In vitro binding assays using ^{3}H nisoxetine and ^{3}H WIN 35,428 reveal selective effects of gonadectomy and hormone replacement in adult male rats on norepinephrine but not dopamine transporter sites in the cerebral cortex

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Abstract

The prefrontal cortices mediate cognitive functions that critically depend on local dopamine levels. In male rats, many prefrontal tasks where performance is disrupted by changes in dopamine signaling are also impaired by gonadectomy, a manipulation that increases cortical dopamine concentration, prefrontal dopamine axon density and possibly extracellular prefrontal dopamine levels as well. Because these actions could be responsible for the impairing effects of gonadectomy on prefrontal function, the question of how they might arise comes to the fore. Accordingly, the present studies asked whether dopamine levels might be increased via a hormone sensitivity of transporter mediated dopamine uptake. Specifically, [3]HWIN 35,428 and [3]Hnisoxetine, ligands selective for the dopamine (DAT)- and norepinephrine transporter (NET) respectively, were used in *in vitro* binding assays to ask whether gonadectomy altered transporter affinity (Kd) and/or binding site number (Bmax) in prefrontal cortex, sensorimotor cortex and/or caudate. Assays performed on tissues dissected from sham-operated, gonadectomized and gonadectomized rats supplemented with testosterone propionate or estradiol for 4 or 28 days revealed no significant group differences or obvious trends in Kd or Bmax for DAT binding or in measures of Bmax for NET binding. However, affinity constants for [3]Hnisoxetine were found to be significantly higher in sensorimotor and/or prefrontal cortex of rats gonadectomized and gonadectomized and supplemented with estradiol for 4 or 28 days but similar to control in gonadectomized rats given testosterone. Because the NET contributes substantially to extracellular prefrontal dopamine clearance, these androgen-mediated effects could influence prefrontal dopamine levels and might thus be relevant for observed effects of gonadectomy on dopamine-dependent prefrontal behaviors. A hormone sensitivity of the NET could also have bearing on the prefrontal dopamine dysfunction seen in disorders like schizophrenia that disproportionately affect males, whose severity correlates with abnormal testosterone levels, and for which the NET is among suspected sites of pathology.

Keywords

prefrontal cortex; estrogen; androgen; working memory; schizophrenia; ADHD

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The prefrontal cortices mediate highest order executive and mnemonic functions including working memory and behavioral flexibility (Goldman-Rakic, 1990, Dalley et al., 2004). It is well known that these complex operations depend on local prefrontal dopamine (DA) levels being maintained within certain limits. In rats, for example, chemical lesions of prefrontal DA afferents, cortical infusions of selective DA D1 receptor agonists and antagonists and pharmacological and stress-induced increases in prefrontal DA turnover have all been shown to produce significant deficits in prefrontal-dependent behaviors and tasks including open field activity, acquisition of T-maze delayed alternation, behavioral flexibility, and novel object recognition (Tassin et al., 1978, Kessler and Markowitsch, 1981, Kalsbeek et al., 1989, Stam et al., 1989, Murphy et al., 1996, Verma and Moghaddam, 1996, Zahrt et al., 1997, Morrow et al., 2000). More recent studies, however, have shown that performance in each of these tasks is also impaired by gonadectomy in adult male rats (Adler et al., 1999, Kritzer et al., 2001, Kritzer et al., 2007, Aubele et al., 2008). Because this is a manipulation that has also been shown to increase cortical DA axon density (Kritzer et al., 1999, Kritzer, 2000) and concentration (Battaner et al., 1987) it is possible that the effects of gonadectomy on cognitive and mnemonic information processing are related to its dysregulation of prefrontal DA systems. Further, more recent evidence of sex differences in extracellular prefrontal DA levels (Shansky et al., 2006) combine with preliminary in vivo microdialysis data from this lab (Aubele et al., 2008) showing that GDX increases basal prefrontal DA overflow to suggest more specifically that the behaviorally deleterious consequences of GDX could be related to stimulation of supranormal extracellular levels of DA in the prefrontal cortex. Although this could come about by several means, this study focused on the possibility that prefrontal DA levels might be affected via hormone regulation/gonadectomy-induced dysregulation of transporter-mediated DA uptake, a process that along with catabolic enzymes and other mechanisms is involved in setting prefrontal DA tone.

In the prefrontal cortex, transporter-mediated DA uptake uniquely involves both the dopamine (DAT) and norepinephrine transporters (NET) whose low local levels (Sesack et al., 1998a, Sesack et al., 1998b, Miner et al., 2003) facilitate the unusually long-lived extracellular DA levels/lifetimes that are hallmark of the frontal cortex and essential for its function (Carboni et al., 1990, Elsworth et al., 1993, Cass and Gerhardt, 1995, Yamamoto and Novotney, 1998, Moron et al., 2002, Valentini et al., 2004, Carboni et al., 2006). As key controllers of the functionally pivotal parameter of prefrontal DA level, it may not be surprising that both the DAT and the NET have also been implicated in disorders including ADHD and schizophrenia (Cook et al., 1995, Gill et al., 1997, Laakso et al., 2001, DeLisi et al., 2002, Hsiao et al., 2003, Madras et al., 2005, Rybakowski et al., 2006) where prefrontal deficits and prefrontal hyper- and hypodopaminergia, respectively, are prominent parts of the clinical scenario (Shaywitz et al., 1977, Davis et al., 1991). That in both disorders the DA-dependent prefrontal processes at risk are not only disproportionately so males (Goodman and Stevenson, 1989, Seeman and Lang, 1990, Leviton et al., 1993) but are linked to anomalous adult, adolescent and/or intrauterine levels of testosterone (Shirayama et al., 2002, Goyal et al., 2004, Taherianfard and Shariaty, 2004, Akhondzadeh et al., 2006, de Bruin et al., 2006) gives further impetus to focus investigation of the hormone sensitivity of the DAT and NET specifically on males. To begin this line of inquiry, this study used the selective ligands ^{3}H WIN 35,428 and ^{3}H nisoxetine and *in vitro* homogenate binding assays to examine the effects of gonadectomy and hormone replacement in adult male rats on the affinity and maximal numbers of ligand binding sites of the DAT and NET, respectively, in the medial prefrontal cortex, the sensorimotor cortex and in the caudate nucleus. For purposes of comparison, these assays were conducted on tissue samples micro-dissected from separate groups of rats that were sham-operated, gonadectomized and gonadectomized and supplemented with testosterone propionate or estradiol for 4 or 28 days according to methods used in previous studies that have identified highly selective and differentially estrogen- and androgen-sensitive effects of long and short term gonadectomy on the anatomical organization, physiology and function of

mesoprefrontal DA systems in adult male rats (Adler et al., 1999, Kritzer et al., 1999, Kritzer, 2000, Kritzer, 2003, Kritzer et al., 2007). Some of this work has appeared in abstract form (Aubele et al., 2008).

EXPERIMENTAL PROCEDURES

Animal Subjects

Adult male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used. All animals were housed with food and water freely available under a 12-hour light/dark cycle and were gonadectomized, with and without replacement with 17- β-estradiol or testosterone propionate (below), or sham operated 4 or 28 days prior to euthanasia. Pilot studies in gonadally intact animals determined that due to the small sizes of the prefrontal samples that could be discretely dissected from individual animals and the low numbers of cortical transporter sites, the DAT assays required tissue pooled from 8 rats per group and the NET assays required tissue pooled from 5 rats per group. All procedures involving animals are approved by the Institutional Animal Care and Use Committee of Stony Brook University, and minimize their use and discomfort.

Surgical Procedures

Gonadectomy and sham surgeries were performed under aseptic conditions using ketamine (. 09ml/100g) and xylazine (.05ml/100g) for anesthesia. For both operations, the sac of the scrotum and underlying tunica were incised; for gonadectomies, the vas deferens was also bilaterally ligated and the testis were removed. For gonadectomized, hormone-replaced rats, slow-release pellets (below) were implanted within the scrotal sac. Incisions were closed using sterile surgical staples; for rats with longer survival times these were removed after 10 days.

Hormone Treatments

Subsets of gonadectomized animals were implanted with slow release pellets that contained either testosterone propionate or 17-β-estradiol (Innovative Research of America, Toledo, OH). The testosterone propionate-containing pellets used have been used in previous studies in this laboratory and others (Kritzer, 2000, Turvin et al., 2007) and have been shown to release between 0.5–2ng of TP per milliliter of blood per day; likewise, the estradiol-containing pellets used were also identical to those used previously (Kritzer, 2000, Turvin et al., 2007) and known to release between 10–40pg of E per milliliter of blood per day.

Euthanasia

Four or twenty eight days after gonadectomy or sham surgery, rats were weighed and euthanized by rapid decapitation. Immediately afterward the brains were removed. For the NET assays, brains were rapidly frozen in powdered dry ice and stored at −80 deg. C until assays were performed. For DAT assays, brain regions of interest (medial prefrontal cortex, sensorimotor cortex and caudate nuclei, see Fig. 1) were dissected out over ice and used immediately. To maximize consistency, all dissections were performed by the same person (MFK). The bulbospongiosus muscles (BSM) were also dissected out and weighed in all of the subjects at the time of euthanasia.

NET: ^{3}H Nisoxetine Binding Assay

a. Tissue preparation: Frozen brains were partially thawed and bilateral samples of medial prefrontal cortex, sensorimotor cortex and caudate nucleus were dissected out over ice (Fig. 1). Tissues from 5 rats per group were pooled and homogenized using a motor-driven Ultra Turrax homogenizer (IKA Works, Inc, Wilmington, NC) for 10 sec, in 10 ml of ice-cold 50 mM phosphate buffer, pH 7.4. The homogenate was then centrifuged at 20,000g for 10 min at

4°C and the supernatant was discarded. The pellet was then re-suspended and re-centrifuged as above in 10 ml of ice-cold 50 mM phosphate buffer, pH 7.4 and recollected. After this process was repeated, the final, washed pellet was re-suspended at a concentration of 60mg wet weight/ml in incubation buffer (50mM Tris Hcl, pH 7.4, 120 mM $NaCl_2$, 5 mM KCl).

b. Assay conditions: Uptake sites were analyzed by full saturation isotherms according to methods adapted from Tejani-Butt (1992) and optimized in pilot studies using tissue from gonadally intact sham-operated control animals and initially using 8 concentrations of ^{3}H nisoxetine (Perkin Elmer, Boston, MA, 87.2 Ci/mmol) ranging from 0.25–40 nmol. Because specific binding plateaued at ligand concentrations of less than 10 nmol, subsequent saturation assays used an 8-ligand concentration range of 0.25–14 nmol. Each assay point was run in triplicate with replicates measuring total binding consisting of 100μl tissue homogenate, 50μl radioligand, and 350 μl cold incubation buffer and those measuring non-specific binding also including 1μM tomoxetine (Sigma Chemical Co, St. Louis, MO). Each saturation isotherm was repeated a total three times for each brain region using tissue from three different sets of animal subjects.

Assays were conducted at 4deg C, and were terminated after 4 hours by rapid filtration using a 48-well cell harvester (Brandel Inc, Gaithersburg. MD) and Whatmann GF/B filter mats followed by 3 rapid rinses with ice-cold incubation buffer. The filters were then dissolved in 10 ml of scintillation fluid (EcoLite, MP Biomedicals, Irvine, CA) and radioactivity was counted in a Beckmann-Coulter Liquid scintillation counter (Beckman Instruments, Inc. Fullerton, CA).

DAT: [3]H WIN35,428 Binding Assay

a. Tissue preparation: Immediately upon brain removal, bilateral samples of medial prefrontal cortex, sensorimotor cortex and caudate nuclei were dissected out over ice (Fig. 1). Tissues were pooled from 8 animal subjects and were homogenized and centrifuged as per the NET assay above. The final pellet was re-suspended at a concentration of 100mg wet weight/ml in ice-cold incubation buffer consisting of 15mM HEPES, 127mM NaCl, 5 mM KCl, 1.2 mM Mg_2SO_4, 2.5mM $CaCl_2$, 1.3 mM NaH_2PO_4 and 10 mM D-glucose- pH 7.4.

b. Assay Conditions: Full saturation isotherm assays were performed according to methods adapted from Izenwasser et al., (1993) and optimized in pilot studies using tissue from gonadally intact, sham-operated control rats and 8 concentrations of ^{3}H WIN35,428 (Perkin Elmer, 85.9Ci/mmol) that ranged from 0.25–40 nmol. These analyses showed that specific binding plateaued at ligand concentrations of 10 nmol or less. Subsequent assays thus used an 8-ligand concentration range of 0.25–14 nmol. Each assay point was run in triplicate with replicates measuring total binding consisting of 350μl incubation buffer, 50μl tissue homogenate, and 100μl ligand; non-specific binding was determined from replicated that additionally included 1μM nomifensine. All assays were conducted at 4deg C for 1.5 hours and were terminated by rapid filtration and rinsing with the filters subsequently dissolved in 10 ml of scintillation fluid (EcoLite) and radioactivity counted as per the NET assay above. Each saturation isotherm assay was repeated a total of three times using tissue from three independent groups of animal subjects.

Protein Quantification

Protein content was measured according to the method of Bradford (1976) using a Bio Rad Protein Assay Kit (BioRad Life Sciences, Hercules, CA). Samples of tissue homogenates used in binding assays were diluted 8-fold and measured at 595 nm in a Nanodrop 1000 Spectrophotometer (Nanodrop Technologies, Inc. Wilmington, DE). Sample protein concentrations were calculated in relation to standard curves generated using 0.2–1.4 mg/ml

of bovine gamma globulin (provided with the kits); these values were used to normalize all binding data prior to analysis (below).

Data Analysis

Specific binding, equilibrium dissociation constants (Kd), and maximum number of binding sites (Bmax) were all calculated in three separate assays from triplicate measures of total and non-specific binding data (normalized for protein, above) determined at each of eight ligand concentrations for each animal group and tissue. These values were obtained using a commercial nonlinear regression analysis/iterative curve fitting routine and always included comparisons of fit for one- versus two-site binding models (Prism, GraphPad Software Inc, San Diego, CA). Affinity and Bmax values obtained from the three separate assays were evaluated using descriptive statistics and compared per tissue across animals and animal groups using the non-parametric Kruskal- Wallis test to identify significant main effects of hormone treatment and the Mann-Whitney U test (with Bonferroni correction) to identify significant between group differences. Bulbospongiosus muscle weights were assessed within and across groups using parametric analyses of variance and allowed post-hoc testing that used the Fisher's PLSD. For all statistical assessments, a $p < 0.05$ level was accepted as significant (Stat View, 4.5).

RESULTS

Efficacy of Hormone Treatments

The dissected weights of the androgen-sensitive bulbospongiosus muscles showed expected differences between the GDX and GDX-E groups on the one hand and the control and GDX-TP groups on the other (Fig. 2). Although absolute differences in the lower mean muscle weights of the GDX and GDX-E groups compared to the higher weights of the sham-operated control and GDX-TP animals (Fig, 2A) were greater for the 28-day compared to the 4-day treatment groups (Fig, 2B), analyses of variance (ANOVA) identified significant main effects of hormone treatment on muscle mass at both time points [animals used for DAT assays: 4 day $F_{(3,28)} = 15.28$, $p < 0.0001$; 28 day, $F_{(3,28)} = 174.51$, $p < 0.0001$; animals used for NET assays: 4 day $F_{(3, 16)} = 6.13$, $p < 0.0056$; 28 day, $F_{(3, 16)} = 49.97$, $p < 0.0001$]. Allowed post hoc analyses further identified significant differences in muscle mass between the GDX and GDX-E groups compared to the GDX-TP rats and sham-operated controls, but no significant differences in muscle mass between the GDX and GDX-E groups or between the control and GDX-TP cohorts (Fig. 2).

DAT Binding

All data from the ^3H WIN 35,428 saturation assays were analyzed using both one and two-site binding models (Prism GraphPad Software). For all tissues and all animal groups, comparisons of convergence and goodness of fit with respect to these two models revealed concordance/ congruence only with the one-site model and data that either did not converge or that showed an extremely poor goodness of fit (r^2 values of 0.1 or less) for the two-site model. Graphical representations of the saturation data and their Scatchard transformations were also consistent with binding at single sites (e.g., Fig. 3).

In both the 4- and 28-day control groups, Kd values for ligand binding sites were between 6 and 8 nmol for all three tissues evaluated (Figs. 3, 4A, C). These values were similar to those previously reported for ligand binding affinity in fresh rat brain homongenates (Katz et al., 2000). The corresponding Kd values that were obtained in the gonadectomized and hormone-supplemented groups were all found to be similar to control values. Statistical analyses further confirmed that there were no significant effects of hormone treatment (Kruskal Wallis) or

significant group differences (Mann-Whitney U) aong any of the ligand binding affinity values measured in the either cortical or subcortical tissues examined (Figs 4A, C).

Compared to Kd values, the maximal numbers of binding sites (Bmax) that were calculated for the two control groups showed more assay-to-assay variability, particularly within the especially DAT-sparse cortical tissues (Fig. 4). Visual inspection of the data, however, revealed that variance was driven largely by single outliers in the data (Figs. 4B,D) and that, as expected, maximal numbers of binding sites in both the 4- and 28-day controls were on average roughly 10 times higher in the caudate nucleus (~ 800–1000 fmol/mg protein) than in the prefrontal (~70–100 fmol/mg protein) or sensorimotor cortex (~90–120 fmol/mg protein, Fig. 3, Figs. 4B, D). Within-group variance was also seen in Bmax values for the gonadectomized and hormone-supplemented groups that, like the controls, usually involved single outliers (Figs. 4B,D). Nonetheless, from tissue-to-tissue Bmax values from all of the experimental groups as well as the controls were largely overlapping and showed no obvious group differences. Quantitative analyses supported these observations and showed that tissue-specific measures of Bmax had similar group means, especially among gonadectomized rats and controls groups and that there were neither significant main effects of hormone treatment (Kruskal Wallis) or group differences (Man-Whitney U) in this parameter for any of the cortical or subcortical tissues assessed (Figs. 4B, D).

NET Binding

Binding data for ^{3}H nisoxetine were analyzed using both single- and two-site models (Prism GraphPad Software). For every tissue and animal group convergence, goodness of fit (r^2 values) and graphical representations of the saturation isotherms and their Scatchard transformations (Fig. 5) were all consistent with binding to single sites.

In both the 4- and 28-day sham-operated control groups, Kd values calculated for the three brain regions assessed were between 1.5 and 2 nmol (Figs. 5, 6 A,C). These values are comparable to those previously reported for rat brain homogenates (Tejani-Butt, 1992,Vathy et al., 1997,Shang et al., 1999). Across all of the 4 and 28-day treated groups, values of ligand binding affinity for the caudate nucleus were similar to corresponding controls. However, for cortical tissues, there were noticeable group differences in Kd value (Figs. 6A, C). Specifically, among the 4-day treatment groups, Kd values obtained from both the prefrontal and sensorimotor cortical tissue were consistently higher than control in the GDX and GDX-E but not the GDX-TP groups, and among the 28 day treatment groups, Kd values in the prefrontal cortex alone were higher than control in the GDX and GDX-E but not the GDX-TP rats (Figs. 6A, C). Statistical analyses confirmed that there were also significant main effects of hormone treatment on cortical Kd after 4 days of treatment for prefrontal and sensorimotor cortex (Kruskal -Wallis: sensorimotor cortex, H=7.82, p < 0.049; prefrontal cortex, H=8.95, p < 0.030), near significant main effects of hormone treatment on Kd for prefrontal cortex (Kruskal-Wallis, H=7.21, p< 0.065) and significant differences in the cortical Kd values of GDX and GDX-E compared to GDX-TP rats and controls in every case (Mann-Whitney, 4 day prefrontal cortex, 4 day sensorimotor cortex, 28 day prefrontal cortex: all U=0, p <0.0495, Figs. 6A, C).

Similar to that seen for DAT assays, Bmax values for NET binding were more variable than measures of Kd in all tissues and in both control and experimental groups (Fig. 6). Nonetheless, as expected maximal numbers of binding sites in the controls were higher in prefrontal cortex (~135–300 fm/mg protein) than in sensorimotor cortex (~150–250 fmol/mg protein) or caudate nucleus (~100–135 fmol/mg protein, Figs. 5, 6B, D) and showed values that on average were comparable to those previously reported for rat brain homogenates (Tejani-Butt, 1992,Vathy et al., 1997,Shang et al., 1999). Visual inspection of the data further showed that for the control and experimental groups alike variance in the data was typically accounted for by single outliers

in the data, that for the most part the tissue-specific values that were obtained across these groups were largely overlapping and that there were no obvious group trends in these data (Figs. 6B, D). Quantitative analyses were consistent with these observations, showing that corresponding tissue specific measures of Bmax had roughly similar group means and that there were no significant effects of hormone treatment (Kruskal-Wallis) or group differences (Mann-Whitney U) among these measures for any treatment group or tissue (Figs. 6B, D).

DISCUSSION

Information from microdialysis (Finlay and Zigmond, 1997, Watanabe et al., 1997, Feenstra et al., 2000), voltammetry, electrophysiology (Lavin et al., 2005) and computational models (Compte et al., 2000, Durstewitz et al., 2000, Durstewitz and Seamans, 2002) all indicate that DA's contributions to prefrontal cortical function include a long-term biasing of local cortical networks. Such protracted actions are consistent with DA's uniquely extended extracellular lifetime in the prefrontal cortex (Garris et al., 1993, Garris and Wightman, 1994, Cass and Gerhardt, 1995) which is in part a consequence of the cortical paucity of the co-utilized DAT and NET (Carboni et al., 1990, Elsworth et al., 1993, Sesack et al., 1998a, Sesack et al., 1998b, Yamamoto and Novotney, 1998, Moron et al., 2002, Miner et al., 2003, Valentini et al., 2004, Carboni et al., 2006). The present studies paired methods of *in vitro* binding with classical hormone manipulation paradigms to examine the gonadal hormone sensitivity of the maximal numbers and affinities of ligand binding sites for these two transporters in selected cortical and subcortical DA-enriched regions of adult male rats. Previous findings in this same animal model demonstrating selective androgen-driven stimulatory effects of gonadectomy on cortical DA concentration DA innervation density (Battaner et al., 1987, Kritzer et al., 1999, Kritzer, 2000, Kritzer, 2003, Kritzer et al., 2007) and, according to preliminary data, on extracellular prefrontal DA levels as well (Aubele et al., 2008) suggest that the adverse behavioral effects of gonadectomy may be related to its ability to abnormally increase prefrontal cortical DA tone. Although this could also be achieved via actions on DA release, metabolism or catabolism, this study explored the possibility that gonadectomy modulates prefrontal DA levels by diminishing the capacity and/or efficiency of DA transport in an androgen-sensitive, estrogen-insensitive manner. The results that were obtained from the present binding studies are in keeping with this scenario. Specifically, while no obvious or statistically significant effects of long or short tem gonadectomy on DAT binding were found, both 4- and 28-day gonadectomy did significantly increase Kd values for ^{3}H nisoxetine binding in cortical but not subcortical sites in an androgen-sensitive, estrogen-insensitive manner. Further, the regional and temporal selectivity of these effects were found to mirror those previously described for the effects of 4 and 28 day gonadectomy on DA axon density in that they impacted both sensorimotor and prefrontal cortex in the 4 day groups, and were selective for prefrontal cortex by 28 days of treatment (Kritzer et al., 1999, Kritzer, 2000). Although studies such as those indicating that the effects of mutations on the DAT can independently influence Kd for synthetic versus endogenous ligands, e.g, DA (Schmitt et al., 2008) indicate that caution is needed in interpreting both the positive and negative findings presented here insofar as DA itself may be concerned, given that DA clearance in the prefrontal cortex is known to utilize the NET to a significant degree, the present findings suggest that impact on the NET may part of the emerging story of androgen modulation of the function and physiology of mesocortical/mesoprefrontal DA systems in males. This possibility is considered further below, following separate comparisons of the present findings to those from previous studies of gonadal hormone regulation of DAT and NET binding.

Gonadal Hormone-Sensitivity of the DAT: Comparison to Previous Studies

Unlike the mesocortical system, both the mesolimbic and mesostriatal DA systems rely heavily on high-affinity transmitter reuptake via the DAT to control extracellular DA levels (Garris

and Wightman, 1994). The fact that drugs of abuse such as cocaine and methamphetamine block DA reuptake via the DAT (Ritz et al., 1987, Moghaddam and Bunney, 1989, Kuhar et al., 1991, Giros et al., 1996) coupled with the sex differences that have been identified in humans and animal models in addictive behaviors, other responses associated with these drugs (Becker et al., 2001, Chen and Kandel, 2002, Festa and Quinones-Jenab, 2004) and in DAT function (Morissette et al., 1990, Morissette and Di Paolo, 1993a, Morissette and Di Paolo, 1993b, Rivest et al., 1995, Lavalaye et al., 2000, Walker et al., 2000, Mozley et al., 2001, Staley et al., 2001, Bhatt and Dluzen, 2005) have focused significant attention on the hormone sensitivity of this transporter moiety. However, to date the typically greater sensitivity/ susceptibility of females to cocaine administration (Kosten et al., 1993, Chen and Kandel, 2002, Chin et al., 2002, Chen et al., 2003) and the protective effects of estrogen on nigrostriatal DA systems in, for example, Parkinson's disease (Horstink et al., 2003, Haaxma et al., 2007) and conditions of DA-selective neurotoxin challenge (Dluzen and Horstink, 2003) has led to a body of work that is concentrated mainly on hormone sensitivity of the DAT in female subjects and in subcortical, primarily striatal structures. This literature describes complex and in some cases contradictory effects of estrogen on DAT binding site number (Morissette et al., 1990, Morissette and Di Paolo, 1993a, Morissette and Di Paolo, 1993b, Attali et al., 1997, Bosse et al., 1997, Le Saux and Di Paolo, 2006, McArthur et al., 2007) and/or affinity (Disshon et al., 1998) and also identifies sex differences favoring females in striatal DAT density and activity (Morissette and Di Paolo, 1993b, Rivest et al., 1995, Walker et al., 2000, Bhatt and Dluzen, 2005). However in only two of these studies were examinations of the effects of hormone manipulations in males included. As in the present study, neither found significant differences in Bmax or Kd values for the striatal DAT in intact compared to gonadectomized or hormone-replaced adult male rats (Attali et al., 1997, Chen et al., 2003). To our knowledge, there have also been no prior studies of sex differences or gonadal hormone impact on DAT binding in the cerebral cortex. The present analyses of ^{3}H WIN35,428 binding in sensorimotor and prefrontal cortex, which may thus be a first, revealed no significant effects of long-or short-term gonadectomy or hormone replacement on cortical DAT binding site number or affinity. Although our data cannot exclude the possibility for effects of gonadectomy on DA's affinity for the DAT nor of subtle effects on Bmax, that our negative findings for striatal WIN 35,428 binding were corroborated in two studies in males, one of which used a different ligand (^{3}H-BTCP) and the other which used DA itself, makes these possibilities less likely. This in turn lends support to conclusions of essentially negative findings in the cerebral cortex as well. Thus, it appears that hormone modulation of prefrontal DA levels in adult male rats may not be a consequence of gonadal steroid modulation of the DAT. However, as discussed below, because the prefrontal cortex engages transporter species in addition to the DAT to mediate extracellular DA clearance, the process of receptor-mediated DA transport remains a potentially relevant target of hormone stimulation.

Gonadal Hormone-Sensitivity of the NET: Comparison to Previous Studies

Studies using knockout mice and/or pharmacological transporter blockade have shown that in the prefrontal cortex, extracellular DA clearance is controlled to an equal if not greater extent by the NET than the DAT (Carboni et al., 1990, Yamamoto and Novotney, 1998, Moron et al., 2002, Valentini et al., 2004, Carboni et al., 2006). In terms of contributing to the effects of gonadectomy on cortical DA level, the NET may also be the more likely candidate than the DAT, as previous studies have also shown that this site may be hormone-sensitive. For example, maximal numbers of frontal cortical ^{3}H-nisoexteine binding sites have been shown to be significantly lower in male than in female rats (Vathy et al., 1997), and Kd and Bmax values for ^{3}H-nisoexteine binding have been shown to be significantly higher in the cortex of adult male rats that have been gonadectomized for two weeks compared to intact controls (Shang et al., 1999). Although in this study no obvious effects on maximal numbers of NET binding sites were found, consistent results were obtained regarding the ability of gonadectomy

to significantly raise cortical Kd values for ^{3}H nisotexine that confirmed and extended previous findings in several ways. First, the effects of long and short-term gonadectomy were found to be selective for cortical compared to subcortical sites. Further, differential effects of long versus short term gonadectomy were found in which Kd values were elevated in both sensorimotor and prefrontal cortex following 4 days of treatment and in the prefrontal cortex alone 28 days after gonadectomy. And finally, the effects of gonadectomy were shown to be attenuated by supplementing gonadectomized rats with testosterone propionate but not estradiol. These latter findings provide a previously lacking definitive link between gonadal steroids and NET regulation, and also identify androgens in particular as active species in males. Although not directly examined in these studies, the protracted time courses over which the effects of gonadectomy on cortical NET sites took place makes it likely that underlying mechanisms of hormone regulation are, at least in part, genomic in nature and mediated by intracellular androgen receptors. This scenario fits well with anatomical data showing that in the locus coeruleus, which supplies the cerebral cortex with virtually all of its NET-bearing axons (Miner et al., 2003), 50–85% of constituent norepinephrine (NE) neurons concentrate the androgen-selective ligand ^{3}H dihydrotestosterone (Heritage et al., 1980, Heritage et al., 1981). While much of the work exploring the underpinnings of gonadal hormone influence over the function and physiology of mesocortical DA systems has logically focused on midbrain DA neurons, these data sum to suggest that the cortically projecting NE neurons of the locus coeruleus should be similarly investigated.

Gonadal Hormone Regulation of Cortical NET Binding: Possible Roles in Regulating Prefrontal Cortical DA Function and Tone

Although currently more extensively studied in females (Gibbs and Gabor, 2003, Luine et al., 2003, Korol, 2004), there is growing evidence from clinical studies and animal models that gonadal and especially androgen hormones also influence DA-dependent prefrontal function in males (Christiansen and Knussmann, 1987, Gouchie and Kimura, 1991, Ceccarelli et al., 2001, Kritzer et al., 2001, Daniel et al., 2003, Janowsky, 2006, Thilers et al., 2006, Aubele et al., 2008). While there are likely to be multiple physiological endpoints that are relevant to these actions, recent evidence showing that the effects of gonadectomy on DA-dependent prefrontal constructs of spatial working memory and behavioral flexibility are correlated with gonadectomy-induced increases in prefrontal DA innervation density (Kritzer et al., 2007) combines with independent evidence that gonadectomy also increases cortical DA concentrations (Battaner et al., 1987) and preliminary data showing that gonadectomy increases prefrontal DA overflow *in vivo* (Aubele et al., 2008) to suggest that actions of gonadal steroids on prefrontal DA levels may be especially important. A number of parallels have emerged in this study between the effects of gonadectomy and hormone replacement on cortical ^{3}H nisoxetine binding and on previously identified physiological and functional measures of the mesoprefrontal DA system in adult male rats that suggest that influence over this transporter site could be a means by which the functionally important endpoint of prefrontal cortical DA level is hormonally regulated. First, like the effects of gonadectomy on behavioral and biochemical DA measures, those exerted on cortical ^{3}H nisoxetine binding were androgen sensitive and estrogen insensitive, which is somewhat uncommon even for the male brain where actions of testosterone often requires its enzymatic conversion to estrogenic metabolites (Simpson et al., 1994, Lephart, 1996). Further, exactly mirroring the regionally differential effects of long and short-term gonadectomy on cortical DA axon density (Kritzer et al., 1999, Kritzer, 2000), 4-days after gonadectomy effects on NET binding affinity were observed in both sensorimotor and prefrontal cortex, and 28 days after gonadectomy effects on Kd were found in the prefrontal cortex alone. That gonadectomy has also been shown to increase cortical concentrations of DA but not NE (Battaner et al., 1987) and alter cortical axon densities of DAergic but not NEergic fibers (Kritzer, 2003) may also be congruent with the two-fold decrease in NET ligand binding affinity that this manipulation brings, which to the extent that

these data may reflect the situation for native ligands, could arguably have greater and perhaps even selective adverse impact on the uptake of DA compared to its preferred substrate, NE. Finally, it may be noteworthy that gonadectomy and androgen replacement coincidently increase prefrontal DA levels/innervation and lower cortical NET binding affinity. Because these actions run counter to the reciprocal, homeostatic relationships that normally drive compensatory changes in transporter efficiently and/or capacity in response to changes in substrate levels, they may be most parsimoniously explained by principal effects of gonadectomy on the NET and resultant changes in cortical DA levels. Although questions remain that can only be empirically addressed about transporter function, and while it remains unclear what dictates the temporal and regional selectivity of the effects that have been observed, the data collected do suggest a specific and novel working hypothesis; that androgen-sensitive, gonadectomy-induced decreases in the affinity of cortical NET sites in adult male rats play a mechanistic role in elevating cortical and especially prefrontal cortical DA levels and in turn lead to the deficits in the DA-dependent prefrontal cortical functions that have been repeatedly seen in this animal model. In further exploring this previously unsuspected route of gonadal hormone and more specifically androgen influence over DA levels and DA dependent prefrontal cortical functions in males it will be especially important to make direct assessments of transporter mediated DA uptake. Such continued investigation along these lines should provide insights into neurobiological basis for the sex differences and hormone-sensitivity that are well known to characterize the development (Diamond, 1985, Clark and Goldman-Rakic, 1989, Bachevalier and Hagger, 1991, Overman, 2004) and adult functioning (Einon, 1980, Tees et al., 1981, van Haaren et al., 1990) of normal prefrontal cortical operations in humans and animal models. In view of recent evidence that also links the NET to the pathophysiology in schizophrenia and ADHD (Stober et al., 1996, Rybakowski et al., 2006, Kim et al., 2008, Retz et al., 2008) it is also possible that investigation of the hormone sensitivity of the NET and its role in modulating cortical DA biochemistry and behavior may also help explain the greater vulnerability of the mesocortical DA systems and DA-dependent prefrontal processes at risk in these disorders in males (Goodman and Stevenson, 1989, Seeman and Lang, 1990, Leviton et al., 1993).

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Medial Prefrontal Cortex

Sensorimotor Cortex, Caudate
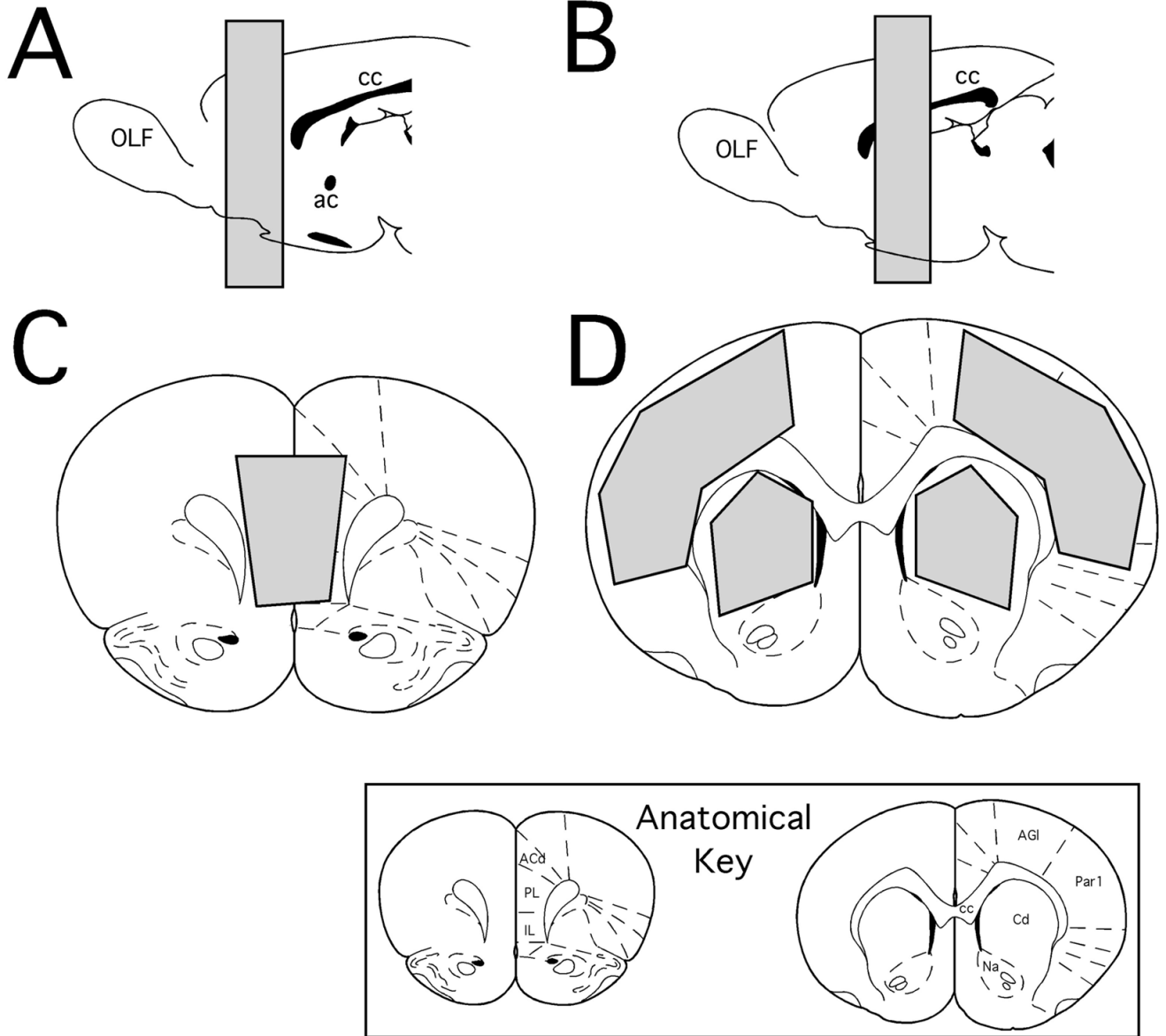
Figure 1.
Schematic diagrams showing the anterior/poster (A, B) and coronal (C,D) locations of brain regions marked in grey that were micro-dissected out for the use in homogenate binding assays. These regions of interest were pregenual portions of the medial prefrontal cortex (A, C) and portions of the sensorimotor cortex and caudate nucleus taken at levels around the anterior commisure (B, D). The templates showing these locations are modified from the atlas of Paxinos and Warson (1998). Anatomical abbreviations: ACd, anterior cingulate cortex; ac, anterior commisure; AGl, primary motor cortex; Cd, caudate nucleus; cc, corpus callosum; IL, infralimbic cortex; Na, nucleus accumbens; OLF, olfactory bulb; Par1, primary somatosensory cortex; PL, prelimbic cortex.
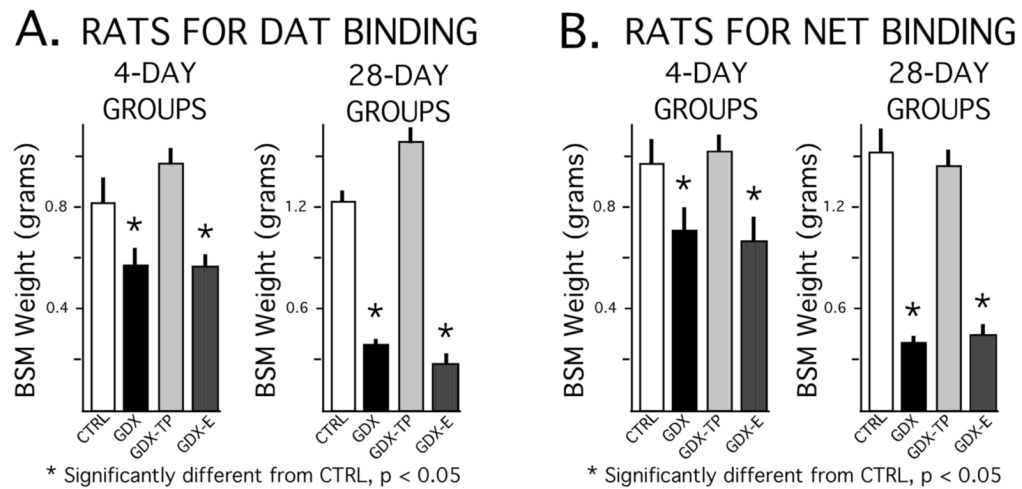
Figure 2.
Bar graphs showing means weights of the bulbospongiousus muscles (BSM) in grams, ±
standard error of the mean, for animal subjects used for dopamine transporter (DAT) binding
assays (A) and norepinephrine transporter (NET) binding assays (B). Data from animals that
were sham-operated (CRTL), gonadectomized (GDX) and gonadectomized and supplemented
with testosterone propionate (GDX-TP) or estradiol (GDX-E) for 4 or 28 days are shown
separately. As expected, there were significant effects of hormone treatment on the weights of
these androgen-sensitive muscles for both time points, and BSM weights in both the 4- and
28-day GDX and GDX-E groups were significantly less (*) than those in the corresponding
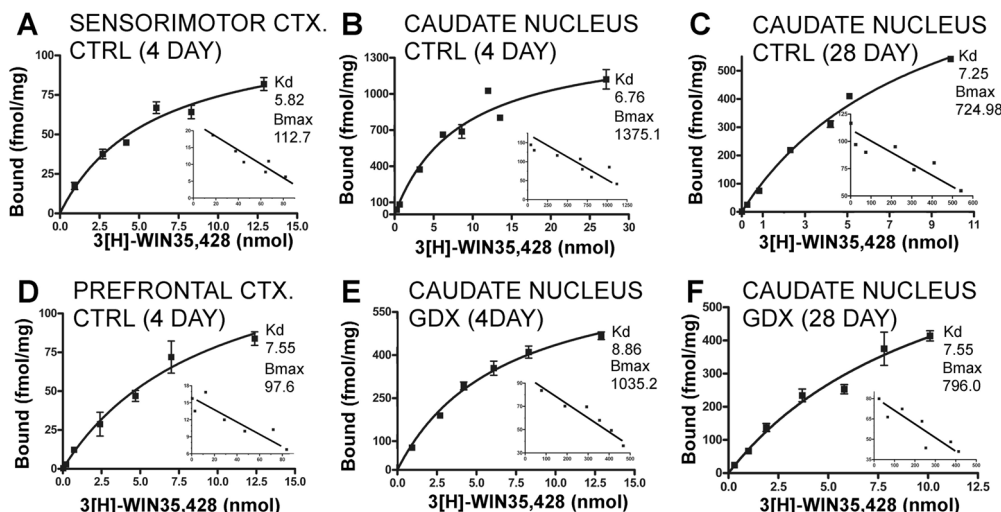CTRL and GDX-TP cohorts.

Figure 3.
Representative saturation isotherms with Scatchard transformations of the data shown as insets for ^{3}H-WIN 35,428 binding. Each graph represents data from a single assay. For the saturation data, points are means of triplicate measures obtained of ligand bound in nmol/mg protein (Y-axis) at each ligand concentration (X-axis) and error bars are standard deviations of these means; the inserted Scatchard transformations plot corresponding measures of bound to bound/free ligand. Data from sensorimotor cortex (A), caudate nucleus (B) and prefrontal cortex (D) dissected from 4-day sham-operated controls (CTRL) are shown. For comparison, binding data for the caudate nucleus dissected from 28-day sham operated controls (C) and from 4- and 28 day gonadectomized animals (E, F) are shown. For each panel, calculated maximal numbers of binding sites (Bmax) and binding affinity constants (Kd) are also shown. These graphs illustrate that there can be variance in the data regarding measures of Bmax, e.g. compare 4 and 28-day controls, and linear regressions in the Scatchard plots that yield single straight lines indicative of binding to a single site.

[3H] - WIN35,428 (DAT)

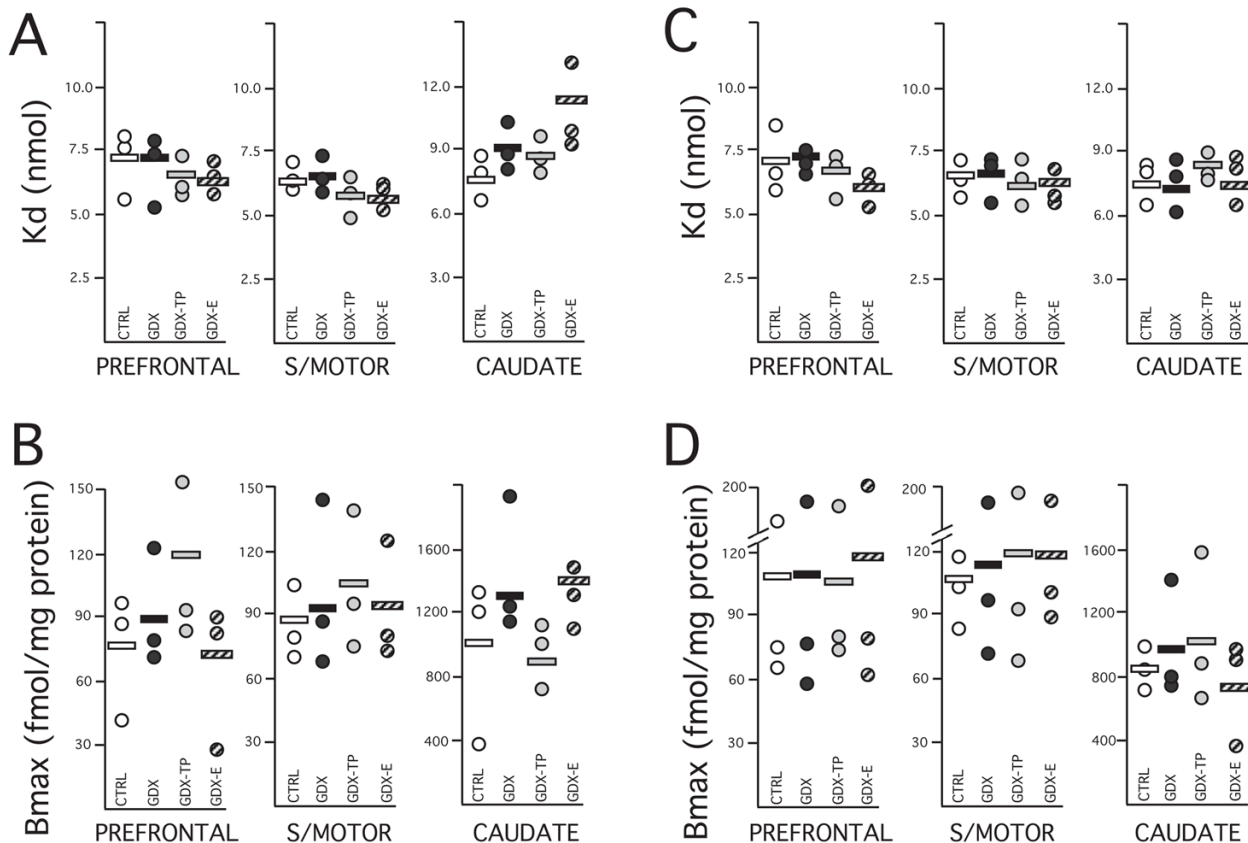## 4-DAY GROUPS   28-DAY GROUPS

Figure 4.
Scatter plots showing data (circles) and means mean (horizontal black bars) for dissociation constants (Kd) in nmol (A, C) and maximal numbers of binding sites (Bmax) in fmol/mg protein (B,D) calculated for the dopamine transporter (DAT) using selective ligand ^{3}H WIN35,428. The Kd and Bmax data obtained from homogenate assays performed on prefrontal cortex, sensorimotor cortex (S/MOTOR) and caudate nucleus tissue dissected from rats that were sham-operated (CRTL, open circles), gonadectomized (GDX, black circles) and gonadectomized and supplemented with testosterone propionate (GDX-TP, light gray circles) or estradiol (GDX-E, dark gray circles) for 4 (left) or 28 days (right) are shown separately. The paucity of the DAT in the cerebral cortex is reflected in Bmax values from both cortical samples that are lower than those obtained in the caudate nucleus. Overall the Bmax measures were also more variable across assays than measures of Kd. There was, however, considerable overlap in both Bmax and Kd values across groups and neither significant main effects of hormone treatment nor significant group differences for either set of values among the 4 or 28 day treatment groups.
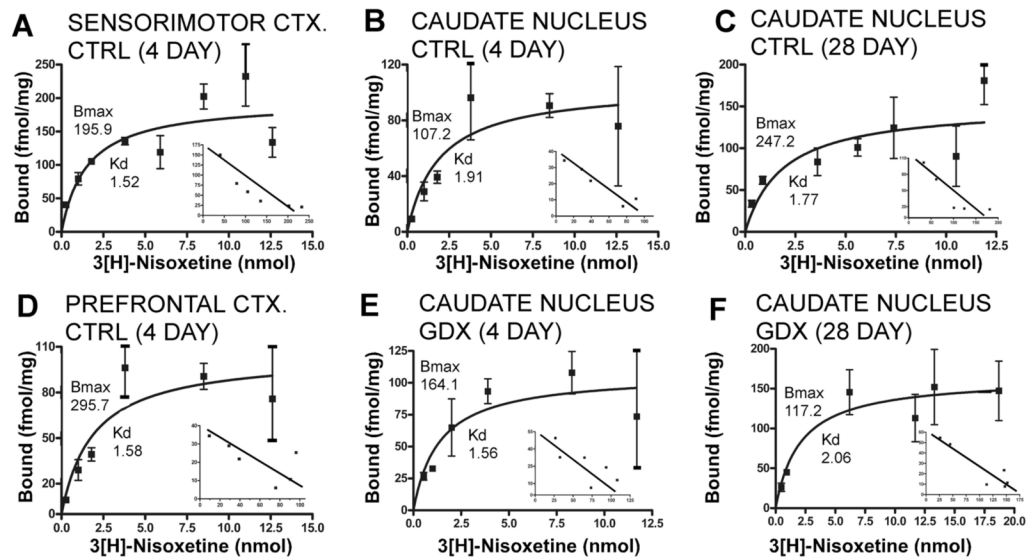
Figure 5.
Representative saturation isotherms with Scatchard transformations of the data shown as insets for ^{3}H-nisoxetine binding. Each graph represents data from a single assay. For the saturation data, points are means of triplicate measures obtained of ligand bound in nmol/mg protein (Y-axis) at each ligand concentration (X-axis) and error bars are standard deviations of these means; the inserted Scatchard transformations plot corresponding measures of bound to bound/free ligand. Data from sensorimotor cortex (A), caudate nucleus (B) and prefrontal cortex (D) dissected from 4-day sham-operated controls (CTRL) are shown. For comparison, binding data for the caudate nucleus dissected from 28-day sham operated controls (C) and from 4- and 28 day gonadectomized animals (E, F) are shown. For each panel, calculated maximal numbers of binding sites (Bmax) and binding affinity constants (Kd) are also shown. These graphs illustrate that there can be variance in the data regarding measures of Bmax, e.g. compare 4 and 28-day controls, and linear regressions in the Scatchard plots that yield single straight lines indicative of binding to a single site.
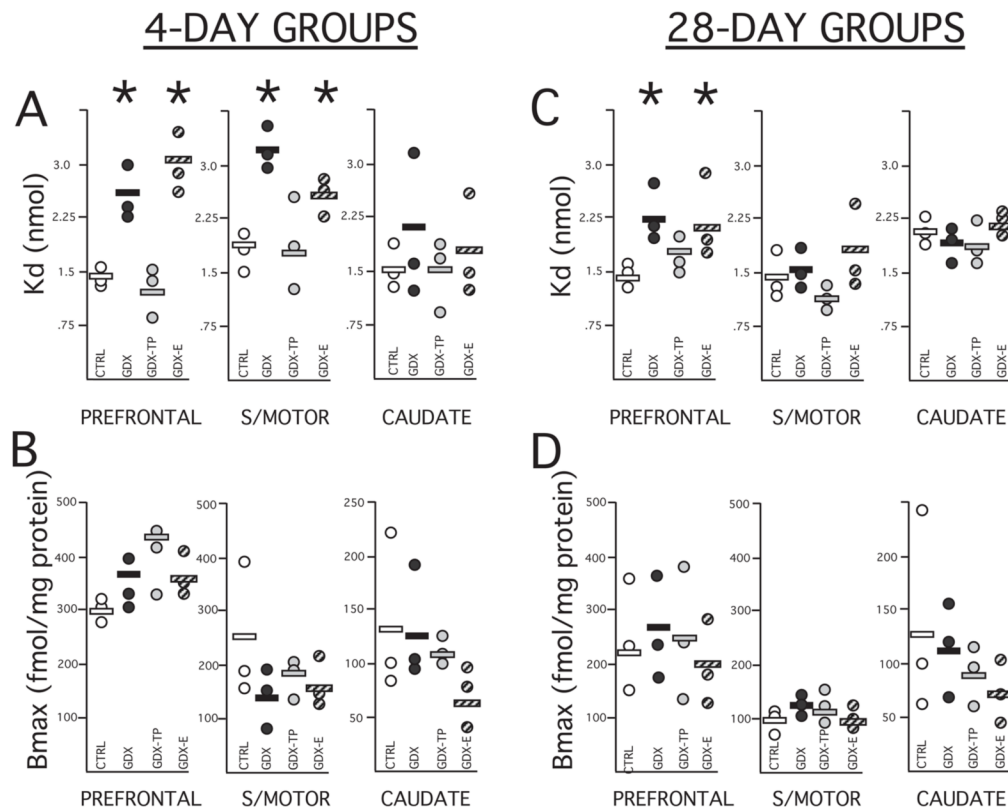
[³H] - NISOXETINE BINDING (NET)

Figure 6.
Scatter plots showing data (circles) and means (horizontal black lines) for dissociation constants (Kd) in nmol (A, C) and maximal numbers of binding sites (Bmax) in fmol/mg protein (B, D) calculated for the norepinephrine transporter (NET) using selective ligand ³H nisoxetine. The Kd and Bmax data collected from homogenate assays performed on the prefrontal cortex, sensorimotor cortex (S/MOTOR) and caudate nucleus tissue dissected from rats that were sham-operated (CRTL, open circles), gonadectomized (GDX, black circles) and gonadectomized and supplemented with testosterone propionate (GDX-TP, light gray circles) or estradiol (GDX-E, dark gray circles) for 4 (left) or 28 days (right) are shown separately. As for the DAT binding, measures of the fairly sparse maximal numbers of NET binding sites (Bmax) were more variable than Kd values, and in all tissues were largely overlapping and not significantly different across any groups. Among the less variable measures of Kd, there were no significant main effects of hormone treatment or group differences seen for values from the caudate nucleus. However, among the 4-day treatment groups, Kd values for the prefrontal and sensorimotor cortex of the GDX and GDX-E groups were significantly higher (*) than those of the control and GDX-TP groups, and at 28 days, Kd values for the prefrontal of the GDX and GDX-E groups were significantly higher than those of the control and GDX-TP cohorts.