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The cortical response to sensory deprivation in adult rats is affected by gonadectomy

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Abstract

The present study investigated the effects of adult-onset sensory deprivation and gonadectomy. Adult male and female rats underwent unilateral transection of the infraorbital nerve. Half of the subjects had been gonadectomized 1 week prior to the nerve injury. We found that the areas of deprived barrels were significantly reduced when compared to barrels in the contralateral control hemisphere, and that this shrinkage was independent of sex and gonadectomy. We also found significant reductions in cytochrome oxidase staining intensity in the deprived barrels. While there were no differences in the magnitude of this effect between males and females, this effect was substantially more pronounced in the gonadectomized subjects. That is, gonadal hormones appeared to play a significant neuroprotective role in the metabolic response of the barrel cortex to deprivation. Thus, either males and females have a common neuroprotective hormonal pathway, or each has a sex-specific hormone pathway that serves an equivalent neuroprotective function.

Introduction

The neuroprotective effects of gonadal hormones have been the focus of a considerable amount of research, and the rat has provided useful models for systematically investigating these effects through both in vitro and in vivo applications. The present study was designed to evaluate the role, if any, of gonadal hormones in the response of adult rat somatosensory cortex to the permanent loss of inputs from one whisker pad. The rat posteromedial barrel subfield (PMBSF) provides an excellent model with which to study somatosensory plasticity

due to the one to one ratio which exists between the mystacial vibrissae and their cortical somatotopic representations (Woolsey et al. 1975). These “barrels” (Simons and Woolsey 1979; Welker 1971; Woolsey and Van der Loos 1970) can be revealed via a number of histological staining techniques, including cytochrome oxidase (CO) staining. It has long been established that CO staining intensity can be used as a measure of long-term metabolic activity due to the direct connection between the mitochondrial oxidative output and the amount of cytochrome *c* oxidase enzymes (Wong-Riley and Welt 1980; Wong-Riley 1989).

The current study evaluated the cortical response to infraorbital nerve (ION) transection by quantifying barrel size and long-term metabolic activity in the deprived and intact barrel cortices of intact and gonadectomized male and female rats. Because both androgen and estrogen receptors (ER) are located within the PMBSF of the somatosensory cortex (Kritzer 2002, 2004; Zsarnovszky and Belcher 2001), and estrogen has been reported to alter functional plasticity within this region (Kis et al. 2001), we hypothesized that estrogens and androgens might play a neuromodulatory role in the functional activity that can alter neuronal states following peripheral nerve injury. While estrogen and progesterone have received a majority of the research attention (Manthey and Behl 2006; Singh et al. 2008), testosterone has also been shown to exert neuroprotective effects after injury (Biaiek et al. 2004). It is important to note that these forms of neuroprotection have traditionally been very acute in nature. By using CO staining intensity as a measure of long-term neuronal metabolism, we planned to determine the extent to which gonadal hormones altered functional properties of chronically deprived neurons. In this way we could begin to investigate “neuroprotection” offered by gonadal hormones outside of acute events.

Despite studies demonstrating that the brain’s hormone receptors function through the intracellular *de novo* synthesis of neurosteroids (Baulieu and Robel 1990; Rune and Frotscher 2005; Prange-Kiel and Rune 2006), many brain receptor systems also rely on normally circulating levels of gonadal hormone (for review see Altman 2004). Therefore, we were interested in how the gonadectomized animal’s sensory cortices might be affected compared to controls as a function of chronic decreases in circulating gonadal hormones. Previous studies (e.g. Andò et al. 1988; Alagwu and Nneli 2005) have demonstrated that circulating levels of gonadal hormones decreased rapidly after gonadectomy and remained at decreased levels over chronic durations. Thus, rats used in this study were given a short adjustment period between gonadectomy and nerve transection (1 week) to ensure that levels of circulating gonadal hormones had decreased prior to nerve injury. Furthermore, the chronic nature of the hormonal decreases demonstrated in these earlier studies suggested that hormone levels would remain lowered throughout the duration of the present study (8 weeks).

Methods

Subjects

Adult male ($n = 25$) and female ($n = 24$) Long-Evans Hooded rats approximately 60 days of age at the start of the experiment (Harlan Laboratories, Indianapolis, IN, USA) were housed in same-sex groups of three with unrestricted access to water and food throughout the experiment. Subjects were maintained in a temperature-controlled environment (20–23°C)

with a 12:12 light/dark cycle. All procedures followed the National Institutes of Health (NIH) guide-lines and were approved by the Bloomington Institutional Animal Care and Use Committee.

Surgeries

Orchidectomy—Adult male Long-Evans Hooded rats ($n = 13$) were orchidectomized (OCX) using methods previously employed in our laboratory (Calvert et al. 2003). Briefly, rats were anesthetized with isoflurane gas, and the scrotum was cut bilaterally. Each testicle was extracted and the blood vessels were ligated. Each testicle was then completely removed, and the scrotal sack was stapled shut.

Ovariectomy—Adult female Long-Evans Hooded rats ($n = 12$) were ovariectomized (OVX) using methods previously employed in our laboratory (Goodman et al. 2004). Briefly, female rats were anesthetized with isoflurane gas, and 2 cm incisions were made bilaterally at the dorsal/ventral midsection just posterior to the ribcage, extending through the muscle wall. Each ovary was fully extracted and ligated. The ovaries were then completely removed. The muscle incisions received two stitches with suture and the skin was stapled closed. All animals received 7 days of recovery prior to ION transection.

Infraorbital nerve transection—All subjects underwent unilateral transection of the ION. Animals were anesthetized with isoflurane gas, the surgical site was shaved and sterilized, and a 1 cm incision was made posterior to the whisker pad at the level of the infraorbital foramen. The ION was located with the use of a dissecting microscope and elevated with a Roth hook. The nerve was then tied with surgical ligature and transected. The nerve was cut anterior to the ligature and any excess nerve was folded on itself and tied with surgical ligature again to prevent reinnervation of the whisker pad. The 1 cm incision was closed with two stitches and topical antibacterial ointment was applied to the surgical area. Post-mortem dissections were conducted on all subjects to confirm the absence of reinnervation.

Histology

Eight weeks after ION transection rats were euthanized by urethane overdose (0.25 g/100 g) via intraperitoneal injection. Rats were then perfused transcardially with 9% saline, 4% paraformaldehyde, 10% sucrose in 0.1 M phosphate buffer (PBS, pH 7.2), and 20% sucrose in PBS. All animals received roughly equal volumes of perfusates. Brains were then removed and submerged in 30% sucrose in PBS until they sank (~2 days). In order to visualize the entire whisker representation in as few slides as possible, the cortex of each hemisphere was removed and placed in the center of a Plexiglas flattener in 4% paraformaldehyde for 1 h prior to sectioning. Flattened cortex was sliced tangentially to the PMBSF in 40 μ m sections on a microtome. Sections were collected in PBS and then reacted in a CO staining solution. The staining protocol was adapted from Wong-Riley (1979), and consists of 600 ml PBS (room temperature), 60 g of sucrose, 0.3 mg of cytochrome *c* (Sigma), 0.1 mg of catalase (Sigma), and 0.075 mg of 3,3'-diaminobenzidine- 4HCl (Sigma). Brains were reacted in groups of 12 that included animals from all experimental groups. All sections were incubated for approximately 1 h before being rinsed in PBS three

times. Following treatment, tissue was mounted on pig-gel subbed slides, dehydrated in ascending alcohols, and cover-slipped. Great care was taken to ensure that staining solutions were exact and tissue sections were incubated for the same amount of time across staining sessions.

Tissue analysis

Tissue sections were analyzed at 140× under bright field illumination using a microscope (Nikon Eclipse 80i; Nikon Instruments; Melville, NY, USA) with the Stereo-Investigator (MBF Bioscience; Williston, VT, USA) software. This software allowed the assessment of histological parameters including area measurements and tissue luminance. The luminance function provides assessment of the amount of light passing through a given area of tissue. Great care was taken to set the base light level (luminance through the blood vessels) at a consistent setting between users and user sessions (~119 on a scale of 0–256). Generated data had extremely small variance in this regard. Consistency in staining procedure produced similar levels of staining within non-barrel tissue, despite variation in staining quality that can arise through differences during perfusion. Data was collected by users blind to the sex and experimental group of the animals. Barrels one through five across rows A–E of the (PMBSF) were analyzed in the left (control) and right (deprived) whisker barrel representations as revealed by CO staining. Both barrel area and staining intensity measurements were generated by manually tracing around the entire perimeter of the barrel ($0.138/\text{mm}^2$). This border is salient because of the much lighter septal regions that surround the barrel. Single barrels were never observed on more than two sections. Because the cutting plane was never perfectly tangential, when necessary barrel contours were carefully reconstructed over these two sections. The software allows the modification of contours. Barrel areas constructed over adjacent sections were first aligned using (1) blood vessel contours, which remain consistent between adjacent sections, and (2) any previously drawn barrel contours. The contour of interest would then be modified by manually adjusting vector points generated by the software. Barrel areas were calculated by the software through the display contour measurements function after all barrels were drawn. Intensity measurements were generated by tracing the contour of each barrel while using the luminance function. For reconstructed barrels, intensity measurements were quantified using the average staining intensity between the two sections. Staining artifacts were always avoided. Due to lighter staining along the perimeter of the slices, intensity measurements were never taken from barrels located adjacent to the edge of the tissue. These barrels were consistently available for proper intensity quantification on the next slice. Tissue thickness was consistent due to stringent parameters surrounding slicing and dehydration during cover-slipping. Barrel areas and staining intensities were analyzed using ANOVA with sex and gonadal status (intact or gonadectomy) as between-subject variables and deprived versus non-deprived status as a within-subject variable.

Results

Figure 1 presents representative lower and higher power photomicrographs of topographically matched portions of deprived and non-deprived barrel field cortex from a

castrated male subject. The barrel representing whisker C3 is denoted for comparison. The quantified data reported below are reasonably well-represented by these sections.

Whisker barrel area

Whisker barrels were measured by manually tracing barrels one through five in rows A through E. Statistically, we find a significant main effect of deprivation [$F(1,36) = 46.43$, $P < 0.001$] reflecting the overall reduction in deprived barrel area of $13.8 \pm 1.7\%$. As illustrated in Fig. 2a, this effect was relatively stable across experimental groups, with reductions of $15.1 \pm 2.8\%$ in intact males ($t = 5.09$, $P < 0.05$), $16.9 \pm 6.0\%$ in OCX males ($t = 2.78$, $P < 0.05$), $12.2 \pm 2.5\%$ in intact females ($t = 3.81$, $P < 0.05$), and $11.2 \pm 2.7\%$ in OVX females ($t = 2.47$, $P < 0.05$). Thus, the deprivation-induced decrease in area of deprived barrels relative to control barrels was neither sex [$F(1,36) = 0.23$, $P > 0.10$] nor hormone specific [$F(1,36) = 0.024$, $P > 0.10$]. There were also no significant interactions.

Staining intensity

For analyzing CO staining intensity, raw values in the contralateral barrel fields were scaled relative to ipsilateral barrel fields to control for the reductions in barrel areas caused by the deprivation. Overall, we found a $28.2 \pm 3.5\%$ reduction in CO staining intensity in deprived barrels, and this difference is reflected by a statistically significant main effect for deprivation status [$F(1,37) = 88.29$, $P < 0.001$]. As illustrated in Fig. 2b, the effect of deprivation on CO staining intensity was not stable across experimental groups. Whereas the mean percent reductions were 22.0 ± 6.14 and $18.0 \pm 6.23\%$ for gonadally intact males and females, respectively, the comparable values are 46.0 ± 8.07 and $31.2 \pm 6.98\%$ for the gonadectomized males and females, respectively. These differences are reflected in a significant interaction between hormonal condition and deprivation status [$F(1,37) = 4.55$, $P < 0.05$]. The strength of this hormonal effect is underscored by the outcome of an ANOVA on the percent difference values whose means are illustrated in Fig. 2b. In this case, the main effect for hormone status is statistically significant [$F(1,37) = 7.97$, $P < 0.01$]; however, it should be noted that there was neither a significant main effect for sex [$F(1,37) = 2.04$, $P > 0.10$] nor a sex by hormonal status interaction [$F(1,37) = 0.68$, $P > 0.10$]. Finally, in order to verify that changes in barrel staining intensity were due to a deprivation effect in the contralateral hemisphere and not to increased staining intensity in the intact, ipsilateral barrel cortex, barrels from the hindlimb region were compared to whisker barrels from the PMBSF in both intact and experimental hemispheres. There was a clear pattern of decreases in staining intensity in the deprived whisker barrels relative to hindlimb barrels across all groups [$F(1,27) = 14.2$, $P < 0.01$]. Alternatively, in the ipsilateral, control hemisphere, staining intensities were very comparable between hindlimb and PMBSF [$F(1,27) = 0.08$, $P > 0.10$].

Discussion

We report area measurements and CO staining intensity measurements within the PMBSF of male and female Long-Evans Hooded rats that received infra-orbital nerve transections with or without gonadectomy. We find that there is a decrease in whisker barrel area relative to controls following sensory deprivation through ION transection, and that this effect is not

modulated by the sex or the hormonal status of the subject. We also find CO staining intensity is reduced in deprived barrels relative to controls. Finally, this latter effect is modulated by the hormonal status of the subjects. Gonadectomized animals show substantially greater reductions in CO staining intensities in deprived barrels than do the gonadally intact animals.

Whisker barrel area

In an earlier report, Wong-Riley and Welt (1980) reported no changes in barrel area in a small number of adult mice following peripheral whisker cauterization or plucking. The incongruity in these results could be due simply to the small ($n = 2$ in 3 experimental groups) sample sizes employed by Wong-Riley and Welt (1980), as they were principally interested in evaluating the developmental consequences of early onset deprivation. Alternatively, the apparently different outcomes could be due to differences in anatomy that exists between the mouse and rat PMBSF. In mice, barrels consist of cell dense sides with dendritic branches extending into the cell sparse barrel hollow (Pasternak and Woolsey 1975; Woolsey and Van der Loos 1970). In rats, these features are organized in a completely different way. Cell bodies are packed in the center of the barrels with their dendritic branches extending outward toward the cell sparse barrel sides (Welker and Woolsey 1974). It seems most likely that the reduced barrel areas reported here for rats reflects the retraction of the dendritic arbors of deprived neurons, as this is a typical anatomical reaction to chronic activity loss following disease and injury (e.g. Luo and O'Leary 2005). If so, it is interesting that we find no effect of the hormonal manipulations on barrel size given studies that report a potent effect of gonadal hormones on dendritic formation and retraction in male and female animals (for reviews see Cooke and Woolley 2005; Parducz et al. 2006).

CO staining intensity

We report reductions in CO staining intensity in the deprived barrels of all subjects. In contrast to the barrel area data, however, we find that the hormonal status of the subjects contributes substantially to the magnitude of this effect. The reduction in CO staining intensity caused by the deprivation is exacerbated by gonadectomy in both male and female subjects. Thus, it appears that hormones were playing a neuroprotective role in the deprived barrels of the gonadally intact subjects.

Previous studies have reported that males and females have non-specific localizations of androgen and ER throughout somatosensory cortex (Kritzer 2002, 2004; Zsarnovszky and Belcher 2001). These neuromodulatory receptors act through fast membrane receptor activation (Moore and Evans 1999) as well as genomic pathways (Spelsberg et al. 1989). After injury or disease onset, steroid hormones exert their neuroprotective effects through these genomic and non-genomic pathways (Bryant et al. 2006). In the brain, these effects mainly occur in the form of decreases in cell death, the preservation of neural activity, and attenuation of neurodegenerative processes.

In the current study there was no significant difference in CO intensity decreases seen for gonadectomized males over gonadectomized females. This could mean that males and females have a common neuroprotective hormone pathway or that each has a gender specific

hormone pathway that serves an equivalent neuroprotective function. Both the testosterone and ER are reported to mediate oxidative processes in the mitochondria, but this depends on cell type and location within the body (Gavrilova-Jordan and Price 2007; Psarra and Sekeris 2008). While testosterone serves cytoprotective functions through the mitochondria in non-neural cells (Cardiac Tissue—Er et al. 2004), it is less clear as to whether it serves the same function within the brain. Estrogen on the other hand does seem to exert mitochondrial neuroprotective functions within the brain for both males and females (Razmara et al. 2007).

A connection can be made between the preservation of long-term metabolic activity, as revealed by CO staining intensity (Wong-Riley 1989) and the ER, alpha and beta, that are localized within the mitochondria (Chen et al. 2004; Chen and Yager 2004; Yang et al. 2004). Under acute durations of neuronal stress (e.g. infarct or seizure) mitochondrial ER serve beneficial oxidative functions that protect the cell from excitotoxicity and neurodegeneration by suppressing transcriptional pathways involved with apoptosis and free radical oxidation. On a longer timeline, such as the one used in this experiment, one could hypothesize that ER modulation of oxidative functions would continue to facilitate mitochondrial function. This would presumably increase CO staining intensity through increased long-term metabolic activity, as these have been shown to be related to one another (Wong-Riley 1989). This could account for the “preservation” of metabolic activity reported in our intact animals if somatosensory hormone receptor activation normally requires circulating gonadal hormones, as opposed to neurosteroids, and if both males and females have a common ER pathway. Further studies are underway to address these questions.

Conclusion

We report hormone dependent changes in CO staining in male and female rats that received unilateral ION transections under conditions of hormone depression (gonadectomy). Given that CO staining is an indirect measure of long-term metabolic activity (Wong-Riley 1979), the results have strong implications toward the consequences of hormone decreases on metabolic function. Based on the recent discovery of the connection between the neuroprotective effects of hormone receptor activation and mitochondrial modulation of oxidative output (for review see Klinge 2008), our results suggest that apart from the traditionally investigated paradigms involving acute effects, such as apoptosis (specifically for ER see Amantea et al. 2005), hormones can also serve neuroprotective functions under conditions of chronic cortical deprivation. Support for this comes from some human studies that demonstrate the benefits of hormone replacement/treatments on neurodegenerative disease and stroke (Garcia-Segura et al. 2001; Behl 2002). Taken together, the rat whisker barrel field offers a powerful scientific tool with which to investigate gonadal hormone-mediated neuroprotective processes both acutely and chronically, and could prove to have far reaching scientific applications toward the understanding of aging, injury, and neurodegenerative diseases.

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Fig. 1. PMBSF of deprived and non-deprived hemispheres. The upper images are taken from the intact (*left side*) and deprived (*right side*) hemispheres of a gonadectomized male. Corresponding higher power images are displayed on the *bottom*. Barrel *C3* is labeled in all micrographs to permit visual comparisons of the two hemispheres

Fig. 2.

a The relative changes in barrel size between deprived and intact PMBSF. Deprivation via transection of the infraorbital nerve resulted in a significant reduction in whisker barrel area that did not significantly differ between groups. **b** The relative change in PMBSF cytochrome oxidase staining intensity between gonadectomized and intact animals. There is a significant decrease in CO staining intensity across all groups; however, gonadectomized male and female CO staining intensities are also significantly decreased compared to

controls. This difference is not sex-related, as the apparent neuroprotective effects of hormones were equivalent in males and females (* $P < 0.05$)