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Glucocorticoid receptor blockade inhibits brain cell addition and aggressive signaling in electric fish, *Apteronotus leptorhynchus*

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Abstract

When animals are under stress, glucocorticoids commonly inhibit adult neurogenesis by acting through glucocorticoid receptors (GRs). However, in some cases, conditions that elevate glucocorticoids promote adult neurogenesis, and the role of glucocorticoid receptors in these circumstances is not well understood. We examined the involvement of GRs in social enhancement of brain cell addition and aggressive signaling in electric fish, *Apteronotus leptorhynchus*. In this species, long-term social interaction simultaneously elevates plasma cortisol, enhances brain cell addition and increases production of aggressive electrocommunication signals (“chirps”). We implanted isolated and paired fish with capsules containing nothing (controls) or the GR antagonist, RU486, recorded chirp production and locomotion for 7d, and measured the density of newborn cells in the periventricular zone. Compared to isolated controls, paired controls showed elevated chirping in two phases: much higher chirp rates in the first 5 h and moderately higher nocturnal rates thereafter. Treating paired fish with RU486 reduced chirp rates in both phases to those of isolated fish, demonstrating that GR activation is crucial for socially induced chirping. Neither RU486 nor social interaction affected locomotion. RU486 treatment to paired fish had a partial effect on cell addition: paired RU486 fish had less cell addition than paired control fish but more than isolated fish. This suggests that cortisol activation of GRs contributes to social enhancement of cell addition but works in parallel with another GR-independent mechanism. RU486 also reduced cell addition in isolated fish, indicating that GRs participate in the regulation of cell addition even when cortisol levels are low.

Keywords

electrocommunication; cortisol; cell addition; RU486; swimming behavior; glucocorticoid receptor; electric fish; *Apteronotus leptorhynchus*; neurogenesis; social behavior

Glucocorticoids were among the first hormones discovered that influence neurogenesis in the adult brain (Gould et al., 1992). In most cases, exogenous glucocorticoid treatment or stressful conditions that elevate endogenous glucocorticoids inhibit both the production and survival of newborn cells (Mirescu and Gould, 2006; Wong and Herbert, 2005; Wong and Herbert, 2006). However, the relationship between glucocorticoids and neurogenesis is not always inhibitory (reviewed in Lucassen et al., 2008). For example, in rodents and primates,

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moderate increases in glucocorticoid levels that occur during environmental enrichment, mild stress or physical exercise are positively associated with neurogenesis (Kempermann et al., 1997; Lucassen et al., 2008; Lyons et al., 2010; Parihar et al., 2011). Although the mechanisms of glucocorticoid action in stress-induced inhibition of neurogenesis has been explored at length, relatively little is known about how glucocorticoids might mediate environmentally induced enhancement of neurogenesis. Here we examine the causal role of glucocorticoid receptors (GRs) in social enhancement of brain cell addition and communication behavior in adult weakly electric fish.

Brown ghost knife fish, *Apteronotus leptorhynchus*, communicate with weak electric discharges produced by modified motoneurons in the tail (Zakon and Smith, 2009). The electric organ discharge (EOD) frequency is very stable within an individual and conveys the fish's sex and individual identity. This continuous EOD is controlled by the spontaneous rhythmic firing of the pacemaker nucleus in the hindbrain. During social interaction, particularly during male-male aggression, fish transiently elevate EOD frequency to produce communication signals termed chirps (Dunlap, 2002; Hagedorn and Heiligenberg, 1985; Larimer and MacDonald, 1968). Chirps are emitted when the prepacemaker nucleus (PPn-C) located in the diencephalon briefly increases the pacemaker firing rate.

Social interaction, chirping behavior, glucocorticoid levels and brain cell addition are all interrelated in *Apteronotus leptorhynchus*. Previously, we showed that both short-term and long-term social interaction influenced chirp production. In short term interactions, chirp rate increased in the first 2 min and decreased over the next 3 min, though still remaining elevated above background chirp rate (Dunlap, 2002). Long-term interaction potentiated chirp rate (Dunlap et al., 2002). That is, fish paired with another fish for 7d chirp more than isolated fish to a standardized, synthetic EOD playback. This latter study showed that long-term interaction modified the propensity to chirp, but nothing was known about chirp production toward another fish in long-term pairings.

In addition to changing chirping behavior, long-term social interaction increases plasma cortisol levels (Dunlap et al., 2002) and promotes cell addition to the periventricular zone (PVZ) of the brain (Dunlap et al., 2006). (We define cell addition as the two part process of cell birth plus 4d survival.) The effect of social interaction on cell addition is regionally and temporally specific. It enhances cell addition in the PVZ region that contributes adult-born cells to the PPn (the brain region that controls chirping), but has no effect on neighboring PVZ regions. Moreover, enhanced cell addition coincides with the period that social interaction potentiates chirping behavior. This regional and temporal specificity suggest that social enhancement of cell addition may contribute to socially induced changes in chirping behavior.

Cortisol treatment to isolated fish mimics many aspects of social interaction on chirping behavior and cell addition. Fish implanted with cortisol show potentiated chirping (Dunlap et al., 2002) and enhanced brain cell addition (Dunlap et al., 2006). The primary difference between cortisol treatment and social interaction is that cortisol treatment, at least at certain time scales, seems to have a more generalized effect on cell addition, increasing cell addition in all the PVZ examined, not just the region adjacent to the PPn (Dunlap et al., 2006).

Given the association between endogenous cortisol levels, chirping and brain cell addition in socially-interacting fish and the similar effects of exogenous cortisol in isolated fish, we hypothesized that cortisol plays a causal role in mediating the effect of social interaction on cell addition and chirping behavior. However, it is possible that socially induced changes in cortisol are epiphenomenal and do not directly influence cell addition and chirping. To

address this possibility, we treated fish with RU486, a GR antagonist (Bury et al., 2003; Scott et al., 2005; Shaw et al., 2007), to see if socially induced changes in brain and behavior still occur without GR activation. Given that glucocorticoids can influence locomotor behavior in fish (Gregory and Wood, 1999; Overli et al., 2002), and physical activity can enhance brain cell production in mammals (van Praag et al., 1999; van Praag, 2008), we also examined swimming behavior in RU486-treated animals to evaluate whether GR blockade might affect brain cell production *via* its influence on physical activity. Our study is presented in two parts: first a behavioral comparison of paired and isolated fish to characterize electrocommunication and locomotor behavior in social conditions that promote brain cell addition and, secondly, an experimental analysis of the effect of GR blockade on electrocommunication, locomotion and brain cell addition.

Materials and methods

Animals

Apteronotus leptorhynchus were obtained from commercial dealers and housed in individual tanks that were part of a 1520-L circulating system. All fish were males, as determined by EOD frequency (> 880 Hz at 28°C). Water temperature ($26 - 28^{\circ}\text{C}$), water conductivity ($400 - 500$ $\mu\text{S}/\text{cm}$), and light cycle (12L:12D) were held constant. Fish were fed frozen brine shrimp and blood worms every 2d. All fish were housed in individual 38-L tanks for at least 7d before the beginning of the experiment. All procedures used in this study adhered to ethical standards of animal use specified by the National Institutes of Health (DHEW Publication 80-23) and the protocol was approved by Institutional Animal Care and Use Committee.

Experimental Design

The treatment groups and timecourse of the experiment are illustrated in Fig 1. To assess the role of GR in socially induced changes brain cell addition and electrocommunication behavior, we divided fish into four treatment groups (Fig 1A): 1) implanted with a blank silastic tube and isolated, 2) implanted with a tube filled with RU486 and isolated 3) implanted with a blank and paired with another blank-implanted fish 4) implanted with a tube filled with RU 486 and paired with another fish implanted with RU486. Hereafter, we term fish implanted with blank tubes as “control” fish. Each group contained 6–7 fish.

Because brain cell addition depends on body size (Zupanc and Horschke, 1995), fish were distributed equally among treatment groups according to body mass (within 1g). Because chirping behavior depends on the difference in EOD frequency between two interacting individuals (difference frequency) (Hupé et al., 2008), we matched all paired fish according to EOD frequency. EOD frequencies for paired fish were within 35Hz of each other.

Experimental fish were implanted with RU486, which specifically blocks GR in teleost fish (Bury et al., 2003). Control fish were implanted with an empty tube of equal length. Fish were implanted 3d before the onset of the experiment (day -3) to allow time for all GRs to become blocked and for the fish to recover from implantation prior to behavioral recordings (Fig 1B). At the beginning of the experiment (day 0) all fish were removed from their original tank and placed in a new tank that was divided into two equal compartments by a mesh divider. The divider consisted of a plastic grid (1×1 cm) covered with nylon mesh (1×1 mm) and allowed electrical, chemical and limited visual communication, but prevented physical contact. Within a pair, fish were placed on either side of the divider. (Paired fish quickly injure each other though biting if they are not separated by a divider.) Isolated fish were placed alone on one side of the divided tanks.

Three days after pairing (day 3 of experiment), all fish were injected with bromodeoxyuridine (BrdU), a thymidine analog that is incorporated into dividing cells. Fish were then sacrificed 4 d later (day 7 of experiment). This stimulus period and post-BrdU survival give sufficient time for social stimulation to promote cell proliferation and potentiate chirping behavior, and for newborn cells to differentiate into neurons in the PPN (Dunlap et al., 2006; Dunlap et al., 2008; Zupanc and Zupanc, 1992).

Administration of RU486 and BrdU

Implants consisted of silastic tubes (0.63 mm i.d. $\times$ 1.19mm o.d.; Konigsberg Instruments) sealed at one end with silicone sealant (Dow Corning) and filled with RU 486 (mifepristone, Sigma M-8046) or left blank (control). Implant length varied from 9–13 mm, depending on the length of the fish; each implant contained 0.8–1.4mg RU486. Fish were anesthetized with 2-phenoxyethanol (0.075%; Sigma, P-1126), and the implant was inserted into the peritoneal cavity through a hole made by an 18G syringe needle. Three days after pairing, fish were injected i.p. with 50–100 μ l solution of BrdU (Sigma, B-9285, 10mg/ml) at a dosage of 100 mg/kg to label newborn cells.

Recording of and analysis of electric organ discharge

To record the fish's chirping behavior and EOD frequency, all tanks were fitted with silver-coated electrodes. Signals were collected and amplified using a 16-channel audio board (MacKie 1604-VL23) and sampled at 44.1 kHz using Audacity software. EODs were recorded for 3 min periods at 29 time points over 7 d. On day 1, we recorded at 1–3 min and 4 h after pairing. Thereafter, we recorded at two time points during the daytime (5 and 9 h after lights on) and two timepoints during the night (1 and 5 h after lights off).

To detect chirps, EOD recordings were analyzed using a spectrogram in Sonic Visualizer software. We defined a chirp as an abrupt frequency rise and fall of at least 30 Hz excursion that occurred in a period < 100 ms. Usually, we identified chirps from traces of the fundamental frequency. When EOD frequencies of paired fish were too similar to distinguish which individual fish produced a chirp, we examined traces of the higher harmonics, which showed greater separation. We did not quantify chirp structure, but the vast majority ($> 90\%$) appeared to be Type 2 chirps (duration ~ 15 ms) that predominate in male-male aggression (Hupé et al. 2008).

Video recordings and analysis of locomotion

We conducted two additional experiments to quantify locomotor behavior during social interaction. The first experiment compared movement in isolated and paired fish. As above, paired fish ($n=8$) were placed on either side of a mesh barrier and isolated fish ($n = 8$) were placed alone in one half of the divided aquarium.

The second experiment examined the effect of RU486 on movement in socially interacting fish. Fish were implanted with a blank tube (controls; $n = 6$) or a tube containing RU486 ($n = 6$). The size of the implants and the implantation procedure were identical to that described above. Three days following implantation, fish were placed in size- and EOD frequency-matched pairs as above and video recorded for 5 min intervals, four times per day for 7d. Just as for the EOD recordings, video samples were taken at 5 and 9 h after lights on and 1 and 5 h after lights off.

Locomotion was recorded through a camera (Panasonic, WV-BP334) mounted above six test aquaria. For night recordings, aquaria were illuminated by four infrared lights (Clover, IR045). Within the video recordings, fish were tracked and their movement quantified using EthoVision® software. EthoVision computed the two dimensional positional coordinates of

the fish every 300 ms and, for each 5 min recording, calculated the total distance moved and the mean movement velocity. Because the distance moved in the vertical dimension was not detectable by our system, our measures likely underestimate the actual distance swam. However, in our observations, it seemed fish spend little time swimming vertically.

Immunohistochemistry and quantification of immunopositive cells

We used an immunohistochemical protocol identical to that described previously (Dunlap et al., 2006; Dunlap et al., 2008). Briefly, brains were fixed in paraformaldehyde (4%, 80 min, 4°C), frozen and sectioned by cryostat. To expose the BrdU epitope, sections were first treated with citrate buffer (5mM, 10 min, 95°C), and pepsin solution (2.5% pepsin in 0.1N HCl, 3 min, 37°C). Slides were treated sequentially with sheep anti-BrdU (Capralogics Inc.; 1:100; overnight, 4°C), biotin-conjugated donkey anti-sheep IgG (Chemicon International; 1:800; 2 h, RT), and streptavidin conjugated to Alexa 488 (Molecular Probes; 1:800; 1 h, RT).

We examined the periventricular zone (PVZ) of the diencephalon, sections 13–23 of the *Apteronotus* brain atlas (Maler et al., 1991) using a Nikon E600 epifluorescence microscope. To quantify the abundance of BrdU+ cells, we counted all labeled cells in the PVZ, which we defined as a 100 µm band surrounding the ventricle. We estimated the PVZ cross-sectional area using NIH Image J 4.0. The volumetric density of BrdU + cells per section was then calculated as the number of BrdU+ cells in each section divided by PVZ area and section thickness (30 µm).

We calculated the mean BrdU+ cell density in the PVZ of 3–5 sections in the region adjacent to the PPn (sections 17–19 in the *Apteronotus* brain atlas [Maler et al., 1991]), and in the regions just anterior and posterior to the PPn (sections 14 – 16 and 20 – 22). Each of these three regions spans an axial distance of ~100 µm. The regions anterior and posterior to the PPn served as control regions to determine the specificity of the treatment effect.

Statistical Analysis

We analyzed behavioral variables (chirp rate, EOD frequency, total distance moved and mean movement velocity) using repeated measures ANOVA, with treatment (isolated vs. paired, RU486 vs. control) and light phase (day vs. night) as independent variables, and time as the repeated measure. We used Bonferroni Multiple Comparison post-hoc test to distinguish significant differences between groups. We compared density of BrdU+ cells among treatment groups using a Kruskal-Wallis test, with a Dunn's Multiple Comparison post-hoc test to distinguish significant differences between treatment groups. All data are presented as mean ± SE, and $P < 0.05$ was considered statistically significant.

Results

Long-term social interaction influences chirp production, but does not affect EOD frequency or locomotor behavior

Paired control fish exhibited both habituation and diel cyclicity in their chirp production (Fig 2A, B). In these fish, chirp production can be divided into two phases: a) the first 5h (day 1) during which chirp rates were very high, and b) the remaining seven nights and six days, during which chirp rates were lower overall, but nevertheless higher at night than during the day. Fish chirped at highest rates in the initial sampling (1–3 min after fish were paired) and decreased over the next 4h. The mean daytime chirp rate (defined as the mean chirp rate in two day-time samples) was higher on the first day than in the remaining six days ($F = 9.3$, $P < 0.0001$). In the last 6 d, there was no significant change in mean daytime chirp rate ($F = 0.4$, $P > 0.05$). The mean nocturnal chirp rate was equivalent on all seven nights of the

experiment, indicating that fish had fully habituated within the first 9 h of pairing. However, in this second phase, fish showed significant diel changes, chirping significantly more at night than during the day ($F = 2.4$, $P < 0.05$).

Isolated control fish showed lower rates of chirp production than control paired fish (Fig 2A, B; $F = 8.2$, $P < 0.0001$). Summing over all time points, isolated fish chirped about five times less than paired fish (Fig 5B). Isolated control fish showed no change in chirp production over the 7d experiment. In the first 24h, there was no day-night difference ($F = 0.2$, $P > 0.05$), but thereafter, fish chirped more at night than in day ($F = 2.9$, $P < 0.05$). Most of the chirping in isolated fish occurred during day 4, at the two time points immediately following BrdU injection. Elevated chirp rates were also evident in paired fish at this time, suggesting that the disturbance associated with the injection transiently increased chirp rates.

Both control paired and control isolated fish showed a slight, but significant decrease in EOD frequency over the 7d experiment (mean change = -6.2 ± 1.6 Hz; $F = 1.9$, $P > 0.05$), but this decrease was similar in both groups (no significant treatment X time interaction; $F = 0.8$, $P > 0.05$). There were no diel differences in EOD frequency in paired or isolated fish ($P > 0.05$).

Both paired and isolated fish showed significant diel changes in locomotor behavior (Fig 3A, B). The total distance moved ($F = 10.7$, $P < 0.0001$) and mean velocity ($F = 9.9$, $P < 0.0001$) were 2–3 times higher during night than during day. Across the 7d experiment, locomotor behavior did not change significantly between days ($P > 0.05$), and there was no significant difference between paired and isolated fish in total distance moved ($F = 1.0$, $P > 0.05$) or mean velocity ($F = 1.4$, $P > 0.05$).

RU486 inhibits chirp production without affecting EOD frequency or locomotor behavior

Overall, paired RU486-treated fish chirped less than paired control fish (Fig 2C, D, Fig 5B; $F = 8.7$, $P < 0.0001$) and at levels that were statistically equivalent to isolated control fish ($F = 1.6$, $P > 0.05$). The complete reduction of chirping in paired RU486-treated fish indicates that socially induced changes in chirping require GR activation. Because both fish in a pair were treated with RU486, we cannot determine whether this effect only results from RU486 directly affecting the motor output of each fish independently or whether its effect is also attributable to impairment of the stimulus value of the partner fish.

In contrast to paired control fish, paired RU486-treated fish showed no significant change in chirp rate over time (Fig 2C). Thus, compared to control fish, RU486-treated fish had significantly lower chirp rates during the initial 5h daytime phase and thereafter during the night. Chirp rates did not differ significantly between night and day in RU486-treated fish (Fig 2D; $F = 1.8$, $P > 0.05$), indicating that GRs also participate in circadian patterns of chirping. Isolated RU486-treated fish did not show any significant changes in chirping behavior over time and did not differ significantly from isolated control fish (Fig 2D, $P > 0.05$).

RU486 had no effect on EOD frequency in either paired or isolated fish. Among paired fish, RU486 had no effect on difference frequency ($P > 0.05$) or any aspect of locomotor behavior (Fig 3C, D; $P > 0.05$).

RU486 inhibits brain cell addition

In paired fish, RU486 inhibits cell addition in a regionally specific manner (Fig 4A). RU486 treated fish had significantly lower BrdU+ cell density than control fish in the PVZ adjacent to the PPn (Fig 4A, 5A; $P < 0.01$), but in anterior and posterior flanking PVZ regions, there was no difference between treated and control groups (Fig 4A; $P > 0.05$). Although RU486

inhibits socially induced cell addition in the PVZ adjacent to the PPn, the effect is only partial. That is, paired RU486-treated fish still have greater BrdU+ cell density in this region than isolated control fish (Fig 5A; $P < 0.05$). Thus, social interaction is still able to have some stimulatory effect even when GRs are blocked with RU486 treatment.

In isolated fish, RU486 inhibits cell addition throughout the PVZ (Fig 4B). RU486-treated fish had lower BrdU+ cell density than control fish in both PVZ adjacent to the PPn ($P < 0.01$) and in axial regions anterior and posterior to the PPn ($P < 0.05$).

Discussion

For many species, glucocorticoid treatment and many experimental manipulations that increase endogenous glucocorticoid levels suppress cell birth or survival in the brain (Lucassen et al., 2008; Mirescu and Gould, 2006). However, in weakly electric fish, *Apteronotus leptorhynchus*, both cortisol implants and social interactions that elevate plasma cortisol enhance cell addition to the PVZ (Dunlap et al., 2002; Dunlap et al., 2006). Here we demonstrate that GR activation is a crucial step that positively links socially induced increases in plasma cortisol and brain cell addition. However, social interaction also has a partial positive effect on brain cell addition even when fish were under GR blockade, indicating social interaction also acts through other GR-independent pathways.

In contrast to its partial effect on cell addition, GR blockade apparently caused full suppression of socially induced chirping behavior and had no effect on locomotor behavior. Together, these results indicate that GR activation during social interaction can have potent and specific effects on behavior while working in parallel with other mechanisms to promote cell addition.

Our experiment was also designed to characterize the behavioral context in which social interaction influences cortisol secretion and brain plasticity. Below, we first discuss social and diurnal influences on chirping and locomotor behavior in untreated fish and then discuss the effect of GR blockade on these behaviors and cell addition.

Social interaction, electrocommunication and locomotion: the behavioral context of enhanced brain cell addition

When living in social conditions known to increase plasma cortisol levels and brain cell addition, fish produced chirps in two phases: an initial phase in the first 5 h of pairing with high rates of chirping followed by second phase (days 2–7) during which chirp rates were overall lower (Fig 2A, B). In the second phase, fish showed small but significant nocturnal increases in chirp rate. Although we previously reported that such long-term interaction potentiates the chirp response to artificial stimuli, this is the first study tracking chirping behavior in long-term interactions between electric fish.

Paired social interaction promotes both an increase in both chirping behavior (Fig 2A, B) and in cell addition adjacent to the brain region that controls chirping (Dunlap et al., 2006). However, it is clear from our present results that this socially enhanced cell addition does not cause the observed changes in chirping. Chirp rate increased immediately at the time of pairing (within seconds to minutes) and nocturnal chirp rate increased within the first 24h (Fig 2A, B), yet enhanced cell addition requires 2–3 days of social interaction (Dunlap et al., 2006). Moreover, there is no change in the diel cycles of chirping that corresponds to the onset of enhanced cell addition (Fig 2A), which occurs on days 4–7 of social interaction (Dunlap et al., 2006). Nevertheless, our previous work showing a temporal correspondence between socially enhanced cell addition and potentiated chirping to a standardized, artificial

EOD indicate that cell addition may nonetheless influence chirp response to new social stimuli.

Although enhanced cell addition does not cause the observed changes in chirping behavior, it could be the effect. That is, cell proliferation or survival might be stimulated by socially induced chirping. Such activity-dependent stimulation of neurogenesis has been reported in birds (Li et al., 2000) and mammals (Kempermann, 2009). Compared to isolated fish, paired fish chirped at higher rates throughout the 7 d period, and, in principle, either the initial pulse of intense chirping and/or the subsequent elevated daily fluctuations of chirping could stimulate cell addition. The difference between chirp rates in paired and isolated fish is much greater in the first day (5–20 fold) than in the remaining 6 d (2–3 fold), suggesting this initial phase may be particularly important for inducing cell addition. However, since only one day of social interaction is ineffective in promoting cell addition (Dunlap et al., 2006), the smaller daily differences after day 1 may be the crucial stimuli for promoting cell addition.

We examined the effect of pairing on locomotion because studies in rodents have indicated that physical activity promotes brain cell proliferation (van Praag et al., 1999; van Praag, 2008). It is conceivable that social interaction affects cell addition in *Apteronotus* by stimulating locomotor activity, rather than through its effects on electrocommunication. However, we found that paired and isolated fish differed in neither the distance nor velocity at which they swam (Fig 3). We cannot rule out the possibility that locomotion in some other experimental conditions might influence brain cell dynamics, but our results indicate that, in our experimental model, the positive effect of social interaction on brain cell addition is not attributable to changes in locomotion.

Glucocorticoid receptor blockade inhibits chirping behavior

RU486 treatment to paired fish blocked both the initial pulse of chirping and the subsequent nocturnal increases in chirping. The effect of RU486 was apparently complete: paired fish treated with RU486 chirped at levels statistically equivalent to isolated control fish at all time points of the experiment. Thus, GR binding is apparently necessary for socially induced changes in chirping behavior.

Previously, we found that, after 7 d, paired fish have higher cortisol levels than isolated fish, and that cortisol treatment to isolated fish potentiates chirping to an artificial stimulus (Dunlap et al., 2006). These results combined with those reported here indicate that socially induced elevation of endogenous cortisol contributes to elevated chirp rates. Because the chirping response to pairing is immediate, it is unlikely that cortisol directly activates chirping through a typical genomic action in this initial pulse of chirping. Rather, it likely acts at longer time scales to influence the responsiveness of the chirp-producing circuits or the production of fast-acting regulators of chirping, such as catecholamines or AVT (Bastian et al., 2001; Maler and Ellis, 1987). RU486 administered 3 d before pairing completely blocked the immediate pulse of chirping in the first minutes of pairing (Fig 2A). This suggests that blocking the action of basal cortisol prior to pairing affected the rapid chirping response at the time of pairing. Alternatively, socially induced increases in cortisol may quickly activate chirp-producing circuits through a non-genomic mechanism, as has been demonstrated for the steroid-sensitive circuits regulating vocal communication in the teleost fish, *Porichthys notatus* (Remage-Healey and Bass, 2004; Remage-Healey and Bass, 2007). Distinguishing between these possibilities will require a more detailed temporal profile of circulating cortisol levels during social interaction.

Beyond the initial phase of chirping, RU486 also blocked nocturnal increases in chirping behavior in both paired and isolated fish, indicating that both socially induced and basal

cortisol secretion participate in regulating the diurnal pattern of chirp production. Many teleost fish show diurnal changes in behavior and glucocorticoid production, and some studies indicate that in fish, as in other vertebrates, glucocorticoids affect the production of melatonin (Larson et al., 2004; Nikaido et al., 2010), an important modulator of diurnal behavioral rhythms. In electric fish, several electrocommunication and locomotor behaviors increase at night (McAnelly et al., 2003; Silva et al., 2007; Stoddard et al., 2007; Zupanc et al., 2001), but little is known about the circadian production of cortisol or its influence in regulating these behaviors.

Our measurements of EOD frequency and locomotor behavior indicate that the effect of RU486 is specific to chirping and not simply a generalized effect on activity. First, although RU486 had a strong inhibitory effect on chirp production, it had no influence on EOD frequency in paired or isolated fish. This is consistent with our previous study in which cortisol treatment to isolated fish potentiated chirping behavior, but had no effect on EOD frequency (Dunlap et al., 2002). Thus the firing rate of the pacemaker nucleus is apparently insensitive to glucocorticoids. RU486 also had no effect on frequency difference within pairs of fish, which is one of the most important determinants of chirp rate (Hupé et al., 2008). So, lower chirp rates in RU486-treated fish are not secondary to modifications in EOD frequency. Finally, RU486 had no effect on the quantity or diel pattern of locomotor behavior in paired fish, indicating its effect is not simply to depress the overall motor output or diel cyclicity of the fish. The apparent insensitivity of locomotor activity to glucocorticoid receptor blockade suggests that circadian changes in chirping and locomotor behaviors are distinctly regulated. In summary, these results indicate that GR activation is required for socially induced and nocturnal increases in chirp rate, but does not participate in other electrocommunication or locomotor behaviors.

Glucocorticoid receptor blockade inhibits socially induced brain cell addition

In paired fish, GR blockade decreased cell addition in a regionally specific manner. RU486 inhibited BrdU+ cell density in the PVZ adjacent to the PPn but not in PVZ anterior and posterior to the PPn. This result, combined with the previous report of social interaction and cortisol-treatment enhancing cell addition in a similar regionally specific manner (Dunlap et al., 2006), indicate that socially induced cortisol secretion contributes causally to social enhancement of cell addition. However, it is important to note that, while RU486 inhibits cell addition in paired fish, it does not reduce it to the low levels found in isolated fish. Our results suggest that about half of the social enhancement of cell addition is attributable to cortisol binding to GR and half is attributable to some other mechanism. Below we consider three explanations for this partial effect of RU486 on socially induced cell addition.

First, perhaps our administration of RU486 through implants did not achieve adequate dosage at the proper time to fully block the GR from cortisol activation. Our rationale for using silastic implants to deliver RU486 was to assure that the drug was available to bind GR for the duration of the experiment. Post-mortem dissection of the fish showed that, in almost all cases, the silastic implants still contained residual RU486, indicating that the delivery of RU486 continued to the end of the experiment. We implanted the RU486 3 d prior to pairing to assure that the drug had adequate time to reach stable levels and that the fish had sufficient time to recover from the procedure before entering the experiment. Our dosage was based on another study in which this dosage affected cell proliferation in the gill of another teleost fish (Scott et al., 2005). We do not know the concentration of circulating RU486 achieved by our implants and whether it completely blocked all relevant GRs. However, this dosage was apparently adequate to fully suppress socially induced chirping behavior for the duration of the experiment, demonstrating that it was strong enough to drastically alter at least some neuronal processes. If the partial effect of RU486 on cell

addition were explained by insufficient dosage, it would mean mechanisms regulating cell proliferation/survival are more sensitive to RU486 than those regulating chirp production.

A second possibility is that our administration of RU486 fully blocked GRs, but endogenous cortisol acted through an alternative receptor. Fish have multiple GRs and a mineralocorticoid receptor (MR) to which cortisol can bind (Bury et al., 2003; Greenwood et al., 2003); RU486 selectively binds GRs (Remage-Healey and Bass, 2004; Schjolden et al., 2009; Wong and Herbert, 2005). In rodents, treatment with specific receptor agonists showed that, although GR activation alone has a significant effect on hippocampal cell proliferation, co-activation of GR and MR has a much larger effect (Wong and Herbert, 2005). By analogy, it is possible that in electric fish socially induced elevation of cortisol normally stimulates cell addition through both GR and MR activation, and our treatment of RU486 yielded a partial effect because only GRs were blocked. In paired RU486 fish, endogenous cortisol may have acted through MRs to have a small positive effect. Although nothing is known about the relative contribution of GRs and MRs to regulating cell proliferation in the fish brain, both receptors participate in glucocorticoid-mediated cell proliferation in the gill (Scott et al., 2005), and thus this possibility merits further research.

Finally, although cortisol binding to MR might explain the partial effect of RU486 on cell addition, our previous work indicates that an additional socially induced factor outside the glucocorticoid system may also contribute to social regulation of brain cell addition. If cortisol activation of GR and/or MR were the only mechanism underlying socially enhanced cell addition, cortisol treatment would quantitatively mimic social interaction. However, earlier we showed that cortisol treatment to isolated fish was only about half as effective as social interaction: cortisol implants caused about a ~2-fold increase in cell addition (compared to blank-implanted fish) while paired interactions caused a four fold increase (compared to isolated fish), even though cortisol treatment yielded plasma cortisol levels comparable to those in paired fish (Dunlap et al., 2002). This “half-potency” of cortisol in boosting cell addition is quantitatively similar to the effect of RU486 in inhibiting cell addition. We do not know what this other factor might be, but we previously suggested that an activity-dependent neurotrophin produced by the PPn might link socially induced chirping and cell addition in the nearby periventricular zone (Dunlap et al., 2006). Activity-dependent production of brain-derived neurotrophic factor mediates the neurogenic response to environmental change in many species and may play a role in electric fish (Wissman and Brenowitz, 2009; Rossi et al. 2006). However, we also found that socially enhanced cell addition is still present (albeit at lower levels) in RU486-treated fish, which showed no socially induced chirping. Thus, there does not seem to be a direct relationship between PPn activity and cell addition that points to a simple activity-dependent mechanism.

Glucocorticoid receptor blockade inhibits basal rates of cell addition in a regionally non-specific manner

GR blockade also inhibited basal cell addition in isolated fish. However, in contrast to the effect in paired fish, the effect in isolated fish was equivalent across all axial levels examined. This result is consistent with previous studies showing that cortisol treatment to isolated fish tended to produce regionally non-specific effects (Dunlap et al., 2006). Although one week of cortisol treatment enhanced PVZ cell addition only adjacent to the PPn, a longer treatment increased cell addition at all axial levels. Similarly, cortisol treatment had a regionally non-specific effect on the growth of radial glia in the PVZ. Together these results indicate that the PVZ is broadly responsive to treatments (i.e. chronic implantation of cortisol implantation and RU486) that modify the tonic GR activation, but more regionally responsive social interaction, which yields greater activity in the nearby PPn.

Glucocorticoid receptors in behavioral and brain plasticity

Glucocorticoid receptors participate in the regulation of aggressive behavior in many vertebrate classes (Summers et al., 2005). In some fish species, exogenous cortisol and social interactions that increase cortisol levels inhibit aggression and locomotion (Overli et al., 2002), and it is often thought that cortisol exerts its inhibitory influence through GR binding (Gilmour et al., 2005). Indeed, DiBattista et al. (2005) showed that trout implanted with cortisol 48 h before social interaction increased the probability of subordinate behavior, which included reduced aggression and locomotion, but simultaneous implantation with RU486 reversed this effect. However, Schjolden et al. (2009) subsequently found that oral administration of RU486 inhibits aggression of trout tested 1h later. In our study RU486 inhibited aggressive signaling in electric fish when implanted 3 d before social interaction. It thus appears that the behavioral effect of GR blockade depends critically on the species, route of administration or duration of treatment.

No one has previously examined the effect of GR blockade on brain cell proliferation or survival in fish, but studies in birds and mammals show differing results. In zebra finches, as in electric fish, RU486 appears to inhibit brain cell proliferation. However, unlike electric fish, its effect is similar to that of glucocorticoid treatment, indicating that RU486 acts as a GR agonist in finches (Katz et al., 2008). In mammals, RU486 generally enhances cell proliferation (Mayer et al., 2006; Wong and Herbert, 2005), an effect consistent with the inhibitory actions of glucocorticoids on brain cell proliferation in mammals and an effect opposite to that in electric fish. In rodents, RU486 has little effect when glucocorticoids are at low concentrations, but it blocks the negative effects of glucocorticoids when their levels are high (Wong and Herbert, 2005). This indicates that stress-induced inhibition of cell proliferation is mediated through GR (Oomen et al., 2007). In contrast to the high glucocorticoid levels associated with stress, modest glucocorticoid increases in rodents during physical exercise are positively associated with brain cell proliferation (Lucassen et al., 2008). Under this treatment, which might be more analogous to the positive correlation between social interaction, cortisol and brain cell interaction in electric fish, the effect appears to be mediated through MRs. The elevated glucocorticoids during exercise act to downregulate MR, whose activation normally suppresses cell proliferation (Chang et al., 2008).

Conclusion

The results presented here combined with our previous work (Dunlap et al., 2006) show that chirping behavior and the proliferative zone that contribute cells to the brain region that controls chirping behavior are influenced by manipulations of the glucocorticoid system. Socially induced elevation of endogenous cortisol or experimental elevation of cortisol increase both chirping and cell addition to the PVZ adjacent to the PPn while GR blockade causes the opposite effect. Importantly, these treatments have specific effects, with no influence on other behaviors (i.e. EOD frequency and locomotion) or other brain proliferative zones (i.e., the PVZ anterior and posterior to the PPn). While these results provide strong evidence for the causal role of glucocorticoids in mediating socially induced changes in chirping behavior and brain cell addition, we also found that some other glucocorticoid independent mechanism also contributes the social regulation of brain cell addition. Future research should describe the profile of socially induced increases in plasma cortisol levels in more temporal detail and examine the role of MR in regulating behavioral and brain cell dynamics.

Highlights

- Electric fish living in pairs produced chirps at high rates.

- Treating paired fish with glucocorticoid receptor blocker, RU486, decreased chirp rate.
- RU486 treatment had no effect on EOD frequency or locomotion.
- RU486 decreased cell addition to the periventricular zone.
- The effect of RU486 on cell addition was partial.

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Fig.1.

Experimental design showing (A) the treatment groups and (B) the time course of the experiment. Fish were implanted with silastic capsules containing nothing (blank, controls) or the glucocorticoid receptor blocker, RU486. Paired fish were placed together in the same aquarium, but separated by a mesh barrier to prevent them from injuring each other. Isolated fish were also placed on one side of a barrier so that all fish in the experiment had the same amount of space.

Fig. 2.

Effect of treatment on chirping behavior as a function of time. Figures A & B compare paired and isolated fish; Figs C & D show the effect of RU486 in paired fish; Figs E & F show the effect of RU486 in isolated fish. Figures A, C & E show all samples (four per 24h) collected throughout the 7 d experiment. Bars along the x-axis indicate the dark phase of the light cycle. Figs B, D, & F summarize the data to illustrate the effects of habituation, diel cyclicity and treatment on chirp rate. In Fig B, “a” denotes significantly greater than all other values, indicating that fish significantly habituated to pairing after the first day. “b” denotes significantly greater than daytime values on days 2–7, indicating that both paired and isolated fish showed nocturnal increases in chirp rate after day 1. “c” denotes significantly lower values than those of paired fish at the same time point, indicating that social interaction stimulated chirp rate at all time points. In Fig D, “a” denotes significantly lower than values of paired control fish at the same time point, indicating that RU486 inhibited chirp rate throughout the first 24h and during the nights 2–7. In Fig F, “a” denotes significantly greater than chirp rates on days 2–7, indicating that isolated fish show greater spontaneous chirp rates at night than during the day. There were no significant differences between controls and RU486 treated fish at any time point.

Fig. 3.

Effect of light cycle, social interaction and RU486 treatment on locomotor behavior. All fish swam greater distances (A, C) and at greater mean velocities (B, D) at night than during the day. There was no significant effect of pairing (A, B) or RU486 (C, D) on any measurement.

A. Paired fish

B. Isolated fish

Fig 4.

Effect of RU486 on cell addition in paired (A) and isolated (B) fish at two axial region of the periventricular zone. Cell addition is the number of cells per brain volume that are born and survive for four days. The region adjacent to the PPn generates cells that migrate to the PPn; the adjacent regions serve as control areas to assess whether the effect of RU486 is regionally specific. Asterisk denotes significantly lower than control fish.

Fig 5. Effect of RU486 and social interaction on cell addition and chirping behavior. Bars with different letters are significantly different. (A) Density of BrdU+ cells in the periventricular zone adjacent to the prepacemaker nucleus. (B) Total number of chirps recorded in 29 three min samples over 7 d.