Conversion from testosterone to oestradiol is required to modulate respiratory long-term facilitation in male rats

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Sex hormones modulate plasticity in the central nervous system, including respiratory long-term facilitation (LTF), a form of serotonin-dependent respiratory plasticity induced by intermittent hypoxia. Since gonadectomy (GDX) attenuates LTF in male rats, we tested the hypotheses that: (1) testosterone replenishment restores LTF in gonadectomized male rats, and (2) that the conversion of testosterone to oestradiol (under the influence of aromatase) is required for these effects. Intact and sham operated male F344 rats were compared to gonadectomized rats implanted with Silastic tubing containing testosterone (T), T plus an aromatase inhibitor (ADT), or 5α-dihydrotestosterone (DHT), a form of testosterone not converted to oestradiol. Seven days postsurgery, LTF was studied in anaesthetized, neuromuscularly blocked and ventilated rats while monitoring integrated phrenic and hypoglossal (XII) motor output. LTF was elicited by three 5 min hypoxic episodes ($P_{a,O_2} = 35-45$ mmHg). Although significant phrenic and XII LTF were observed in all rat groups, GDX reduced both phrenic and XII LTF, an effect reversed by T. In contrast, LTF was not restored in T + ADT or DHT-treated gonadectomized rats. We conclude that the conversion of testosterone to oestradiol modulates phrenic and XII LTF in male F344 rats.

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Long-term facilitation (LTF) is a form of respiratory plasticity expressed as a persistent augmentation of phrenic and/or hypoglossal (XII) nerve activity following intermittent hypoxia. The fundamental mechanisms of LTF have been studied extensively in recent years (Mitchell *et al.* 2001; Feldman *et al.* 2003). Spinal serotonin (5-HT) receptor activation during hypoxic episodes initiates LTF (Fuller *et al.* 2001; Baker-Herman & Mitchell, 2002), whereas new synthesis of brain derived neurotrophic factor (BDNF) maintains it (Baker-Herman *et al.* 2004). Thus, factors that affect serotonergic function and BDNF synthesis may have the capacity to modulate LTF.

Sex hormones influence respiratory plasticity. For example, hypoglossal LTF is (1) reduced by gonadectomy in male rats (Zabka *et al.* 2005), (2) correlated with serum testosterone levels in male rats (Zabka *et al.* 2005), and (3) correlated with serum oestradiol and progesterone levels in female rats (Zabka *et al.* 2003). Sex hormones enhance serotonergic function in many brain regions (Aylward, 1973; Poirier *et al.* 1985; Lopez-Jaramillo & Teran, 1999; Bethea *et al.* 2000; Klink *et al.* 2002; Kugaya *et al.* 2003). Thus, gonadectomy in male rats reduces 5-HT concentration in the caudal raphe nuclei (Long *et al.* 1983), and decreases 5-HT immunoreactivity in the XII motor nucleus (M. Behan, unpublished observations). In female rats, serotonin concentrations vary with the oestrus cycle in phrenic and hypoglossal motor nuclei, and are associated with oestrus-cycle related differences in phrenic and hypoglossal LTF (Zabka *et al.* 2001*b*; Behan *et al.* 2003). Consequently, sex hormones have the potential to modulate hypoglossal and phrenic LTF via effects on serotonergic function within the respective motor nuclei.

Sex hormones also influence BDNF gene expression (Gibbs, 1999; Scharfman *et al.* 2003; Zhao *et al.* 2004; Scharfman & MacLusky, 2005). Since BDNF plays a key role in LTF (Baker-Herman *et al.* 2004), oestradiol and progesterone may influence LTF by altering BDNF expression in respiratory motor nuclei. Furthermore, since BDNF exerts complex trophic effects on serotonergic neurons (Mamounas *et al.* 1995, 2000; Mattson *et al.* 2004), and serotonin receptor activation increases BDNF synthesis within respiratory motor nuclei (Baker-Herman *et al.* 2004), sex hormone effects on BDNF and serotonin may have complex, interactive effects on respiratory plasticity.

Since oestrogen modulates many forms of neuroplasticity (Woolley & McEwen, 1994; Murphy *et al.* 1998; Brinton, 2001; Scharfman *et al.* 2003), and exerts potent effects on both 5-HT and BDNF, an important question is whether the effect of testosterone on respiratory

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LTF in male rats is direct, or indirect by conversion to oestradiol due to aromatase activity. For example, testosterone influences 5-HT2A receptor expression in the rat forebrain only through the conversion of testosterone to oestrogen (Sumner & Fink, 1998). In this study, we tested the hypotheses that decreased LTF following gonadectomy in male rats can be reversed by restoring normal circulating testosterone levels, and that this effect requires the conversion of testosterone to oestrogen under the influence of aromatase activity. Differential influences of testosterone and oestrogen on respiratory plasticity may contribute to an understanding of sexual dimorphisms in age-related human breathing disorders, such as age and sex-specific manifestations of obstructive sleep apnoea (Redline *et al.* 1994; Ware *et al.* 1999; Bixler *et al.* 2001).

Methods

Experimental groups

Experiments were performed on young adult male Fischer 344 rats (National Institutes of Health, National Institute of Ageing Colony). Six groups of male rats (3–4 months old) were used for this study: intact, unoperated (Intact, n (phr) = 7, n (XII) = 6), sham operated (Sham n (phr) = 6, n (XII) = (8), gonadectomized (GDX n (phr) = 9, n (XII) = (6), gonadectomized with testosterone supplement (T, n (phr) = 8, n (XII) = 8), gonadectomized with testosterone and an aromatase inhibitor (ADT, n (phr) = 8, n (XII) = 11), and gonadectomized with 5α-dihydrotestosterone supplement (DHT, n (phr) = 10, n (XII) = 8), a form of testosterone that cannot be converted to oestradiol. n differed between phrenic and XII analyses since only one neurogram was usable for data analysis in some rats. All experimental procedures were approved by the University of Wisconsin–Madison Animal Care and Use Committee.

Gonadectomy

Anaesthesia was induced with isoflurane in an induction chamber and maintained (2.0–2.5% isoflurane in 30% O_2) using a nose cone. All operated rats received 0.05 mg kg^{-1} buprenorphine I.M. prior to surgery to prevent postsurgical pain. Rats were positioned in dorsal recumbency on a heated pad. The scrotum was clipped and cleaned with an antiseptic detergent. A skin incision of 1 cm was made on the raphe scroti followed by a 0.5 cm incision in the muscle layer over the testicles. Testicles were removed by separating them distally from a ligature around the ductus deferens and accompanying blood vessels with a scalpel. The muscle layer and the skin incision were closed with reabsorbable suture and staples, respectively.

Hormone implants

Silastic® laboratory tubing (Dow Corning, Midland, MI, USA) was used for testosterone (crystalline testosterone (T), Steraloids, Inc. (Newport, RI, USA) and aromatase inhibitor (crystalline 1,4,9-androstatriene-3,17-dione (ADT), Steraloids implants. Prior to implantation, T and ADT implants were submerged in 0.1 mol l^{-1} phosphate buffered saline at room temperature for 24 h. GDX and Sham rats received one empty Silastic implant (T implant size). DHT rats received one pellet of 5α-dihydrotestosterone (10.0 mg per pellet, 21 day release, Innovative Research of America, Sarasota, FL, USA) subcutaneously.

Two pilot studies were conducted to determine the number of implants that would produce serum hormone levels in the middle of the physiological range 7 days following surgery (GDX and hormone replacement). In pilot studies, physiological serum testosterone levels in intact animals were 11.7 ± 4.7 and 11.6 ± 3.5 ng ml^{-1}, respectively (range 1.7–27.7 and 1.8–26.8 ng ml^{-1}, respectively; $n = 3$). In gonadectomized rats, two testosterone implants (inner diameter 0.16 cm, outer diameter 0.32 cm, length 3 cm) per rat resulted in testosterone levels of 5.3 ± 0.3 ng ml^{-1}. One ADT (inner diameter 0.15 cm, outer diameter 0.20 cm, length 3 cm) and one testosterone implant resulted in testosterone levels of 5.0 ± 0.2 ng ml^{-1}. Two ADT implants and one testosterone implant resulted in testosterone levels of 7.3 ± 0.4 ng ml^{-1}. Based on these pilot experiments, for the T group of rats we implanted three testosterone implants per rat. For the ADT group of rats, we implanted three ADT and one testosterone implant per rat.

Hormone replacement

Following gonadectomy or sham surgery, rats were placed in ventral recumbency. One cm distal to the shoulder blades, the skin was clipped and cleaned with an antiseptic detergent in an area 2 cm × 2 cm. A skin incision of 1 cm was made parallel to the spine. The skin was undermined bluntly and Silastic implants or DHT pellets were inserted. The incision was closed using reabsorbable suture (Vicryl, 3–0). Seven days postgonadectomy, after initiating anaesthesia for the acute neurophysiological experimental protocol (LTF protocol), blood was collected to assess levels of testosterone, 5α-DHT, oestrogen and progesterone. Hormone levels were compared to levels in intact, unoperated animals that were used for the neurophysiological experiments.

Experimental preparation

Methods have been extensively described in previous publications (Bach & Mitchell, 1996; Baker & Mitchell,

2000; Fuller *et al.* 2001; Zabka *et al.* 2001*a*,*b*, 2003, 2005). In brief, animals were anaesthetized with urethane, subjected to neuromuscular blockade (pancuronium bromide, 2.5 mg/kg I.V.), bilaterally vagotomized and pump ventilated. Blood samples (0.2 ml in a 0.5 ml heparinized glass syringe) were drawn to determine arterial blood gases (P_{a,O_2} and P_{a,CO_2}), pH and base excess (ABL 500; Radiometer, Copenhagen, Denmark). Body temperature was maintained between 37 and 38°C using a heated table. End-tidal CO_2 was measured with a flow-through capnograph (Capnogard, Novametrix, Wallingford, CT, USA). The left phrenic and XII nerves were isolated via a dorsal approach, cut distally, desheathed, submerged in mineral oil and placed on bipolar silver wire electrodes. Nerve activities were amplified (×10 000), band pass filtered (100 Hz to 10 kHz) (Model 1700, A-M Systems, Inc., Carlsborg, WA, USA) and integrated (time constant = 50 ms, Model MA-821RSP, CWE Inc., Ardmore, PA, USA).

Experimental protocol

Before recording, phrenic and XII nerve activities were allowed to stabilize for approximately 90 min following surgery under hyperoxia and normocapnia ($P_{a,O_2} >$ 150 mmHg). Subsequently, the CO_2 apnoeic/recruitment threshold was determined and baseline nerve activities were established 2 mmHg above this threshold. Baseline blood gas values were assessed before starting the protocol. All subsequent blood samples were compared to this initial baseline value. Strict isocapnia (± 1 mmHg from baseline P_{a,CO_2}) was maintained throughout an experiment. An LTF protocol started with three 5 min hypoxic episodes ($F_{I,O_2} = 0.11$, target P_{a,O_2} 35–45 mmHg) separated by 5 min intervals, and followed by 60 min of isocapnic hyperoxic baseline conditions. A protocol ended with 5 min of hypercapnia ($P_{ET,CO_2} = 80-90$ mmHg) to assess maximal hypercapnia-stimulated nerve activity. Arterial blood samples were drawn during the last minute of the first hypoxic episode to determine the severity of hypoxia, and 15, 30 and 60 min after the final hypoxic episode to confirm isocapnic conditions. Rats were excluded from analysis if P_{a,CO_2} deviated from baseline by more than 3 mmHg during hypoxia or 1 mmHg during post-hypoxic episodes from baseline. Therefore, changes in P_{a,CO_2} had minimal impact on the results of this study. Experiments were also excluded if arterial blood pressure dropped by more than 30 mmHg from baseline at the end of a protocol. At the end of each experiment, rats were killed with an overdose of urethane (I.V.).

Sex hormone levels

Arterial blood samples (1 ml) were taken as soon as the arterial catheter was placed. Subsequently, blood samples were centrifuged to collect serum. Serum was immediately frozen at −80°C. After collection of all serum samples, total testosterone, oestradiol and progesterone levels were analysed using radioimmunoassay (RIA) (Testosterone Coat-a-Count, Oestradiol Coat-a-Count, Progesterone Coat-a-Count; Diagnostic Products, Los Angeles, CA, USA). 5α-Dihydrotestosterone was analysed using enzyme-linked immunosorbent assay (ELISA) (Immuno-Biological Laboratories Inc., Minneapolis, MN, USA). Prior to analysing the samples, the assays were validated with pooled serum from 10 rats to create a standard curve.

Data analysis

Phrenic and XII nerve activities were recorded throughout the protocol. Peak integrated amplitude (ΔPhr and ΔXII), burst frequency (bursts min^{-1}), and mean arterial blood pressure (MAP) were measured at the following time points: baseline, last minute of first hypoxic episode (short-term hypoxic response), 15, 30 and 60 min after the final hypoxic episode, and the last minute of the hypercapnic response (max CO_2 response; Fig. 1). Nerve activity was averaged over approximately 60 s at each measurement. Changes in amplitude from baseline were normalized as a percentage of baseline nerve activity (% baseline), and as a percentage of the hypercapnic response (% maximum). All conclusions were the same, regardless of the normalization used. Thus, only percentage baseline data are presented in this paper. Changes in burst frequency were expressed as a difference from baseline in bursts per minute.

Depending on the variable, either a one-way or a two-way ANOVA with a repeated measures design (SigmaStat v. 2.0, Jandel Corporation, San Rafael, CA, USA) was performed, followed by *post hoc* inferences for individual comparisons using the least significant difference test (Fisher's LSD method). If the normality test failed, a Kruskal–Wallis one-way ANOVA on ranks was performed followed by an all pair-wise multiple comparisons test (Dunn's Method) if differences were significant. Differences were considered significant if $P < 0.05$. All data reported are means ± S.E.M. Serum levels of testosterone, 5α-DHT, oestradiol and progesterone in individual rats were related to the magnitude of phrenic and XII LTF via multiple and/or simple linear regressions. A variable was considered to contribute significantly to the model if $P < 0.05$.

Results

Experimental animals

Mean weights of rats in all groups were statistically indistinguishable, either at the time of surgery, or at the time of acute LTF studies (range: 263–300 g; $P > 0.05$).

Sex hormone levels

After gonadectomy, serum testosterone levels were reduced, although to a lesser extent than in previous studies from our laboratory (Behan *et al.* 2003; Zabka *et al.* 2005). Serum testosterone levels were at the high end of the physiological range in T and ADT rats (2.34–18.2 and 5.03–11.4 ng ml^{-1}, respectively) (Fig. 2*A*). Serum testosterone levels were significantly higher in T and ADT rats than in GDX or DHT rats ($P < 0.05$), but not different from Intact or Sham animals ($P > 0.05$; Fig. 2*A*). Serum oestradiol and progesterone levels were not significantly different among groups ($P > 0.05$; Fig. 2*A*). Serum 5α-DHT levels were similar in T and ADT rats but significantly greater than in Intact, Sham, GDX and DHT rat (Fig. 2*B*).

Apnoeic/recruitment threshold, baseline conditions and CO_2 regulation

Baseline conditions were standardized through individual determinations of individual CO_2 apnoeic/recruitment thresholds, and establishing baseline conditions 2 mmHg above this level. Overall, the CO_2 apnoeic/recruitment threshold did not differ among rat groups (Intact = 41 ± 2; Sham = 41 ± 2; GDX = 41 ± 1; T = 40 ± 1; ADT = 39 ± 1; DHT = 41 ± 1 mmHg; $P > 0.05$). Throughout protocols, strict isocapnic conditions were maintained to prevent CO_2-based changes in phrenic or XII nerve activity. The ratio of baseline/maximal CO_2 response was similar in all rat groups for phrenic and XII nerve activity indicating a similar dynamic range and baseline ventilatory drive in all rats (Phrenic: $P > 0.05$; XII: $P > 0.05$).

Short-term hypoxic responses

P_{a,O_2} during hypoxic episodes did not differ among rat groups ($P > 0.05$). Phrenic and XII short-term hypoxic response amplitude when expressed as a percentage change from baseline (Table 1), and frequency when expressed as a change from baseline (bursts min^{-1}) were not different among groups (all $P > 0.05$). Thus, sex hormone changes have little effect on phrenic or XII short-term hypoxic responses in anaesthetized rats.

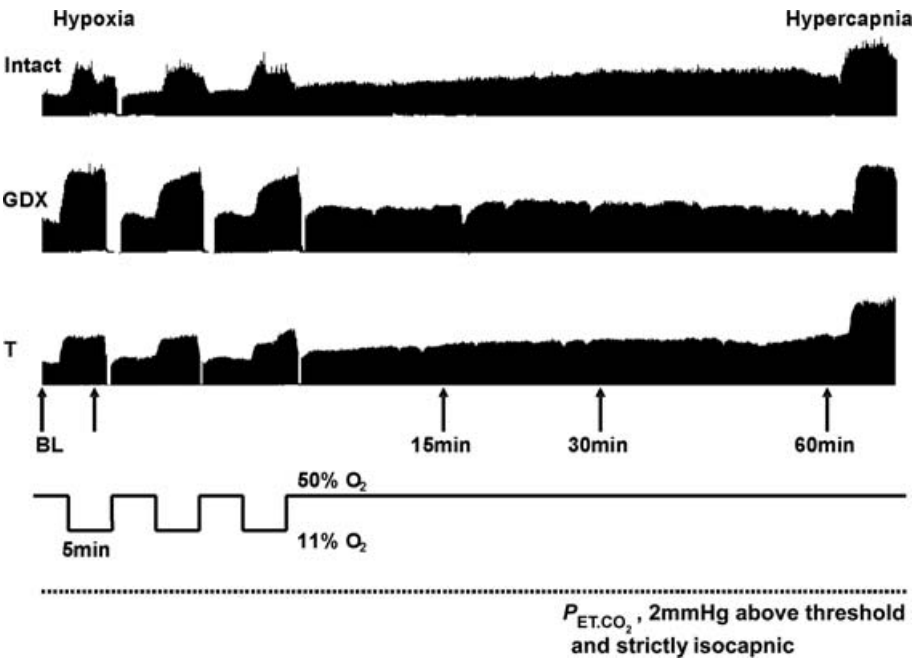

Figure 1. Representative phrenic LTF protocols from Intact, GDX and GDX-T rats
After establishing baseline conditions, three episodes of hypoxia (11% O_2) were applied. Arterial blood samples for blood gas analysis (arrows) were taken under baseline conditions (BL), during the last minute of the first hypoxia, and 15, 30 and 60 min after the last hypoxic episode. A protocol ended with 5 min of hypercapnia to assess maximal nerve activity. The upper panel shows a compressed tracing from a young adult male rat revealing an LTF magnitude typically seen in young male rats. Tracings from Intact and Sham rats were comparable (not shown). The middle tracing shows reduced LTF in a gonadectomized (GDX) young male rat. Tracings from GDX and T/ADT or DHT rats were comparable (not shown). The bottom tracing shows LTF in a gonadectomized rat supplemented with testosterone (T). P_{ET,CO_2}: end-tidal CO_2

Table 1. Short-term hypoxic responses

	Phrenic amplitude (% change from baseline)	XII amplitude (% change from baseline)
Intact	65 ± 11	161 ± 22
Sham	48 ± 2	143 ± 17
GDX	64 ± 14	130 ± 21
T	76 ± 18	169 ± 16
ADT	59 ± 9	150 ± 18
DHT	75 ± 11	155 ± 23

Phrenic long-term facilitation (LTF)

All six groups of rats revealed phrenic LTF (ΔPhr significantly increased *versus* baseline; Δ percentage BL) and showed a significant time–treatment interaction ($P = 0.003$). In individual groups, LTF was observed in Intact, GDX, T and DHT animals at 15, 30 and 60 min, in Sham animals at 30 and 60 min, and in ADT animals at 15 and 30 min following episodic hypoxia ($P < 0.001$; Fig. 3*A*). The magnitude of phrenic LTF was significantly different among groups at 30 and 60 min post-episodic hypoxia ($P < 0.05$) such that T rats had significantly greater LTF compared to Sham, GDX, ADT, and DHT rats at 30 and 60 min ($P < 0.05$). Furthermore, at 60 min post-hypoxia, Intact rats developed greater LTF than ADT and DHT rats ($P < 0.05$); LTF in Sham animals was greater than ADT ($P = 0.027$). Intact and T animals were not different at any time post-episodic hypoxia.

XII long-term facilitation

All six groups of rats revealed XII LTF (ΔXII significantly increased *versus* baseline; Δ percentage BL) and showed a significant time–treatment interaction ($P = 0.008$). In individual groups, LTF was observed in Intact, Sham, GDX, T and DHT animals at 15, 30 and 60 min, and in ADT animals at 15 and 30 min following episodic hypoxia ($P < 0.001$; Fig. 3*B*). The magnitude of XII LTF was significantly different among groups at 30 and 60 min post-episodic hypoxia ($P < 0.05$). T rats had significantly greater LTF compared to GDX and DHT rats at 30 min ($P < 0.03$), and compared to GDX, ADT and DHT rats at 60 min ($P = 0.001$). Furthermore, at 30 min, Sham showed greater LTF than GDX rats ($P = 0.018$) and at 60 min, Intact and Sham rats developed greater LTF than GDX, ADT and DHT rats ($P < 0.04$). Intact, Sham and T animals were not different at any time post-episodic hypoxia.

Burst frequency LTF

There was a small but significant increase in burst frequency *versus* baseline post-hypoxia (i.e. frequency LTF) including all groups at 30 and 60 min and in T animals only at 15 min ($P < 0.05$; data not shown). LTF in T animals was significantly greater *versus* DHT at 15 min, *versus* ADT at 30 min, and *versus* GDX at 60 min ($P < 0.05$).

Sex hormone levels correlate with LTF

There was no correlation between the magnitude of phrenic LTF and any sex hormone measured in serum at the beginning of experiments (Fig. 4*A*). However, there was a weak, but significant positive correlation between XII LTF and serum testosterone in Intact, Sham, GDX and T rats, but not in DHT and ADT rats (Fig. 4*B*). The linear model expressing this relationship was:

$$\text{Xii ltf} = 52.635 + (3.595t)r^2 = 0.208,\ P = 0.015$$

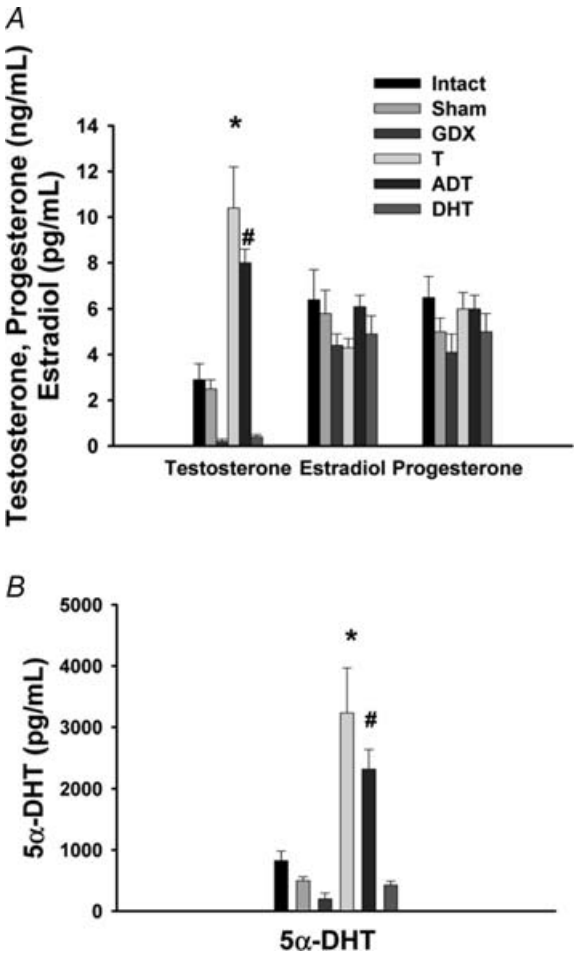

Figure 2. Serum levels of testosterone, oestradiol, progesterone and 5α-DHT during baseline conditions
A, testosterone levels (ng ml^{-1}) were significantly greater in T (*) and ADT (#) rats than in GDX or DHT rats ($P < 0.05$), but not different from Intact or Sham animals ($P > 0.05$). Oestradiol levels (pg ml^{-1}) and progesterone levels (ng ml^{-1}) did not differ among groups ($P > 0.05$). *B*, 5α-DHT levels (pg ml^{-1}) were significantly greater in T (*) and ADT (#) rats than all other groups ($P < 0.05$). Values are means ± S.E.M. from all animals used for XII LTF analysis.

Mean arterial blood pressure (MAP)

MAP did not differ between rat groups at any time before, during or following hypoxic episodes ($P > 0.05$; data not shown). During hypoxic episodes, MAP decreased significantly in all groups, as typically observed during hypoxia in anaesthetized rats.

Discussion

The major finding of this study is that phrenic and XII LTF are restored by testosterone replacement in gonadectomized male rats, and that this effect requires the conversion of testosterone to oestrogen by the actions of aromatase. This latter conclusion is supported by observations that testosterone replacement with an aromatase inhibitor, and application of a testosterone derivate that is not converted to oestrogen, fail to restore LTF in either neurogram. We speculate that testosterone-derived oestrogen exerts indirect effects on the serotonergic system and/or BDNF within the respective respiratory motor nuclei, thereby restoring phrenic and XII LTF.

Sex hormone levels

In this study, gonadectomy failed to decrease serum testosterone levels significantly as observed previously in our laboratory (Behan *et al.* 2003; Zabka *et al.* 2005). Such interstudy variations may result from: (1) incomplete gonadectomy, particularly in animals with testosterone levels > 1 pg ml^{-1}; or (2) relatively low serum testosterone levels in the Intact and Sham rats used in this study (range

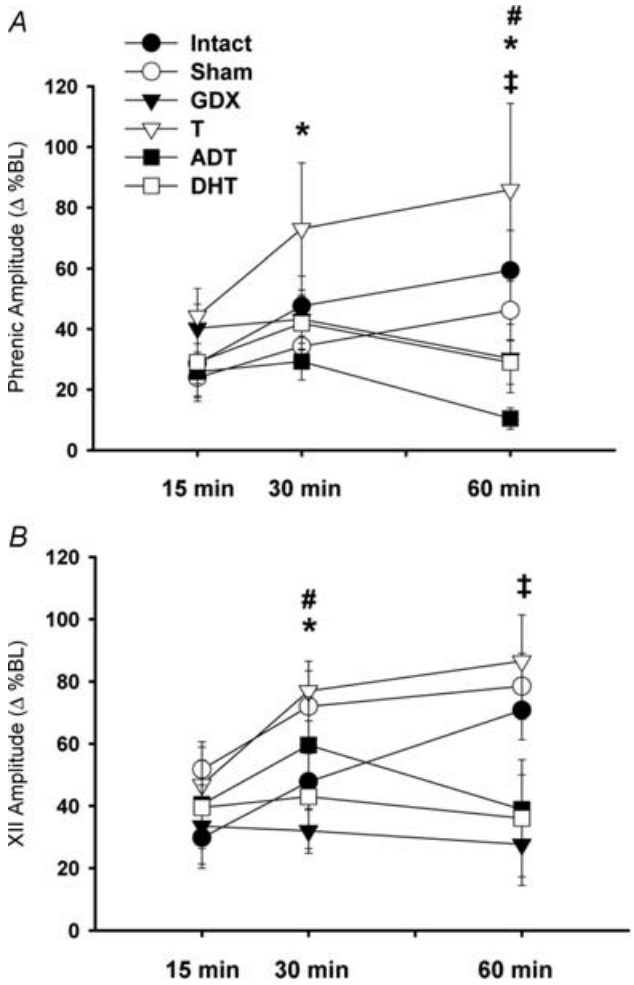

Figure 3. Long-term facilitation (LTF) measured at 15, 30, and 60 min post-episodic hypoxia
A, at 30 and 60 min, phrenic LTF in T (*) was significantly greater than in Sham, GDX, ADT and DHT rats. At 60 min, phrenic LTF in Intact (#) was significantly greater than in ADT and DHT rats, and LTF in Sham (‡) was significantly greater than in ADT rats (all $P < 0.05$). *B*, at 30 min, XII LTF in T (*) was significantly greater than in GDX and DHT rats. LTF in Sham (#) was significantly greater than in GDX rats (all $P < 0.05$). At 60 min, XII LTF in Intact, Sham and T (‡) was significantly greater than in GDX, ADT and DHT rats (all $P < 0.05$).

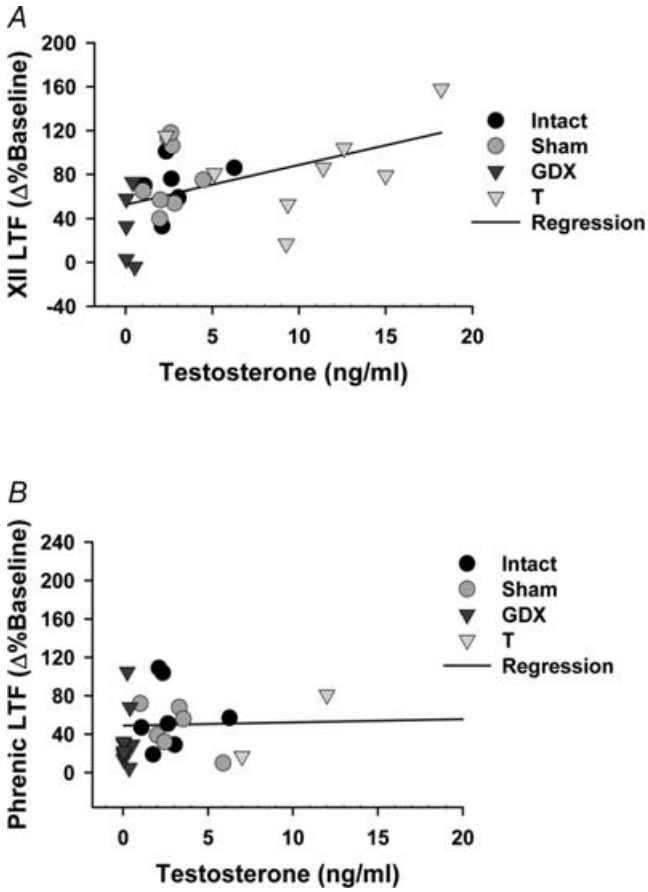

Figure 4. Correlation of Phrenic and XII LTF with serum testosterone levels
A, the magnitude of XII LTF measured in Intact, Sham, GDX, T and DHT rats was positively correlated with serum testosterone levels ($R^2 = 0.208$; $P = 0.015$). *B*, no correlation was observed between phrenic LTF in and serum testosterone levels ($R^2 = 0.0495$; $P = 0.237$).

1.08–6.27 and 1.01–5.89 ng ml^{-1}, respectively) compared with levels measured in two pilot studies (see Methods), although serum testosterone levels in the present study are similar to levels reported in several other studies (Dohler & Wuttke, 1974; Smith *et al.* 1992; Neill, 2006). Nonetheless, significantly greater serum testosterone levels in T and ADT rats compared with GDX or DHT rats that were not different from Intact or Sham animals suggest that reasonable and appropriate manipulations of sex hormone levels were achieved in this study (Fig. 2*A*). Since serum oestradiol and progesterone levels were not significantly different among groups, differences in circulating levels of these hormones cannot explain differences caused by testosterone replacement. However, this finding does not rule out localized differences in oestrogen and progesterone levels in other body compartments, such as the central nervous system. On the other hand, serum oestradiol levels are quite low in male rats, and are at the edge of the detectable range using our assay procedures. Thus, potential increases in serum oestradiol may have been obscured by a lack of sensitivity in the bioassay and/or by oestradiol binding to globulin and albumin (Davison *et al.* 2005). In a study by Robaire *et al.* (1979), testosterone implants of differing strengths failed to alter serum oestradiol levels in male rats.

Short-term hypoxic responses

Phrenic and XII short-term hypoxic responses were unaffected by sex hormones, as shown previously in this rat strain (Zabka *et al.* 2005). Other studies have demonstrated that the short-term ventilatory and/or carotid chemoafferent neuron responses to hypoxia are influenced by sex hormones in some species (Schlenker & Goldman, 1985; Hannhart *et al.* 1990; Tatsumi *et al.* 1994, 1997). Apparent discrepancies between our study and previous reports might arise from species and/or experimental preparation differences, or may be due to the direct assessment of respiratory motor output in the phrenic and XII nerves in this study *versus* measurements of carotid sinus nerve activity and breathing.

Long-term facilitation (LTF)

Earlier studies on Sprague-Dawley and F344 rats report that XII LTF is reduced by gonadectomy (Behan *et al.* 2003; Zabka *et al.* 2005) whereas phrenic LTF is unaffected (Zabka *et al.* 2005). Consistent with these earlier studies, gonadectomy reduced XII LTF in the present experiments. On the other hand, in contrast to these earlier reports, we found that phrenic LTF is also reduced following gonadectomy in the present study. This discrepancy in the phrenic LTF response to gonadectomy in the two studies is difficult to explain. In general, there is greater variability in reported phrenic motoneuron responses to age and/or sex hormones in male and female rats, whereas XII responses have been more consistent (Zabka *et al.* 2001*a*,*b*, 2003, 2005; Behan *et al.* 2002, 2003). Both motoneuron pools have robust expression of androgen and oestrogen receptors (Behan & Thomas, 2005), leading us to speculate that their differing responses to sex hormones may be regulated at the level of neuromodulatory inputs such as 5-HT in the caudal raphe nuclei. Since increased androgen levels enhance aromatase activity (Roselli, 1991; Wagner & Morrell, 1997; Roselli *et al.* 1998; Bourguiba *et al.* 2003), pharmacological testosterone administration may enhance its own conversion to oestradiol, and the possibility exists that this response is differentially expressed in phrenic and XII motor nuclei, thereby explaining differential responses in phrenic and XII LTF.

Correlation between sex hormones and LTF

Serum testosterone levels correlated more robustly with XII *versus* phrenic LTF, suggesting that testosterone has a greater impact on plasticity in motoneurons innervating upper airway *versus* respiratory pump muscles. These male F344 rats demonstrated positive correlations between XII LTF and testosterone, the ratio testosterone/oestradiol and the ratio testosterone/progesterone. Female rats show a stronger correlation of XII (*versus* phrenic) LTF with serum oestrogen and progesterone levels (Zabka *et al.* 2003). In contrast to XII LTF, phrenic LTF was not significantly correlated with any sex hormone or hormone ratio (this study; Zabka *et al.* 2005). Reasons for this difference in the correlation with sex hormones in XII and phrenic LTF are not known. However, an important caveat is that sex hormone levels measured in serum may not accurately reflect hormone levels in respiratory-related motor nuclei. Measurements of aromatase activity or measurements of testosterone and oestradiol levels in XII and phrenic nuclei could provide important insights concerning the testosterone-derived oestradiol levels achieved. Thus, additional investigations are necessary to define the mechanistic basis of differential hormone influence on respiratory motoneuron pools.

Possible mechanisms

Serotonergic neurons in the caudal raphe nuclei project to the XII and phrenic motor nuclei (Manaker *et al.* 1992; Manaker & Tischler, 1993), and there is some suggestive evidence that sex hormones modulate respiratory plasticity indirectly via the serotonergic system. For example, gonadectomy decreases 5-HT immunoreactivity in the XII nucleus of young male Sprague-Dawley rats (M. Behan, unpublished observations). Thus, depletion of gonadal hormones may decrease serotonergic input to the XII and/or the phrenic motor nuclei, thereby

diminishing LTF. Androgen effects on the serotonergic system are thought to be indirect, reflecting the conversion of testosterone to oestradiol in the CNS (Celotti *et al.* 1991; Fink *et al.* 1998; this study). Depletion of oestradiol decreases the synthesis, release, reuptake and degradation of serotonin, and decreases serotonin terminal and receptor density in several brain regions (Aylward, 1973; Poirier *et al.* 1985; Sohrabji *et al.* 1995; Gibbs, 1998, 1999; Lopez-Jaramillo & Teran, 1999; Kugaya *et al.* 2003; Scharfman *et al.* 2003; Blurton-Jones *et al.* 2004). Thus, oestradiol may be a key player in modulating serotonergic function, thereby influencing respiratory LTF.

BDNF is another molecule critical to respiratory LTF (Baker-Herman *et al.* 2004) that is regulated by oestradiol. Oestradiol directly affects BDNF gene expression via an oestrogen response element encoding sequence in the BDNF gene (Sohrabji *et al.* 1995). Hippocampal BDNF protein fluctuates in parallel with oestradiol levels during the oestrus cycle in female rats (Scharfman *et al.* 2003). Oestrogen and progesterone supplementation elevates BDNF in multiple brain regions of ovariectomized rats (Gibbs, 1999). In male rats, increases in spinal BDNF protein due to chronic pain are attenuated by gonadectomy (Zhao *et al.* 2004). Thus, the regulation of BDNF expression by testosterone-derived oestradiol has the potential to modulate LTF. Testosterone-derived oestradiol could alter BDNF expression directly, or indirectly by actions on serotonergic function (Baker-Herman *et al.* 2004).

Oestradiol could also affect respiratory plasticity by direct effects on motoneurons or interneurons or glia in respiratory motor nuclei. Androgen and oestrogen receptors are present in identified XII and phrenic motoneurons (Behan & Thomas, 2005). By acting on these receptors, sex hormones could enhance synaptic density, as shown in other CNS regions (Woolley & McEwen, 1994; Brinton, 2001; Leranth *et al.* 2004). Oestrogen also causes changes in GABAergic interneurons, transiently inhibiting the limiting enzyme for GABA synthesis, and up-regulating glutamatergic and GABAergic synapses onto hippocampal neurons (Murphy *et al.* 1998). A similar mechanism is possible in respiratory motor nuclei, as both GABAergic and glutamatergic inputs play key roles in regulating respiratory motoneuron activity (McCrimmon *et al.* 1997).

Significance

Respiratory LTF provides an important model, enabling the investigation of sex hormone effects of on neuroplasticity in general, and respiratory plasticity in particular. By studying mechanisms underlying the effects of sex hormones on the control of breathing, we may gain new insights into breathing disorders with distinct age/sex patterns, such as obstructive sleep apnoea (OSA). OSA is most prevalent in middle-aged men and postmenopausal women who are not taking hormone replacement therapy (Hla *et al.* 1994; Redline *et al.* 1994; Ware *et al.* 1999; Bixler *et al.* 2001; Young *et al.* 2003). Besides anatomical factors such as narrowed airways and obesity, OSA is caused by relaxation of upper airway muscle tone during sleep, accompanied by prolapse of the tongue and soft palate, which obstructs the upper airway (Partinen *et al.* 1988). By stiffening the upper airway muscles, respiratory LTF may serve to stabilize breathing under conditions that would otherwise lead to upper airway obstruction and apnoea.

Although a specific role for LTF (or diminished LTF with age) has not been clearly identified as a causal factor in OSA, diminished capacity for LTF due to decreasing sex hormone levels with ageing may contribute to the progression of this syndrome. The occurrence of OSA is increased in men with low serum testosterone levels (Luboshitzky *et al.* 2002; Meston *et al.* 2003), in middle aged men, and in women with low oestrogen and progesterone levels (Netzer *et al.* 2003). Interestingly, administration of testosterone to individuals with low testosterone levels actually worsens the symptoms of OSA (Matsumoto *et al.* 1985; Cistulli *et al.* 1994), possibly suggesting a defect in the conversion of testosterone to oestradiol in these individuals. In conclusion, low androgen levels are correlated with greater occurrence of OSA in men, but testosterone application does not protect against OSA. In contrast, oestrogen appears to protect against OSA in women, as postmenopausal women on hormone replacement therapy (HRT) are less likely to have OSA than similarly aged women without HRT (Bixler *et al.* 2001; Young *et al.* 2003). Ultimately, testosterone may be essential to prevent OSA in men, but only if it can be converted to oestradiol. Thus, it may be that the critical deficit in middle-aged men with OSA is an inability to produce oestradiol in the CNS due to inadequate aromatase activity. This hypothesis remains to be tested.

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