamitve obravnava predvsem samce, rezultati vedenjskih raziskav kaģejo na veļjo obļutljivost samcev v primerjavi s samicami (pregledno v 70, 71).

Socialna osamitev med puberteto pri miģih lahko glede na objavljene raziskave zmanjģa izraģanje obnaģanja, podobnega anksioznemu in depresivnemu, tako pri samicah (72) kot samcih (73), ne spremeni pa anksioznosti pri nobenem od spolov pri miģih (74, 75) ali celo okrepi izraģanje anksioznosti pri samcih (76, 77). Socialni osamitveni stres med puberteto pri podganah lahko okrepi izraģanje anksioznemu in ne spremeni depresivnemu podobno obnaģanje pri samcih (78, 79, 80), okrepi depresivnemu podobno obnaģanje pri samicah (81) ter ne vpliva na anksioznost pri samicah (78, 80, 82). Pri samcih prerijskih voluharic socialna osamitev okrepi obnaģanje, podobno anksioznemu (83). Dosedanje raziskave vpliva socialne osamitve tako podajajo zelo raznolike, vļasih celo nasprotujoļe si rezultate, kar je lahko posledica razliļnih pogojev reje, trajanja socialne osamitve in stresnega ravnanja, uporabe razliļnih linij ģivali, uporabe razliļnih vedenjskih testov, testiranja samic v razliļnih fazah spolnega ciklusa in mnogih drugih dejavnikov (npr. 84).

Osamitev med puberteto pri miģih lahko glede na dosedanje raziskave poveļa spontano fiziļno aktivnost tako pri samicah (72) kot pri samcih (73), pri drugih raziskavah pa se lahko poveļa aktivnost pri samcih, ne pa tudi pri samicah (74). Pri podganjih samcih in samicah socialni stres ne vpliva na spontano aktivnost (78), ļeprav ģtevilne druge raziskave kaģejo na poveļano aktivnost zaradi socialne osamitve med puberteto (pregledno v 85). Osamljeni samci prerijskih voluharic imajo poveļano spontano aktivnost (83). Socialna osamitev pri samcih nima veļjega vpliva na agresivno obnaģanje pri miģih (86), je pa zaradi osamitve agresivnost okrepljena pri podganah (87, 88).

Miģji samci, ki so osamljeni med puberteto, kaģejo slabģe predmetno prepoznavanje, vendar pa sposobnost uļenja ni okrnjena (73), medtem ko sta pri samicah okrnjena tako spomin kot tudi uļenje (72). Socialno prepoznavanje je mnogo slabģe pri osamljenih samcih miģi (76) in podgan (88) kot tudi pri samicah podgan (89). Za z razmnoģevanjem povezana obnaģanja je bilo opisano, da osamitev med puberteto ne vpliva na starģevsko (90) niti na spolno obnaģanje podganjih samic (91), da pa osamljeni podganji samci slabģe izraģajo moģko spolno obnaģanje kot samci, nastanjeni v druģbi (92, 93, 94).

2.1.2.3 Socialna osamitev med puberteto ter oksitocin in vazopresin v odraslem obdobju

Raziskave o vplivu socialne osamitve med puberteto na nevrohipofizne hormone razkrivajo, da imajo osamljeni samci prerijskih voluharic poveļano izraģenost mRNK za AVP in OT v paraventrikularnem jedru (83), po drugi strani pa imajo nespremenjeno ģtevilo tako AVP- kot tudi OT-imunoreaktivnih nevronov v paraventrikularnem jedru in tudi nespremenjeno koncentracijo AVP in OT v krvni plazmi (95). Osamljene samice prerijskih voluharic pa kaģejo zmanjģano ģtevilo AVP-imunoreaktivnih nevronov v paraventrikularnem jedru, vendar pa nespremenjeno koncentracijo v plazmi. OT v paraventrikularnem jedru in plazmi pri samicah ni spremenjen (95). Osamljeni miģji samci imajo zmanjģano ģtevilo AVP-imunoreaktivnih nevronov v paraventrikularnem jedru in posledičļno je sproģļanje AVP iz paraventrikularnega jedra ob socialnem stiku manjģe (76). Osamljeni samci podgan imajo v paraventrikularnem jedru nespremenjeno ģtevilo OT-imunoreaktivnih nevronov (96) ter zmanjģano (89) ali celo nespremenjeno ģtevilo AVP-imunoreaktivnih nevronov (96), samice pa zmanjģano ģtevilo OT-imunoreaktivnih nevronov (89).

Veliko veļ je znanega o vplivu socialne osamitve v odraslosti. Dolgotrajna socialna osamitev v odraslem obdobju pri samicah, ne pa tudi pri samcih prerijskih voluharic, poveļa ģtevilo nevronov, imunoreaktivnih na OT v paraventrikularnem jedru (97, 98) in posledilļno tudi koncentracijo OT v krvi (98, 99). Izraģenost mRNK za OTR v celotnem hipotalamusu je zmanjģana tako pri osamljenih samcih kot pri osamljenih samicah prerijskih voluharic (99). Po drugi strani pa osamitev v odraslosti lahko pri samicah poveļa koncentracijo AVP v krvni plazmi (97) ali celo nima vpliva nanjo, tako kot pri samcih prerijskih voluharic (99). Izraģenost mRNK za vazopresinski receptor 1a (V1aR) v celotnem hipotalamusu ob socialni osamitvi ni bila spremenjena niti pri samcih niti pri samicah prerijskih voluharic (99).

2.2 SOCIALNO PREPOZNAVANJE

Socialno prepoznavanje je sposobnost posameznika prepoznati, zapomniti si ter razlikovati znani osebek od neznanega. Pri glodavcih ima kljuļni pomen pri medsebojnem prepoznavanju voh (pregledno v 22). Glodavci raziskujejo/ ovohavajo nove osebke dljeļasa kot znane osebke, kar kaģe na sposobnost socialne prepoznave. Ļe prepoznajo ģe videne/ ovohavane osebke, jih z vsakim naslednjim sreļanjem raziskujejo in ovohavajo krajģiļas, kar imenujemo socialna habituacija ali prilagoditev (opisano v 100, 101). Beljakovina c-Fos, ki je pokazatelj trenutne aktivnosti nevronov, se ob socialnem prepoznavanju najmoļneje izraģa v vohalnih betiļih, piriformni skorji, medialnem in kortikalnem delu mandlja (CoA), stranskem septumu ter v spodnjem jedru konļne proge in v medialnem delu predoptiļnega podroļja (MPOA) (pregledno v 102, 103). Vzpostavitev in vzdrģevanje socialnih razmerij je zapleten proces, ki vkljuļuje veļ ravni obdelave podatkov v moģganih. Sistema OT in AVP spadata med kljuļne sisteme v moģganih, ki uravnavajo socialno prepoznavanje (opisano v 101, 104). OT (predvsem v mandlju) je bolj pomemben pri samicah (105, 106), AVP (predvsem v stranskem septumu) pa pri samcih (27, 107). Vohalni draģljaj ob socialnem prepoznavanju vzdraģi vohalne betiļe, od koder potekajo ģivļna vlakna vse do medialnega dela mandlja, kjer se pod vplivom OT vzpostavlja socialni spomin. Ģivļna vlakna iz medialnega dela mandlja se nadaljujejo do spodnjega jedra konļne proge ter iz medialnega dela mandlja in spodnjega jedra konļne proge vse do stranskega septuma, kjer se pod vplivom AVP predelujejo socialni draģljaji. Stranski septum je povezan s hipokampusom, ki ima kljuļno vlogo pri uļenju in

pomnjenju. Spodnje jedro konļne proge in stranski septum sta povezana z medialnim delom predoptiļnega podroļja, ki uravnava spolno obnaģanje (opisano v 101).

2.2.1 Socialno prepoznavanje in spolni hormoni med puberteto

Ģtevilne raziskave so pokazale pomembnost spolnih hormonov ter njihovih receptorjev v odraslem obdobju pri uravnavanju socialnega prepoznavanja (pregledno v 104), in sicer preko uravnavanja sistemov OT (estradiol) (pregledno v 108) in AVP (testosteron) (109). Zelo redke in zgolj posredne raziskave o nemoteni izpostavljenosti estradiolu v ļasu pubertete kaģejo na dobro socialno prepoznavanje pri odraslih samicah (npr. 110), medtem ko testosteron med puberteto pri samcih slabģa socialno prepoznavanje v odraslosti (111).

Pri odraslih samicah miģi in podgan je socialno prepoznavanje boljģe v proestrusu, ko je raven estrogenov in progesterona v plazmi visoka v primerjavi z ostalimi fazami spolnega ciklusa (112). Odstranitev jajļnikov pri odraslih miģih in podganah oslabi socialno prepoznavanje, dodajanje estradiola pa ga ponovno izboljģa tako pri samicah miģi (113, 114) kot podgan (114). Miģje samice in samci z izbitim genom za ERŭ ali ERŏ slabģe izraģajo socialno prepoznavanje kot miģi z nespremenjenim genomom (108, 115). Socialno prepoznavanje pri samicah brez gena za oba estrogenska receptorja je podobno kot pri samicah brez gena za OT (108), kar dokazuje, da uravnavanje socialnega prepoznavanja pri samicah poteka preko uravnavanja sistema OT (pregledno v 108). Estradiol uravnava izraģanje OT v paraventrikularnem jedru preko ERŏ (116) ter izraģanje OTR v medialnem delu mandlja preko ERŭ (117, 118).

Odstranitev mod pri odraslih samcih izzove poslabģanje socialnega prepoznavanja, dodajanje testosterona pa ga ponovno izboljģa. Ļe testiranje obnaģanja opravimo kmalu po kastraciji ali pa ga izvajamo redno vsak drugi dan po kastraciji, se socialno prepoznavanje ne razlikuje bistveno od tistega pri intaktnih samcih (109). Antagonist za AVP enako uspeģno oslabi socialno prepoznavanje tako pri intaktnih kot tudi pri kastriranih samcih, ki prejemajo testosteron, ne pa tudi pri kastriranih, ki testosterona ne prejemajo (109). Uravnavanje socialnega prepoznavanja pri samcih poteka s strani testosterona preko uravnavanja sistema AVP, kar ne presenļa, saj je AVP v spodnjem jedru konļne proge, medialnem delu mandlja in stranskem septumu veliko bolje izraģen pri samcih kot samicah (119). Ģtevilo AVP-

imunoreaktivnih teles nevronov v spodnjem jedru konļne proge in vlaken v stranskem septumu se moļno zmanjģa po kastraciji, dodajanje testosterona pa te uļinke izniļi (120).

2.2.2 Socialno prepoznavanje in socialna osamitev med puberteto

Socialna osamitev med puberteto moļno poslabģa sposobnost razlikovanja znane od neznane ģivali kakor tudi sposobnost prepoznave ģe znane ģivali pri podganjih samcih (88) in samicah (89). Nesposobnost socialnega prepoznavanja socialno osamljenih ģivali sovpada z zmanjģanjem ģtevila OT- pri podganjih samicah kakor tudi ģtevila AVP-imunoreaktivnih nevronov v paraventrikularnem jedru pri samcih podgan (89) in miģi (76). Socialna osamitev med puberteto pri samcih zmanjģa sproģļanje AVP iz paraventrikularnega jedra po socialni izpostavljenosti v primerjavi s skupinsko nastanjenimi miģjimi samci (76). Socialno osamljene miģje samice (brez spolnih ģlez ali brez spolnih ģlez, vendar z dodanim estradiolom) imajo slabģo socialno habituacijo kakor tudi slabģo, vendar vseeno prisotno sposobnost razlikovanja znane od neznane ģivali (113). Po drugi strani pa Gatewood in sodelavci (121) poroļajo, da so socialno osamljeni miģji samci in samice (brez spolnih ģlez z dodanim testosteronom) vseeno sposobni dobrega socialnega prepoznavanja kakor tudi moļne socialne habituacije, brez razlik med spoloma.

2.2.3 Socialno prepoznavanje ter oksitocin in vazopresin

Raziskave velikokrat kaģejo na kontradiktornost delovanja OT glede na uporabljen odmerek, in sicer podkoģno dajanje visokih odmerkov OT poslabģa socialni spomin (122), medtem ko ga niģje doze OT izboljģajo (123). Dodajanje niģjih odmerkov OT v moģganske kletke ali medialni del mandlja bistveno ne spremeni ali pa celo izboljģa socialno prepoznavanje, medtem ko dodajanje antagonista OT oslabi socialno prepoznavanje tako pri samicah podgan (124) in miģi (108) kot tudi pri samcih podgan (125) in miģi (126). Miģje samice (108) in samci brez gena za OT (126) imajo slabģe socialno prepoznavanje v primerjavi z miģmi z nespremenjenim genomom ali spremenjenim genomom in dodanim OT v moģganske kletke ali v medialni del mandlja (102, 126). Tudi miģi brez gena za OTR niso sposobne razloļevati znane od neznane ģivali (127, 128). OT izboljģa socialno prepoznavanje tudi, ļe ga dodajamo

v stranski septum (129), ventralni hipokampus (130) in v medialni del predoptiļnega podroļja (131).

Ģtevilne raziskave pri podganjih samcih so pokazale, da AVP, dodan v moģganske kletke ali v stranski septum, izboljģuje socialno prepoznavanje, medtem ko ga AVP ali V1aR antagonisti slabijo (120, 132, 133, 134, 135, 136). Po eni strani AVP izboljģa socialno prepoznavanje tudi pri samicah, po drugi strani pa AVP antagonist nima vpliva (135). AVP je torej pomembnejģi pri samcih (pregledno v 120), saj je AVP v moģganih veliko bolj izraģen pri samcih kot pri samicah (pregledno v 117). Samci miģi brez gena za V1aR imajo moļno zmanjģano sposobnost socialnega prepoznavanja in tudi ne kaģejo nobene prilagoditve ģe znani ģivali po veļkratni izpostavljenosti (137), kar pa ponovno izraģanje gena za V1aR v stranskem septumu izniļi (107). Podobno zmanjģanje socialnega spomina kaģejo tudi podganji samci po dodajanju antagonista V1aR v supraoptiļno jedro ali v stranski septum, ki prejema ģivļna vlakna iz supraoptiļnega jedra in v katerem so moļno prisotni V1aR (138), podobno pa je bilo opisano pri podganjih samcih linije Brattleboro, ki niso sposobni proizvajati AVP (139). Socialno prepoznavanje pri samcih je torej v veliki meri odvisno od zadostnega izraģanja AVP in V1aR predvsem v stranskem septumu (pregledno v 104, 107). Tudi hipokampus ima pomembno vlogo pri socialnem spominu, saj dodajanje protiteles proti AVP neposredno v hipokampus poslabģa socialno prepoznavanje pri podganjih samcih (130).

2.3 ĢENSKO SPOLNO OBNAĢANJE

Ģensko spolno obnaģanje lahko na sploģno opiģemo kot zapleten nabor razliļnih obnaģanj pri samici, ki so potrebna in zadostna, da pride do zdruģitve ģenske in moģke spolne celice (10). Pri glodavcih razlikujemo dva osnovna elementa ģenskega spolnega obnaģanja: proceptivno obnaģanje ali spolna razvnetost in receptivno obnaģanje ali spolna sprejemljivost (140). Spolna razvnetost je skupek razliļnih obnaģanj, ki so potrebna za zaļetek kot tudi nadaljevanje spolnega odnosa s samcem in zajemajo poskakovanje z izmeniļnim pribliģevanjem in oddaljevanjem od samca, ovohavanje samca in miganje z uģesi (22, 140). Spolna sprejemljivost je trenutno zavzetje telesne drģe, ki omogoļa nemoten spolni odnos s samcem in zajema usloļenje hrbtenice (lordoza) z dvigom glave in medenice ter odklonom repa na stran. Spolna razvnetost in spolna sprejemljivost sta veliko izrazitejģi pri podganah kot pri miģih (10, 22, 140). Ģensko spolno obnaģanje v glavnem uravnavajo spolni hormoni

(estradiol in progesteron) in nevroni v mnogih podroļjih moġganov, ki so obļutljivi na delovanje spolnih hormonov (v glavnem na estradiol) (npr. 141, 142, 143). Beljakovina c-Fos, ki je pokazatelj trenutne aktivnosti nevronov, se ob spolnem obnaġanju najmoļneje izraġa v medialnem delu predoptiļnega podroļja, ventromedialnem jedru hipotalamusa in v medialnem delu mandlja (141). Estradiol z delovanjem preko estrogenskega receptorja Ŭ (ERŬ) v nevronih teh podroļij moġganov uravnava izraġanje spolne sprejemljivosti pri samicah (142).

2.3.1 Žensko spolno obnašanje in spolni hormoni med puberteto

Zelo malo je znanega o vplivu ġenskih spolnih hormonov med puberteto na spolno obnaġanje pri samicah, in ġe to je omenjeno le v preglednih ļlankih (npr. 1, 13). Presenetljivo je, da ġenski spolni hormoni med puberteto kaġejo na nasprotujoļi si, vļasih celo nesmiselni uļinek, kot sta slabġe izraġanje ġenskih (defeminizacija) in celo spodbujanje moġkih (maskulinizacija) oblik spolnega obnaġanja pri samicah. Prisotnost jajļnikov ali estradiola med puberteto pri samicah zlatega hrļka poslabġa izraġanje spolne sprejemljivosti ali lordoze v primerjavi s samicami z odstranjenimi jajļniki pred puberteto (pregledno v 21). Po drugi strani pa prisotnost jajļnikov ali estradiola med puberteto pri samicah podgan ne spremeni izraġanja ġenskega spolnega obnaġanja, vendar pa hkrati spodbuja izraġanje moġkih oblik spolnega obnaġanja ob prisotnosti samice ter veļje zanimanje za samice (26).

2.3.2 Žensko spolno obnašanje in socialna osamitev med puberteto

Edina raziskava pri samicah o vplivu socialne osamitve med puberteto in po njej ugotavlja, da se socialno osamljene podganje samice ne razlikujejo od skupinsko nastanjenih v stopnji izraġanja spolne sprejemljivosti (lordoza) (91). Po drugi strani pa pri istih samicah dodajanje testosterona v odraslem obdobju izzove moġko spolno obnaġanje (naskakovanje spolno sprejemljivih samic in potiskanje z medenico), ki je bolje izraġeno pri socialno nastanjenih kot pa osamljenih, kar sovpada z ugotovitvami pri socialnih in osamljenih sorojencih moġkega spola (91).

Maloġtevilne dosedanje raziskave pri samicah laboratorijskih glodavcev (144, 145, 146, 147) dokazujejo, da doloļene vrste stresorjev v obdobju pubertete lahko izzovejo trajne morfoloġke spremembe v moġganih in posledilno spremenijo nevroendokrini odgovor na estradiol in progesteron, kar se kaġe kot zmanjġana stopnja izraġanja ġenskega spolnega obnaġanja v odraslem obdobju. Stresorji, kot so transport ġivali od vzrejne/ dobaviteljske do uporabniġke organizacije (144, 145) ter enkratno injiciran bakterijski endotoksin lipopolisaharid (144) zmanjġajo spolno sprejemljivost, medtem ko tridnevna skrajnostna prostorska omejitev (146), dvodnevna popolna odtegnitev hrane (146) ter tridnevni sestavljen stres (skrajnostna prostorska omejitev z moļno osvetlitvijo in posledilno poveļano temperaturo prostora) (146) ne spremeni spolnega obnaġanja pri odraslih miġjih samicah. Ġkodljivi uļinki transporta in lipopolisaharida na spolno sprejemljivost pri miġih so mnogo moļnejġi, ļe delujejo med puberteto (pri starosti 4, 5, 6 ali 8 tednov) kot pa v odraslosti (pri starosti 10 tednov) (144, 145, 146).

2.3.3 Žensko spolno obnašanje ter oksitocin in vazopresin

Poleg estradiola in progesterona so za sproġitev spolne sprejemljivosti potrebni ġe ġtevilni drugi nevropeptidi in nevrotransmiterji, ki imajo pomembno vlogo pri spreminjanju izrazitosti, trajanja in pogostosti lordoze (148, 149). Nevropeptid OT nastaja v paraventrikularnem in supraoptilnem jedru, od koder se ob spolnem odnosu sproġa po ġivļnih vlaknih preko nevrohipofize v krvoġilni sistem. Po drugi strani pa so vlakna nevronov iz paraventrikularnega jedra povezana z ventromedialnim jedrom hipotalamusa (150), v katerem so moļno izraġeni OTR in ki ima kljuļno vlogo pri izraġanju spolne sprejemljivosti in razvnetosti pri samicah v pojatvi (151, 152). Poveļane vrednosti estradiola med pojatvijo tako poveļajo sproġanje OT iz paraventrikularnega jedra (153) ter posledilno poveļajo OT-imunoreaktivnost nevronov in vezavo na OTR v ventromedialnem jedru hipotalamusa (154, 155).

Raziskave o uļinkih nevropeptida AVP na ġensko spolno obnaġanje kaġejo ravno nasprotne rezultate kot pri OT. AVP, apliciran v moġgansko kletko pri podganah (156) ali v medialni del predoptilnega podroļja pri hrļicah (157), zavira tako spolno sprejemljivost kot tudi spolno razvnetost, nasprotno pa antagonist V1aR spodbudi obe obnaġanji. AVP lahko celo zavira pozitivne uļinke OT na izraġanje ġenskega spolnega obnaġanja (158).

2.4 STARĠEVSKO OBNAĠANJE

Rosenblatt je starġevsko obnaġanje na splošno opisal kot vsako obnaġanje, ki je izraġeno do lastnega potomstva (159). Bolj natanļno pa ga lahko opiġemo kot vrsto razliļnih aktivnosti, ki neposredno pripomorejo k boljġemu preġivetju oplojene jajļne celice ali skotenih mladiļev. Starġevskemu obnaġanju samic pravimo materinsko, samcev pa oļetovsko obnaġanje (9). Oļetovsko obnaġanje je med sesalci dokaj redko, vendar prisotno pri nekaterih mesojedih, prvakih in glodavcih (pregledno v 9), med slednjimi predvsem pri kalifornijski miġi, zlatem hrļku, hiġni miġi ter prerijski voluharici. Pri glodavcih poznamo dva osnovna vzorca starġevskega obnaġanja: obnaġanje, ki je usmerjeno k mladiļem, in obnaġanje, ki ni usmerjeno k mladiļem (160). K mladiļem usmerjeno obnaġanje vkljuļuje zadrġevanje mladiļev v gnezdu, negovanje, gretje pod telesom in druge dejavnosti, ki vkljuļujejo telesni stik z mladiļi. Obnaġanje, ki ni usmerjeno k mladiļem, pa zajema gradnjo gnezda, zauġitje posteljice in varovanje mladiļev (pregledno v 161). Pri podganah in miġih ob kotitvi pride do hitrega padca progesterona in hkrati dviga estradiola, prolaktina in laktogenih hormonov posteljice, kar spodbudi zaļetek materinskega obnaġanja, za vzdrġevanje obnaġanja pa so dovolj ġe draġljaji mladiļev (160, 162, 163). Pri odraslih samicah in samcih podgan lahko starġevsko obnaġanje izzovemo z veļkratnih izpostavljanjem tujim mladiļem oziroma z razvojem t.i. senzitizacije (opisano v 9, 164). V nasprotju s podganami lahko miġje samice in samci starġevsko obnaġanje izraġajo ġe po prvi izpostavljenosti tujim mladiļem (163, 165) in celo neodvisno od estrogenih hormonov (166). Samci tako podgan kot miġi so naļeloma bolj nagnjeni k detomoru in slabġe izraġajo starġevsko obnaġanje kot samice (165). Beljakovina c-Fos, ki je pokazatelj trenutne aktivnosti nevronov, se po materinskem obnaġanju ob izpostavljenosti mladiļem najmoļneje izraġa v medialnem delu predoptiļnega podroļja in spodnjem jedru konļne proge, manj v medialnem in kortikalnem delu mandlja, v stranskem septumu ter v nekaterih drugih delih moġganov (167, 168). Estrogeni v medialnem delu predoptiļnega podroļja in v spodnjem jedru konļne proge spodbujajo materinsko obnaġanje pri podganah (163, 168, 169, 170).

2.4.1 Starševsko obnašanje in spolni hormoni med puberteto

Mlade podganje samice in samci pred puberteto hitro pristopijo k mladiļem, jih nosijo v gnezdo, liġejo in stiskajo k telesu, kar se drastiļno zmanjġa z nastopom pubertete in pokaġejo

se razlike med spoloma (171). Pri podganjih samcih je najpogostejġa ugotovitev, da odstranitev mod pred zaļetkom pubertete naļeloma izboljġa oļetovsko obnaġanje in zmanjġa detomore (pregledno v 165, 172), po drugi strani pa odstranitev jajļnikov pri podganjih samicah pred puberteto in med njo lahko zmanjġa (173) ali pa nima bistvenega uļinka na materinsko obnaġanje v odraslosti (174).

Mnogo veļ je znanega o vplivih spolnih hormonov v odraslem obdobju. Odstranitev jajļnikov ter s tem ġenskih spolnih hormonov pri neizkuġenih odraslih samicah miġi (175) in podgan (176) nima bistvenega vpliva na starġevsko obnaġanje, medtem ko pri miġjih samcih po nekaterih raziskavah odstranitev mod izboljġa starġevsko obnaġanje (177), po nekaterih drugih pa pri samcih miġi in podgan nima vpliva na starġevsko obnaġanje (121, 178, 179). Samice miġi v glavnem hitreje pristopijo k mladiļem, jih prenesejo v gnezdo in negujejo pod telesom ter so veliko manj nagnjene k detomoru kot samci (121), ne glede na prisotnost ali odsotnost jajļnikov v odraslosti (175) ali nadomeġļanje estradiola po odstranitvi jajļnikov (177). Po drugi strani pa odstranitev mod ne glede na dodajanje ali nedodajanje estradiola pri odraslih miġjih samcih skrajġa ļas do zaļetka prenaġanja mladiļev v gnezdo ter spodbudi obļutljivost do mladiļev v primerjavi s samci z modi ali brez njih in ne glede na nadomeġļanje testosterona (177).

2.4.2 Starševsko obnašanje in socialna osamitev med puberteto

Socialna osamitev med puberteto nima vpliva na oļetovsko obnaġanje pri odraslih miġjih samcih (180), saj ne spremeni nagnjenosti k detomoru niti stopnje izraġanja oļetovskega obnaġanja (prenaġanje mladiļev v gnezdo) v primerjavi s skupinsko nastanjenimi samci. Po drugi strani pa so socialno osamljeni samci po puberteti (od 60. dneva naprej) manj nagnjeni k detomoru in hkrati moļneje izraġajo oļetovsko obnaġanje (180). Socialno osamljene miġje samice med puberteto in po njej zaļnejo hitreje prenaġati mladiļe v gnezdo in jih prenesejo veļ v primerjavi z osamljenimi samci (121). Podobna razlika med spoloma se kaġe tudi pri nastanitvi v skupine po 3 do 5 miġi od odstavitve naprej (177).

2.4.3 Starševsko obnašanje ter oksitocin in vazopresin

Ob koncu brejosti, ko moļno pade raven progesterona in narastejo vrednosti estrogenov, se nevroni v medialnem delu predoptiļnega podroļja moļneje odzovejo na estrogene, kar spodbudi nevrone k tvorbi in sproģļanju nevroaktivnih substanc (nevrotransmiterji, nevropeptidi), ki spodbudijo starģevsko obnaģanje (podrobno opisano v 163). Eden od teh nevropeptidov je OT (181), ki se iz paraventrikularnega jedra med drugim sproģļa tudi do medialnega dela predoptiļnega podroļja, v katerem je izraģeno veliko OTR (182). Ļe pride do poģkodb nevronov v paraventrikularnem jedru, se poslabģa tudi izraģanje starģevskega obnaģanja (183, 184). V primerjavi z OT se zdi, da je nevropeptid AVP manj pomemben pri uravnavanju starģevskega obnaģanja in zato tudi manj prouļevan (185, 186), ļeprav dodajanje AVP v moģganske kletke ali v medialni del predoptiļnega podroļja lahko pripomore k izboljģanju starģevskega obnaģanja pri podganah (pregledno v 187).

OT, dodan v cerebrospinalno tekoļino stranskih moģganskih kletk, sproģi popolno materinsko obnaģanje pri podganjih samicah, poprej tretiranih z estradiolom (185) ali v fazi pojatve (186), medtem ko blokiranje OTR podaljģa in oslabi zaļetek materinskega obnaģanja. Podobno je pri ovcah (188). V primerjavi s podganami raziskave pri miģih razkrivajo, da OT ni tako nujno potreben za popolno izraģanje materinskega obnaģanja (razen dojenja), saj se samice brez OT ne razlikujejo bistveno v obnaģanju od samic brez te mutacije (189), medtem ko je OTR nujno potreben, saj miģje samice (po kotitvi ali nebreje) z izbitim genom za OTR okrnjeno izraģajo materinsko obnaģanje (127). Po drugi strani pa kastracija kot tudi dodajanje estradiola pri miģjih samcih primerljivo poveļa ģtevilo OT-imunoreaktivnih nevronov v paraventrikularnem jedru, kar sovpada z izboljģanjem starģevskega obnaģanja (prenaģanje mladiļev v gnezdo), ki je primerljivo z obnaģanjem pri samicah (intaktnih, z odstranjenimi jajļniki ali z odstranjenimi jajļniki in dodanim estradiolom) (177).

2.5 DEPRESIVNE IN ANKSIOZNE MOTNJE TER RAZLIKE MED SPOLOMA

Depresija je raznotera, veļplastna razpoloģenjska oziroma ļustvena motnja, ki se izraģa na duģevni, vedenjski in fiziološki ravni (190). Depresivne motnje razvrģļamo glede na stopnjo resnosti kliniļne slike in trajanja v 3 glavne skupine: veliko depresivno motnjo, distimijo in bipolarno motnjo (191). Za veliko (ali hudo) depresivno motnjo so znaļilni moļno depresivno

razpoloģenje, obļutek neugodja ter nevrovegetativne in kognitivne spremembe, ki trajajo najmanj 2 tedna (190, 192). Distimija (ali prikrita depresija) zajema zmerno ali blago depresivno razpoloģenje, ki traja vsaj 2 leti, prekinitve simptomov pa niso daljģe od 2 mesecev. Bipolarna motnja (ali maniļna depresija) je razpoloģenjska motnja, pri kateri se menjavata razpoloģenjski stanji depresije in manije (191, 192, 193). Epidemioloģke in kliniļne ģtudije ugotavljajo razlike med spoloma z nastopom pubertete (194), kjer je obolevnost za depresijo pri ģenskah najmanj dvakrat pogostejģa kot pri moģkih (195, 196). Raziskave zadnjih let razkrivajo vpletenost ģtevilnih hormonskih sistemov pri razvoju motenj v razpoloģenju, predvsem pa poudarjajo pomen motenj v osi HPA in HPG (191, 197) pri razvoju depresivnih bolezenskih znakov. Receptorji tako za glukokortikoidne kot spolne hormone so moļno izraģeni prav v podroļjih moģganov (hipotalamus, osrednji mandelj, hipokampus, nekatera podroļja moģganske skorje), vkljuļenih v razvoj depresivnih motenj (192, 197).

Anksiozne motnje so ļustvene motnje, ki jih lahko natanļneje opiģemo in opredelimo glede na okoliģļine, predmet ali misli, ki so izzvale strah, avtonomne, kognitivne in motoriļne znaļilnosti strahu ter glede na razliļne odzive v vedenju pri spopadanju s strahom (190). Odzivi anksioznosti se lahko razlikujejo v jakosti, pogostnosti, vztrajnosti, sproģilnih dejavnikih, resnosti in posledicah ter v ģtevilnih drugih znaļilnostih (198). Diagnostiļni in statistiļni priroļnik duģevnih motenj DMS-5® Ameriģkega zdruģenja psihiatrov podrobno opredeljuje veļ kot 12 razliļnih anksioznih motenj, ki jih uvrģļa v 5 glavnih skupin: fobije, paniļna motnja, obsesivno kompulzivna motnja, postravmatska stresna motnja in sploģna anksiozna motnja (190). Epidemioloģke in kliniļne ģtudije ugotavljajo razlike med spoloma, saj je obolevnost za anksioznimi motnjami pri ģenskah najmanj dvakrat pogostejģa kot pri moģkih (195, 196). Anksiozne motnje se pogosto prepletajo z depresivnimi (199), saj se prepletata tudi njuna regulacijska sistema (200).

2.5.1 Zdravljenje depresivnih in anksioznih motenj ter razlike med spoloma

Pri zdravljenju depresivnih motenj se uporabljajo razliļni antidepresivi, kot so SSRI, triciklиļni antidepresivi (TCA) in zaviralci oksidaz monoaminov (MAOI). Med njimi se najpogosteje uporabljajo in imajo tudi najmanj stranskih uļinkov antidepresivi SSRI (199), ki jih pogosto predpisujejo tudi pri zdravljenju anksioznih motenj (201). Antidepresivi SSRI

zviģujejo koncentracijo ģivļnega prenaģalca serotonina (5-HTT ali SERT) v sinapsah ter posledilļno zviģujejo zunajcelilļno koncentracijo serotonina (5-HT) (199, 202). Fluoksetin je bil prvi SSRI, ki je bil dostopen na trģiģļu (203, 204).

Vedno veļ raziskav ugotavlja razlike med spoloma v farmakokinetiki in/ ali farmakodinamiki antidepresivnih zdravil, vendar zdravljenje ostaja ģe zmeraj enako pri obeh spolih (pregledno v 205). Raziskave so pokazale, da fiziološke razlike med spoloma in spolni hormoni lahko vplivajo na vsrkavanje, prerazporeditev, presnovo, farmakodinamiko in pojav stranskih uļinkov mnogih zdravil (pregledno v 206), tudi fluoksetina (207, 208, 209). Ģenske se bolje odzivajo na zdravljenje s fluoksetinom kot moģki in k temu naj bi prispevali prav estrogeni hormoni (209), kar dokazujejo tudi raziskave pri podganjih samicah (v fazah proestrusa/ estrusa s poveļanimi vrednostmi estradiola) (210) in samcih (211).

2.5.2 Živalski modeli depresivnih in anksioznih motenj ter razlike med spoloma

Popoln ģivalski model za prouļevanje razliļnih kliniļnih stanj pri ļloveku mora izpolnjevati 3 merila: napovedno, priļakovano in temeljno vrednost (212), kar pa je pri modelih za depresivne in anksiozne motnje teģko, saj doloļenih kliniļnih znakov, znaļilnih za ljudi, ni mogoļe izzvati in ocenjevati pri ģivalih (obļutki krivde, samomorilne misli) (190). Pri prouļevanju uļinka antidepresivnih zdravil se pri modelnih organizmih najpogosteje uporabljajo 3 testi obnaģanja, ki pri laboratorijskih ģivalih delujejo kot nenaden in kratkotrajen stresor: test prisilnega plavanja (FST) (213, 214, 215), dvignjen labirint v obliki kriģa (EPM) (212, 216, 217) in test odprtega polja (OF) (212, 218). Pri FST se ocenjuje stanje negibnosti ģivali v brezizhodnem poloģaju, kar kaģe na stanje, podobno obupanosti in vdanosti v usodo (219). Pri testih EPM in OF se ocenjuje izogibanje dvignjenim, odprtim in svetlim predelom labirinta, kar kaģe na stanje, podobno bojazljivosti in prestraģenosti (216, 217). Pri testu OF pa se dodatno preverja ģe sposobnost gibanja oziroma motivacija za gibanje testirane ģivali (220).

Dosedanje raziskave delovanja fluoksetina in razlik med spoloma pri testih FST, EPM in OF pri miģih in podganah so dale zelo raznolike, vļasih celo nasprotujoļe si rezultate, najverjetneje prav zaradi uporabe razliļnih ģivalskih vrst, linij ģivali, stresnega ravnanja, testiranja samic v razliļnih fazah spolnega ciklusa, uporabe razliļnih odmerkov, naļina in

ļasa dajanja fluoksetina ter mnogih drugih dejavnikov (pregledno v 84). Kljub tej raznolikosti se lahko izpostavi nekaj skupnih toļk. Dolgotrajnejġe dajanje fluoksetina kaġe na enotnejġe uļinke kot kratkotrajno ali enkratno dajanje, kar kaġe na potrebo po daljġem prilagoditvenem obdobju na poveļane vrednosti 5-HT (192). Samice v proestrusu ali estrusu so naļeloma manj depresivne (221, 222) in manj anksiozne (223, 224, 225) v primerjavi s samci in samicami v medestrusu ali diestrusu, medtem ko v gibalnih aktivnostih med spoloma ni bistvenih razlik (220, 223). Samice v proestrusu ali estrusu so v glavnem bolj odzivne na antidepresivno delovanje fluoksetina (220, 222) in se nanj odzivajo z obnaġanjem, podobnim anksioznemu, (225) v primerjavi s samci in samicami v medestrusu in diestrusu. Fluoksetin zmanjġa spontanost po gibanju pri miġjih samcih (226, 227, 228) in samicah (228, 229), pri podganah pa nima vpliva (220, 230).

3 OBJAVLJENI ZNANSTVENI ČLANKI

3.1 ADOLESCENT SOCIAL ISOLATION CHANGES SOCIAL RECOGNITION IN ADULT MICE

SOCIALNA OSAMITEV V MLADOSTI SPREMENI SOCIALNO PREPOZNAVANJE PRI ODRASLIH MIŠIH

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Izvleček

Socialna osamitev moļno vpliva na ģtevilna obnaģanja odraslih glodavcev, vendar je malo znanega o uļinkih osamitvenega stresa pri socialnem obnaģanju. Pri naģi raziskavi smo prouļevali socialno prepoznavanje pri miģjih samcih in samicah, ki smo jih trajno socialno osamili vse od starosti 30 dni in jih testirali okrog 80. dneva starosti ali jih zaļasno osamili od 30. do 60. dneva starosti in jih nato od 60. dneva do testiranja pri 80 dnevih starosti ponovno skupinsko nastanili. Miģi obeh osamljenih skupin smo primerjali s kontrolno skupino miģi, ki je bila nastanjena v socialnih skupinah med celotno raziskavo. Izvedli smo standardni test socialnega prepoznavanja, kjer smo kot stimulusne ģivali uporabili odrasle miģje samice z odstranjenimi jajļniki. Pri testnih ģivalih smo beleģili in ocenjevali ļas ovohavanja stimulusne miģi. Stimulusno samico smo dodajali testnim ģivalim v domaļo kletko osemkrat po 1 minuto vsakih 9 minut, devetiļ pa uporabili novo, neznano stimulusno samico. Kontrolne skupinsko nastanjene miģi obeh spolov so izraģale moļan vzorec socialnega uļenja in sposobnost prepoznavanja znane od neznane ģivali, medtem ko trajno socialno osamljene miģi niso kazale nobenega socialnega prepoznavanja. Tudi zaļasna osamitev miģi od 30. do 60. dneva starosti je zmanjģala sposobnost socialnega uļenja v primerjavi s skupinsko nastanitvijo ter prizadela socialno prepoznavanje predvsem pri samicah. Naģe ugotovitve kaģejo, da ima socialna osamitev moļan vpliv na socialno obnaģanje miģi, saj ģe zaļasna osamitev lahko privede do trajnih sprememb pri obnaģanju.

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Research report

Adolescent social isolation changes social recognition in adult mice

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ABSTRACT

Rearing in social isolation has profound effects on several aspects of behavior in adult rodents. However, little is known about effects of social stress on social behavior in these animals. In the present study, we examined social recognition in mice of both sexes that were individually housed from 30 days of age until testing at approximately 80 days of age, individually housed from day 30 until day 60, followed by group housing from day 60 until testing at around 80 days of age and in control mice that were group housed throughout experiment. A standard social recognition test was performed with ovariectomized female conspecifics introduced into the home cage of tested mice for 1 min, eight consecutive times with 9 min breaks between tests, and in the ninth test, new, unfamiliar females were introduced. The time spent investigating stimulus mice during each of the nine tests was recorded. Group housed male and female mice showed strong pattern of social learning, whereas mice reared in isolation from day 30 until testing did not show evidence of social recognition. Interestingly, mice reared in isolation from 30 until 60 days of age and then group housed again, also showed reduced ability for social learning in comparison to the controls housed in groups through the entire period. These results therefore show that social isolation has a profound effect on social behavior in mice, and that even isolation for a limited period can produce lasting behavioral deficits.

1. Introduction

Early life stress can have long lasting deleterious effects on brain development and consequently on behavior in adult life [1]. Rearing in isolation either in infancy or in adolescence can produce severe behavioral consequences in adult life in rodents that are social animals by nature [2]. Such studies provide for better understanding of animal behavior and may have implications for human behavior and psychiatric disorders where social isolation is a contributing factor [1].

Mice and rats are social mammals living in large social groups in natural conditions [3,4]. In laboratory conditions, however, males are sometimes housed individually to prevent intermale aggression, or unwanted mating if males and females would be housed together. Therefore, males (and even females) that have been used in behavioral testing are sometimes housed individually [5]. Unfortunately, such studies do not account for possible effects of social isolation on the outcome of behavioral testing. This is a potential problem because previous studies have shown that rearing in social isolation during the pubertal period can lead to hyperactivity and reduced habituation, reduced novel object recognition, and reduced floating time in forced swim tests. Interestingly, the effects on anxiety-like behavior are somewhat conflicting since social isolation decreased anxiety-like behavior assessed by elevated plus maze testing, but increased anxiety-like behavior in light–dark field testing in mice; and similar effects of social isolation have been reported for rats [6–9]. Although the molecular mechanisms that lead to these behavioral deficits are mostly unknown, several studies have shown that social isolation directly affects brain development in juvenile rodents. Rearing in isolation has been reported to result in reductions of medial prefrontal cortex volume [10], cytoskeletal alterations in hippocampus [11] and changes in CREB expression and dopamine and serotonin turnover in different parts of the brain [6,12].

Social recognition is critical for establishing and maintaining social structures in groups of animals living together. Tests for social recognition were first described by Thor and Holloway [13] and are based on monitoring the time that tested animals spend investigating conspecifics introduced multiple times into the cages of tested animals. In rats and mice, the time of investigation normally decreases with exposure to the same animal, and is increased upon exposure to novel unfamiliar animals, usually to the time as observed during the first exposure to the novel animal [5]. Interestingly, social recognition is usually sexually dimorphic with the

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reduction in time during the test with same animal being more prominent in males than in females [14,15]. The influence of social isolation on performance in social recognition tests has not been thoroughly investigated. Recently, Zhao et al. reported abnormalities in social recognition in male rats, housed individually [16], but we are not aware of any reports concerning the effect of social isolation on social recognition in mice of both sexes, and particularly whether social isolation for a limited time period could have a lasting effect on social learning. The present study, therefore, examined social recognition in mice of both sexes that were reared in groups or in isolation from day 30 (beginning of puberty) until testing or for a limited period of time to explore whether social isolation could cause long-lasting alterations in murine social behavior.

2. Material and methods

2.1. Animals

C57BL/6J mice were bred in standard conditions with 12–12 LD cycle (lights on at 5 am and off at 5 pm) and food (phytoestrogen free diet; Harlan Teklad Diet 2016, Harlan, Milan, Italy) and water ad libitum. C57BL/6J strain of mice was chosen as this is a strain frequently used in genetic analyses of brain and behavior. Mice were weaned at 21 days of age and mice from the same litters were divided into three groups. Mice from the first group (social group) were divided into groups of three mice of same sex after weaning at 3 weeks of age, mice from second group (isolated) were isolated into individual cages at 30 days of age and mice from third group (isolated/social) were isolated into individual cages at 30 days of age and grouped into social groups of three mice (of same sex) at 60 days of age. Group housed mice were housed in 16 cm high cages with floor area 38 cm × 22 cm and individually housed mice were kept in 13 cm high cages with 35 cm × 15 cm floor area. For stimulus mice, ovariectomized female mice of the same strain were used. Ovariectomized stimulus female mice were used to prevent any possible influence of estrus cycle on duration of sniffing of tested mice, and to make stimulus mice neutral to both male and female test mice. The goal was to present the stimulus mice as conspecifics but not possible mating partners or aggressive opponents. Stimulus mice were ovariectomized around 60 days of age. Mice were anaesthesized with the mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 μg/g BW), acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) and xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μg/g BW) and both ovaries were excised through small wounds. Wounds were stitched and mice received two injections of butorfanol (Turbogesic, Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) after surgery to ease any potential pain. Mice were allowed to recover for at least 10 days before being used as stimulus mice. All animal experiments were approved by Veterinary commission of Slovenia (VURS) and were done according to ethical principles and NIH guidelines.

2.2. Social recognition test

The ability to recognize familiar conspecifics was tested in 80-day-old mice. Estrous cyclicity was evaluated in experimental female mice by examining vaginal cytology. Vaginal smears were stained with haematoxyline and eosine using standard procedures and examined using brightfield microscopy (Nikon Eclipse 80i). Only female mice in diestrus (identified by the presence of large numbers of leucocytes) were used for behavior testing to control for differences in behavior due to hormonal changes. Diestrus was chosen as a period of the estrus cycle with most reproductive hormones (estradiol, progesterone, LH and FSH) at low levels [17]. All tests were done at the beginning of the dark period under dim red illumination. Prior to testing (24 h), socially housed mice were put individually into new cages (small cages 35 × 15 × 13 ($L \times W \times H$) with filtertops) with bedding from their old cage; isolated mice were tested in their home cages with at least 3-day-old bedding. On the day of testing, ovariectomized females were put into the cage for 60 s and then removed. The same female was put into the cage again after 9 min and this was repeated 8 times. After a final 9 min break, a new, unfamiliar ovariectomized female was put into the cage for the ninth test. During each 1 min trial, the duration of sniffing by the tested mice was recorded using 'stopwatch' software (Center for Behavioral Neurosciences, Atlanta, GA). All tests were performed in the presence of an observer who remained still and quiet during the test to minimize potential observer effects. All tests were done by the same investigator (J.K.) who was blinded to group assignment at the time of testing.

2.3. Statistical analyses

All statistical analyses were done using NCSS software (NCSS Statistical Software, Kaysville, UT). At least nine mice of each sex were tested in each group (social, isolated, isolated/social). To test differences between groups over the trials, repeated measures ANOVA was performed with housing conditions and sex as independent variables followed by post hoc Bonferroni test. To assess differences between test 1 and 8 and tests 8 and 9 within individual groups, repeated measures ANOVA followed by post hoc Bonferroni testing was performed. Differences were considered statistically significant with $p < 0.05$.

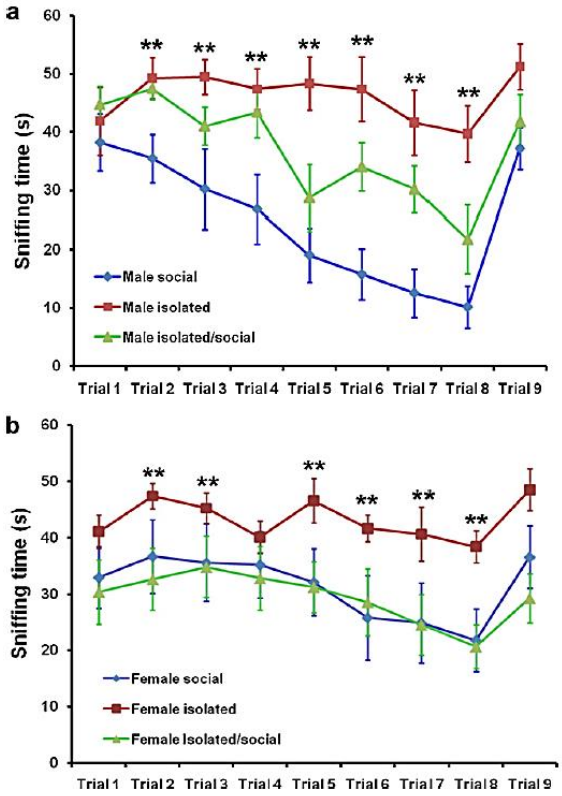

Fig. 1. A pattern of social habituation was observed in the male social group, where time spent sniffing stimulus mice decreased significantly from trials 1 to 8 (a). The pattern of social habituation was also noted with isolated/social males (a) and in female social group and female isolated/social group (b) while social habituation to the same stimulus mice was not noted in male (a) and female (b) mice that were isolated from day 30 until testing (isolated group). Significant difference in sniffing time between three groups of mice was noted in most trials in both sexes (**$p < 0.01$).

3. Results

3.1. Differences in time spent sniffing between groups

All mice were exposed 8 times for 1 min to the same stimulus mouse followed by ninth test with a new stimulus mouse. No aggressive (attacks, bites, charges) or sexual behavior (mounting, lordosis) was observed between test mice and stimulus mice during any test. Statistical analysis using repeated measure ANOVA with housing condition and sex as independent variables revealed a significant interaction between housing conditions and test ($F(16,51) = 5.11$, $p < 0.01$) and between sex and test ($F(8,51) = 3.55$, $p < 0.001$). As shown in Fig. 1, the greatest amount of habituation to the repeated presentation of the same stimulus female and the greatest response to a new female was observed in male mice grouped socially throughout the experiment. Time spent sniffing declined significantly with each test ($p < 0.001$) and recovered to levels observed in test 1 during the ninth test (test with new mice; $p < 0.001$). In socially housed female mice as well as in male and female mice isolated from day 30 until day 60 (returned to social housing on day 60), a pattern of habituation to the repeated presentation of the same stimulus female was also apparent. Nonetheless,

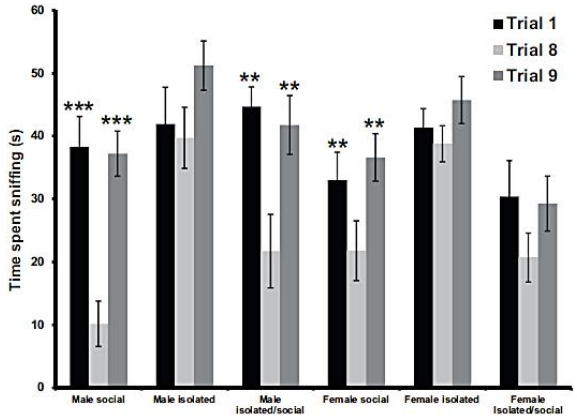

Fig. 2. Time spent sniffing stimulus mice differed significantly between tests 1 and 8 and tests 8 and 9 in group housed male mice, group housed females and isolated/social males while there was no significant difference between tests 1 and 8 and tests 8 and 9 in male and female mice reared in isolation and in isolated/social females, suggesting that in these three groups of mice, social isolation affected their ability to differentiate between familiar and unfamiliar conspecifics (**$p < 0.01$; ***$p < 0.001$; all different from test 8 within the same group).

the reduction in time spent sniffing between tests 1 and 8 was much lower in comparison to males housed socially, suggesting reduced habituation or learning in these mice (Fig. 1). By contrast, in both male and female mice housed individually from day 30 until testing at around 80 days of age (i.e., isolated), animals failed to habituate to the stimulus female (Fig. 1). Even though the time spent sniffing increased from tests 8 to 9 in both males and females, this difference was not statistically significant nor was the minimal reduction in time sniffing between tests 1 and 8, suggesting that these mice did not habituate to the stimulus mice and did not recognize them as familiar during test 8 (or any of the previous tests).

3.2. Difference in time spent sniffing within groups

Analysis of the difference in time spent sniffing between trials 1, 8 and 9 within individual groups of mice revealed a significant effect of housing conditions as social recognition was indicated in both groups of socially housed mice. In socially housed mice there was a significant difference between tests 1 and 8 in both males ($p < 0.001$) and females ($p < 0.05$) and between tests 8 and 9 in males ($p < 0.001$) and females ($p < 0.01$). In isolated groups of mice, only isolated/social males showed habituation with a significant difference between tests 1 and 8 ($p < 0.01$) and tests 8 and 9 ($p < 0.01$) while time spent sniffing the stimulus mice in the other three groups of mice (isolated males, isolated females, isolated/social females) did not differ significantly between tests 1, 8 and 9 (Fig. 2).

4. Discussion

Previous studies have shown that rearing in social isolation during development leads to long lasting changes in brain structure or gene expression [6,10–12], and causes behavioral changes in adult life such as increased locomotor activity, decreased anxiety-like behavior (elevated plus maze), impaired novel object recognition and increased aggression [6,7,12,18]. However, with the exception of one recent study [16], we are not aware of other reports concerning the effect of prolonged social isolation on social learning in rodents. Therefore, the results of the present study provide data indicating that either long-term or transient social isolation impacts social recognition in mice, particularly in males.

In the current study, the strongest pattern of social recognition was noted in male mice that were housed socially in groups of three. For this group of mice, time spent investigating familiar ovariectomized female mice during 8 tests linearly decreased to reach the lowest levels during the eighth test, but reverted to the initial time during the ninth test with novel females. This suggests that male mice, reared in groups, have strong ability for social learning and successfully discriminated between familiar and unfamiliar ovariectomized female mice. However, in male mice that were housed individually from day 30 until testing around day 80, there was little reduction in time spent sniffing the same mice during the 8 tests and no significant difference between tests 8 and 9, suggesting that these mice did not distinguish familiar female from the unfamiliar one (presented in test 9). This is in agreement with the results by Zhao et al. [16], where similar deficits in social recognition were reported in male rats housed individually from day 30 until testing in adulthood, suggesting that social isolation affects both mice and rats similarly with respect to this behavior. The current study contains an important additional group; mice that were reared in isolation from day 30 until day 60 and then were housed in groups of three (same sex) until testing around day 80 (isolated/social group). Interestingly, in this group of male mice, there was a social recognition pattern of behavior. The reduction in time sniffing familiar females, however, was significantly lower than in male mice housed in groups continuously. This might suggest that social isolation for a transient period caused changes that were evident as a behavioral deficit in a form of reduced habituation later in adult life, after mice were resocialized. This reduction was evident even though isolated/social male mice were still able to distinguish familiar from unfamiliar females as demonstrated by the significant increase in times spent sniffing stimulus mice between tests 8 and 9.

As expected, social recognition was also observed in female mice, although the reduction in time during 8 tests was much smaller in comparison to male mice, in agreement with previously published data for rats [19,20]. Nevertheless, socially housed female mice showed significant differences in time spent sniffing between tests 8 and 9 suggesting that they could distinguish familiar from unfamiliar females. Similar to male mice reared in isolation, female mice reared in isolation from day 30 until testing did not show evidence of social habituation or recognition. Interestingly, however, the time spent sniffing stimulus mice did not change between test 8 and 9 also in isolated/social females suggesting that in female mice, transient isolation was sufficient to cause an effect on their ability to distinguish familiar and unfamiliar conspecifics even some time after they were resocialized.

The influence of sex hormones on the ability to recognize conspecific animals has been shown previously [14,15]. Therefore, the finding of a sex difference in the social recognition in social group of mice in the current study was not surprising. However, all females were tested in the diestrus phase of the estrous cycle when hormone levels are lowest. Therefore, this finding does not necessarily mean that social recognition and/or habituation are worse in female than in male mice at all times. It is quite possible that during estrus phase, when more estradiol is in the female circulation (perhaps similar in action to males circulating testosterone), social recognition/habituation would be more comparable to males. Although a previous study [19] did not find differences in social recognition between proestrus and estrus female rats, they only looked at two phases (proestrus and estrus).

Social recognition is a form of learning and previous studies have shown that some kinds of learning behaviors (novel object recognition, fear conditioning) are affected by social isolation while others like water maze performance are not [7]. This could imply that similar mechanisms as reported in previous studies could be responsible for reduced social recognition observed in the

current study. However, the learning ability of mice in other learning paradigms such as novel object recognition, water maze and others were not addressed in the current study. The social learning process is connected with both short and long term memory and interestingly, previous studies have shown that social isolation could induce changes in hippocampal development [11]. Although lesions of central hippocampus do not produce deficit in social recognition [21], the hippocampus in general plays important roles in the formation of short term memory [22] that are important for the learning process, and lesions of areas around the hippocampus produce some deficits in social recognition [23].

Social recognition is an important behavioral feature that is needed in social groups to differentiate individuals belonging to the same social group from unknown, potentially harmful individuals. Many studies have implicated vasopressin and oxytocin in the regulation of different social behaviors including social recognition (reviewed in [24]). Vasopressin acting through its receptor V1a in lateral septum is thought to be particularly important since V1aR knockout mice do not show social recognition, but this deficit could be rescued by replacing the V1aR gene into the lateral septum alone [25]. Interestingly, however, vasopressin may be more important for social recognition in males than females, since a vasopressin antagonist blocked social learning in male but not in female rats [14]. This is probably due to much higher levels of vasopressin peptide in the lateral septum that is dependent upon exposure to testosterone. Social-learning in females that have much less vasopressin in the lateral septum may rely more on other neurohormones or neuropeptides, perhaps oxytocin, which has also been shown to be important in this behavior [26,27]. Several studies have shown that oxytocin influences social recognition in mice [28,29] and this is most likely regulated by estrogens [28,30,31]. In the present study, all female mice were therefore tested in the diestrus part of the estrous cycle with the lowest levels of gonadal estrogens to minimize their possible effects, and importantly minimize confusion due to changing levels of estrogens at different cycle stages. In a preliminary experiment using immunocytochemical detection of vasopressin and oxytocin (data not shown) there were no obvious differences in either immunoreactive vasopressin in the lateral septum or oxytocin in the paraventricular nucleus of the hypothalamus between groups of mice in different housing regimes. At the same time, as expected, there was a robust sex difference in immunoreactive vasopressin in fibers of the lateral septum in all experimental groups. Sex differences in vasopressin content in the lateral septum might help explain sex differences in social recognition [32]. However, it may be difficult to relate the differences in behavior to a simple static immunocytochemical view of the vasopressin system as opposed to determining the more active measures of peptide release or turnover.

The lasting effect of transient social isolation observed in the current study is particularly interesting since it suggests that brain circuitry regulating social recognition has been altered in a lasting fashion. Previous studies have shown that social isolation could induce changes in hippocampal development [11], but beside structural changes, long lasting effects on behavior could be due to epigenetic regulation of certain genes, achieved either by histone modifications and/or DNA methylation [33]. In this context, it might be useful to examine potential epigenetic regulation of vasopressin/oxytocin systems in future studies.

In conclusion, the current study shows that social isolation strongly influences subsequent social behavior in mice and that even transient social isolation resulted in lasting effects on social behavior. Results of this study contribute to our understanding that social isolation, which is often used in breeding of laboratory mice, causes long-lasting changes in social behavior and should be taken into account when interpreting results from such studies.

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3.2 SOCIAL ISOLATION DURING PUBERTY AFFECTS SOCIAL BEHAVIOR IN ADULT MICE

SOCIALNA OSAMITEV MED PUBERTETO PRIZADENE SOCIALNO OBNAŠANJE ODRASLIH MIŠI

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Izvleček

Zgodnja socialna osamitev, ki povzroļa spremembe tako v strukturi moģganov kot tudi v izraģanju genov, ima lahko pomemben vpliv na razliļna socialna obnaģanja. Vpliv osamitve pri miģih na socialno prepoznavanje ali izraģanje vazopresina (AVP) in oksitocina (OT) pa ģe ni bil raziskan. Prouļevali smo socialno prepoznavanje miģi obeh spolov, ki so bile nastanjene individualno vse od starosti 30 dni do testiranja pri starosti 80 dni, individualno nastanjene od 30. do 60. dneva in nato od 60. dneva ponovno skupinsko nastanjene, in miģi iz kontrolne skupine, ki so bile ves ļas raziskave nastanjene skupinsko. S standardnim testom socialnega prepoznavanja smo ugotavljali sposobnost testnih miģi, da loļijo znano miģ od neznane. Skupinsko nastanjene miģi so kazale neokrnjen, moļan socialni spomin, medtem ko ga miģi, nastanjene individualno, niso. Zanimivo je, da so individualno nastanjene miģi za doloļeno ļasovno obdobje (od 30. do 60. dneva) kazale slabģi socialni spomin, kar pomeni, da ima lahko tudi osamitev za doloļen ļas trajne posledice pri socialnem obnaģanju, ģe posebej pri samicah. Z imunohistokemijsko metodo smo ugotavljali izraģanje vazopresina in oksitocina v moģganih in ugotovili spolno razliko v izraģanju vazopresina v stranskem septumu (LS). Samci so imeli namreļ veļ vazopresina v ģivļnih vlaknih kot samice. V izraģanju tako vazopresina kot oksitocina glede na naļin nastanitve nismo naģli razlik.

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Review Article

SOCIAL ISOLATION DURING PUBERTY AFFECTS SOCIAL BEHAVIOUR IN ADULT MICE

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Summary: Early social isolation can have profound consequences on different social behaviours due to alterations in brain structures or gene expressions, but its influence on social recognition or vasopressin (AVP) and oxytocin (OXT) expression has not been thoroughly investigated in mice. We examined social recognition in mice of both sexes that were individually housed from 30 days of age until testing at around day 80, individually housed from day 30 until day 60 and regrouped from day 60 until testing at day 80 and in control mice that were group housed throughout experiment. The ability to recognize familiar mouse was tested using standard social recognition test. Group housed mice showed strong social memory, whereas individually housed did not. Interestingly, mice reared in isolation for a limited period showed reduced social memory, suggesting that even isolation for a limited period can have lasting behavioural deficit, especially in female mice. Using immunohistochemistry we examined vasopressin and oxytocin expression in the brain. As expected, immunohystochemical detection of AVP in lateral septum (LS) revealed robust sex difference with males having much more AVP in fibers than females. However, there were no obvious differences in either vasopressin or oxytocin between groups in different housing regimes, suggesting that social isolation in mice has no effect on the expression of these two neurohormones.

Key words: mice; social stress, isolation; social behaviour, social recognition; vasopressin, oxytocin

Introduction

In the natural conditions, mouse *(Mus musculus)* is a social species living in large social groups establishing group territories (1). The ability to recognize familiar conspecifics, social recognition memory, is critical for many forms of social interactions (2). But in laboratory conditions they are often individually housed to prevent intermale aggression or unwanted matings (1, 3). Many studies have shown that early social deprivation, not only in rodents but also in primates and humans, can induce different behavioural, brain structure and gene expression abnormalities (4, 5). It can cause hyperactivity, reduction in habituation and reduction in anxiety-like behaviour in the elevated plus maze (EPM) test, but an opposite effect in the dark-light (3) and staircase test (6), impairment in novel object recognition (7), aberrant self-manipulation, frequent chasing and biting of the tail (1) and higher levels of aggressive attacks in males (8).

Isolation in rats induces enlargements in different stress-sensitive brain regions (5), cytoskeletal microtubular alterations in the hippocampus (9) and reduction in the size of medial prefrontal cortex (10). It also alters peripheral vasopressin (AVP) and oxytocin (OXT) concentrations, and a lack of social stimuli adversely affects development of these two systems in rats (11).

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Social isolation and social recognition

Social recognition in rodents is critical for the formation and maintenance of all social relationships. The influence of social isolation on performance in social recognition tests has not been thoroughly investigated. There are only two studies that reported impairments in social recognition in individually housed male (12) and female rats (11). Our

study revealed that the strongest pattern of social recognition is present in socially housed males. Social recognition was also observed in socially female mice with much smaller reduction in sniffing time (lower habituation), but still with significant difference between last two trials (the last trial with a new unfamiliar female), suggesting that they could distinguish familiar from unfamiliar mouse. In contrast, both male and female mice that were isolated throughout the test did not show either habituation during the first 8 tests and neither social recognition as there was no significant difference between tests 8 and 9. In male mice isolated for a limited period the habituation was reduced, although social recognition was still present as evident by significant difference between tests 8 and 9. However, in female mice that were isolated for a limited period, there was no social recognition (although habituation was similar to social female mice), suggesting that even isolation for a limited period can have lasting effect on this behaviour (13).

Social isolation and expression of AVP and OXT

Social isolation has been reported to affect expression of hypothalamic OXT and AVP (11), which are important in modulating the social recognition and other social behaviours (reviewed in (14, 15). Lateral septum, medial amygdala (MeA), hippocampus, hypothalamus, olfactory bulbs and vomeronasal organ have all been demonstrated as regions critical for OXT and AVP effects on social recognition (16). Previous studies have shown that administration of AVP agonists into LS have improved (17), while AVP antagonists have blocked normal social recognition in rats (18). Post-weaning social isolation can decrease number of AVP cells in male or OXT in female rats in the paraventricular nucleus (PVN), what coincides with the impairment in social recognition in isolated rats (11) and with the suggestion that AVP is more important in male (14), and OXT in female behaviour (15).

In our study, immunoexpression of AVP in LS, which contains axons from the MeA and bed nucleus of the stria terminalis (BNST), and OXT in PVN was not altered by social isolation. However, since we only used immunocytochemistry that could only detect proteins stored in the nerve fibers, it is still possible that there are differences in either of these two peptides at the level of protein secretion or turnover, or even at the level of their receptors expression, therefore, we were not able to either confirm or reject the hypothesis that dysregulation of AVP and/or OXT system in the brain is responsible for alterations in social recognition behaviour in socially isolated mice (16).

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3.3 SOCIAL ISOLATION DURING PUBERTY AFFECTS FEMALE SEXUAL BEHAVIOR IN MICE

SOCIALNA OSAMITEV MED PUBERTETO VPLIVA NA ŽENSKO SPOLNO OBNAŠANJE ODRASLIH MIŠI

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Izvleček

Izpostavljenost stresnim dogodkom med puberteto lahko vodi do dolgotrajnih sprememb pri obnaģanju v odraslem obdobju. To sovpada s preoblikovanjem in reorganizacijo moģganov med puberteto, ki sta pogojena z aktivnostjo spolnih hormonov. Za socialne ģivali, kot so miģi, predstavlja socialna osamitev stresno izkuģnjo, vendar pa je malo znanega o vplivu takģne stresne izkuģnje med puberteto na spolno obnaģanje v odraslem obdobju. Prouļevali smo spolno obnaģanje miģjih samic, ki smo jim odstranili spolne ģleze ter jim dovajali estradiol in progesteron. Miģi smo trajno socialno osamili vse od starosti 25 dni in jih testirali okrog 95. dneva starosti ali jih zaļasno osamili od 25. do 60. dneva starosti in jih nato od 60. dneva ponovno skupinsko nastanili. Miģi obeh osamljenih skupin smo primerjali s kontrolno skupino samic, ki je bila nastanjena v socialnih skupinah med celotno raziskavo. Pri testnih samicah smo beleģili in ocenjevali spolno sprejemljivost, pri spolno izkuģenih stimulusnih samcih pa posamezne aktivnosti moģkega spolnega obnaģanja. Kontrolne skupinsko nastanjene samice so spolno sprejemljivost izraģale moļneje kot pa obe skupini socialno osamljenih samic. Socialne samice so namreļ imele viģji koliļnik lordoze (LQ) in so pogosteje zavzele drģo moļne usloļenosti hrbtenice, ki je pogojena s spolno sprejemljivostjo, v primerjavi z osamljenimi miģmi. Nastanitveni dejavnik pri samicah ni imel vpliva na spolno obnaģanje pri stimulusnih samcih, iz ļesar lahko sklepamo, da socialna osamitev ni prizadela spolne privlaļnosti samic. Skupinsko nastanjene samice so imele manj ģivļnih celic v anteroventralnem delu periventrikularnega jedra (AVPV) in v ventromedialnem jedru hipotalamusa (VMH), ki v primerjavi z obema socialno osamljenima skupinama izraģajo estrogenski receptor (ER) Ŭ. Naģe ugotovitve dokazujejo, da socialna osamitev miģjih samic med puberteto vpliva na ģensko spolno obnaģanje, saj ga niti ponovna socialna nastanitev v odraslem obdobju ni uspela povrniti na raven obnaģanja, ki so ga izraģale skupinsko nastanjene samice.

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Social isolation during puberty affects female sexual behavior in mice

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Exposure to stress during puberty can lead to long-term behavioral alterations in adult rodents coincident with sex steroid hormone-dependent brain remodeling and reorganization. Social isolation is a stress for social animals like mice, but little is known about the effects of such stress during adolescence on later reproductive behaviors. The present study examined sexual behavior of ovariectomized, estradiol and progesterone primed female mice that were individually housed from 25 days of age until testing at approximately 95 days, or individually housed from day 25 until day 60 (during puberty), followed by housing in social groups. Mice in these isolated groups were compared to females that were group housed throughout the experiment. Receptive sexual behaviors of females and behaviors of stimulus males were recorded. Females housed in social groups displayed greater levels of receptive behaviors in comparison to both socially isolated groups. Namely, social females had higher lordosis quotients (LQs) and more often displayed stronger lordosis postures in comparison to isolated females. No differences between female groups were observed in stimulus male sexual behavior suggesting that female "attractiveness" was not affected by their social isolation. Females housed in social groups had fewer cells containing immunoreactive estrogen receptor (ER) α in the anteroventral periventricular nucleus (AVPV) and in the ventromedial nucleus of the hypothalamus (VMH) than both isolated groups. These results suggest that isolation during adolescence affects female sexual behavior and re-socialization for 1 month in adulthood is insufficient to rescue lordosis behavior from the effects of social isolation during the pubertal period.

Keywords: mice, social isolation, female sexual behavior, estrogen receptor α, puberty/adolescence

INTRODUCTION

Puberty is a period during which an individual attains sexual maturity following the re-activation of the hypothalamic-pituitary-gonadal (HPG) axis and elevated secretion of gonadal steroid hormones (Sisk and Zehr, 2005; Schulz and Sisk, 2006). During puberty, the brain undergoes remodeling and reorganization which is partially influenced by gonadal steroid hormones (Schulz et al., 2009). There is growing evidence that exposure to stressors in adolescence can cause profound long-lasting alterations in the brain and subsequently in behavior in adulthood, perhaps due to interactions between sex hormones and hypothalamic-pituitary-adrenal (HPA) function (McCormick and Mathews, 2007; McCormick et al., 2010; Blaustein and Ismail, 2013). In social mammals like mice and rats, isolation can be stressful (Dixon, 2004; Koolhaas, 2010). Post-weaning social isolation behavioral studies have frequently been done in males, although some studies performed in both sexes suggest differences in the effects of social stress during adolescence, with males appearing to be more vulnerable (reviewed in Blanchard et al., 2001; Fone and Porkess, 2008).

Female sexual behavior is a complex set of behaviors that are necessary and sufficient to achieve fertilization of female ova by male sperm (Nelson, 2005). Two types of female sexual behaviors are often distinguished in rodents: receptive (reflexive postural changes at copulation - lordosis) and proceptive (attracting and initiating copulation) behaviors (Crusio et al., 2013). However, proceptive behaviors are more pronounced in rats than in mice and are difficult to evaluate in mice (Nelson, 2005). Female sexual behavior is mostly regulated by action of sex steroid hormones and neurons in many brain regions sensitive to the action of ovarian steroid hormones (mainly estradiol) are thought to be involved in the regulation of female sexual behavior including those in the preoptic area and ventromedial hypothalamic nuclei (Flanagan-Cato and McEwen, 1995; Rissman et al., 1999; Musatov et al., 2006). The estrogen receptor (ER) α present in neurons in these regions is essential for the effects of estradiol on the expression of sexual receptivity (Rissman et al., 1999). The medial amygdala (MeA) is also rich in estradiol receptors and is another region that may be involved in the regulation of female sexual behavior (DiBenedictis et al., 2012).

For example, Fos expression was much higher in the MeA of mated than non-mated females (Flanagan-Cato and McEwen, 1995). ERα can be autoregulated with estradiol down-regulating the expression of ERα in many brain areas (e.g., Simerly and Young, 1991; DonCarlos et al., 1995; Gréco et al., 2001). Stress activates the HPA axis and this can suppress HPG axis activity. Stressed animals may therefore be exposed to lower levels of gonadal hormones (reviewed in Kalantaridou et al., 2004) and this could cause an increase in ERα expression and detection of immunoreactive ERα.

In female mice, some stressors such as LPS injections during the peripubertal period (around 42 days of age) caused reductions in the level of receptive behavior lordosis quotient (LQ). This effect was stronger if mice were stressed peripubertally than if they were stressed in adult life (Laroche et al., 2009a,b). However, not all stressors were equally effective in decreasing receptive behaviors. For example, restraint stress and food deprivation did not have strong effects on these behaviors (Laroche et al., 2009a). The current study reports on the influence of social isolation during pubertal period on sexual behavior in adult female mice, and whether social re-housing in adulthood could eliminate behavioral alterations provoked by social isolation during this vulnerable adolescent period. Sexual behavior was examined in female mice that were group housed, socially isolated (from 25 days of age onwards), or isolated only during the pubertal period (25–60 days of age) followed by group housing. The goal was to explore whether the social isolation during pubertal period might have long-lasting effects on sexual behavior in adult female mice and on the expression of ERα in brain regions important for the regulation of female sexual behavior.

MATERIALS AND METHODS

ANIMALS

C57BL/6J mice were originally obtained from Harlan (Italy) and bred at the University of Ljubljana, Veterinary Faculty, in standard conditions with 12-12 light/dark cycle (lights on at 3 am and off at 3 pm) and food (phytoestrogen free diet; Harlan Teklad Diet 2016, Harlan, Milan, Italy) and water *ad libitum*. Mice were weaned at 21 days of age and at 25 days of age females were divided into three groups:

1. group-housed with at least 3 mice per cage (Social),
2. socially isolated with 1 mouse per cage (Isolated),
3. socially isolated and regrouped at 60 days of age (Isol/Social).

Social females (Social, $n = 8$) were housed in larger 15 cm high cages with floor area of 37.5 × 22 cm, socially isolated females (Isolated, $n = 8$) in smaller 14 cm high cages with 35 × 15 cm floor area, and the transiently isolated females (Isol/Social, $n = 8$) first in smaller cages and after regrouping in larger cages. Sexually experienced stimulus males of the same strain ($n = 9$) were individually housed in 13 cm high cages with 28.5 × 10.5 cm floor area that had been previously used for mating (at least 3 successful matings with weaned litters). The C57BL/6J mice were chosen because this is a commonly used reference strain for behavioral phenotyping studies with high rates of copulatory behaviors displayed (reviewed in Crawley et al., 1997).

All animal experiments were approved by the Veterinary Administration of the Republic of Slovenia and were done according to ethical principles, EU directive, and NIH guidelines.

SURGERY AND HORMONAL TREATMENTS

All female mice were ovariectomized bilaterally at 60 days of age (after puberty) to eliminate endogenous gonadal steroids. Mice were anesthetized with the mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 μg/g BW), acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) and xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μg/g BW) and gonads were excised through small incisions. The incisions were stitched (absorbable sutures; Safil, Braun, Aesculap, Tuttlingen, Germany) and mice received two injections of butorfanol (Turbogesic, Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) after surgery to alleviate potential pain. To regulate circulating estradiol levels in adulthood at approximately 80 days of age mice received subcutaneous implants containing estradiol benzoate. Silastic implants (1.02 mm inner diameter, 2.16 mm outer diameter) were filled 5 mm in length with crystalline β-estradiol 3-benzoate (EB; Sigma), diluted 1:1 with cholesterol (Sigma) (Wersinger et al., 1999) and closed on both ends by medical silastic adhesive (Dow Corning). Implants were inserted subcutaneously in the midscapular region under anesthesia (mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 μg/g BW), acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) and xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μg/g BW). These implants yield plasma estradiol levels close to the physiological range normally observed during estrus (Wersinger et al., 1999). Behavior tests were performed at least 10 days after implantation. Approximately 4–8 h before each test the females were injected subcutaneously with 0.8 mg of progesterone (P; Sigma) dissolved in corn oil (Sigma). All mice were initially tested for sexual behavior between 90 and 100 days of age, and were sacrificed by transcardial perfusion fixation with 4% paraformaldehyde 4 days after the last test, around 125 days of age, and the brains were dissected and stored in 0.1 M PB at 4°C until further processing for immunohistochemistry.

FEMALE SEXUAL BEHAVIOR TEST

Female sexual behavior tests were performed in clear glass aquaria (17 cm high with 41.5 × 26 cm floor area) with a mirror positioned under the testing arena to obtain better views of facets of sexual behaviors (Wersinger et al., 1997). Females were tested during the first 2–4 h of the dark period of the circadian cycle, under dim red light illumination, and the test sessions were videotaped for subsequent scoring. Each female was tested 6 times, every 4–5 days to mimic the normal physiological estrus cycle. The first trial served for animals to gain sexual experience prior to testing, the next five trials were scored. The stimulus males were placed into the aquarium at least 4 h prior behavior testing with at least 3 day old bedding and food and water *ad libitum* to acclimate to the novel environment. Food and water were removed during the behavior tests.

Hormonally-primed females were placed in the middle of aquaria with a stimulus male for 20 min (Park, 2011), or until

the female received an ejaculation. The following behaviors were recorded: lordosis posture, total number and latency of attempted mounts, successful mounts, pelvic thrusts and intromissions, and the latency of ejaculation. Behaviors were recorded by "stopwatch" software (Center for Behavioral Neuroscience, Atlanta, GA) and were observed by the same investigator (Jasmina Kercmar) who was blinded to the group assignment at the time of testing.

If the stimulus male did not try to mount the female, after 5 min of testing the tested female was moved to another aquaria with a new, previously habituated stimulus male. Ejaculating males were not used again for the remainder of the trial day. Mounts were counted when the female had all four limbs on the floor. Female lordosis posture as an index of sexual receptivity was scored from 0 (no receptive behavior with no lordosis reflex) to 5 (completely receptiveness with strongest lordosis reflex as described previously (Bakker et al., 2002). Lordosis was defined using the following stipulations: all four paws are grounded, hind region is elevated off the floor of the test chamber, and the back is slightly arched (Takasugi et al., 1983; Kudwa et al., 2007). A LQ was calculated by the following formula: number of mounts during which the female stood still (lordosis 4 and 5)/total number of attempted and successful mounts × 100.

ERα IMMUNOHISTOCHEMISTRY

Brains were embedded in 5% agarose (Sigma) and sectioned at 50 μm in cold 0.05 M PBS using a vibrating microtome (Integraslice 7550 MM, Campden Instruments, UK). Sections were incubated in 0.1 M glycine (Sigma) in cold 0.05 M PBS for 30 min followed by incubation in 0.5% sodium borohydride (Sigma) for 15 min at 4°C. Glycine and sodium borohydride were washed out with 15 min and 20 min washes (every 5 min) in cold 0.05 M PBS. Sections were blocked in 5% normal goat serum (Jackson Immunoresearch, West Grove, PA, USA) containing 0.5% Triton X-100 (Sigma) and 1% H_2O_2 (Merck, Darmstadt, Germany) for 30 min at 4°C. Rabbit primary antiserum against ERα (1:5000, Cat.#06-935, Upstate, Lake Placid, NY, USA) were diluted in 0.05 M PBS containing 1% bovine serum albumin (Sigma) and 0.5% Triton X-100. Sections were incubated with primary antibodies over 2–3 nights at 4°C with shaking. Sections were after then washed in 0.05 M PBS containing 1% normal goat serum and 0.02% Triton X-100 four times for 15 min at room temperature. Biotinylated secondary antibodies (Jackson Immunoresearch) against primary rabbit antiserum were diluted 1:500 in 0.05 M PBS containing 1% normal goat serum and 0.5% Triton X-100. Sections were incubated with biotinylated secondary antibodies for 2 h, followed by 4 washes for 15 min in 0.05 M PBS buffer containing 0.02% Triton X-100. Streptavidin–HRP complex (Jackson ImmunoResearch) was diluted 1:2500 in 0.05 M PBS solution containing 0.5% Triton X-100. Sections were incubated with Streptavidin–HRP for 1 h at room temperature and then washed in Tris-buffered saline (0.05 M Tris–HCl/0.9% NaCl; pH 7.5; Sigma) for 1 h (four times for 15 min) at room temperature. Antigen–antibody complexes were visualized as a black reaction product by incubating sections in 0.025% 3, 3′-diaminobenzidine/0.2% ammonium nickel (II) sulfate substrate (Sigma) in Tris-buffered saline containing 0.02% H_2O_2 for 5 min at room temperature. Sections were finally washed in Tris-buffered saline three times every 10 min. After mounting, sections were dried and coverslipped using hydrophobic medium (Pertex; Burgdorf, Germany).

DATA COLLECTION AND ERα QUANTIFICATION

Digital images of brain regions of interest were obtained using a Nikon Eclipse 80i microscope with Nikon DS-Fi1 camera. Images were enhanced for contrast using Adobe Photoshop software (Version 8.0). The number of immunoreactive cells or total area that was immunoreactive for ERα was analyzed in coronal sections containing the anteroventral periventricular region (AVPV) between 0.26 and 0.14 mm rostral from Bregma, the ventromedial hypothalamic region (VMH) 1.70 mm caudal from Bregma, and MeA 1.22 mm caudal from Bregma according to stereotaxic coordinates (Franklin and Paxinos, 2008). All digital images were taken under 100x magnification. The base of the brain was considered as a reference boundary with the third ventricle in the center of the images for AVPV, the third ventricle and the base of the brain as reference boundaries for VMH, and the junction of the optic nerve and cortex-amygdala transition as reference boundary with this junction in the middle of the lateral sides of the images for MeA. Due to the possibility of asymmetry in antigen detection in VMH and MeA between the left and right sides of the brain, the side (unilateral) with more immunopositive cells was always chosen for analysis. Immunoreactivity in AVPV was assessed on both sides (bilateral) of the third ventricle, extending approximately 640 μm laterally from the third ventricle and 960 μm dorsally from the base of the brain. Due to the large number of overlapping immunopositive ERα cells in AVPV, the immunoreactive area was quantified using custom software (Surfkvad; made by Dr. Marko Kreft, Institute of pathophysiology, Faculty of Medicine, Ljubljana) that divides an image into 6 × 8 squares (measuring 160 × 160 μm each under 100x magnification) and calculates a percentage of dark area for each square (Büdefeld et al., 2008). To standardize the collection of immunoreactive area data, all images were taken under the same illumination and converted to grayscale. Grayscale images were subjected to threshold conversion to selectively identify immunoreactive elements using Photoshop software. Black and white images were then analyzed with Surfkvad. The number of immunopositive cells in the VMH and MeA was counted with the help of Image J software (NIH, Bethesda, MD). The grayscale images were divided into a grid of 6 × 8 squares for VMH (measuring 160 × 160 μm each), and a grid of 8 × 10 squares for MeA (measuring 120 × 120 μm each). Only the grid delimiting the VMH region (4 × 4 squares; extending approximately from 480 μm to 1120 μm laterally from the third ventricle and 640 μm dorsally from the base of the brain), and the grid delimiting the MeA region (3 × 3 squares; extending approximately from 120 μm to 480 μm laterally, 120 μm ventrally and 240 μm dorsally from the lateral boundary) were analyzed (**Figures 2B,C**).

STATISTICAL ANALYSES

All data were statistical analyzed using NCSS software (NCSS statistical software, Kaysville, UT, USA). To test differences between

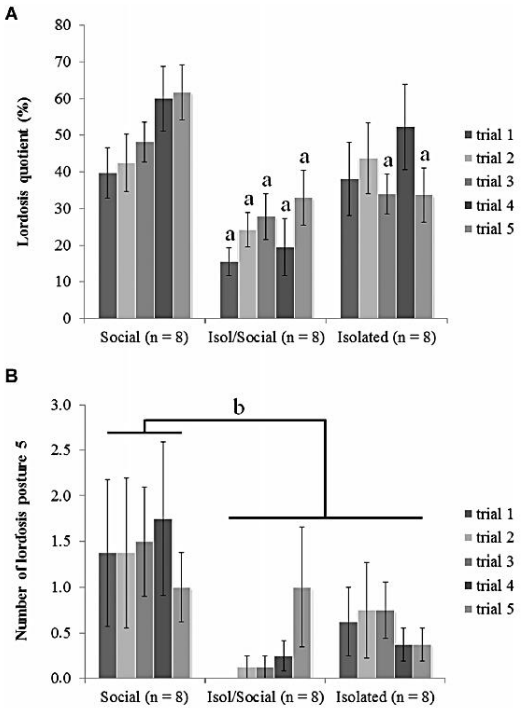

FIGURE 1 | Mice that were socially isolated during puberty showed impaired receptive female sexual behavior. (A) LQ (^{a} $p < 0.01$), **(B)** number of displayed lordosis posture scored with 5 (^{b} $p < 0.05$). Data are reported as mean ± SEM; ^{a,b} Significant difference between females housed in social groups (Social) and other two isolated groups (Isol/Social, Isolated).

groups in sexual behavior tests, repeated measures ANOVA was performed with housing condition as independent variable, and trial as a repeated measure (within) factor, followed by *post hoc* Fisher LSD tests. Eight mice in each group were tested for female sexual behaviors (Social, $n = 8$; Isolated, $n = 8$; Isol/Social, $n = 8$). Differences between groups in the number or area of immunoreactive ERα were analyzed by ANOVA followed by Fisher LSD *post hoc* (for VMH and amygdala) and by repeated measures ANOVA followed by Fischer LSD *post hoc* test (for AVPV). At least 4 brains in each group were analyzed and differences were considered statistically significant at $p < 0.05$.

RESULTS

SEXUAL BEHAVIOR OF FEMALE MICE

Social isolation during adolescence reduced female sexual behaviors, and re-socialization in adulthood was insufficient to rescue receptive lordosis behavior from the effects of social isolation during the pubertal period. ANOVA revealed a significant overall effect of housing condition [$F_{(3,24)} = 7.57$, $p < 0.01$] on the LQ between all three groups (**Figure 1A**). The *post hoc* tests indicated that mice housed in social groups (Social) had higher LQs in comparison with mice isolated for the limited period (Isol/Social) in all five trials while mice isolated throughout the experiment differ from the socially housed group selectively in trials 3 and 5.

ANOVA also showed significant effect of housing condition on the number of displayed lordosis reflexes scored 5 (the highest receptiveness with strongest lordosis reflex) [$F_{(3,24)} = 5.32$, $p < 0.05$] (**Figure 1B**). *Post hoc* analysis showed that socially housed mice (Social) displayed lordosis reflexes scores of 5 more often than did other two groups of female mice (Isol/Social, Isolated). There were no statistically significant differences between permanently isolated mice and mice isolated only during the pubertal period, suggesting that 1 month re-socialization could not rescue from the effects of social isolation during puberty.

SEXUAL BEHAVIOR OF STIMULUS MALE MICE

No differences between female groups (Social, Isol/Social and Isolated) were observed in stimulus male sexual behavior, suggesting that female "attractiveness" was not affected by social isolation.

Repeated measures ANOVA did not show any significant effect of housing conditions (Social, Isol/Social and Isolated; means ± SEMs of all five trials) in the total number of mounts (19.1 ± 2.2, 19.9 ± 1.9 and 13.7 ± 1.4), thrusts (326.3 ± 33.1, 299.0 ± 30.3 and 268.3 ± 26.3), or intromissions (16.7 ± 2.0, 15.5 ± 1.7 and 11.8 ± 1.3) nor in the latencies to mount (54.8 ± 9.1, 56.4 ± 7.7 and 69.9 ± 12.0), intromit (82.8 ± 14.4, 112.6 ± 17.4 and 109.7 ± 17.2), or ejaculate (793.9 ± 65.9, 923.1 ± 60.2 and 767.5 ± 68.9).

EXPRESSION OF ERα IN AVPV, VMH AND MeA

Statistically significant differences in ERα immunoreactivity were found in the AVPV, VMH, but not in the MeA (**Figures 2**, **3**). Socially housed mice had less ERα immunoreactive area than isolated mice of both groups. Repeated measures ANOVA with columns as the within factor showed a significant effect of housing condition in the total immunoreactivity for ERα in cells in the AVPV [$F_{(3,14)} = 6.72$, $p < 0.01$] (**Figures 2A**, **3A**). ANOVA revealed a significant effect of housing condition in the number of ERα immunoreactive cells in the VMH [$F_{(3,16)} = 3.93$, $p < 0.05$] (**Figures 2B**, **3B**), but not in the MeA (**Figures 2C**, **3C**). *Post hoc* analysis revealed that socially housed mice (Social) had less ERα immunoreactive area in the AVPV and less ERα immunoreactive cells in the VMH than the other two housing groups (Isol/Social, Isolated; **Figures 3A,B**). Mice isolated throughout the experiment or only during puberty had more immunoreactive area or more cells in the AVPV and VMH, respectively, than socially housed mice, and there was no statistical difference in total immunoreactivity or number of cells in both AVPV and VMH between permanently isolated mice and mice isolated for transient time only (**Figures 3A,B**).

DISCUSSION

Beside the pre- and early postnatal period, the pubertal period is important for the appropriate development of specific behaviors displayed in adulthood (Sisk and Zehr, 2005). Early life stress can have profound influences on brain development and subsequently on behavior later in life (reviewed in McCormick et al., 2010). Particular stressors during the pubertal period in female mice (Laroche et al., 2009a,b; Ismail et al., 2011) may

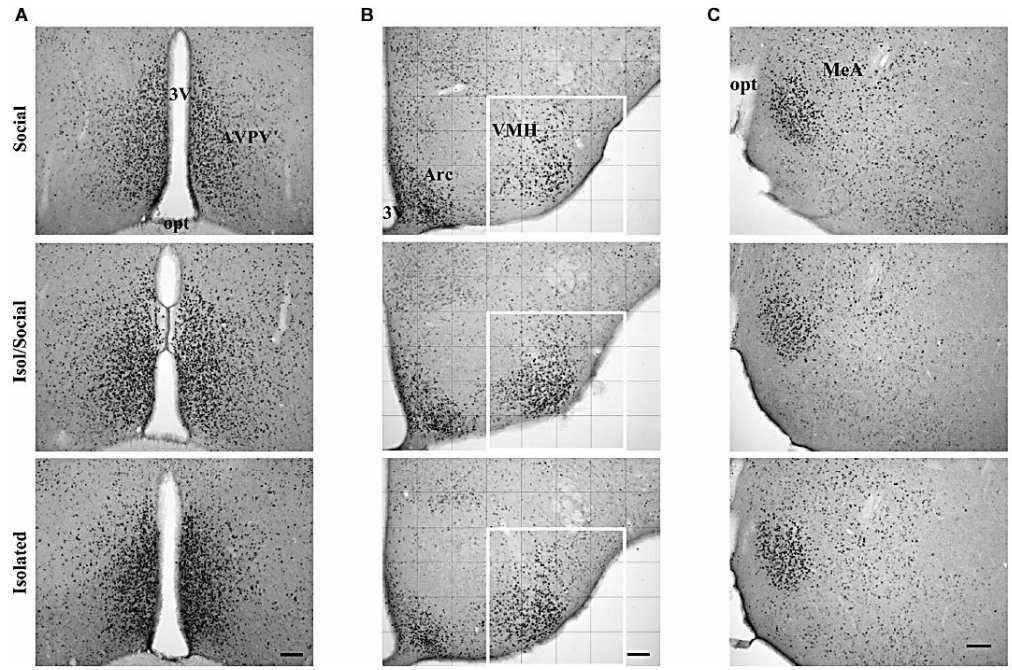

FIGURE 2 | Digital images showing ERα immunoreactive cells. **(A)** In the anteroventral paraventricular nucleus (AVPV), **(B)** ventromedial nucleus of the hypothalamus (VMH) and **(C)** in the medial amygdala (MeA) in socially housed mice (Social), isolated for transient time (Isol/Social) and in isolated throughout the experiment (Isolated). In the VMH images, the white square denotes the area analyzed. Bar = 100 μm. 3V—third ventricle, opt—optic nerve, Arc—arcuate nucleus.

cause enduring changes in behavioral responsiveness of the brain to estradiol and progesterone. The current study provides new information about the effects of a different source of stress, post-weaning social isolation, on female sexual behavior. Sexual behavior was examined in female mice that were individually or group housed from 25 days of age throughout the experiment, or individually housed from day 25 until day 60 (during puberty), followed by housing in social groups. The results of the current study suggest the importance of the social environment during puberty for the display of sexual behaviors in adult female mice.

In the current study socially housed female mice displayed stronger lordosis behavior in comparison to mice that were socially isolated during puberty (Isol/Social or Isolated). There were no significant differences between mice that were isolated during puberty vs. re-socialized afterward. Therefore, 1 month re-socialization was insufficient to rescue the behavior from the deleterious effects of social isolation during puberty. This is consistent with previous reports about sexual behavior in female mice that were exposed to different stressors during the peripubertal adolescent period (Laroche et al., 2009a,b; Ismail et al., 2011). In the current study there was a significant overall effect of housing on LQ. However, *post hoc* tests revealed significant differences between social and social/isol groups in all five trials while the difference in LQ was significantly different between social and isol groups more selectively in trials 3 and 5. This is intriguing, as it suggests a potentially stronger effect of temporary isolation than permanent isolation. Perhaps this could be explained by the possibility that re-socialization after isolation during puberty might present additional stress for female mice, while persistent isolation possibly force mice to adapt to the isolation and in longer period of isolation somehow compensate some of the effects of isolation stress. The observation that isolation stress selectively during puberty had a stronger effect on LQ than isolation throughout the experiment is partially in agreement with the previous study by Laroche and coworkers that reported mice exposed to stress (shipping or LPS injection) at 6 weeks of age (peripubertally) show lower levels of sexual receptivity than mice exposed to the same stressors at 12 weeks of age (in adulthood) or control mice (Laroche et al., 2009a,b). Thus, the pubertal period might be an especially vulnerable period for stress to cause alterations in the circuitry regulating female sex behavior. In contrast, sexual behavior of socially housed or isolated female rats after weaning did not differ (Duffy and Hendricks, 1973), suggesting that female mice might be more vulnerable to social isolation stress during puberty than female rats. As expected, there were too few proceptive behaviors (sniffing or following the male) seen in any female mice to analyze impact. Interestingly, the sexual behaviors of stimulus males (mounts, thrusts, intromissions and ejaculation) were not altered by the

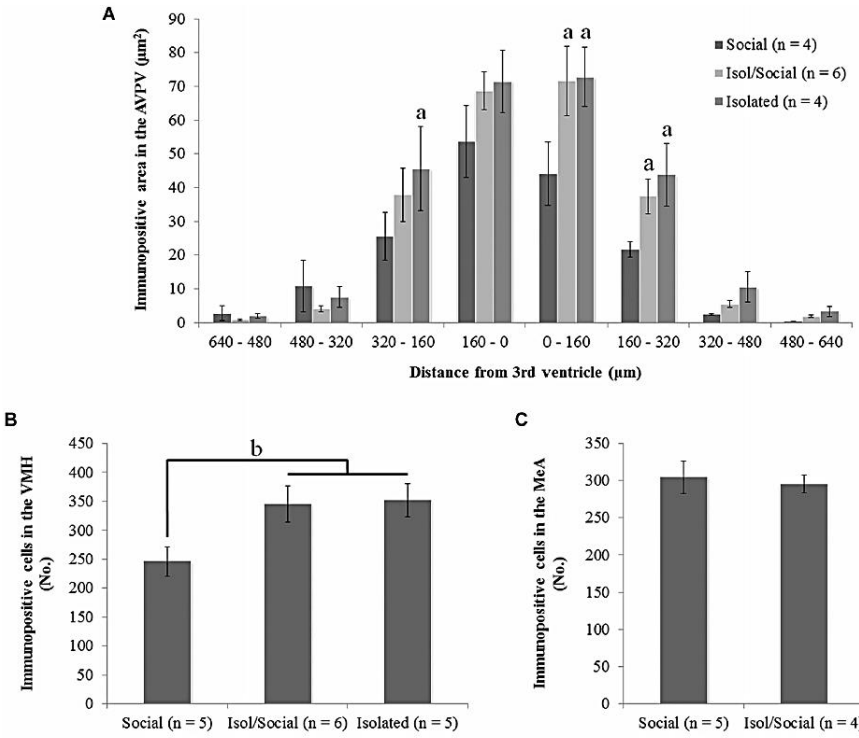

FIGURE 3 | Social isolation during puberty increases ERα immunoreactivity in the anteroventral paraventricular nucleus (AVPV) and in the ventromedial nucleus of the hypothalamus (VMH), but not in the medial amygdala (MeA). (A) Area of representing immunopositive ERα cells in the AVPV on both sides of the third ventricle (^{a} $p < 0.01$), **(B)** total number of immunopositive ERα cells in the VMH (^{b} $p < 0.05$), **(C)** total number of immunopositive ERα cells in the MeA. Data are reported as mean ± SEM; ^{a} Significantly different from females housed in social groups (Social). ^{b} Significant difference between females housed in social groups (Social) and other two isolated groups (Isol/Social, Isolated).

different housing regimes of the test females, suggesting that female "attractiveness" was not affected by social isolation during puberty.

Estradiol effects in the brain are mediated via interactions with ERs and for the regulation of female sexual behavior by estradiol, ERα is essential (Ogawa et al., 1998; Rissman et al., 1999). MPOA, MeA, and VMH and other sites are rich in ERs that likely contribute to sexual behaviors (Flanagan-Cato and McEwen, 1995; Rissman et al., 1999; DiBenedictis et al., 2012). In the current study, social isolation (permanent or for a specifically limited period) increased the area of ERα immunoreactivity in the AVPV and the number of ERα immunopositive cells in the VMH in comparison to socially housed mice. This contrasts with previous studies in mice (Ismail et al., 2011) and prairie voles (Ruscio et al., 2009), where females stressed during puberty had reduced numbers of ERα immunopositive cells in different brain areas (MPOA, BNST, VMH and arcuate nucleus) involved in the regulation of female sexual behavior in comparison to control animals. One report (Ismail et al., 2011) showed reduced levels of ERα in the MPOA, VMH and arcuate nucleus of adult mice (at 16 weeks of age), but not in the AVPV, after exposure to shipping stress during the pubertal period at 6 weeks of age. Another report (Ruscio et al., 2009) showed no differences in immunoreactive ERα in the MeA and VMH, but reduced expression in MPOA and BNST, between isolated and pair housed (different sex pairs) prairie voles. These differences among studies may be due to the use of different stressors, the timing of stress, and species or strain differences. All of these factors have been shown to cause differences in stress effects (reviewed in McCormick et al., 2010). Estradiol has been shown to auto-regulate its receptor expression with increased levels of estradiol having suppressive effects on ERα mRNA or protein (DonCarlos et al., 1995; Gréco et al., 2001). In the present study, all mice were exposed to estradiol prior to sacrifice and therefore the differences between groups are presumably due to differences in housing/exposure to social stress. The difference between our study and study by Ismail et al. (2011) which reported reduction of ERα cell numbers in the VMH and no differences in AVPV could be explained by the duration of stress. Namely, in the current study, the stress was prolonged whereas in the prior study in mice (Ismail et al., 2011) the duration of stress was limited and short. Perhaps reduced

expression of ERα in certain brain areas presents a rebound effect following short stress and consequent suppression of HPG axis, followed perhaps by a compensatory period with increased estradiol levels. By contrast, prolonged stress could more permanently affect/reduce estradiol levels, causing an increase in receptor expression. Future studies should consider determining the time course of changes in ERα in the relevant brain areas and possible mediating factors.

As noted in the Introduction section, MeA is also thought to be involved in female sexual behavior, since mating increases c-Fos expression in this brain region (Flanagan-Cato and McEwen, 1995; Sah et al., 2003; DiBenedictis et al., 2012). However, another study in female rats suggested that impairment of sexual behavior is not due to ERα knockdown in MeA (Spiteri et al., 2010a), indicating that female sexual behavior is not modulated by the ERα in MeA in rats. As for many characteristics, it is difficult to know whether this is true for rats and mice (Bonthuis et al., 2010). Indeed, in the present study there was not a significant alteration in the number of ERα immunopositive cells in the MeA regardless of different housing regimes during puberty.

The amygdala is a major brain region mediating emotional and hormonal responses to stress (mainly the basolateral complex of amygdaloid nuclei and central nucleus of the centromedial complex) (reviewed in Sah et al., 2003). Regardless of the lack of influence of social isolation on immunoreactive ERα, alterations of lordosis behavior in socially isolated mice in the current study could be due to increased general anxiety or impaired social behaviors. Anxiety-like behaviors were not directly assessed. In the absence of proceptive behaviors or group differences in attractiveness to stimulus males, impaired lordosis behavioris perhaps less likely due to impaired social interactions. In agreement, a study in socially isolated female rats during puberty showed no differences in social interactions in comparison to socially grouped rats (Lukkes et al., 2012). Another study in mice found no differences in aggressive behavior in female mice of different housing conditions (Ouchi et al., 2013). Other studies of housing isolation stress after weaning in female C57BL/6J mice reported no effect on anxiety-like behavior in comparison to socially housed mice (∼4 weeks of isolation (Pietropaolo et al., 2008) or ∼8 weeks of isolation (Kulesskaya et al., 2011)). Interestingly, one study in socially housed female rats suggested that anxiety-like behavior might be modulated by ERα in the MeA based on reductions of anxiety-like behaviors after silencing ERα in the MeA (Spiteri et al., 2010b). The lack of differences in MeA ERα immunoreactivity in the current study might be taken to suggest that all three groups of females in the current study were in similar anxiety-like states.

In conclusion, the results of the current study suggest that social isolation stress during puberty/adolescence can have a profound effect on female sexual behavior and on the detection of immunoreactive ERα in brain regions important for behavior regulation in adult female mice without impacting female attractiveness to stimulus males. Re-socialization for 1 month in adulthood could not reverse the effects of social isolation during the pubertal period. These results highlight the importance of social environment during puberty on the development of sexual receptivity in female mice and this should be taken into account when planning or interpreting results of such behavior assessment studies.

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3.4 GONADECTOMY PRIOR TO PUBERTY DECREASES NORMAL PARENTAL BEHAVIOR IN ADULT MICE

ODSTRANITEV SPOLNIH ŽLEZ PRED PUBERTETO POSLABŠA STARŠEVSKO OBNAŠANJE ODRASLIH MIŠI

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Izvleček

Steroidni hormoni iz spolnih ģlez vplivajo tako na razvoj kot tudi na izraģanje ģtevilnih obnaģanj, vkljuļno s starģevskim obnaģanjem. Razvoj sposobnosti izraģanja ģtevilnih obnaģanj se odvija pod vplivom spolnih hormonov, ki se zaļne s spolno diferenciacijo in se nadaljuje v puberteto. Ļas odstranitve spolnih ģlez ima zato lahko pomemben in dolgotrajen vpliv na organizacijo in aktivacijo doloļenih delov ģivļnega sistema, ki so pomembni pri uravnavanju izraģanja razliļnih vrst obnaģanja. Naģa raziskava je prouļevala pomembnost nemotenega delovanja spolnih ģlez med puberteto/ adolescenco na sposobnost izraģanja starģevskega obnaģanja miģi v odraslem obdobju. Samcem in samicam miģi divjega tipa smo odstranili moda in jajļnike bodisi pred puberteto (starost 25 dni) ali po njej (starost 60 dni) ter jih v odraslem obdobju testirali za starģevsko obnaģanje brez dodanega in z dodanim estradiol benzoatom. Dodatnim skupinam miģi smo odstranili moda in jajļnike pri starosti 25 dni ter jim med puberteto (do starosti 60 dni) dodajali testosteron (samci) oziroma estradiol (samice). Samice s prisotnimi ali z odstranjenimi jajļniki med puberteto ter tretiranimi z estradiolom med puberteto so moļneje izraģale starģevsko obnaģanje, usmerjeno k mladiļem, v primerjavi z miģmi brez spolnih ģlez med puberteto, ne glede na dodajanje estradiola v odraslem obdobju. Miģi samci in samice, ki so prejemali estradiol v odraslosti, so moļneje izraģali starģevsko obnaģanje, ki ni usmerjeno k mladiļem (gradnja gnezda), kot pa pred prejemanjem estradiola, ne glede na ļas odstranitve mod oziroma jajļnikov. Samice smo dodatno testirali ģe za ģensko spolno obnaģanje z namenom, da bi ugotovili morebitne spremembe v obļutljivosti odziva na spolne hormone glede na dolģino ļasa brez jajļnikov. Med samicami z odstranjenimi jajļniki pred puberteto ali po njej v spolnem obnaģanju ni bilo statistiļno znaļilnih razlik, kar pa popolnoma ne izkljuļuje moģnosti, da starģevsko obnaģanje ni bolj obļutljivo na daljģo ļasovno odsotnost spolnih hormonov. Izsledki kaģejo na slabģe starģevsko obnaģanje do mladiļev najverjetneje zaradi odsotnosti spolnih ģlez in s tem odsotnosti spolnih steroidnih hormonov med puberteto/ mladostniģtvom.

J. Kerļmar: Vpliv socialnega stresa, spolnih hormonov med puberteto é na obnaġanje odraslih miġi. Ljubljana: UL, Veterinarska fakulteta, 2015. Doktorska disertacija

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Regular article

Gonadectomy prior to puberty decreases normal parental behavior in adult mice

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ABSTRACT

Sex steroid hormones secreted by gonads influence development and expression of many behaviors including parental behaviors. The capacity to display many behaviors develops under the influence of sex steroid hormones; it begins with gonadal differentiation and lasts through puberty. The timing of gonadectomy may have important and long lasting effects on the organization and activation of neural circuits regulating the expression of different behaviors. The present study investigated the importance of exposure to endogenous gonadal steroid hormones during pubertal period/adolescence on parental behavior in adult mice. Male and female WT mice were gonadectomized either before puberty (25 days of age) or after puberty (60 days of age) and tested for parental behavior with and without estradiol benzoate (EB) replacement in adulthood. Additional groups of mice were gonadectomized at P25 and supplemented with estradiol (females) or testosterone (males) during puberty. Female mice gonadectomized after puberty or gonadectomized before puberty and supplemented with estradiol during puberty, displayed better pup directed parental behaviors in comparison to mice gonadectomized at 25 days of age regardless of treatment with estradiol in adulthood. However, mice treated with EB in adulthood displayed better non-pup directed nest building behavior than when they were tested without EB treatment regardless of sex and time of gonadectomy. To examine whether the sensitivity to sex steroid hormones was altered due to differences in time without gonads prior to the testing, mice were also tested for female sex behavior and there were no differences between mice gonadectomized at P25 or P60, although this could not completely rule out the possibility that parental behavior is more sensitive to prolonged absence of steroid hormones than female sex behavior. These results suggest that the absence of gonads and thereby the absence of appropriate gonadal steroid hormones during puberty/adolescence may have a profound effect on pup directed parental behaviors in adult mice.

Introduction

Gonadal steroid hormones influence the development of brain and consequently behavior. Steroid hormones affect brain development and activity through two relatively separable processes characterized as organizational versus activational (Arnold and Breedlove, 1985; Schulz et al., 2009; Sisk and Foster, 2004). Usually, more permanent organizational effects occur perinatally, while activational effects occur later in life when gonadal hormones act on specific neural circuits to trigger specific aspects of physiology or the expression of various adult behaviors (reviewed in (Arnold, 2009; Majdic and Tobet, 2011; McCarthy and Arnold, 2011)). A growing number of studies also show that gonadal hormones can have "organizational" effects on the brain later in life, during puberty and possibly in adult life (Ahmed et al., 2008; De Lorme et al., 2012; Romeo, 2003; Romeo et al., 2000; Schulz and Sisk, 2006; Schulz et al., 2009). Puberty is a period during which the hypothalamic–pituitary–gonadal axis reactivates with the elevated secretion of gonadal steroid hormones (Sisk and Zehr, 2005). Many brain regions are sensitive to the action of gonadal steroid hormones, including parts of the brain that are thought to be involved in the regulation of parental behavior (Kalinichev et al., 2000).

Parental behavior is broadly defined as any behavior performed in the relation to one's offspring, or as any behavior that contributes directly to the survival of fertilized eggs or newborns. In mammals, maternal care is more common than paternal, although paternal behavior is also present in several mammalian species including mice and humans (Nelson, 2005). In many species, maternal behavior is triggered by the exposure to steroid hormones during pregnancy. However, nulliparous

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(virgin) female rats and mice exhibit parental behavior when presented with foster pups even without circulating gonadal steroids (Numan and Insel, 2003). By contrast, male rats and mice are commonly infanticidal (Lonstein and De Vries, 2000), although a recent study by Tachikawa et al. (2013) demonstrated that male mice are not aggressive towards pups if cohabited with female mice for two weeks after mating. In this study, it was suggested that pheromonal cues are responsible for aggressive behavior of males towards pups and this response is diminished if fathers are cohabited with pregnant females or the vomeronasal organ in sexually naïve males has been surgically removed. Many studies of parental behavior in mice and rats, and influences of gonadal steroid hormones on parental behavior, have been done in parturient rodents (e.g. (Lonstein et al., 1999; Stolzenberg and Rissman, 2011)) or in virgin, gonadally intact or gonadectomized rodents in adulthood (e.g. (Gatewood et al., 2006; Koch and Ehret, 1989; Lonstein et al., 1999; Ogawa et al., 1998a; Okabe et al., 2013; Okabe et al., 2010; Stolzenberg and Rissman, 2011)). Gonadectomy in adult life seems to have little effect on parental behavior in adult virgin female mice (Ogawa et al., 1998a) and rats (Lonstein et al., 1999) while in male mice, published results differ with one study reporting improvement of paternal behavior after gonadectomy (Okabe et al., 2013) and another finding no influence of adult gonadectomy on paternal behavior (Gatewood et al., 2006). Although pup retrieval behavior can improve with gonadectomy (or social experience) in adulthood in mice of both sexes (Okabe et al., 2010), we were not aware of reports concerning the possible consequences of juvenile gonadectomy on the development of parental behavior in mice. Therefore, the present study examined parental behavior in mice of both sexes that were gonadectomized at 25 days of age (before puberty) or at 60 days of age (after puberty) to explore whether the prolonged absence of gonads during pubertal/adolescent period can impact parental behaviors directed towards foster pups in adult mice. To check whether exposure to sex steroid hormones during puberty is necessary for the development of parental behavior directed towards foster pups in adult mice, some mice were also gonadectomized before puberty and supplemented with sex steroid hormones during puberty.

Materials and methods

Animals

C57BL/6J male and female mice were originally obtained from Harlan, Italy and bred at the University of Ljubljana, Veterinary Faculty, in standard conditions with 12–12 LD cycle (lights on at 3 am and off at 3 pm) and food (phytoestrogen free diet; Harlan Teklad Diet 2016, Harlan, Milan, Italy) and water ad libitum. Mice were weaned at 21 days of age and mice from the same litters were group-housed (3 mice of same sex per cage). Mice were housed in 15 cm high cages with floor area of 37.5 × 22 cm. First groups of male (n = 11) and female mice (n = 10) were gonadectomized (GDX) at 25 days of age (before puberty), the second groups of male (n = 5) and female (n = 8) mice were gonadectomized at 60 days of age (after puberty) and additional groups of male (n = 4) and female mice (n = 4) were gonadectomized at 25 days of age (before puberty) and had inserted testosterone (T) or estradiol benzoate (EB) silastic implants, respectively, from 25 to 60 days of age (during puberty).

Foster pups for assessing parental behavior were obtained from the mice of the same strain, bred in the same conditions.

Sexually experienced stimulus males (C57BL/6J), previously used for mating (at least 3 successful mating with weaned litters), were used for assessing sexual behavior in female mice. Stimulus males were housed individually in 13 cm high cages with 28.5 × 10.5 cm floor area and bred also in the same conditions.

All animal experiments were approved by Veterinary Administration of Slovenia (license no. 34401-32/2012/8) and were done according to ethical principles, EU Directive 2010/63/EU and NIH guidelines.

Surgery and hormone replacement

Male and female mice were gonadectomized bilaterally at 25 or 60 days of age to remove endogenous gonadal steroids. Mice were anesthetized with the mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 μg/g BW), acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) and xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μg/g BW) and gonads were excised through small incisions. Incisions were stitched and mice received two injections of butorfanol (Turbogesic, Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) after surgery to ease any potential pain. The additional group of males and females, gonadectomized at 25 days of age, received during gonadectomy subcutaneous silastic implants (1.02 mm inner diameter, 2.16 mm outer diameter) filled with sex steroid hormones according to sex. Males received implants filled 10 mm in length with crystalline testosterone (T; Sigma, Taufkirchen, Germany; (Gatewood et al., 2006)) and females received implants filled with 5 mm in length with crystalline 17β-estradiol 3-benzoate (EB; Sigma, Taufkirchen, Germany) diluted 1:1 with cholesterol (Wersinger et al., 1999) and closed on both ends by medical silastic adhesive (Dow Corning, Lakeside, AZ, USA). Implants were inserted subcutaneously in the midscapular region. Mice from this group had silastic implants removed in general anesthesia at 60 days of age, after 35 days of hormone exposure. Mice were left to recover for at least 10 days (Seitz et al., 2010) before behavior assessments were performed. Mice were tested as hormonally naïve first and then later after priming with estradiol benzoate (EB; only mice without gonadal hormones priming during puberty). All mice were initially tested twice on two consequent days for parental behavior between 70 and 75 days of age. Two to 3 days after the second test, mice without gonadal hormone priming during puberty received subcutaneous implants of EB and were tested again 10 days after implantation. Silastic implants (1.02 mm inner diameter, 2.16 mm outer diameter) were again filled with 5 mm in length with crystalline 17β-estradiol 3-benzoate (EB; Sigma, Taufkirchen, Germany) (diluted 1:1 with cholesterol) (Wersinger et al., 1999) and closed on both ends by medical silastic adhesive (Dow Corning, Lakeside, AZ, USA). Implants were inserted subcutaneously in the midscapular region under anesthesia. Similar implants filled with 17β-estradiol have yielded plasma estradiol levels within the physiological range in female mice (Bakker et al., 2002) and rats (Seale et al., 2004). For female sex behavior tests, implants inserted in adulthood were left in situ and mice were injected subcutaneously with 0.8 mg of progesterone (P; Sigma) approximately 4 to 8 h before each test. All mice were initially tested for female sexual behavior after conclusion of parental behavior tests between 90 and 100 days of age.

Parental behavior test

Each mouse was tested in a standard parental behavior test 4 times, twice without hormone supplement and twice primed with EB (only mice without gonadal hormones priming during puberty) to examine the potential effect of estradiol on parental behavior directed towards foster pups, although this is not mimicking physiological situation during pregnancy where mice are exposed first to high levels of progesterone followed by high estradiol exposure. First two (without hormones) and last two trials (with hormones) were performed on two consecutive days; on the first day the first trial lasted 20 min and the next day the second trial lasted for 15 min as mice were experienced and had shorter latencies to initiate parental behavior. Mice were transferred to smaller test-cages (14 cm high with 35 × 15 cm floor area) with at least three day-old bedding from their home cage 24 h prior to behavioral testing. After the first and last two trials, mice were returned to their larger home cages in their respective social groups.

Parental behavior tests were performed during the light cycle 1 to 2 h before the start of the dark cycle as described by Gatewood et al. (2006). This is supported by previously published study reporting that

parental behavior in mice has been reported to be more frequent during the light phase (Shoji and Kato, 2006). Three male foster pups 1 to 3 days old and torn tissue paper for nest building were placed in separate corners of the test-cage on the sides furthest from the nest of the test mouse. Male foster pups were chosen due to their potentially greater attraction to mothers in comparison to neonatal female rats (Moore, 1981, 1985), although in mice differences in maternal behavior, depending on the sex of pups have not been observed (Keller et al., 2010). If pups were attacked, all pups were immediately removed and infanticidal behavior was recorded. During testing, the following parental activities were recorded: latency to visit and lift pups, latency to retrieve the first, second and third pup and the number of pups retrieved into the nest, latency and total time licking and sniffing pups, latency and total time spent crouching over pups, aggression towards pups, latency, number and total time spent building the nest and the quality (zero to three points) of the nest.

All tests were video recorded and an observer (J.K.), blinded to the group assignment at the time of testing, scored all behaviors directly using 'stopwatch' software (Center for Behavioral Neurosciences, Atlanta, GA).

Female sexual behavior test

Female sexual behavior was tested to check the hormone sensitivity of mice with extended absence of endogenous steroids. Behavior tests were performed in a clear glass aquarium (17 cm high with 41.5 × 26 cm floor area) with a mirror positioned under the testing arena to obtain views of all facets of sexual behaviors (Wersinger et al., 1997). Females were tested during the first 2 to 4 h of the beginning of the dark period of the circadian cycle, under dim red light illumination, and the test sessions were videotaped for subsequent scoring. The timing of testing was chosen to mimic the timing of the normal occurrence of mating in mice. As ovulation in intact female mice tends to occur during the late hours of the dark or early hours of the light period (Bingel and Schwartz, 1969) and the female is sexually receptive in the late proestrus/early estrus portion of the cycle (e.g. (Zinck and Lima, 2013)), mating in mice normally occurs during the first half of the dark period. Each female was tested 6 times; every 4 to 5 days to mimic the timing of the normal physiological estrous cycle. The first trial served to provide the animals with sexual experience prior to testing the next 5 trials were scored. The stimulus males were placed into the aquarium at least 4 h prior to behavior testing with at least 3 day old bedding and food and water ad libitum to acclimate to the novel environment. Food and water were removed during the behavior testing.

Hormonally-primed females (EB implants & subcutaneous injection of P 4 to 8 h prior to behavioral assessment) were placed in the middle of aquaria with stimulus males for 20 min (Park, 2011), or until the females received an ejaculation and the following behaviors were recorded: lordosis posture, total number and latency of attempted mounts, successful mounts, pelvic thrusts and intromissions, and the latency of ejaculation. Behaviors were recorded by 'stopwatch' software (Center for Behavioral Neuroscience, Atlanta, GA) and were performed always by the same investigator (J.K.) who was blinded to the group assignment at the time of testing.

If the stimulus male did not try to mount the female after 5 min of testing, the female was transferred to a different cage with a new stimulus male. Ejaculating males were not used again for the remainder of the trial day. Mounts were counted when the female had all four limbs on the floor, exhibited lordosis posture and the male successfully clasped its hindquarters with forepaws. Attempted mounts were counted when the female resisted and failed to display lordosis postures when males were clearly trying to mount. Female lordosis posture as an index of sexual receptivity was scored from 0 (no receptive behavior with no lordosis reflex) to 5 (complete receptiveness with strongest lordosis reflex; modification of the 4 scale protocol in Bakker et al., 2002). Lordosis was defined using the following stipulations: all four paws are grounded, hind region is elevated off the floor of the test chamber, and the back is slightly arched (Kudwa et al., 2007; Takasugi et al., 1983). A lordosis quotient (LQ) was calculated by the following formula: number of mounts during which the female stood still (lordosis 4 and 5) / total number of attempted and successful mounts × 100.

Blood collection and hormone assay

Four days after the last sexual behavior test, around 125 days of age or approximately 45 days after receiving the EB implants, females were anesthetized with the mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 μg/g BW), acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) and xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μg/g BW) and a blood sample was collected by cardiac puncture. Plasma estradiol concentration was determined by commercial IBL (Hamburg, Germany) ELISA kit following the instructions for users and performed at the Institute of Physiology, Pharmacology and Toxicology, Veterinary Faculty, University of Ljubljana. Intra and inter assay coefficients of variation (CVs) were 7.91% and 10.12% for low (62 ± 0.34 pg/mL) and 4.65% and 8.57% for high (430 ± 0.81 pg/mL) estradiol values, respectively.

Statistical analyses

All statistical analyses were done using NCSS software (NCSS statistical software, Kaysville, UT). To test differences between groups in parental behavior, repeated measures ANOVA was performed with sex and gonadectomy as independent variables, and tests as repeated measures, followed by post hoc Fisher LSD test. To test differences between groups in female sexual behavior, repeated measures ANOVA was performed with gonadectomy/ovariectomy as an independent variable, and tests as a repeated measure, followed by post hoc Fisher LSD test. At least four mice (4 to 9) of each sex were tested in both parental and female sexual behavior tests. Size effect was estimated by calculating eta squared (η^2) using formula $\eta^2 =$ SSbetween/SStotal. Differences were considered statistically significant with $p < 0.05$.

Results

Parental behavior

Most of the mice tested showed parental behavior directed towards foster pups at some point. The analyses indicated that earlier gonadectomies led to less parental behavior in females and this could be rescued in females by pubertal treatment with estradiol, but not in males treated pubertally with testosterone. Components of a 3-way ANOVA (time of gonadectomy × sex and test as a repeated measure) revealed a significant effect of time of gonadectomy/pubertal EB treatment [$F(8, 83) = 5.39$, $p < 0.01$; $\eta^2 = 0.13$] on latency to lift the first pup (Fig. 1a) and a significant effect of time of gonadectomy/pubertal EB treatment [$F(8, 83) = 7.06$, $p < 0.01$; $\eta^2 = 0.16$] on the latency to retrieve the first pup into the nest (Fig. 1b). Statistical analysis also revealed a significant effect of sex [$F(8, 83) = 6.35$, $p < 0.05$; $\eta^2 = 0.08$] on the latency to lift the first pup (Fig. 1a) and on the latency to retrieve the first pup into the nest [$F(8, 83) = 8.89$, $p < 0.01$; $\eta^2 = 0.10$] (Fig. 1b). The post hoc test revealed that male and female mice gonadectomized at P25 had significantly longer latencies to lift and to retrieve the first pup into the nest in comparison to females gonadectomized at P60 and females gonadectomized at P25 and supplemented with EB, suggesting that treatment during puberty could restore pup lifting and retrieving latency to the levels observed in P60 gonadectomized female mice. Males gonadectomized at P60 or gonadectomized at/treated with T during puberty needed almost twice as long to lift and to retrieve the first pup into the nest in comparison to females from the same group (Fig. 1a,b).

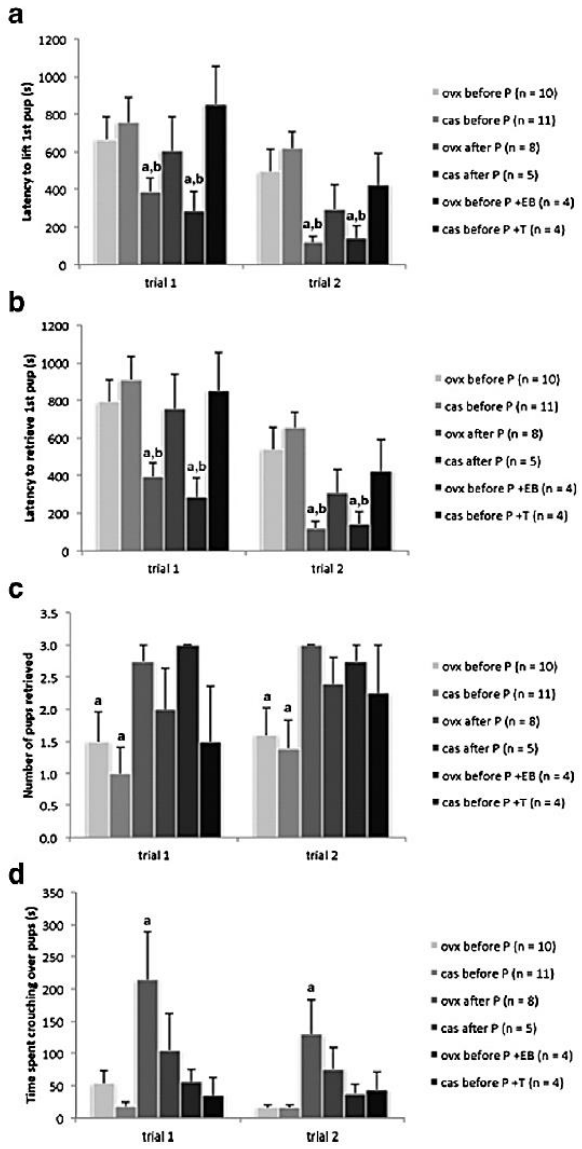

Fig. 1. Mice gonadectomized at P25 displayed reduced parental behavior in comparison to female mice gonadectomized at P60 and female mice gonadectomized at P25 and treated with EB during puberty in most parental behavior activities directed towards pups: a. latency to lift the first pup ($^{a}p < 0.01$; $^{b}p < 0.05$), b. latency to the retrieval of the first pup into the nest ($^{a}p < 0.01$; $^{b}p < 0.05$), c. number of retrieved pups into the nest ($^{a}p < 0.01$) and d. total time spent crouching over the pups ($^{a}p < 0.01$). Data are reported as mean + SEM; ^{a}Significant difference between mice gonadectomized before puberty, after puberty, or mice gonadectomized before puberty and treated with EB during puberty. ^{b}Significantly different from all three groups of males.

Statistical analyses also revealed a significant effect of time of gonadectomy [$F(8, 83) = 5.44$, $p < 0.01$; $\eta^2 = 0.18$] on the number of retrieved pups with female mice gonadectomized at P60/gonadectomized at P25 and treated with EB during puberty retrieving almost twice as many pups into the nest in comparison to male and female mice gonadectomized at P25 (Fig. 1c). There was a trend towards statistical significant effect of sex [$F(8, 83) = 3.52$, $p = 0.07$; $\eta^2 = 0.06$] in the number of retrieved pups into the nest (Fig. 1c) with females retrieving more pups than males. The post hoc analysis revealed that mice of both sexes gonadectomized at P25 retrieved significantly fewer pups into the nest than female mice gonadectomized at P60 and gonadectomized at P25 and supplemented with EB during puberty (Fig. 1c). However, there was no difference between males and females gonadectomized at P25 and males gonadectomized at P60 and males gonadectomized at P25 and treated with testosterone through puberty.

A significant effect of time of gonadectomy [$F(8, 83) = 8.00$, $p < 0.01$; $\eta^2 = 0.19$] was found also in the time spent crouching over the pups (Fig. 1d) with mice gonadectomized after puberty spending almost four times as much time crouching over pups than mice gonadectomized before puberty. Post hoc analyses revealed that mice gonadectomized at P25 regardless of pubertal sex steroid treatment spent significantly less time crouching over the pups than female mice gonadectomized at P60 (Fig. 1d).

EB treatment in adulthood did not affect a majority of pup-directed parental behaviors in mice of both sexes with the exception of time spent crouching over pups which was reduced in mice treated with EB in both sexes (34.5 ± 10.8 s in P25 GDX females without adult EB treatment, 24.7 ± 10.3 s in P25 GDX females with adult EB treatment, 171.7 ± 45.9 s in P60 GDX females without EB treatment, 49.3 ± 24.0 s in P60 GDX females with adult EB treatment, 17.0 ± 3.9 s in P25 GDX males without adult EB treatment, 9.9 ± 4.8 s in P25 GDX males with EB treatment, 90.6 ± 31.6 s in P60 GDX males without EB treatment and 8.9 ± 3.7 s in P60 GDX males with adult EB treatment; [$F(8, 108) = 7.23$, $p < 0.05$; $\eta^2 = 0.07$]).

There was an effect of EB treatment on non-pup oriented behaviors. Statistical analyses revealed a significant effect of EB treatment on the duration of nest building (non-pup-directed behavior) in males and females [$F(8, 108) = 12.40$, $p < 0.01$; $\eta^2 = 0.15$] with mice treated with EB spending almost twice as much time building nests as mice

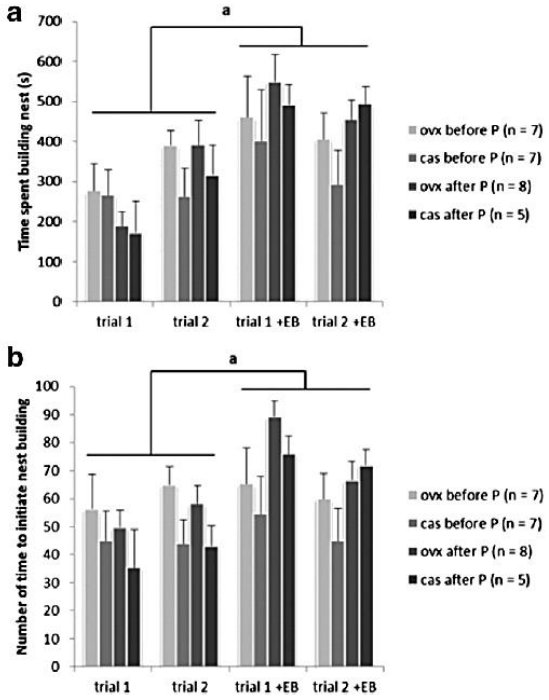

Fig. 2. Mice without adult EB treatment displayed less nest building behavior than mice supplemented with EB in adulthood (during testing) regardless of sex or time of gonadectomy: a. total time spent building the nest ($^{a}p < 0.01$), b. the number of the time to initiate nest building ($^{a}p < 0.05$). Data are reported as mean + SEM. ^{a}Significant difference between mice without and with EB treatment.

without EB supplementation (Fig. 2a). Similarly, there was a significant difference in the number of times to initiate building the nest (non-pup-directed behavior) [$F(8, 108) = 7.06$, $p < 0.05$; $\eta^2 = 0.09$] regardless of sex (Fig. 2b) with mice supplemented with EB initiating nest building up to twice as often as mice without EB treatment. Post hoc test revealed that mice of both sexes without EB treatment spent less time to build nest and less often initiated this non-pup-directed behavior than mice of both sexes treated with EB.

Seven of the total 49 mice tested displayed aggressive behaviors and there were no significant differences in the number of aggressive mice between groups (data not shown).

Female sexual behavior

When female mice from the parental behavior tests were tested for female sexual behavior, there were no differences between female mice ovariectomized at P25 versus those ovariectomized at P60 (Fig. 3). Lordosis quotients (LQs) were similar for both groups suggesting that long absence of exposure to sex steroid hormones did not affect the sensitivity to EB and progesterone for the induction of female sexual behavior.

Plasma estradiol levels

Plasma estradiol level in ovariectomized (before & after puberty) females ($n = 14$) after behavior assessments (~125 days of age) and after approximately 45 days of 17β-estradiol 3-benzoate supplementation in the form of subcutaneous implants was 224 ± 19 pg/mL. This was higher than previously reported levels for implants with crystalline 17β-estradiol without benzoate esterification: from ~80 pg/mL (Wersinger et al., 1999) to ~130 pg/mL (Bakker et al., 2002) and also from levels obtained using EB implants in rats that were approximately 140 pg/mL (Febo et al., 2002). However, the levels were within the range reported in a previous study measuring levels of estradiol during pregnancy, which were around 200 pg/mL on day 6 of pregnancy (with individual levels up to 250 pg/mL; Jacquet et al., 1977).

Discussion

Parental behavior is expressed in most mammalian species from marsupials (Russell, 1982), monotremes (Nicol, 2013) to eutherians. The widest variety of parental care patterns has evolved among the eutherians with significant differences in the amount and quality of parental behavior, and which sex engages in the care of the offspring (e.g. (Dwyer, 2008; Lonstein and De Vries, 2000; Root Kustritz, 2005; Saltzman and Maestripieri, 2011)). Although it is well established that parental behavior is provoked by exposure to hormones during pregnancy in rats (reviewed in (Rosenblatt et al., 1988)) and sheep (reviewed in (Dwyer, 2008)), the development of capacity to express parental behavior in mice is not yet fully understood (Kuroda et al., 2011). The current study examined whether the timing of gonadectomy during the adolescent period affects parental behavior in adult mice. In general, several measures of parental behavior were more prominent in mice that were gonadectomized after puberty and tested shortly thereafter in comparison to mice gonadectomized before puberty and tested after 7 weeks, mostly regardless of sex. Interestingly, only estradiol but not testosterone treatment during puberty could restore several measures of pup-directed parental behavior to the levels observed in P60 GDX mice. The results in the current study affirm the importance of gonads and thereby the continuous presence of appropriate gonadal steroid hormones in young mice for normal parental behaviors. This is consistent with the suggestion that gonadal steroids have organizational effects on the brain during puberty in addition to their activational roles, as has been described for other behaviors like sexual, agonistic (aggressive behavior and flank marking, submissive behavior) and anxiety-related (social interaction in a novel environment, open-field ambulation) behaviors (reviewed in (Romeo, 2003; Sisk and Zehr, 2005)).

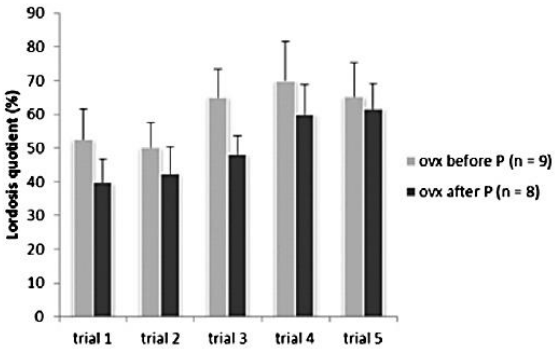

Fig. 3. Female mice gonadectomized at P25 displayed similar lordosis quotient as females, gonadectomized at P60 after EB and progesterone treatment in adulthood. Data are reported as mean ± SEM.

Most studies of parental behavior in mice were conducted with mice that were gonadectomized postpubertally (e.g. (Gatewood et al., 2006; Koch and Ehret, 1989; Ogawa et al., 1998a; Ogawa et al., 1998b; Okabe et al., 2013; Okabe et al., 2010)). In virgin mice, gonadectomy after puberty seems to have little or no effect on parental behavior in adult females (Ogawa et al., 1998a) and males (Gatewood et al., 2006; Ogawa et al., 1998b), although gonadectomy in male mice may moderately improve parental behavior in adulthood (Okabe et al., 2013). To examine the importance of continuous exposure to gonadal sex steroids during the pubertal period on the development of parental behavior in adult virgin mice of both sexes, the mice in the present study were gonadectomized at 25 days of age (before puberty) or at 60 days of age, after the normal pubertal period. An additional group of male and female mice was gonadectomized at 25 days of age and treated either with testosterone (males) or EB (females) in the form of subcutaneous implants only during puberty. The results revealed that the mice of both sexes gonadectomized before puberty had significantly longer latencies to lift a pup, retrieve pups to the nest, retrieved less pups into the nest and spent less time crouching over pups in comparison to mice gonadectomized after puberty. In most of these pup-directed parental behaviors, with the exception of the time crouching over pups, estradiol given to prepubertal ovariectomized females rescued the behavior, but testosterone given to prepubertally castrated males had no discernible effect. These results suggest that the continuous presence of gonadal hormones (especially estradiol) during puberty is likely to be important for the development of the capacity to display these behaviors in adulthood either by causing permanent alterations in brain circuitry responsible for parental behavior (Kalinichev et al., 2000) or perhaps maintaining the sensitivity to sex steroid hormones by continuous exposure to such hormones. Although estradiol levels measured in mice in the current study were higher than previously reported preovulatory levels, these levels were within the physiological range of late pregnancy stages (Jacquet et al., 1977) when capacity to display maternal behavior is thought to develop at least partially under the influence of high levels of estradiol.

Sex differences were detected in the latency to lift and to retrieve the first pup into the nest, similar to a previous report (Gatewood et al., 2006) where it was also reported that females were faster to retrieve the first pup into the nest in comparison to males. However, there was no statistically significant effect of sex in some other behaviors. In particular, there was very little infanticidal behavior in males, suggesting that in mice, unlike rats, parental behavior is not strongly sex-dependent and is present in both sexes, although a recent study by Tachikawa shows that pup priming is necessary to display paternal behavior (Tachikawa et al., 2013). However, in the study by Tachikawa,

gonadally intact males were used while in the present study, gonadectomized males were tested.

In comparison to the time of gonadectomy, EB treatment in adulthood did not have a major effect on parental behavior in mice of both sexes, although previous studies have suggested that EB stimulates maternal behavior in adult female mice (Koch and Ehret, 1989; McCarthy, 1995) and rats (Sheehan and Numan, 2002; Siegel and Rosenblatt, 1975). In contrast, a recent study by Okabe et al. (2013) reported no significant effect of estrogen implants (presumably estradiol, but not stated) after gonadectomy in adult female mice on retrieving behavior or pup sensitization, which is in agreement with the results in the current study. The only significant effect of EB treatment in the current study was found in the duration of nest building, in the number of times tested mice initiated nest building and in the time spent crouching over pups, suggesting that EB treatment in adulthood mainly affects non-pup oriented behavior, while EB is not necessary to provoke the onset of parental behavior directed directly towards pups even in gonadectomized mice. Interestingly, estrogens have been implicated in the thermoregulation and perhaps nest building behavior reflects some effects of estrogens on this mechanism, especially as two previous studies have suggested that in mice and rats, estrogens lower tail skin temperature (Opas et al., 2004, 2006). It has to be noted that the mice in the present study were treated only with EB, while during normal pregnancy mice are exposed to both progesterone and estradiol. Therefore, the hormone "replacement" conditions in the present study were not identical to the hormonal status during pregnancy.

The timing of gonadectomy relative to testing is an important question. The time after gonadectomy and before behavioral testing can be a significant factor as sex steroid hormones can have long lasting effects (Becker et al., 2008). Although circulating plasma levels of testosterone or estradiol rapidly decline within 1 to 3 days after gonadectomy, testosterone or estradiol are cleared from the bloodstream by about 14 days after gonadectomy in male (Wichmann et al., 1996) and female (Ingberg et al., 2012) adult mice. Therefore, in hormonally naïve mice, the time between gonadectomy and testing (at least 10 to 15 days), especially in mice gonadectomized after puberty, could influence the results of such studies. Therefore, it is possible that in the current study, the observed alterations in the parental behavior resulted through 2 mechanisms. First, they could have arisen due to the alteration of neural circuits that develop during puberty. Alternatively, they could result as a consequence of the longer-term absence of exposure to sex steroid hormones (~50 days versus ~15 days). Postcastration deficiency in sex steroid sensitivity has previously been reported in rats (Clark et al., 1995). However, it seems less likely that sensitivity to the steroid hormones in a context of parental behavior was different between the groups in the present study. Treatment with EB did not result in any significant differences in parental behavior between mice gonadectomized before or after pubertal period. Furthermore, testing females for sexual behavior did not reveal any differences between mice ovariectomized before or after puberty. As female sex behavior is dependent on estrogen exposure, this could suggest that the female mice in the current study did not differ in their sensitivity to sex steroid hormones. Although of course, female sexual behavior and parental behavior are two different behaviors, regulated differently and therefore, similar sensitivity to estradiol in female sex behavior does not conclusively suggest that effects would be the same in parental behavior.

In conclusion, the current study suggests that the absence of gonadal steroid hormones during puberty/adolescence can have a significant effect on several aspects of pup directed parental behaviors in adult mice of both sexes, that can be reversed by estradiol supplementation of GDX mice during puberty. Steroid hormone supplementation in adulthood could not reverse the effects of a hormonal deficit during puberty on parental behavior. Adult hormone supplementation was equally effective in parental and sexual behaviors between groups, gonadectomized before or after puberty, suggesting that sensitivity to estradiol was not altered due to a longer absence of sex steroid hormones. These results therefore highlight a requirement of a pubertal period with appropriate gonadal hormonal stimulation for adequate development of parental behavior in mice.

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3.5 SEX-SPECIFIC BEHAVIORAL EFFECTS OF FLUOXETINE TREATMENT IN ANIMAL MODELS OF DEPRESSION AND ANXIETY

RAZLIKE MED SPOLOMA V DELOVANJU ZDRAVILA FLUOKSETIN PRI ŽIVALSKIH MODELIH ZDRAVLJENJA DEPRESIVNIH IN ANKSIOZNIH MOTENJ

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Izvleček

Bolezenski znaki in uspeģnost zdravljenja z razliļnimi zdravili se pri ģtevilnih duģevnih boleznih med spoloma moļno razlikujejo. Depresivne in anksiozne motnje se 2- do 3-krat pogosteje pojavljajo pri ģenskah kot pri moģkih, vseeno pa se veļina predkliniļnih raziskav in preizkuģanj novih zdravil opravi samo pri samcih poskusnih ģivali. Veļ raziskav je nakazalo, da je odziv ģensk in ģenskih ģivali na selektivne zaviralce prevzema serotonina (SSRI) boljģi kot pri moģkih, kar kaģe, da spolni hormoni vplivajo na odzivnost organizma na tovrstna zdravila. Uvedba prvega zdravila iz skupine SSRI, in sicer prozaca v osemdesetih letih prejģnjega stoletja, je bil pomemben napredek pri zdravljenju motenj depresivnosti od odkritja zaviralcev monoaminskih oksidaz in triciklińnih zdravil proti depresiji trideset let prej. Fluoksetin je danes v ģiroki uporabi za zdravljenje depresivnih motenj, pa tudi za zdravljenje motenj anksioznosti. Poskusne ģivali predstavljajo dober model za prouļevanje vpliva zdravil proti depresivnim in anksioznim motnjam in tudi za prouļevanje spolnih razlik v delovanju tovrstnih zdravil. V preglednem ļlanku smo predstavili razliļne ģivalske modele za prouļevanje motenj depresivnosti in anksioznosti, in sicer test prisilnega plavanja, test dvignjenega labirinta in test odprtega polja. Poleg tega je predstavljen vpliv fluoksetina na obnaģanje ģivali v teh testih s poudarkom na razlikah med spoloma. Ģtevilne raziskave v preteklosti so pokazale, da so laboratorijski glodavci primeren model za prouļevanje tovrstnih motenj, v prihodnosti pa bo treba veļji poudarek nameniti raziskavam razlik med spoloma pri nastanku tovrstnih obolenj in pri njihovem zdravljenju.

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Original Scientific Article

SEX-SPECIFIC BEHAVIORAL EFFECTS OF FLUOXETINE TREATMENT IN ANIMAL MODELS OF DEPRESSION AND ANXIETY

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Summary: There are strong sex differences in clinical characteristics and in responses to treatment of several psychiatric diseases. Depressive and anxiety disorders are 2 to almost 3 times more common in women, but the majority of experiments examining the biological basis of these disorders and pharmacological agents for treatments are conducted in male animals. Several studies suggest that females respond better than males to the action of selective serotonin reuptake inhibitors (SSRIs), suggesting that gonadal hormones modulate mood and the response to these drugs. The beginning of clinical use of SSRI fluoxetine (Prozac) in late 80-ies was the frst major breakthrough in the treatment of depression since the introduction of tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) nearly 30 years earlier. Fluoxetine is today widely prescribed for the treatment not only of depression but also of some anxiety related disorders. Animal models of depression and anxiety represents a useful tool for the investigation of sex differences of pharmacokinetics and pharmacodynamics of antidepressants. In this review the animal models of depression/anxiety using three most common performed acute stressor behavior tests (forced swim test – FST, elevated plus maze – EPM and open field – OF) will be introduced, followed by presenting behavior alterations after fluoxetine treatment in male and female rodents. In addition, data from our lab in C57BL/6J mice of both sexes on the behavioral effects of chronic fluoxetine treatment in comparison to other studies will be presented. Given the overlap between human and rodent findings, rodents provide a good model for further research on the sex-dependent effects of SSRIs and other antidepressants.

Key words: depression and anxiety; SSRI antidepressants; fluoxetine; sex differences; animal models

Introduction

Decreased serotonergic activity has been implicated in depressive and anxiety disorders, and antidepressants directly increase the long-term activity of the serotonin system (1). Selective serotonin reuptake inhibitors (SSRIs) are commonly prescribed antidepressants in the treatment of depressive and some anxiety disorders (2). This predominance is due in part to their limited side-effects and high selectiveness to serotonin transporter inhibitor, in comparison to tricyclic antidepressants (3). Fluoxetine was the first of SSRIs and is the most studied antidepressant (4), mostly in men and male animal models. Results obtained in men have been often uncritically generalized to women, therefore exact response to SSRIs in women is still not well known. A growing amount of data shows that differences in pharmacokinetics, pharmacodynamics, and physiology exist between women and men and that they contribute to the occurrence of sex-gender differences in drugs response (reviewed in 5).

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Depressive disorders

Depression is a heterogeneous, multifaceted disorder with symptoms manifested at the psychological, behavioral and physiological levels (6). There are three frequent types of depressive disorders that vary in severity of symptoms and persistence: *major depression* (also called *unipolar depression*) where symptoms interfere with the ability to eat, sleep, work and enjoy life and last chronically for at least 2 weeks; *dysthymia* which is a long-term or chronic disease lasting for at least 2 years and is characterized by less severe, non-disabling symptoms; and *bipolar disorder*, which is characterized by wide mood swings ranging from deep lows to manic highs (1, 7, 8). Both major depression and dysthymia occur in the absence (*primary depression*) or presence (*secondary depression*) of the other psychological or physical problems beside the reduced mood, low self-esteem, feelings of worthlessness, general fatigue, feelings of guilt, disturbances in sleep, sex drive and food intake, anger, absence of pleasure and agitated or retarded motor symptoms (6). Depressive disorders are the fourth leading cause of disease burden worldwide (9, 10). Epidemiological and clinical studies have consistently observed significant sex-specific differences among patients with depression, with women outnumbering men at least 2:1 (11, 12) and this sex difference becomes evident after the onset of puberty (13). While in recent years a number of hormonal systems have been demonstrated to be associated with depression (i.e., appetite-regulatory, thyroid and growth hormones; reviewed in (7), evidence overwhelmingly supports the involvement of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes in the development of mood dysregulation (7, 14). Co-morbidity with HPA-HPG-axis dysregulation is not surprising, as depression is a disorder that involves hypothalamic nuclei (paraventricular and ventromedial), central amygdala, hippocampus, subgenual anterior cingulate cortex, and medial and orbitofrontal cortex, regions that have dense expression of glucocorticoid and sex steroid hormone receptors (reviewed in (1, 14).

Anxiety disorders

Anxiety disorders can be described in terms of the situation, object or thoughts which provoke anxiety, the specific expression of anxiety in terms of autonomic, and cognitive or motoric features, as well as the specific behaviors used to cope with the provoked anxiety (6). Anxiety reactions can vary in intensity, frequency, persistence, trigger situations, severity and consequences and other qualifying features (15). DMS-5® specifies over 12 different anxiety disorders (6), classified in five types: phobias, panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, and generalized anxiety disorder. Anxiety is reported to be the most prevalent disorder among all psychiatric diseases (16). Data from epidemiological studies have consistently shown that anxiety disorders are at least twice as common in women as in men (11, 12). Anxiety is also a common symptom of depression. Many individuals with major depression disorder experience severe anxiety and many individuals with anxiety disorders develop major depression disorder (3), what is not surprising as it is known that neural circuits thought to regulate both conditions do overlap (17). Corticotropin-releasing hormone (CRH), a strong anxiogenic neuropeptide, and its receptors are localized within the serotonergic raphe nuclei suggesting that interactions between the CRH system and serotonin may play a role in fear and anxiety (reviewed in 18).

SSRI antidepressants

In the treatment of depression, different antidepressant such as selective serotonin reuptake inhibitors (SSRIs), tricyclics (TCAs), and monoamine oxidase inhibitors (MAOIs) are used. Today, the most widely prescribed antidepressants with a minimum of side effects are SSRIs (3). SSRI antidepressants are also effective in treating some anxiety disorders (2).

Serotonin (5-HT) is produced by serotonergic cell bodies in the raphe nuclei, which form a cluster of nuclei in the brain stem (3) and send their axons to many brain regions throughout the brain and affect multiple central processes, including emotion, learning and memory, feeding, sleep, sexual and other social behaviors and sensory perception (19). Serotonin at the synapses may undergo several different molecular processes after release into synaptic cleft, one of them is reuptake by a presynaptic serotonin transporter channel (5-HTT or SERT). The targets

of SSRIs are 5-HTTs, which are located at the plasma membrane of serotonergic neurons, and are responsible for 5-HT reuptake (3). SSRIs inhibit the 5-HTT, resulting in increased extracellular 5-HT levels, and thereby sustained activation of pre- and postsynaptic 5-HT receptors (3, 19). However, the therapeutic action of SSRI antidepressants is dependent on long-term administration, suggesting that adaptations to the upregulation of 5-HT are required for therapeutic responses (mood improvement) (1).

The mostly prescribed SSRIs are fluoxetine, sertraline, paroxetine, citalopram and escitalopram (3, 19, 20). Fluoxetine was first synthesized in 1971 (21) and the United States Food and Drug Administration (FDA) approved fluoxetine in 1987. In 1988 it was launched on the market under the trade name Prozac as a first SSRI to be marketed in the United States (reviewed in 22).

Sex differences in treatment of depression and anxiety disorders

Women are clearly different from men in clinical appearance and characteristics of many psychiatric illnesses (12, 23), and also in therapy responses. An increasing number of studies have reported differences in the pharmacokinetics and/ or pharmacodynamics of antidepressants between women and men, although the clinical treatment at present is still identical in both sexes (reviewed in 24). Physiological differences in women and men that may affect pharmacokinetics include average body weight, body composition, and the affinity and/or capacity of metabolizing enzymes for the administered drug. Many studies have shown that sex hormones could influence absorption, distribution, metabolism, pharmacodynamics, and adverse effects of many different drugs (reviewed in 5).

Several studies have identified sex differences in fluoxetine treatment with women of reproductive age responding to fluoxetine better than men (25, 26). Estrogens may boost the effects of SSRIs, as postmenopausal women taking estrogens and treated with fluoxetine responded significantly better than women treated with fluoxetine only (27). Some laboratory studies in rodents also suggest that gonadal hormones modulate mood and the response to SSRIs (e.g., 28, 29) with inducing changes in the serotonin systems (30). Antidepressant effects in female rats are reported to be weaker during phases with lower levels of gonadal hormones (metestrus/diestrus) in comparison to females in higher gonadal hormone phases (proestrus/estrus) or to males (31). Gonadal hormone responsible for these differences seems to be estradiol, as orchidectomized male rats treated simultaneously with 17β-estradiol (10 µg/rat) and fluoxetine had much better behavioral response in comparison to males treated with fluoxetine only (29).

Animal models

The ideal animal model for any human clinical condition must fulfill three criteria (16): [1] pharmacological treatments effective in patients should induce comparable effects in the animal model (predictive validity); [2] the responses/ symptoms in patients should be the same in the animal model (face validity); [3] the underlying rationale should be the same in both humans and animal models (construct validity). Meeting all three validity criteria is difficult for an animal model of depressive/ anxiety disorders. Namely, many of the human symptoms of depression/ anxiety like recurring thoughts of death or suicide or excessive thoughts of guilt are impossible to be modeled in laboratory rodents (6). However, the physiological and behavioral responses to aversive stimuli, similar in both humans and animals, are allowing animal models to be used for at least two distinct purposes: as behavioral tests to screen for potential antidepressant/ anxiolytic properties of drugs and as tools to investigate specific pathogenetic aspects of cardinal symptoms of disease (reviewed in 16).

Behavioral data from our laboratory (32) in C57BL/6J mice of both sexes as a potential animal model to study depression/anxiety in comparison to behavioral data of other studies in mice and rats is presented. C57BL/6J male and female mice were originally obtained from Harlan Italy and bred at the University of Ljubljana, Veterinary Faculty, in standard conditions with 12-12 LD cycle (lights on at 3 am and off at 3 pm) and food (phytoestrogen free diet; Harlan Teklad Diet 2016, Harlan, Milan, Italy) and water *ad libitum*. Mice were weaned at 21 days of age and group-housed (3 mice of same sex per cage) in 15 cm high cages with floor area of 37.5 x 22

cm. At 55 days of age mice were divided into four groups with 9 mice per group: Control males and females, Fluoxetine males and females. Fluoxetine (Sigma-Aldrich®) was delivered in drinking water (10 mg/kg/day) as described elsewhere (33). At approximately 70 days of age (or at least 14 days of fluoxetine treatment) the behavior assessment using "stopwatch" software (Center for Behavioral Neuroscience, Atlanta, GA, USA) began with at least 2 days break between each behavioral test in the following order: elevated plus maze (EPM), open field (OF) and forced swim test (FST). Females were tested in the diestrus phase what was checked before each behavior assessment by vaginal smears as described previously (34). All animal experiments were approved by Veterinary Administration of the Republic of Slovenia and were done according to ethical principles, EU directive 2010/63/EU, and NIH guidelines. Statistical analyses were done using NCSS software (NCSS statistical software, Kaysville, UT, USA). To test differences between groups, repeated measures ANOVA was performed with sex and treatment as independent variables, followed by post hoc Fisher LSD test. Differences were considered statistically significant with $p < 0.05$ (32).

Depression-related behavioral assessments

Forced swim stress is one example of acute stressors that was developed as a tool to test the efficacy of antidepressant compounds (35) and is probably the most used tool among all animal models for screening antidepressants in mice and rats (36, 37). The critical response measured is immobility in an inescapable situation, which is believed to measure despair-like behavior (38).

Forced swim test (FST)

The first forced swim test (FST), also termed as behavioral despair test, was developed by Porsolt and coworkers in the rat (35) and subsequently in the mouse (39). In this animal model of depression animals are forced to swim in a tall cylinder and the time spent swimming or climbing (active behavior) versus the time spent floating (passive behavior) is measured. Session durations between 4 and 20 minutes have been used in mice, with 2 to 5 minutes of pre-exposure period (36, 40). If the animals cease all movements (active swimming motions), except those necessary for survival (keeping the head above the water), the behavior is considered to be immobile (floating). This immobile behavior is considered as an index of despair in response to the stressor or as an index of coping with the stressful procedure (41) and is diminished by antidepressant administration (38).

In our lab the FST was performed as described elsewhere (33), with 5 minutes session duration and 2 minutes of pre-exposure period (32).

Sex differences in FST

Studies of sex differences in the FST in rats and mice have shown highly controversial results likely due to several causes such as strain, different behaviors analyzed, exposure to various conditions prior to testing, estrous cycle phase and others (42). Some studies have shown that female Wistar rats in estrus phase are showing lower immobility and higher active behaviors in comparison to males (28, 43) what could be the result of high estrogens levels in females. However, some other studies that did not control for the phase of the estrus cycle showed higher levels of despair (longer immobility periods) during the FST in female rats (Wistar, Sprague-Dawley) in comparison to males (44, 45, 46). The second important difference between these contradictory results is that in the latter studies rats were exposed to at least two other stressors/ behavior tests (open field, light and dark transitions) prior to the exposure to the FST, suggesting that expositions to other stressors might increases the vulnerability of female rats to develop depressive-like behaviors (45).

In another study, chronic fluoxetine treatment (10 mg/kg) reduced immobility and increased active behaviors in male rats (Sabra strain derived from Wistar) only, and had no effects in females (estrous cycle phase was not reported; 47). However, some newer studies have shown that acute or chronic fluoxetine treatment (10 or 20 mg/kg) produced an antidepressant-like effect (reduced immobility) in both male and female rats (Wistar; females tested in estrus phase; 28, 48) and in females this effect was observed at lower doses (5 mg/kg) in comparison to males (10 mg/kg) (28), suggesting that estrus females are more sensitive to the antidepressant-like effects of fluoxetine.

In our laboratory, similar studies were performed with socially housed adult C57BL/6J mice, chronically treated with fluoxetine in drinking water (10 mg/kg for at least 14 days). All females were tested in the diestrus phase what was checked by vaginal smears, taken before each behavior assessment. Although we did observe fluoxetine effect in both male and female mice, no sex difference in immobility/ swimming time in the FST was observed (Figure 1), even after exposure to three other behavioral tests (elevated plus maze, open field, social recognition test) prior to FST (32), suggesting that female rats might be more vulnerable to the acute stress caused by FST than female mice.

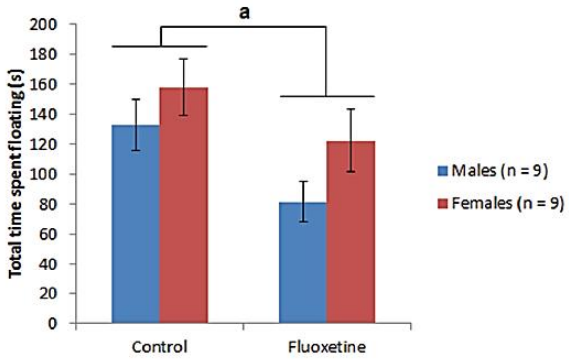

Figure 1: FST in male and female mice did not reveal any significant sex difference in response to fluoxetine or in behavior in FST, although fluoxetine treatment significantly reduced immobile time in both sexes. Data are reported as mean ± SEM; [a] Significant effect of treatment, $p < 0.05$

Anxiety-related behavioral assessments

Anxiety in rodents is defined as a high level of avoidance of novel and unfamiliar environment and increased fear reaction (16). Probably the most widely used test to assess the anxiety is the elevated plus maze (EPM), and less often the open field test (OF). OF test is mostly used to check whether changes in immobility observed in FST are associated with alterations in the motor activity (e.g., 48).

Elevated plus maze (EPM)

Probably the most frequently used test for unconditioned anxiety assessment, widely used in pharmaceutical companies, is the elevated plus maze (EPM), which was first introduced by File and coworkers in rats (49) and later in mice (50). The plus maze, elevated above the ground, consists of four arms arranged in a cross formation: two opposing non-anxiogenic closed arms with walls and other two anxiogenic open arms without walls (40). Rodents tend to avoid elevated, brightly lit areas, and avoidance of the open arms is interpreted as anxiety like behavior (49, 50). The animal is placed in the junction of the open and closed arms, and entries into the each arm and time spent in each arm over a 5-minute test session is scored (40).

In our lab the EPM was performed as described elsewhere (50), with 5 minutes session duration (32).

Sex differences in EPM

Previous reports in male mice are inconsistent, with some studies reporting higher levels of anxiety in C57BL/6J compared to BALB/c mice (51), other reported opposite results (52, 53). A newer study in both sexes showed that C57BL/6J female mice are more anxious, spending less time in open arms, than males, but no sex difference was observed in BALB/c mice when females were tested in the diestrus phase (53). This is in agreement with our study (32) showing that females of C57BL/6J strain are more anxious than males (Figure 2), suggesting that C57BL/6J strain could be a good animal model for studying sex differences in anxiety disorders. In contrast, female rats tested in proestrus phase appear to be less anxious than male rats (54, 55).

Many previous studies of fluoxetine effects were performed only in males and are showing controversial results in behavior responses. Some studies in male rats (mostly used Wistar strain; ~10 mg/kg) of acute fluoxetine administration have shown an anxiolytic (56, 57), anxiogenic (58, 59, 60), or no effect (49, 61). In several studies (55, 57, 59) the chronic treatment (5, 10 or 20 mg/kg) did not affect behavior in EPM of male rats (Wistar-Kyoto, Sprangue-Dawley or Wistar). Interestingly, one study reported that chronic fluoxetine treatment (5 mg/kg) decreased the time spent in the open arms (anxiogenic effect) in female rats during proestrous phase (Sprangue-Dawley), and the stress exposure even potentiated

this effect (55). Our study with C57BL/6J male and female mice showed no treatment difference, neither in the number of entries nor in the total time spent in the open arms (32), what is in agreement with the study by Kobayashi and coworkers in males (62), suggesting that chronic fluoxetine has neither anxiolytic nor anxiogenic effects in EPM in either sex in C57BL/6J mice (Figure 2) and that fluoxetine treatment does not contribute to the major improvement of anxiety behavior like in humans (63).

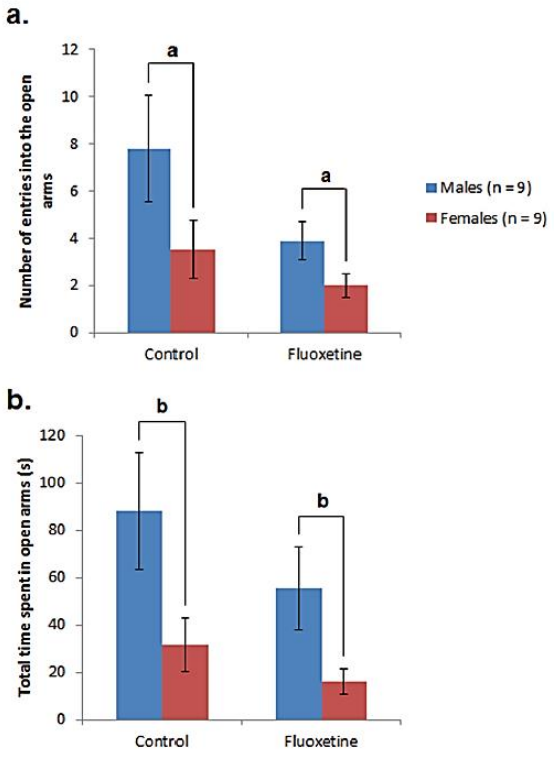

Figure 2: Sex differences are present in C57BL/6J mice (females were tested in the diestrus phase): **(a.)** number of entries into the open arms, **(b.)** total time spent in open arms. No significant effect of fluoxetine treatment in either sex was found ($^{a}p < 0.05$, $^{b}p < 0.01$). Data are reported as mean ± SEM; ^{a,b} Significant difference between males and females

Open field (OF)

In 1934 Calvin Hall designed the first open field test to assess "emotionality" in rats (64) and since then different types of open fields have been used. The modern standard open field is a Plexiglas box with square floor area, surrounded by high walls to prevent animals from escaping, and usually equipped with either photocells or videotracking and computer software to assess locomotor parameters. The animal is placed in the center or in the periphery of the area and the behavior assessment can last from 2 min to several hours. Like in EPM the avoidance of exploratory behavior towards the anxiogenic unprotected area (center zone) is the indicator for anxiety or fear-related behavior (16, 40). OF is mostly used for assessing spontaneous motor activity (distance traveled, average speed, duration of (im)mobility and others), which is the most standardized general measure of locomotor function (40), or to exclude the increased immobility in FST due to reduced locomotor ability (48).

In our lab the OF was performed as described elsewhere (62), with 30 minutes session duration (32).

Sex differences in OF

Previous studies in C57BL/6J and BALB/cJ mice have shown that males and females in diestrus phase did not differ in their locomotor or exploratory activity having similar duration of locomotion and spent similar time in the center area of OF (53), what is in agreement with our study (unpublished results) performed in C57BL/6J strain (Figure 3 and 4b).

There are numerous studies of fluoxetine effects on OF activity in mice but far less in rats. Neither acute (2 and 10 mg/kg) nor chronic (10 and 20 mg/kg) treatment in male rats (Wistar) have shown any effect on locomotor and exploratory activity of center area in comparison to controls (48, 61), and study in both sexes by Ghorpade et al. does not mention any sex differences between treated or control rats (48).

In regard to spontaneous motor activity, previous studies in male mice after chronic fluoxetine treatment (mostly 10 mg/kg) have shown differences between strains, with C57BL/6J mice having reduced, and BALB/cJ mice unchanged distance traveled in comparison to untreated males (62, 33). Indeed, C57BL/6J treated males in our study (unpublished results) also traveled shorter distance (Figure 3a), moved slower (Figure 3b) and had longer immobile periods (Figure 3c) in comparison to controls, and there was no sex difference observed (Figure 3).

Our results in females (unpublished results) are in agreement with the study of Marlatt et al. where chronically treated (18 mg/kg) C57BL/6J females also traveled shorter distance than control mice (65). Similar decrease in traveled distance with no sex difference was reported also after acute fluoxetine administration (15 mg/kg) (66).

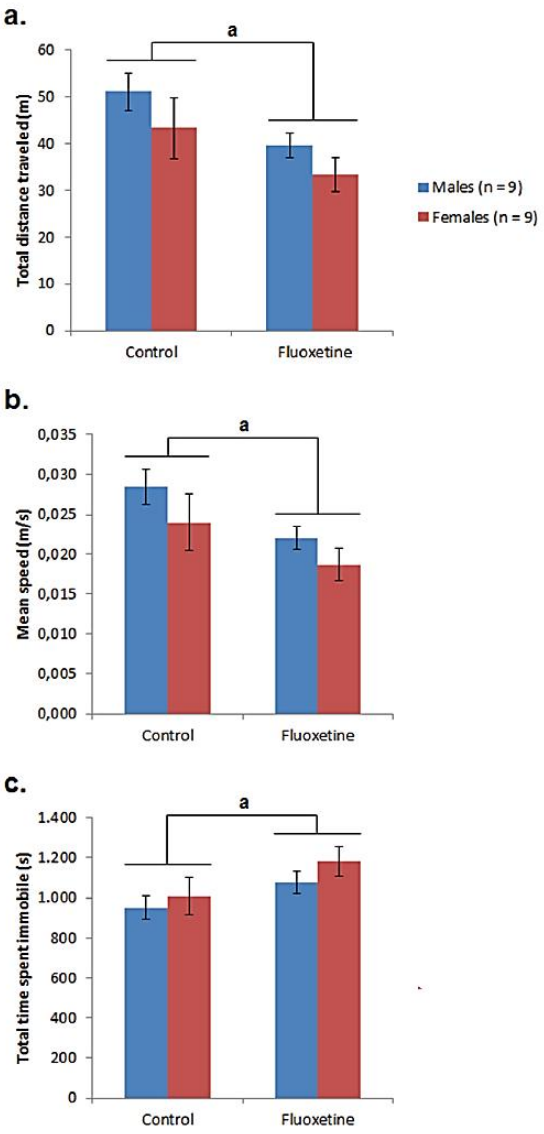

Figure 3: Spontaneous motor activity in OF did not differ between male and female C57BL/6J mice in response to fluoxetine or in behavior in OF, although fluoxetine treatment significantly affected locomotor activity in both sexes: **(a.)** reduced distance traveled, **(b.)** reduced average speed and **(c.)** prolonged time of immobility. Data are reported as mean ± SEM; [a] Significant effect of treatment, $p < 0.05$

In regard to the anxiety like behavior, chronic fluoxetine exposure in previous studies reduced the number of entries or time spent in the center of the OF in C57BL/6J, but not in BALB/cJ males relative to controls (62, 33), and such reduction in time spent in the center zone was revealed also in C57BL/6J females in comparison to controls (65), but there are no previous reports about sex differences in such effects of fluoxetine. However, in contrast to these studies, in our study (unpublished results) there was no effect of fluoxetine treatment on these two parameters in C57BL/6J males and females (Figure 4a and b), although there was a small, but significant sex difference in the number of entries into the central zone that was reduced in females but not in males (Figure 4a).

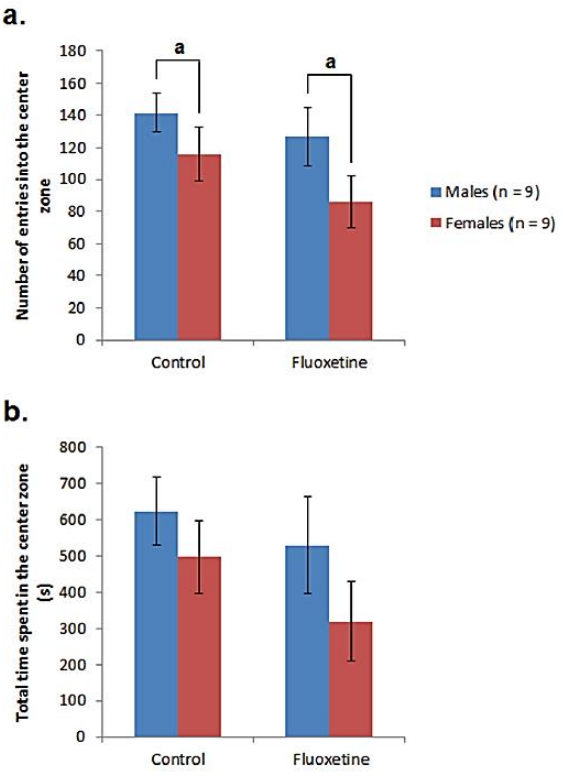

Figure 4: Sex differences in C57BL/6J mice (females were tested in the diestrus phase) were observed in the number of entries into the center zone of OF **(a.)**, but not in the total time spent in the anxiogenic area **(b.)**. No significant effect of fluoxetine treatment in either sex was found. Data are reported as mean ± SEM; [a] Significant difference between males and females, $p < 0.05$

Conclusions

Studies of sex differences in the FST, EPM and OF behavioral tests in mice and rats have yielded controversial results, most likely caused by several factors which are known to influence animal behavior such as species, strain, age, body weight, handling, social isolation or enriched environment, food, various kinds of stress, endocrine manipulations and surgery, schedule and routes of treatment, dosage of the drugs as well as experimental design and others. Consideration of these factors in planning experiments could result in more consistent results. However some common conclusions connected the main findings in the different rodent studies of FST, EPM and OF can be made:

- Proestrus/estrus females are usually less despaired and anxious than males or females in metestrus/diestrus.
- No consistent sex difference in the locomotor or exploratory activity in mice and rats are found.
- Chronic fluoxetine treatment provided more consistent effects than acute treatment.
- Proestrus/estrus females are usually more sensitive to the antidepressant like effects of fluoxetine than males.
- No effect of anxiogenic/anxiolytic treatment is usually found in males and metestrus/diestrus females, but anxiogenic effects of fluoxetine have been described in estrus/proestrus females.
- No sex/treatment difference in locomotor or exploratory activity in rats, but reduced locomotion in treated mice regardless of sex, was found.

Our data (32) with chronic fluoxetine administration in C57BL/6J mice of both sexes revealed that fluoxetine has an antidepressant like effect in FST with decreased immobility time but no effect on latency to float. Males and females did not significantly differ in their despair like performance. In regard to the anxiety like behavior, a chronic fluoxetine treatment had no anxiolytic or anxiogenic effect in the EPM or in the OF, but females behaved more anxiously than males in EPM and OF tests. However, fluoxetine did impair locomotor activity in comparison to control mice of both sexes as tested in OF. Taking together, C57BL/6J mice could be a good animal model for anxiety assessment studies as females were significantly more anxious than males, but not for despair behavior studies as females were equally depressed than males. Chronic fluoxetine treatment might not be a good model to study its effects in anxiety related disorders studies in C57BL/6J mice.

Acknowledgements

We would like to thank Ana Strgar and Ariadna Štorman for performing EPM and OF behavioral assessments.

Ethical statement

Animal experiments from our lab were approved by Veterinary Administration of the Republic of Slovenia and were done according to ethical principles, EU directive, and NIH guidelines.

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4 RAZPRAVA

Puberteta oziroma mladostniģtvo je obdobje, ki zajema ne samo spolno dozorevanje osebka, ampak tudi kognitivno, ļustveno in socialno dozorevanje obnaģanja (1, 12). Izpostavljenost razliļnim stresnim dejavnikom v mladosti lahko vodi do dolgotrajnih sprememb v obnaģanju v odraslem obdobju, kar lahko sovpada s spremembami v preoblikovanju in reorganizaciji moģganov s strani spolnih (1) in/ ali stresnih hormonov (45).

Miģi in podgane so socialne ģivali, ki v naravnem okolju ģivijo v veļjih socialnih skupinah z urejeno druģbeno lestvico (65, 231), zato lahko socialna osamitev predstavlja moļan stresni dejavnik. Za socialne ģivali je znaļilna visoka stopnja socialnih stikov in medsebojnih odnosov, kar jim omogoļa razvoj socialnih veģin, ki so nujno potrebne za vzpostavitev in vzdrģevanje socialne skupine in ne nazadnje za obstoj same ģivalske vrste. Ena od osnovnih veģin socialnega obnaģanja je socialno prepoznavanje, ki predstavlja osnovo za socialno ģivljenje (opisano v 106). Sistema OT in AVP sta eden od kljuļnih sistemov, ki uravnavata razliļna socialna obnaģanja, vkljuļno s socialnim prepoznavanjem (pregledno v 101, 104, 232), medtem ko je sistem OT pomemben predvsem pri uravnavanju z razmnoģevanjem povezanih obnaģanj (150, 151, 152, 182, 183).

V okviru doktorskega dela smo dokazali, da trajna socialna osamitev med puberteto in po njej popolnoma onesposobi odrasle miģi obeh spolov socialnega uļenja ter socialnega prepoznavanja, kar se sklada z opaģanji drugih avtorjev pri samcih miģi (76) in podgan (88, 89) ter pri podganjih samicah (89). Tanaka in sod. so ugotovili, da socialna osamitev med puberteto pri podganah zmanjģa ģtevilo OT-imunoreaktivnih nevronov v paraventrikularnem jedru pri samicah in AVP-imunoreaktivnih nevronov pri samcih (89), kar sovpada s splošno znanim dognanjem, da ima OT veļji pomen pri uravnavanju obnaģanja pri samicah (105), AVP pa pri samcih (27). Nasprotno je pri nadaljnjih raziskavah v naģem laboratoriju bilo ugotovljeno (neobjavljeni podatki), da socialna osamitev pri miģih obeh spolov nima vpliva niti na OT (koncentracija v krvni plazmi, ģtevilo OT-imunoreaktivnih nevronov v paraventrikularnem jedru) niti na sistem AVP (AVP-imunoreaktivnost v ģivļnih vlaknih v stranskem septumu, izraģanje gena za V1aR v hipotalamusu ter epigenetske spremembe v promotorskem območju tega gena), kar je v skladu z rezultati podobnih raziskav pri prerijskih voluharicah (95, 99). Vrednotenje sproģanja OT in AVP iz paraventrikularnega jedra bi morda dalo bolj jasno sliko vpliva socialnega stresa med puberteto (76). Pri naģi raziskavi

smo pokazali, da so socialno nastanjeni miģji samci sposobni boljģega socialnega uļenja in prepoznavanja v primerjavi s socialnimi samicami, kar se sklada s predhodnimi ugotovitvami pri podganah (233, 234). Pri miģjih samcih smo potrdili ģe znano dejstvo, da imajo samci veļjo izraģenost AVP v ģivļnih vlaknih v stranskem septumu v primerjavi s samicami (117), s ļimer bi lahko razloģili ugotovljeno spolno razliko pri socialnem prepoznavanju. Nadalje smo preverili moģnost povrnitve sposobnosti socialnega prepoznavanja pri osamljenih miģih obeh spolov s ponovno socialno nastanitvijo v odraslem obdobju. Dokazali smo, da je sposobnost socialnega uļenja pri zaļasno osamljenih samicah med puberteto primerljiva, pri samcih pa delno zmanjģana, glede na kontrolne socialno nastanjene miģi, medtem ko se je sposobnost socialnega prepoznavanja povrnila le pri samcih. Slednje bi lahko razloģili s predhodnimi dognanji pri podganah, kjer so socialno osamitev ocenili kot bolj stresno za samice (viģje vrednosti kortikosterona) v primerjavi s samci (69, 80).

Obstoj socialne zdruģbe kot tudi obstoj ģivalske in ne nazadnje tudi ļoveģke vrste je v veliki meri odvisen od uspeģnosti razmnoģevanja njenih ļlanov. Ker je vpliv socialne osamitve in drugih stresnih dejavnikov med puberteto na spolno obnaģanje v veliki meri ģe raziskan pri samcih laboratorijskih glodavcev (pregledno v 70), ki v glavnem privede do poslabģanja moģkega spolnega obnaģanja (91, 92, 93, 94), smo se v doktorski disertaciji osredotoļili predvsem na spolno obnaģanje samic.

Pri naģi raziskavi smo tako preverili in dokazali, da socialna osamitev med puberteto in po njej poslabģa sposobnost izraģanja ģenskega spolnega obnaģanja pri miģjih samicah, kar je v nasprotju s starejģo raziskavo pri podganjih samicah, pri katerih osamitveni stresor ni imel vpliva na sposobnost izraģanja spolnega obnaģanja (91). Çe predhodne raziskave vpliva razliļnih kratkotrajnih stresorjev med puberteto (prevoz ali enkratno injiciranje endotoksina lipopolisaharida) pri miģjih samicah pa so pokazale moļno prizadetost izraģanja spolne sprejemljivosti v odraslosti (145, 146), iz ļesar bi lahko sklepali, da so miģji samice bolj obļutljive na socialno osamitev kot podgane. V nasprotju s spolno sprejemljivostjo spolna privlaļnost samic s socialno osamitvijo ni bila spremenjena. Podobno kot pri socialnem prepoznavanju ponovna socialna nastanitev v odraslem obdobju ni povrnila spolne sprejemljivosti osamljenih samic do ravni obnaģanja, ocenjenega pri socialno nastanjenih miģjih samicah. Ugotovili smo, da socialna osamitev med puberteto sovpada s poveļanjem ģtevila nevronov v ventromedialnem jedru hipotalamusa in anteroventralnem delu

periventrikularnega jedra, ki izraģajo ERŭ. To se moļno razhaja z rezultati predhodnih raziskav pri miģjih samicah (enkratni stresor prevoza in injiciranja lipopolisaharida) (144) in pri samicah prerijskih voluharic (stresor osamitve) (147), pri ļemer poroļajo o zmanjģanem ali nespremenjenem izraģanju ERŭ v omenjenih podroļjih moģganov. Do razlik med raziskavami bi lahko priģlo zaradi uporabe razliļnih vrst stresorjev, trajanja stresa, ģivalske vrste, linije miģi in mnogih drugih dejavnikov (opisano v 45). Kratkotrajno delovanje stresorja pri raziskavi Ismaila in sod. bi lahko hitro zavrlo os HPG, nakar bi sledilo poveļanje vrednosti estradiola in posledilno zmanjģano izraģanje ERŭ (144). Medtem ko bi lahko dolgotrajno delovanje stresorja pri naģi raziskavi zaviralo delovanje osi HPG ter drģalo vrednosti estradiola dlje ļasa na niģjem nivoju (pregledno v 235), bi kot protiuteģ lahko sledilo poveļano izraģanje ERŭ.

Spolni hormoni v odraslosti so nujno potrebni za spodbujanje izraģanja ģenskega spolnega obnaģanja pri samicah, saj poveļane vrednosti estradiola ob prisotnosti ali neprisotnosti progesterona izzovejo refleks lordoze (opisano v 148). Ġtevilne raziskave kaģejo na pomembnost spolnih hormonov med puberteto za razvoj ģtevilnih obnaģanj v odraslem obdobju, med drugim tudi spolnega obnaģanja (pregledno v 1, 19). Raziskave pri samcih hrļka in podgan dokazujejo, da je prisotnost mod in s tem tudi testosterona med puberteto nujno potrebna za normalno izraģanje moģkega spolnega obnaģanja odraslih samcev (opisano v 19, 21, 23). Pri naģi raziskavi smo ugotovili, da odstranitev jajļnikov pred puberteto, ne glede na vrsto nastanitve, ne vpliva na izraģanje spolne sprejemljivosti in spolne privlaļnosti miģi v odraslem obdobju v primerjavi z odstranitvijo jajļnikov po puberteti, kar se sklada s predhodnimi ugotovitvami pri samicah podgan (26). Iz tega lahko sklepamo, da obdobje pubertete nima bistvenega pomena pri dozorevanju sposobnosti izraģanja spolnega obnaģanja samic podgan in miģi, kot jo ima pri njihovih samcih. Raziskava Söderstena in sod. pri podganjih samicah izpostavlja obdobje prehoda v puberteto med 15. in 20. dnevom starosti kot kljuļno pri dozorevanju odziva na estradiol in sposobnost izraģanja spolne sprejemljivosti (236).

Z evolucijskega vidika je edino zagotovilo za uspeģno razmnoģevanje uspeģno potomstvo, ki je sposobno preģiveti in ustvarjati novo potomstvo. Da bi se osamosvojili, dosegli spolno zrelost in se razmnoģevali, potrebujejo mladiļi mnogih ģivalskih vrst nego enega ali obeh starģev (9, 164). V sklopu doktorske naloge smo preverili vpliv osamitvenega stresa ģe pri

starģevskem obnaģanju in ugotovili, da socialna osamitev med puberteto ne vpliva na sposobnost izraģanja materinskega in oļetovskega obnaģanja miģi, kar se sklada s predhodnimi opaģanji pri samcih miģi (180) ter pri samicah podgan (90). Pri podganah in miģih se obnaģanje in naklonjenost do mladiļev z nastopom pubertete moļno spremenita in pokaģejo se razlike med spoloma (pregledno v 165). Dosedanje raziskave starģevskega obnaģanja pri miģih in podganah obravnavajo vpliv spolnih hormonov predvsem v odraslem obdobju (npr. 121, 166, 175, 176, 177, 237, 238), v okviru doktorske disertacije pa smo preverili pomen spolnih hormonov med puberteto na razvoj sposobnosti izraģanja starģevskega obnaģanja pri odraslih miģih obeh spolov. Ugotovili smo, da miģji samci in samice brez spolnih ģlez med puberteto slabģe izraģajo k mladiļem usmerjeno starģevsko obnaģanje v primerjavi z miģmi, ki smo jim odstranili spolne ģleze v odraslem obdobju, saj potrebujejo dlje ļasa za dvig in prenos prvega mladiļa v gnezdo, prenesejo manj mladiļev in jih tudi manj ļasa negujejo pod telesom, kar se v veliki meri sklada s podobnimi ugotovitvami pri samicah podgan (173). Dodajanje oziroma nadomeģanje estradiola med puberteto pri samicah z odstranjenimi jajļniki, ne pa tudi testosterona pri samcih z odstranjenimi modi, je pri samicah uspelo povrniti veļino k mladiļem usmerjenih aktivnosti do ravni, primerljive obnaģanju miģi s prisotnimi spolnimi ģlezami med puberteto. Iz tega lahko sklepamo, da je prisotnost spolnih hormonov med puberteto, ģe posebej estradiola, pomembna za razvoj sposobnosti izraģanja starģevskega obnaģanja v odraslem obdobju pri miģih. Predhodne raziskave starģevskega obnaģanja pri laboratorijskih glodavcih poroļajo o moļnejšem in doslednejšem izraģanju starģevskega obnaģanja pri samicah v primerjavi s samci (pregledno v 165). Tudi pri naši raziskavi smo našli razlike med spoloma, vendar le pri nekaterih aktivnostih, usmerjenih k mladiļem, ko so samice hitreje dvignile in prenesle prvega mladiļa v gnezdo v primerjavi s samci, kar je skladno s predhodnimi ugotovitvami pri miģih (121). Nadalje smo preverili moģnost izboljšanja sposobnosti izraģanja starģevskega obnaģanja z dodajanjem estradiola v odraslem obdobju, saj številne raziskave ugotavljajo, da estradiol spodbuja materinsko obnaģanje tako pri odraslih miģjih (163, 181, 237) kot tudi podganjih samicah (163, 168, 239, 240). Ugotovili smo, da prejemanje estradiola v odraslem obdobju pri miģjih samcih in samicah (ne glede na ļas odstranitve spolnih ģlez) ne vpliva na aktivnost prenaģanja mladiļev v gnezdo, kar se ujema z nedavno raziskavo pri samicah miģi (177). Po drugi strani pa je estradiol v odraslosti izboljšal aktivnosti, ki se nanaģajo na gradnjo gnezda (aktivnost, ki ni usmerjena k mladiļem). Iz tega lahko sklepamo, da estradiol v odraslem

obdobju v glavnem izboljģa starģevsko obnaģanje, ki ni usmerjeno k mladiļem, medtem ko za zaļetek in vzdrģevanje izraģanja obnaģanja, usmerjenega k mladiļem, ni nujno potreben. Ugotovitve, da estradiol ugodno vpliva na aktivnosti v zvezi z gradnjo gnezda, bi lahko povezali z vzdrģevanjem telesne temperature, saj estrogeni hormoni zniģujejo telesno temperaturo na telesnih ģrlinah pri miģih (241) in podganah (242), zaradi ļesar bi morda miģi pod vplivom estradiola bolj intenzivno gradile gnezda, v katerih bi laģje ohranjale svojo telesno temperaturo.

Puberteta je torej razvojno ranljivo obdobje, ki je med drugim znano tudi po veļji obolevnosti in dovzetnosti za ģtevilne psihološke motnje, vkljuļno z depresijo in anksioznostjo, ki sta pod vplivom delovanja spolnih hormonov (predvsem estradiola in/ ali progesterona) kot tudi razliļnih stresnih dejavnikov v tem obdobju (pregledno v 46, 56, 197). Epidemiološke in kliniļne ģtudije ugotavljajo razlike med spoloma vse od zaļetka pubertete (194), ko je obolevnost za depresijo in/ ali anksioznostjo pri ģenskah najmanj dvakrat pogostejģa kot pri moģkih (195, 196). Kljub znanim razlikam med spoloma v farmakokinetiki in farmakodinamiki antidepresivnih zdravil (pregledno v 205, 206), tudi SSRI antidepresiva fluoksetina (207, 208, 209), zdravljenje pri ģenskah in moģkih ostaja enako.

Ker glavnina raziskav delovanja antidepresivov pri ģivalskih modelih (podgane, miģi) obravnava predvsem samce (npr. 214, 226, 227, 243), smo v zadnjem sklopu doktorske naloge preverili delovanje dolgotrajnega prejemanja fluoksetina na depresivnemu in anksioznemu podobno obnaģanje tako pri samcih kot pri samicah miģi linije C57BL/6J, ki je v zadnjih letih najpogosteje uporabljena linija miģi pri raziskavah (244). Fluoksetin je v naģi raziskavi zmanjģal izraģanje depresivnemu podobnega obnaģanja tako pri samcih kot pri samicah, brez razlik med spoloma, kar se le delno sklada s podatki iz literature za podgane. Lifschytz in sod. so poroļali o antidepresivnem uļinku fluoksetina le pri samcih (245), vendar pa novejģe raziskave pri podganah kaģejo podoben antidepresivni uļinek fluoksetina pri obeh spolih (220, 222). Vzrok za razhajanja rezultatov bi lahko bil v hormonalnem nihanju pri samicah. Medtem ko Lifschytz in sod. (245) niso preverjali faze spolnega ciklusa, sta novejģi raziskavi (220, 222) testirali samice le v fazi estrusa, kjer bi lahko ģe poveļane vrednosti estradiola izboljģale razpoloģenje pri samicah (221, 222). Ker smo v naģi raziskavi samice miģi testirali v fazi diestrusa, lahko sklepamo, da je k zmanjģanemu izraģanju depresivnega obnaģanja prispeval fluoksetin. Miģji samci in samice v estrusu bi morda lahko bili dober

ģivalski model za prouļevanje depresije, saj se tudi ģenske v plodnih letih bolje odzivajo na fluoksetin v primerjavi z moģkimi (207) ali z ģenskami v menopavzi (209).

Nadalje smo ugotovili, da fluoksetin nima vpliva na izraģanje obnaģanja, podobnega anksioznemu niti pri samcih niti pri samicah, kar je v skladu s predhodno raziskavo pri testu EPM pri samcih miģi (226) in podgan (225, 246, 247), vendar v nasprotju z raziskavami pri testu OF pri miģih obeh spolov (226, 227, 229) ter pri samicah podgan (225), kjer je fluoksetin deloval anksiogeno. Vzrok za razhajanja rezultatov pri miģih bi lahko bil v zaporedju izvajanja testiranj. Medtem ko je test OF pri nekaterih raziskavah (226, 227, 229) bil uporabljen kot prvi, je v naģi raziskavi sledil testu EPM. Pokazali smo, da so miģje samice v diestrusu (ne glede na prejemanje fluoksetina) bolj anksiozne kot samci, kar je potrdila ģe predhodna raziskava pri isti liniji miģi (223). Miģji samci in samice v diestrusu bi lahko bili dober ģivalski model za prouļevanje anksioznosti, vendar pa fluoksetin pri njih ne prispeva k izboljģanju razpoloģenja, kot se dogaja pri ljudeh, kjer se ģenske slabģe odzivajo na fluoksetin kot moģki (248).

Pri naģih raziskavah smo torej ugotovili, da imajo socialna osamitev in spolni hormoni med puberteto lahko moļan in trajen uļinek tako na razvoj moģganov kot na sposobnost izraģanja nekaterih spektrov obnaģanj v odraslem obdobju. Ugotovitve naģih raziskav prispevajo k boljģemu razumevanju neobhodnega pomena odraģļanja v socialni zdruģbi in spolnih hormonov med razvojno obļutljivim obdobjem pubertete, ki narekujeta tako fiziļno kot psihiļno dozorevanje osebka. Motnje pri izloļanju in delovanju spolnih hormonov kot tudi razliļni stresni dejavniki v ļasu pubertete lahko trajno in nepovrnljivo privedejo do nevrokemiļnih in nevrofizioloģkih sprememb v moģganih in posledиļno spremenijo obnaģanje v odraslem obdobju, kar lahko ogroģa obstanek socialne zdruģbe kot tudi obstoj same vrste. Spoznanja naģih raziskav predstavljajo smernice za bolj premiģljeno naļrtovanje ter tolmaļenje rezultatov znanstvenih poskusov pri ģivalskih modelih, naģe ugotovitve pa predstavljajo smernice za nadaljnje raziskave pri ljudeh.

5 ZAKLJUČKI

1. Socialna osamitev med puberteto zmanjģa sposobnost socialnega uļenja in nepovrnljivo okrni sposobnost socialnega prepoznavanja predvsem pri miģjih samicah. K boljģemu socialnemu prepoznavanju pri samcih bi lahko prispevala veļja izraģenost AVP v stranskem septumu. Zato **hipotezo 1**, da socialna osamitev v mladosti vpliva na socialno prepoznavanje odraslih miģi, lahko potrdimo.
2. Socialna osamitev med puberteto nepovrnljivo okrni sposobnost izraģanja spolne sprejemljivosti in trajno spremeni izraģanje ERŭ v anteroventralnem delu periventrikularnega jedra in v ventromedialnem jedru hipotalamusa, vendar pa spolni hormoni med puberteto nimajo bistvenega pomena pri razvoju sposobnosti spolnega obnaģanja pri samicah. Zato del **hipoteze 2**, da zgodnja odstranitev spolnih ģlez in socialna osamitev vplivata na ģensko spolno obnaģanje odraslih miģi, lahko delno potrdimo.
3. Odsotnost spolnih hormonov med puberteto, ģe posebej estradiola, nepovrnljivo okrni sposobnost izraģanja starģevskega obnaģanja, usmerjenega k mladiļem, vendar pa osamitveni stres med puberteto nima bistvenega vpliva na sposobnost izraģanja starģevskega obnaģanja pri samcih in samicah. Zato del **hipoteze 2**, da zgodnja odstranitev spolnih ģlez in socialna osamitev vplivata na starģevsko obnaģanje odraslih miģi, lahko delno potrdimo.
4. Dokazali smo, da dolgotrajno prejemanje antidepresiva fluoksetina pri miģih zmanjģa izraģanje depresivnemu podobnega obnaģanja, vendar ne vpliva na izraģanje anksioznemu podobnega obnaģanja. Uļinki fluoksetina pri samicah so primerljivi z uļinki pri samcih. Zato **hipotezo 3**, da antidepresiv fluoksetin zmanjģa depresivnemu in anksioznemu vedenju podobno obnaģanje, razliļno pri samicah in samcih, lahko zavrnemo.

6 POVZETEK

Puberteta/ mladostniģtvo je obdobje, med katerim posameznik spolno dozoreva in postane sposoben razmnoģevati se, dozoreva pa tudi vedenjsko, kar je posledica poveļanega izloļanja in delovanja steroidnih spolnih hormonov. Ti med puberteto dokonļno preoblikujejo delovanje kortikalnega in limbiļnega dela moģganov, kar vpliva na razvoj sposobnosti izraģanja razliļnih spektrov obnaģanj v odraslem obdobju. Razliļni stresni dejavniki lahko v tem razvojno obļutljivem obdobju trajno prizadenejo dozorevanje moģganov, njihovo nevroendokrino odzivnost in vodijo do dolgotrajnih sprememb pri socialnem obnaģanju ter pri z razmnoģevanjem in s ļustvovanjem povezanih obnaģanjih.

Namen naģih raziskav je bil ugotoviti pomen socialne osamitve in spolnih hormonov med puberteto ter delovanje psihoaktivnih zdravil, kot je fluoksetin, na obnaģanje odraslih miģjih samcev in samic. V prvem sklopu naģega dela smo preverili, ali socialna osamitev med puberteto vpliva na sposobnost socialnega prepoznavanja in ali privede do sprememb v sistemih OT in AVP v konļnih in vmesnih moģganih. V drugem sklopu dela smo ugotavljali pomen socialne osamitve in vpliva odsotnosti spolnih hormonov med puberteto na sposobnost izraģanja starģevskega obnaģanja pri obeh spolih ter spolnega obnaģanja pri samicah. Zanimalo nas je tudi, ali omenjena dejavnika privedeta do sprememb v izraģenosti ERŭ v konļnih in vmesnih moģganih. V zadnjem sklopu doktorske naloge smo preverili, ali dolgotrajno prejemanje fluoksetina zmanjģa obnaģanje, podobno depresivnemu in anksioznemu, razliļno pri samcih in samicah.

Dokazali smo, da socialna osamitev med puberteto zmanjģa sposobnost socialnega uļenja in nepovrnljivo prizadene sposobnost socialnega prepoznavanja predvsem pri samicah. Socialna osamitev nima vpliva niti na sistem OT niti na sistem AVP, v nadaljnjih raziskavah pa bi bilo potrebno preveriti dinamiko sproģļanja OT in AVP iz paraventrikularnega jedra, kar lahko vpliva na delovanje moģganov in na obnaģanje. Socialno nastanjeni samci so bolje izraģali sposobnost socialnega uļenja in socialnega prepoznavanja v primerjavi s socialno nastanjenimi samicami, kar bi lahko razloģili z ugotovitvijo, da imajo samci veļjo izraģenost AVP v ģivļnih vlaknih v stranskem septumu kot pa samice.

Socialna osamitev med puberteto ni vplivala na sposobnost izraģanja materinskega in oļetovskega obnaģanja pri miģih. Ugotovili pa smo, da osamitveni stres med puberteto

nepovrnljivo poslabġa izraġanje spolne sprejemljivosti pri samicah, vendar ne vpliva na njihovo spolno privlaļnost, trajno pa spremeni izraġenost ERŭ v anteroventralnem delu periventrikularnega jedra in ventromedialnem jedru hipotalamusa. Ugotovljeno poveļanje izraġenosti ERŭ bi lahko razloġili s tem, da so ga izzvale niġje vrednosti estradiola med puberteto kot posledica zavrtja delovanja osi HPG zaradi poviġane ravni stresnih hormonov.

Ġenski spolni hormoni v odraslem obdobju so sicer nujno potrebni, da izzovejo izraġanje spolnega obnaġanja pri samicah, vendar pa glede na naġe rezultate njihova odsotnost med puberteto nima bistvenega vpliva na razvoj sposobnosti izraġanja spolne sprejemljivosti in spolne privlaļnosti v odraslem obdobju. Ugotovili pa smo, da odsotnost spolnih hormonov med puberteto, ġe posebej estradiola pri samicah, nepovrnljivo poslabġa sposobnost izraġanja starġevskega obnaġanja, usmerjenega k mladiļem. Oļetovsko obnaġanje pri samcih se ni bistveno razlikovalo od materinskega pri samicah, le da so bile samice hitrejġe pri prenaġanju mladiļev v gnezdo v primerjavi s samci. Nadomeġanje estradiola v odraslem obdobju ni imelo vpliva na aktivnosti prenaġanja mladiļev v gnezdo, je pa izboljġalo aktivnosti gradnje gnezda pri samcih in samicah, kar bi morda lahko razloġili z vpletenostjo estrogenih hormonov v mehanizme uravnavanja telesne temperature.

V zadnjem delu doktorske naloge smo ugotovili, da dolgotrajno prejemanje fluoksetina pri obeh spolih primerljivo zmanjġa izraġanje obnaġanja, podobnega depresivnemu, vendar nima vpliva na anksioznemu podobno obnaġanje. Samice so priļakovano moļneje izraġale anksioznemu podobno obnaġanje v primerjavi s samci, nismo pa ugotovili razlik med spoloma v delovanju fluoksetina.

Naġi rezultati dokazujejo, da imajo socialna osamitev in spolni hormoni med puberteto moļan in trajen uļinek tako na razvoj moġganov kot na sposobnost izraġanja nekaterih spektrov obnaġanj v odraslem obdobju.

7 SUMMARY

Puberty/ adolescence is the period during which an individual attains reproductive as well as behavioral maturation through the elevated secretion and action of gonadal steroid hormones. Steroid hormones during puberty direct proper development of cortical and limbic circuits in the brain and program a variety of adult behaviors. Stressful events during this vulnerable period can have profound consequences on different behaviors, like social, reproductive and emotional behaviors, due to alterations in developing brain and their neuroendocrine responses.

The purpose of our studies was to determine the influence of social isolation stress and sex steroid hormones during puberty and the action of psychoactive drugs such as fluoxetine on the behavior of adult male and female mice. In the first part of the study we explored whether social isolation during puberty affects the ability of social recognition and leads to changes in the OT and AVP system in the forebrain and midbrain. In the second part of the study we explored whether social isolation stress and lack of sex steroid hormones during puberty affects the ability to express parental behavior in both sexes and the ability to express receptive sexual behavior in females. Furthermore, we checked whether these two factors may lead to changes in the expression of ERŭin the forebrain and midbrain. In the last part of the study we explored whether the chronic fluoxetine treatment differently reduced the expression of depressive and anxiety like behavior in male and female mice.

Social isolation during puberty impairs social learning as well as irrecoverably affects the ability of social recognition in females only. Isolation stress did not affect the OT nor the AVP system, although the dynamics of the release of OT and AVP in the paraventricular nucleus was not studied and this could also cause behavioral disturbances. Socially housed males exhibited stronger pattern of social recognition than socially housed females, what could possibly be explained by higher expression of AVP in nerve fibers of the lateral septum in males than in females.

Social isolation during puberty did not affect the ability of expression of maternal and paternal behavior in mice. However, social isolation stress during puberty irrecoverably reduced the receptive sexual behavior in adult females, but did not affect their sexual attractiveness. Furthermore, social isolation stress permanently altered the expression of ERŭ

in the anteroventral periventricular nucleus and ventromedial nucleus of the hypothalamus. The greater expression of ERŭ in socially isolated females could be explained by the lower levels of estradiol during puberty as a result of the suppression of HPG axis activity due to the higher levels of stress hormones.

Female sex hormones in adulthood are necessary to elicit the expression of sexual behavior in females, but according to our results, their absence during puberty does not have a significant impact on the development of the ability to express receptive behavior and sexual attractiveness in adulthood. However, the absence of sex hormones during puberty, especially the lack of estradiol in females, irreversibly reduced the amount of pup-directed parental behaviors. Estradiol replacement in the adulthood had no effect on the pup retrieval into the nest, but improved the nest building activities in males and females, what might be connected with the involvement of estrogens in the thermoregulation.

Studies with chronic fluoxetine treatment revealed that such treatment reduces the expression of depressive like behavior in mice of both sexes, and fluoxetine has no effect on anxiety-like behavior in mice. As expected, anxiety like behavior was more prominent in females than in males, but there was no sex difference in the effect of fluoxetine.

Our results suggest that social isolation and sex hormones during puberty have profound and long-lasting influences on brain development and subsequently on the ability to express different behaviors in adult life.

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