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## Research

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# A mechanism for rapid neurosteroidal regulation of parenting behaviour

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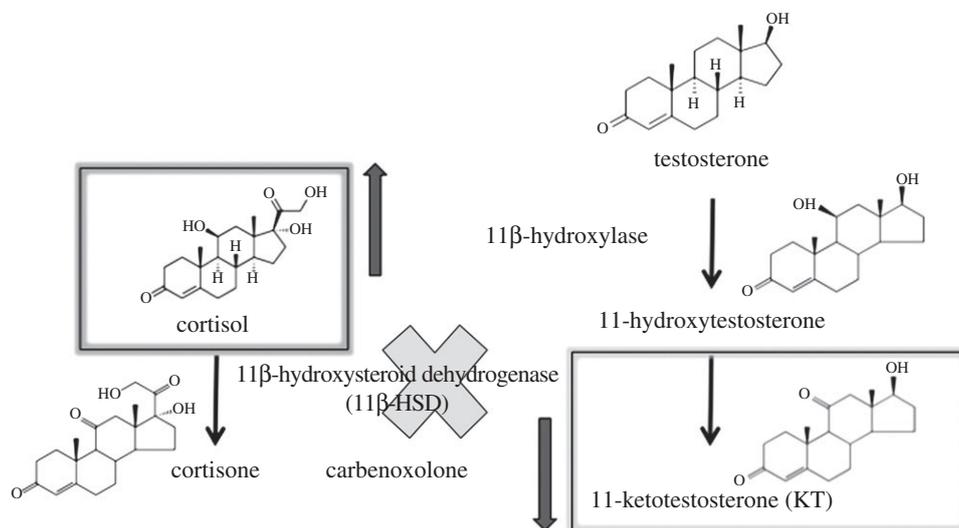
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While systemic steroid hormones are known to regulate reproductive behaviour, the actual mechanisms of steroidal regulation remain largely unknown. Steroidogenic enzyme activity can rapidly modulate social behaviour by influencing neurosteroid production. In fish, the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) synthesizes 11-ketotestosterone (KT, a potent teleost androgen) and deactivates cortisol (the primary teleost glucocorticoid), and both of these steroid hormones can regulate behaviour. Here, we investigated the role of neurosteroidogenesis in regulating parenting in a haremically bidirectionally hermaphroditic fish, *Lythrypnus dalli*, where males provide all requisite parental care. Using an *in vitro* assay, we found that an 11 $\beta$ -HSD inhibitor, carbenoxolone (CBX), reduced brain and testicular KT synthesis by 90% or more. We modulated neurosteroid levels in parenting males via intracerebroventricular injection of CBX. Within only 20 min, CBX transiently eliminated parenting behaviour, but not other social behaviour, suggesting an enzymatic mechanism for rapid neurosteroidal regulation of parenting. Consistent with our proposed mechanism, elevating KT levels rescued parenting when paired with CBX, while cortisol alone did not affect parenting. Females paired with the experimental males opportunistically consumed unattended eggs, which reduced male reproductive success by 15%, but some females also exhibited parenting behaviour and these females had elevated brain KT. Brain KT levels appear to regulate the expression of parenting behaviour as a result of changes in neural 11 $\beta$ -HSD activity.

## 1. Introduction

Steroid hormones are critical proximate determinants of reproductive success in vertebrates because they regulate fundamental aspects of life history and phenotype [1–4]. Many groups of vertebrates exhibit temporally distinct periods of mating and parenting during the breeding season, and predictable differences in systemic levels of steroid hormones are observed during these different phases of reproduction [2]. In some species, territory defence, mating and parenting temporally overlap, and in these cases a clear relationship between specific steroid hormones and behaviour may not be as evident [5,6]. In such cases, rapid and/or local changes in steroid hormone levels might control particular reproductive behaviours. The specific mechanisms by which steroid hormones regulate parenting are largely unknown. Chronically high systemic levels of androgens and glucocorticoids have been associated with adverse effects on parenting in birds [6,7] and primates [8–10]. In some rodents and fish, however, high systemic androgens are positively associated with parenting [11], and glucocorticoids do not appear to inhibit parenting [12,13]. Such opposite patterns suggest that information about systemic steroid levels is incomplete to explain variation in behaviour, and recently it has become clear that rapid behavioural responses to the environment can be regulated by steroids synthesized *de novo* from cholesterol in the brain [14–17], via steroidogenic enzymes and co-substrates present in brain cells [18,19].

Steroidogenic enzymes are active in the brain and function via evolutionarily conserved mechanisms in all groups of vertebrates [19]. In many species,



**Figure 1.** Simplified pathway of steroidogenesis in fish. Testosterone is converted to KT via the sequential action of 11 $\beta$ -hydroxylase, which converts KT to 11 $\beta$ -hydroxytestosterone (11 $\beta$ -OHT), and 11 $\beta$ -HSD, which converts 11 $\beta$ -OHT to KT and cortisol to cortisone. Inhibition of 11 $\beta$ -HSD with CBX (solid grey 'X') can elevate cortisol and reduce KT (solid grey arrows) levels.

neural steroidogenic enzymes are sensitive to changes in seasons [20], life history stages [21,22] and sexual behaviour [23]. Neural steroidogenic enzyme expression also varies during territory acquisition, courtship and parenting [15,22]. In fish, testosterone (T) can be converted to 17 $\beta$ -oestradiol (E<sub>2</sub>) via aromatase and to 11-ketotestosterone (KT, a potent androgen) via the sequential action of 11 $\beta$ -hydroxylase and 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD; figure 1) [1]. 11-Ketotestosterone cannot be converted to any other metabolically active steroid hormone. These steroidogenic enzymes (aromatase, 11 $\beta$ -hydroxylase and 11 $\beta$ -HSD) are expressed in both gonadal and neural tissue [24–26]. 11 $\beta$ -Hydroxysteroid dehydrogenase also catalyses the conversion of the primary potent glucocorticoid cortisol to cortisone, which has low potency. Therefore, the regulation of 11 $\beta$ -HSD is critical because increases in its activity may result both in local elevation of KT and reduction of cortisol [27], and each of these changes could increase reproductive success, because these steroid hormones have been suggested to regulate parenting and aggression. This dual action of 11 $\beta$ -HSD might function as a switch between behavioural states and this idea can be directly tested in fish owing to the position of 11 $\beta$ -HSD in the steroidogenic pathways for the major glucocorticoid and androgen [27–29].

The bluebanded goby, *Lythrypnus dalli*, is a serially hermaphroditic marine fish that offers an ideal opportunity to understand mechanisms regulating parenting. Males exclusively demonstrate parenting, while also expressing territorial aggression and courtship. In a semi-natural laboratory setting, wild-caught fish can be kept under conditions that mimic natural social groups [30]. The male, as the dominant individual, establishes a territory and actively defends his nest, a PVC tube. Males do jerk swims as courtship displays, while females solicit males through postural displays of their gravid abdomen (D. S. Pradhan 2012, unpublished data). During spawning, females lay adhesive eggs on the inner surface of the nest, and the male fertilizes and cares for them until they hatch. Males exhibit high rates of parenting for multiple overlapping broods, including vigorous rubbing and aeration of eggs by fanning. Parenting males also display aggressively towards egg predators, including conspecific females, and leave the nest unguarded for only a few seconds

at a time to feed or interact with females. We examined whether neurosteroids regulate parenting in *L. dalli*, based on the following observations. First, parenting males exude high levels of KT in water compared with males not actively parenting and compared with females [5]. Second, there are no sex differences between females and non-parenting males in levels of systemic, brain and gonad KT [31,32]. Third, brain KT levels are equal to or higher than gonad KT, suggesting that the brain is an important *de novo* producer of KT [32,33]. Finally, brain and gonadal KT levels are not correlated [32].

We conducted a series of four experiments to test the acute neuroendocrine regulation of parenting in *L. dalli* through the use of carbenoxolone (CBX), a potent 11 $\beta$ -HSD inhibitor. The activity of 11 $\beta$ -HSD has been well studied in glucocorticoid conversion pathways in mammalian tissues [34]; however, its role in KT synthesis has received little attention. In the first experiment, we tested whether CBX regulates 11 $\beta$ -HSD by inhibiting the conversion of 11 $\beta$ -hydroxytestosterone (11 $\beta$ -OHT) to KT in brain and testis tissues. In the second experiment, to examine whether *in vivo* systemic CBX treatment reduces systemic KT levels, we intraperitoneally (IP) implanted breeding male *L. dalli* with either CBX or beeswax (vehicle). We predicted that exogenous CBX treatment should inhibit the synthesis of KT, resulting in decreased levels of KT exuded in water. In the third experiment, we tested the hypothesis that neurosteroids directly regulate parenting, by manipulating brain steroid hormone levels via intracerebroventricular (ICV) injection of CBX or vehicle into parenting males. The effects of CBX on parenting could be due to direct effects on glucocorticoids (elevated cortisol) and/or androgens (decreased KT) [27,29] (figure 1). Consequently, we had two predictions regarding the mechanism(s) of CBX action. If reduced parenting was due to CBX-induced decreases in KT synthesis, then delivery of KT along with CBX should rescue parenting. However, if reduced parenting was due to elevated glucocorticoids, then cortisol delivery alone should also reduce parenting. Finally, in the fourth experiment, we tested behavioural and endocrine responses of females and consequent effects on male reproductive success, in response to a social context that resulted from experiment 3 (reduced male attendance in the nest). Bluebanded gobies can undergo socially

controlled sex change [30], and as a result, our manipulations provided females with the opportunity to respond to changes in social context by exhibiting male-typical behaviour.

## 2. Material and methods

### (a) General methods

All fish for these experiments were collected off the coast of Catalina Island, CA, during the months of June–July of 2009, 2011 and 2012 by SCUBA diving and using hand nets (permit numbers SC-10676 and SC-11879). After capture, the fish were kept in 2 l plastic Nalgene bottles, brought to the boat and then placed in a large bucket for transport to a laboratory at the Wrigley Institute for Environmental Studies. Fish were housed in  $60 \times 94 \text{ cm}^2$  aquaria with continuous seawater and exposed to natural ambient light cycles. Animals were fed twice daily, at 08.00 and 16.00 with frozen brine shrimp. Most procedures involving direct handling, such as measurement of standard length (SL), surgeries and necropsies, were conducted under a dissecting microscope. All chemicals used in these experiments were purchased from Sigma-Aldrich, unless specified. All data were analysed using PRISM v. 4.0a for Macintosh. Data were transformed where necessary and presented as mean  $\pm$  s.e.m.; all statistical tests were two-tailed (unless specified), and  $\alpha$  was set at 0.05.

### (b) Experiment 1: effect of carbenoxolone on *in vitro*

#### 11 $\beta$ -hydroxysteroid dehydrogenase activity

To measure the conversion of OHT to KT, we used an *in vitro* approach modified from previous studies [20,25]. Upon euthanization with tricaine methanesulfate (MS-222; 1.0 mg/100 ml water), brains and gonads from adult male *L. dalli* ( $n = 3$ ) were removed within 5 min, rapidly frozen on dry ice and stored at  $-80^\circ\text{C}$  until biochemical assays. Each tissue type was pooled and homogenized with a hand-held homogenizer, using bursts, in ice-cold sucrose–Tris buffer (250 mM, pH 7.4). Samples were then centrifuged using a 5804 R Eppendorf centrifuge at  $1000 \times g$  for 30 min, at  $4^\circ\text{C}$  to obtain supernatants. Samples were divided into aliquots with the following treatments: buffer and drug vehicle only (negative control), tissue supernatants only and tissue supernatants with  $50 \mu\text{M}$  CBX. The samples were then incubated by shaking (60 r.p.m., New Brunswick shaker) at  $25^\circ\text{C}$  for 60 min, with  $0.5 \text{ mM}$  OHT (substrate for 11 $\beta$ -HSD) and  $0.5 \text{ mM}$   $\text{NAD}^+$  (co-substrate for 11 $\beta$ -HSD) for a final reaction volume of  $100 \mu\text{l}$ . Reactions were terminated by immersing the tubes in a methanol dry ice bath, after which  $0.1 \mu\text{M}$  corticosterone (B, internal standard for liquid chromatography mass spectrometry (LC-MS/MS) analyses) was added to each sample and vortexed for 5 min. Steroids were extracted from samples using C18 columns (Sepak 1 cc, 50 mg sorbent, Waters). We used a Triple Quadrupole LC-MS/MS mass spectrometer, Agilent 1200 (Agilent Technologies Inc., USA), equipped with a binary pump, auto sampler and a Gemini 3 $\mu$ . 110A C18 column (Phenomenex). Throughout each run, the pressure was carefully monitored under a flow rate of  $600 \mu\text{l min}^{-1}$  using water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid. ANALYST v. 1.5.1 software was used for data quantitation (calculation of peak area ratio relative to the internal standard), and data were analysed using one-way ANOVA.

### (c) Experiment 2: effect of systemic carbenoxolone implants on 11-ketotestosterone exuded into water

Fish were anaesthetized with MS-222 (0.5 mg/100 ml water) to determine SL. Fish were housed in pairs, consisting of one male

( $\text{SL} = 31.16 \pm 0.54$ ) and one female ( $\text{SL} = 26.24 \pm 0.42$ ). These morphological differences ensure that fish established a robust linear hierarchy within 5 days [30]. Each pair was provided with a PVC tube (7.62 cm in length, 3 cm in diameter) that served as the nest. After 5 days, each male was implanted IP with either beeswax (Natural Cosmetics) only (vehicle,  $n = 5$ ) or beeswax + CBX ( $n = 4$ ) using previously described procedures [33]. Implants were prepared by heat sterilizing beeswax and moulding into small pellets under a microscope. Carbenoxolone was added to beeswax in 1:3 ratio, and each pellet was sterilized again by immersion in 100% ethanol for 1 s and dried. In brief, for the surgery, a small ventral incision was made anterolateral to the vent, the pellet was inserted, the skin was sealed with a cyanoacrylate adhesive (Henkel Loctite) and Stress Coat (API Pharmaceuticals) was applied over the incision site. The fish was kept moist with seawater during the surgery. On average, the surgery took  $432 \pm 51.62 \text{ s}$  for vehicle fish and  $382.5 \pm 64.08 \text{ s}$  for CBX fish ( $t_7 = 0.6096$ ,  $p = 0.54$ ). Post-operatively, each fish was resuscitated and transferred to a clean cup of water for collection of exuded steroid hormone, and then the same pairs of fish were placed back into their tanks. Samples of KT exuded in water were also collected 1- and 4-days post-treatment. Previously validated procedures (using Sepak 2 cc, 200 mg columns, Waters) were used for extraction of steroid hormones from water [31], and KT levels were determined using specific enzymeimmunoassays (Cayman Chemical) [31]. Data were analysed using two-way repeated measures ANOVA, with time as the within subjects factor and treatment as the between subjects factor.

### (d) Experiment 3: effect of intracerebroventricular carbenoxolone on male parenting

This is the first study, to our knowledge, to inject an enzyme-inhibiting drug into the brain of a fish to investigate effects on social behaviour, but our methods of ICV injection and recovery were modified from a previous injection study [35]. Therefore, in a pilot experiment we investigated the appropriateness and efficacy of ICV CBX dose by examining recovery from injections, basic locomotion, social interactions and concentration of KT and cortisol (Steraloids Inc.) to be injected (see below).

During mid-breeding season, social groups of *L. dalli*, each consisting of one male ( $\text{SL} = 41.44 \pm 0.53 \text{ mm}$ ) and two size-mismatched females ( $\text{SL}$ :  $\alpha = 35.13 \pm 0.29 \text{ mm}$ ;  $\beta = 30.72 \pm 0.24 \text{ mm}$ ) were constructed. The male was the largest and dominant member of the group and was at least 3 mm larger than the biggest female ( $\alpha$ , subordinate only to the male); the  $\beta$ -female (most subordinate) was at least 3 mm smaller than the  $\alpha$ -female. Each group was provided with an acetate-lined PVC tube (7.62 cm in length, 3 cm in diameter) that served as the nest. Over a period of four weeks, daily checks were conducted to ensure that males were experienced at parenting (two to four clutches). On the day before injections, groups were chosen for use if their males were actively parenting in the nest. All the fish from the selected group, along with their acetate-lined PVC tube containing eggs, were placed in a different experimental tank that allowed for more convenient behavioural observations and injections. On the day of the injections, observers, blind to the particular treatment, recorded pre-injection behaviour (see below). Within seconds after the baseline observations, the male was removed and anaesthetized.  $\alpha$ - and  $\beta$ -females were placed in separate cups during male injections and recovery, to ensure that they did not have access to the unprotected eggs and that dominant females did not initiate behavioural and physiological transformations associated with sex change in the absence of the male. This experiment consisted of the following treatment groups: (i) vehicle ( $n = 7$ ), (ii) CBX ( $1265 \text{ ng brain}^{-1}$ ,  $n = 9$ ), (iii) CBX + KT ( $1265 \text{ ng CBX} + 0.50 \text{ ng KT brain}^{-1}$ ;  $n = 9$ ) and (iv) cortisol ( $0.05 \text{ ng brain}^{-1}$ ;  $n = 9$ ). The CBX dose was scaled to match the

dose used for an ICV study in rats [34] and the recovery of fish in our pilot studies. The scaled KT and cortisol doses were based on the highest brain levels of these steroid hormones following CBX injections in the pilot experiment. Briefly, in that study, we found that 60 min following ICV injection, brain KT levels did not change significantly (vehicle =  $195.80 \pm 177.40$  pg mg<sup>-1</sup> tissue, CBX =  $20.25 \pm 5.58$  pg mg<sup>-1</sup> tissue;  $t_{12} = 1.11$ ,  $p = 0.29$ ), whereas cortisol levels increased significantly (vehicle =  $13.71 \pm 1.65$  pg mg<sup>-1</sup> tissue, CBX =  $32.53 \pm 4.67$  pg mg<sup>-1</sup> tissue;  $t_{13} = 3.10$ ,  $p = 0.008$ ). Overall, the ratio of KT to cortisol (KT : cortisol) decreased significantly (vehicle =  $17.97 \pm 16.53$  pg mg<sup>-1</sup> tissue, CBX =  $0.59 \pm 0.16$  pg mg<sup>-1</sup> tissue;  $t_{12} = 2.33$ ,  $p = 0.038$ ). These data were converted to the units required for ICV injection for 5 mg tissue, which is the average weight of an *L. dalli* brain (D. S. Pradhan and M. S. Grober 2012, unpublished results).

After recovery, the male was returned to his tank, and females were re-introduced 1 min later. Latency for the male to enter the nest was recorded over a period of 61 min. Immediately after the group was reunited, post-injection rates of social behaviour were measured in 10 min intervals for the first 30 min and then from 50–60 min. Time between the injection and start of behavioural observations did not differ significantly among the four groups (Kruskal–Wallis statistic,  $H = 0.136$ ,  $p = 0.98$ ). Before and after ICV injections, we quantified agonistic behaviours, which consisted of approaches (when one fish came within two body lengths of another fish) and displacements (if the approached fish responded by swimming away). Rates of agonistic behaviour were calculated by dividing the number of occurrences of each type of behaviour by 10 min, which was the length of each observation period. Parenting was quantified by counting the number of bouts of egg care, including vigorous rubbing and fanning of eggs inside the nest using fins. Two independent bouts were separated by at least 2 s, and each bout could last from 1 to several seconds. Previous observations showed that measuring the frequency of fanning bouts was a good predictor of the duration of bouts of parenting ( $r^2 = 0.52$ ,  $p < 0.0001$ ). For logistical reasons, it was not possible to accurately measure bout length in this study; so we only recorded the number of parenting bouts. Before injections, males from all groups spent the majority of their time in the nest ( $91.80 \pm 2.17\%$ ); however, there was no relationship between time in the nest and number of parenting bouts ( $r^2 = 0.02$ ,  $p = 0.46$ ). By dividing the number of parenting bouts by the amount of time spent in the nest, we are able to distinguish high parenting males who did not spend much time in the nest from those who spent a lot of time in the nest, but did not exhibit high rates of parenting. One-way ANOVA was used to compare the treatment groups for latency to enter nest, time in nest from 10 to 20 min, and number of parenting bouts from 10 to 20 min, followed by *post hoc* Tukey Multiple Comparison tests. Additionally, rate of approaches, rate of displacements, number of parenting bouts and number of parenting bouts/time in nest were analysed using a series of two-way repeated measures ANOVA with treatment as the between subjects factor and time as the within subjects factor, followed by *post hoc* Bonferonni tests.

#### (e) Experiment 4: effect of context on male reproductive success, female behaviour and tissue 11-ketotestosterone

The changes in male behaviour caused by some ICV injections (one vehicle, seven CBX and four cortisol) in experiment 3 created a rare social context that permitted females to enter the nest and consume eggs and/or exhibit parenting, even while the male was in close proximity. In other (control) groups, male behaviour inside the nest inhibited females from entering the nest. For females that interacted with the eggs, we recorded

the number of bouts of egg eating and parenting using methods similar to those of experiment 3. When all animals were removed from the home tank for male injections, the acetate was removed from the PVC tube, placed in a frame, immediately photographed (Canon SX150 IS) and replaced in the tube. This process was repeated again at the end of the 60 min post-injection behavioural observation period. All the photographs were uploaded to a Macintosh computer, and eggs were quantified using IMAGEJ. Number of eggs is a direct measure of male reproductive success and we predicted the number of eggs to decrease for two reasons. First, females do not generally lay eggs in the absence of a male, so we did not expect the number of eggs to increase. Second, in our pilot experiment we observed females eating eggs in the absence of a male. Hence, we used a one-tailed *t*-test to compare the percentage of eggs lost between the vehicle- and CBX-treated groups. At the end of the 60 min behaviour observations, females were euthanized, and their brain and gonad tissues were immediately collected, frozen on dry ice and maintained at  $-80^\circ\text{C}$  until assayed. Steroid hormone extractions were conducted using previously described techniques [32,36] (see the electronic supplementary material for details), and differences in tissue KT of parenting versus non-parenting  $\alpha$ -females were analysed using unpaired *t*-tests.

### 3. Results

#### (a) Experiment 1: effect of carbenoxolone on *in vitro* 11 $\beta$ -hydroxysteroid dehydrogenase activity

After 60 min of incubation, the addition of CBX reduced the KT : B peak area ratio (figure 2) by 90% in brain (one-way ANOVA,  $F_{2,5} = 147.1$ ,  $p = 0.001$ ) and 100% in testes (one-way ANOVA  $F_{2,5} = 190.4$ ,  $p = 0.0007$ ).

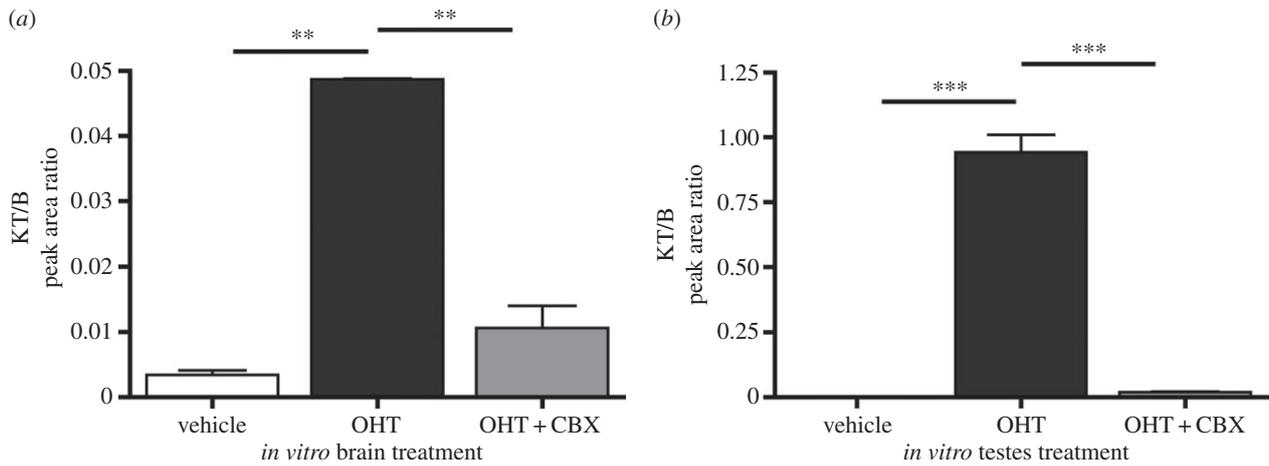
#### (b) Experiment 2: effect of carbenoxolone implants on 11-ketotestosterone exuded in water

Levels of KT exuded in water were transiently affected following IP CBX implants in males (figure 3). Two-way repeated measures ANOVA revealed that there was a significant main effect of time ( $F_{2,7} = 0.17$ ,  $p = 0.018$ ), no main effect of treatment ( $F_{1,7} = 0.17$ ,  $p = 0.69$ ) and no interaction between time and treatment ( $F_{2,7} = 1.43$ ,  $p = 0.27$ ). *Post hoc* Bonferonni tests revealed that 1 day after implanting fish with CBX, KT levels were reduced by 48% compared with 1 h after treatment ( $t = 2.80$ ,  $p < 0.05$ ), and then significantly increased 4 days after treatment (1 day versus 4 days,  $t = 2.68$ ,  $p < 0.05$ ).

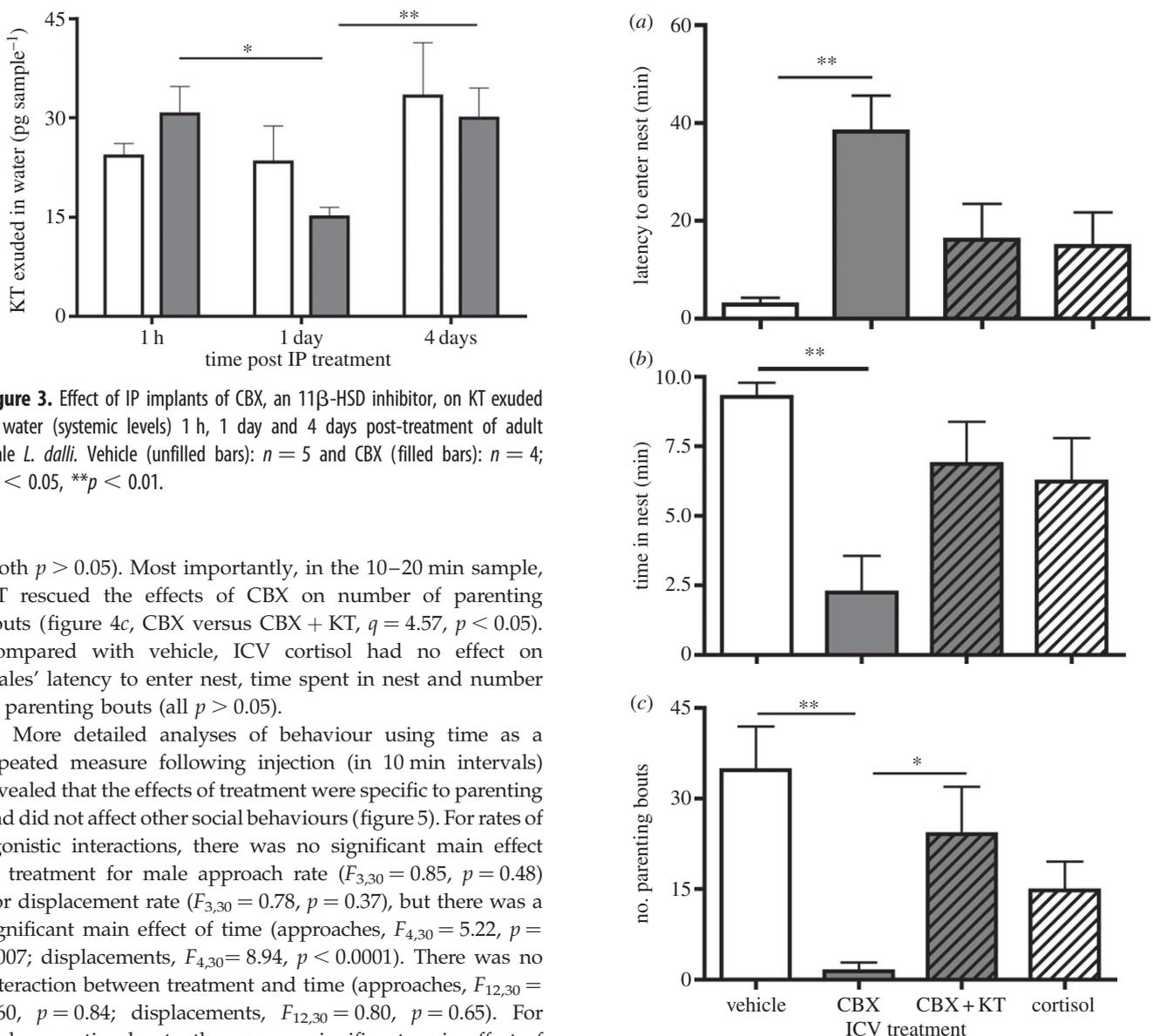
#### (c) Experiment 3: effect of intracerebroventricular carbenoxolone on male parenting

Following ICV injections, one-way ANOVA determined that there was a significant effect of treatment on males' latency to enter the nest (figure 4a,  $F_{3,30} = 3.74$ ,  $p = 0.02$ ), time spent in the nest (figure 4b,  $F_{3,30} = 4.28$ ,  $p = 0.013$ ) and number of parenting bouts (figure 4c,  $F_{3,30} = 6.67$ ,  $p = 0.001$ ). Compared to vehicle-injected males, males injected with CBX took longer to enter the nest ( $q = 4.59$ ,  $p < 0.05$ ), and during the 10–20 min sample period, they spent less time in the nest ( $q = 4.90$ ,  $p < 0.01$ ) and demonstrated much less parenting ( $q = 5.99$ ,  $p < 0.01$ ).

As predicted, males injected with a physiological dose of KT along with CBX did not differ significantly from the vehicle in latency to enter the nest nor time in the nest



**Figure 2.** Effect of CBX and  $11\beta$ -OHT (an endogenous substrate) on  $11\beta$ -HSD activity in adult male *L. dalli* (a) brain and (b) testes. Tissue supernatants were incubated *in vitro* at  $25^{\circ}\text{C}$  with  $1\text{ mM NAD}^{+}$  as a co-substrate for 60 min. Controls were incubated with buffer + vehicle only. KT peak area ratio was calculated by dividing area of the KT peak by that of corticosterone (B), the internal LCMS standard ( $N = 3$  males per group);  $**p < 0.01$ ,  $***p < 0.001$ .

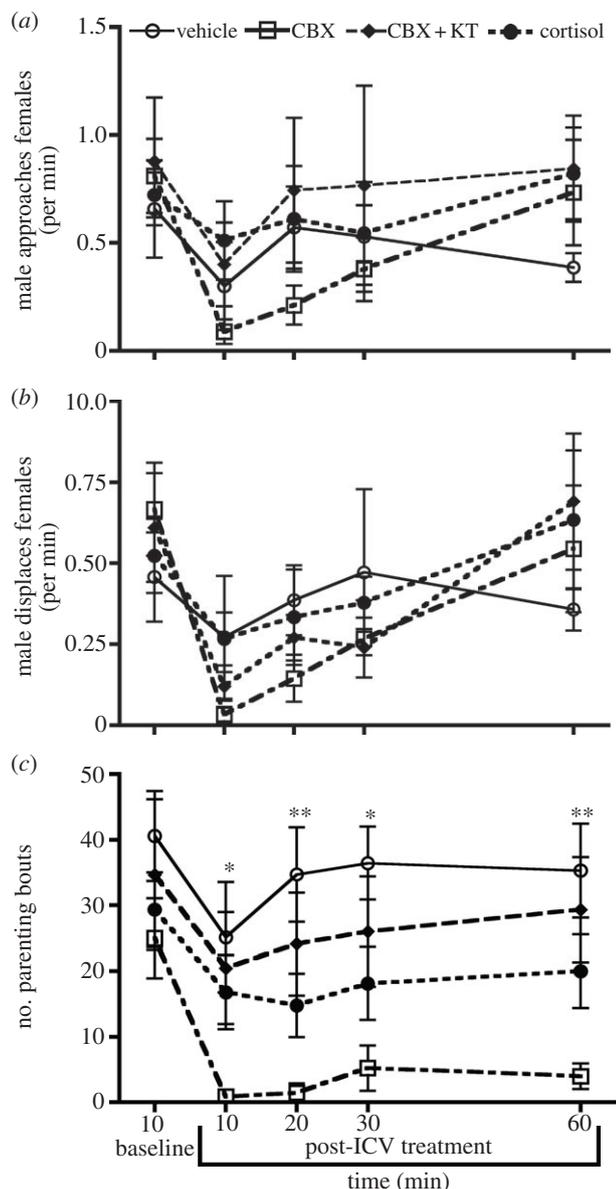


**Figure 3.** Effect of IP implants of CBX, an  $11\beta$ -HSD inhibitor, on KT exuded in water (systemic levels) 1 h, 1 day and 4 days post-treatment of adult male *L. dalli*. Vehicle (unfilled bars):  $n = 5$  and CBX (filled bars):  $n = 4$ ;  $*p < 0.05$ ,  $**p < 0.01$ .

(both  $p > 0.05$ ). Most importantly, in the 10–20 min sample, KT rescued the effects of CBX on number of parenting bouts (figure 4c, CBX versus CBX + KT,  $q = 4.57$ ,  $p < 0.05$ ). Compared with vehicle, ICV cortisol had no effect on males' latency to enter nest, time spent in nest and number of parenting bouts (all  $p > 0.05$ ).

More detailed analyses of behaviour using time as a repeated measure following injection (in 10 min intervals) revealed that the effects of treatment were specific to parenting and did not affect other social behaviours (figure 5). For rates of agonistic interactions, there was no significant main effect of treatment for male approach rate ( $F_{3,30} = 0.85$ ,  $p = 0.48$ ) nor displacement rate ( $F_{3,30} = 0.78$ ,  $p = 0.37$ ), but there was a significant main effect of time (approaches,  $F_{4,30} = 5.22$ ,  $p = 0.007$ ; displacements,  $F_{4,30} = 8.94$ ,  $p < 0.0001$ ). There was no interaction between treatment and time (approaches,  $F_{12,30} = 0.60$ ,  $p = 0.84$ ; displacements,  $F_{12,30} = 0.80$ ,  $p = 0.65$ ). For male parenting bouts, there was a significant main effect of treatment ( $F_{3,30} = 5.38$ ,  $p = 0.004$ ) and a main effect of time ( $F_{4,30} = 6.66$ ,  $p < 0.0001$ ), but there was no interaction between treatment and time ( $F_{12,30} = 0.52$ ,  $p = 0.90$ ). For male parenting bouts/time in nest, all patterns were the same, such that

**Figure 4.** Effect of ICV injection of parenting male *L. dalli* on (a) latency to enter nest, (b) time spent in nest between 10 and 20 min and (c) number of parenting bouts between 10 and 20 min. Vehicle:  $n = 7$ ; CBX:  $n = 9$ ; CBX + KT:  $n = 9$ ; cortisol:  $n = 9$ ;  $*p < 0.05$ ,  $**p < 0.01$ .



**Figure 5.** Effect of ICV injection of parenting male *L. dalli* on agonistic interactions and parenting behaviour; (a) male approach rate, (b) male displacement rate and (c) male parenting. Vehicle:  $n = 7$ ; CBX:  $n = 9$ ; CBX + KT:  $n = 9$ ; cortisol:  $n = 9$ ; \* $p < 0.05$ , \*\* $p < 0.01$ .

there was a significant main effect of treatment ( $F_{3,30} = 7.10$ ,  $p = 0.001$ ) and a main effect of time ( $F_{4,30} = 9.56$ ,  $p < 0.0001$ ), but there was no interaction between treatment and time ( $F_{12,30} = 1.10$ ,  $p = 0.37$ ). While overall rates of all social behaviours in other treatment groups increased to pre-injection levels as a function of time, parenting behaviour in 89% of CBX-treated males did not recover to baseline within 60 min of ICV injection (figure 5c).

#### (d) Experiment 4: effect of context on male reproductive success, female behaviour and tissue 11-ketotestosterone

Manipulation of some males (one vehicle, seven CBX and four cortisol) produced a social context that permitted females to opportunistically enter the nest tube without the male. In the first 30 min,  $\alpha$ -females (44%) and  $\beta$ -females (15%) that entered the nest exhibited egg consumption. The highest consumption was observed between 10 and 20 min ( $p < 0.05$ ), reducing

number of eggs by  $15.76 \pm 5.74\%$  ( $t_{12} = 2.03$ ,  $p = 0.03$ ). A subset of the  $\alpha$ -females (34%) that entered the nest while the male was in close proximity, but not in the nest, also demonstrated low and variable levels of male-typical parenting behaviour at some point during the 60 min post-treatment observation period. Compared with  $\alpha$ -females that did not exhibit parenting, those that parented had significantly elevated brain KT (non-parenting:  $3.67 \pm 0.37$  mg tissue<sup>-1</sup>, parenting:  $6.35 \pm 1.58$  mg tissue<sup>-1</sup>,  $p < 0.01$ ) and reduced ovarian KT (non-parenting:  $3.06 \pm 0.33$  mg tissue<sup>-1</sup>, parenting:  $1.70 \pm 0.13$  mg tissue<sup>-1</sup>,  $p < 0.05$ ). In  $\alpha$ -females demonstrating parenting, brain KT : cortisol was  $0.03 \pm 0.01$ . Although female parenting was exhibited at a very low frequency compared with male parenting, this association is intriguing because this effect was specific to KT and not to other steroid hormones (T, cortisol, E<sub>2</sub>; all  $p > 0.05$ , data not shown).

## 4. Discussion

We demonstrate that exogenous manipulation of a single steroidogenic enzyme in the brain, at the site of both its synthesis and action, can rapidly regulate parenting behaviour and consequently reduce male reproductive success. We provide the first direct evidence that neural synthesis of KT is a pivotal mechanism for regulation of male parenting and that cortisol does not decrease parenting in a species of teleost fish. We also show that brain KT is elevated in females that display parenting, a male-typical phenotype. We speculate that rapid modulation of local steroidogenesis allows for regulation of dynamic changes in behaviour in an environment that requires an organism to successfully coordinate multiple activities to enhance fitness [16,17], including interaction with conspecifics, territory defence, foraging and parenting. Our data indicate that neural synthesis of steroid hormones can be just as important for regulation of reproductive behaviour as other sources of steroid hormones (e.g. gonads and inter-renal tissue [37]).

### (a) Effect of carbenoxolone on steroid hormone conversion pathways

Like other steroidogenic enzymes, 11 $\beta$ -HSD participates in the conversion of more than one steroid hormone (figure 1). As a result, changes in 11 $\beta$ -HSD activity can potentially affect hormones in multiple pathways [29]. Previous studies have reported that CBX dramatically inhibits 11 $\beta$ -HSD activity in deactivation pathways for corticosterone [34] and cortisol [38]. However, CBX has not been previously studied in the regulation of KT synthesis. We showed that CBX inhibits 11 $\beta$ -HSD activity and thus the production of KT in brain and testes (figure 2). Our study demonstrates that it is possible to pharmacologically inhibit KT production and highlights the utility of CBX as a powerful tool for studies of the phenotypic effects of KT in a variety of species. We also demonstrated that IP CBX implants in males produced a transient decline in KT exuded in water, such that KT levels were reduced after 1 day, but recovered to control levels after 4 days (figure 3). A previous study showed that following IP injections, CBX was not detectable in the cerebrospinal fluid of rodents, although it was detectable in plasma [39]. The authors concluded that CBX does not penetrate the blood brain barrier, and they recommend ICV

injections to induce brain effects of CBX [39]. If CBX similarly does not cross the blood brain barrier in fish [40], our data suggest that lower levels of KT 1 day after IP CBX implants are due to a reduction in peripheral KT synthesis. Finally, ICV CBX delivery allowed us to manipulate neurosteroids. Our pilot studies showed that whole brain KT:cortisol decreased significantly in CBX-treated males. This approach allowed us to use a local manipulation to investigate the role of neurosteroids in regulating parenting.

### (b) Neurosteroidal regulation of parenting behaviour

Within only 20 min, ICV CBX transiently eliminated parenting behaviour, but not other social behaviour, suggesting an enzymatic mechanism for rapid neurosteroidal regulation of parenting. The rapid and specific effects of CBX on male parenting are likely due to its inhibitory effects on neural 11 $\beta$ -HSD activity, which could have direct effects on glucocorticoids and/or androgens [27] and result in a local decrease in KT and/or elevation in cortisol. Consistent with our proposed mechanism, elevating KT levels rescued parenting when paired with CBX, while cortisol alone did not affect parenting.

By manipulating brain 11 $\beta$ -HSD activity and relative concentrations of KT and/or cortisol, we demonstrate that these steroid hormones do not decrease parenting, but rather can promote parenting. Causative relationships between changes in levels of systemic steroid hormones and reproductive phase-dependent changes in behaviour have not been investigated extensively. In many bird species, systemic T levels decline in males at the onset of parenting [6,7,41]. In a paternal anuran, plasma androgen levels decline between the sexually active stage and parenting stage, and levels then remain low throughout all stages of parenting [42]. In fish, the pattern of androgen involvement in parenting is less straightforward. In male sticklebacks, circulating KT levels correlate with maturation of eggs: KT is high during courtship but lower during parenting. Systemic androgen treatment, however, does not decrease parenting rates [43,44]. In the peacock blenny (*Salaria pavo*), brood size is positively correlated with both plasma T and KT levels [45]. However, in bluegills (*Lepomis macrochirus*), KT mediates a trade-off between aggressive and nurturing components of paternal care [46]. In some mammals, circulating T is elevated during parenting [3,42], but this is associated with increased brain aromatization of T to E<sub>2</sub> [15]. In *L. dalli*, circulating levels of KT in parenting males are higher compared with females and non-parenting males [5]. In many vertebrates, the role of androgens in the regulation of parenting remains elusive because the correlative patterns seen in most species may be a consequence of studying systemic steroid hormones and this might not reflect their action in the brain. This could be resolved by examining local steroid hormone and receptor levels within specific target tissues. This experiment is the first to provide a mechanism by which locally synthesized neural KT regulates parenting behaviour.

The role of circulating glucocorticoids in regulating various aspects of reproduction and life-history trade-offs has been widely studied due to the general interest in the harmful impacts of chronic stress. Here, neural cortisol treatment reduced parenting in 55% of the males, but overall there were no significant effects. From the literature, the role of systemic cortisol in regulating parenting in fish is not resolved [13], but long-term cortisol treatment may increase the rate of nest desertion [47]. Our data support the hypothesis that

short-term ICV treatment with glucocorticoids does not reduce parenting. It is possible that glucocorticoids may support allocation of energetic reserves towards caring for the present clutch to maximize reproductive success [48].

### (c) Role of neural steroidogenic enzymes in regulating transitions between behavioural states

The integration of environmental stimuli (e.g. social factors, food availability or stress), endocrine cues from extra-neural sources and the local ratio of steroid hormones within the brain can generate a highly context-specific means of rapidly gating behavioural output. Male *L. dalli* maintain the highest rank in the social group, while interacting with conspecifics in a variety of ways, including territory defence, courtship, spawning and parenting. Moreover, these behavioural states are dynamic and fluctuate within seconds. We propose a neurosteroidogenic mechanism for the inhibition and release of these behaviours via temporally (rapid) and spatially (local) controlled changes in the activity of a single enzyme. We showed that decreased 11 $\beta$ -HSD activity can potentially depress brain KT and simultaneously increase brain cortisol, thereby providing a means to rapidly modify the local ratio of these two steroid hormones. Consistent with this idea, female *L. dalli* generally do not provide parental care, and brain KT:cortisol is two orders of magnitude lower in females than in parenting males (see below). However, changes in conversion of either of these steroid hormones can independently regulate parenting. Our data do not provide evidence for local cortisol regulation of parenting on a short time-scale. We suggest that brain 11 $\beta$ -HSD activity functions as an enzymatic switch to shift males between behavioural states via changes in local KT levels, such that high KT increases parenting and low KT reduces parenting.

In addition, the reproductive plasticity of *L. dalli* permits bidirectional adult sex change—a process that involves multiple changes throughout the body axis [30]. Initiation of sex change involves rapid increases in rates of aggressive behaviour in the dominant female undergoing sex change [30]. This is accompanied by rapid reduction in brain aromatase activity, thus providing a mechanism for a local reduction in brain E<sub>2</sub> [21]. Brain KT also increases in females during sex change and this could occur via an increase in brain 11 $\beta$ -HSD activity [21,32]. Here, in the absence of males occupying the nest and caring for eggs, opportunistic females rapidly monopolized these resources. In addition to engaging in agonistic interactions outside the nest, several  $\alpha$ - and  $\beta$ -females gained access to the nest, leading to consumption of 15% of the eggs within 30 min.  $\alpha$ -females from 12 groups also exhibited substantial rates of parenting. These females were likely demonstrating the onset of behavioural sex change in the absence of male domination and had high brain KT and low ovarian KT relative to females that did not care for eggs. These data provide evidence that females that exhibit parenting (albeit for a short amount of time) also have high KT, similar to parenting males [5]. A rapid increase in brain 11 $\beta$ -HSD activity can be a mechanism for elevated brain KT, but it is also possible that these females had naturally high brain KT prior to the social manipulation. Female *L. dalli* appear to be extremely sensitive to changes in social context [33], and this behavioural plasticity ultimately has the potential to increase fitness. Our data provide a clear demonstration of social dynamics having differential

effects on local steroid hormone levels and behavioural output. Our data also demonstrate that elevated neural KT is associated with parenting in both males and females.

## 5. Conclusion

Our results support the hypothesis, posited by Perry & Grober [27], that regulation of 11 $\beta$ -HSD, can play a key role in the induction of major life history transitions. This local mechanism of neural steroidogenesis could be advantageous for the speed of action, thus reducing the inappropriate expression of behaviour in unpredictable environments. Although our study focused on the role of neural 11 $\beta$ -HSD in regulating parenting, we suggest that this mechanism of local regulation could have broad importance in the context

of other enzymes, such as aromatase, 5 $\beta$ -reductase and 3 $\beta$ -hydroxysteroid dehydrogenase and other classes of social behaviour [17,20,21,23].

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