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ANDROGEN METABOLITES IMPACT CSF AMINES AND AXONAL SEROTONIN VIA MAO-A AND -B IN MALE MACAQUES

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Abstract

A number of studies have shown that mutations or deletions of the monoamine oxidase-A (MAO-A) gene cause elevated CNS serotonin and elevated impulsive aggression in humans and animal models. In addition, low cerebrospinal fluid (CSF) 5-hydroxyindole acetic acid (5HIAA) has been documented in a limited number of violent criminal populations and in macaques that exhibit impulsive aggression. To reconcile these different analyses, we hypothesized that CSF 5HIAA reflected degradation of serotonin by the activity of MAO-A; and that low MAO-A activity would result in lower CSF 5HIAA, but overall higher serotonin in the CNS. To test this hypothesis, male Japanese macaques (*Macaca fuscata*) were castrated, rested for 5–7 months, and then treated for 3 months with [1] placebo, [2] testosterone (T), [3] dihydrotestosterone (DHT; non-aromatizable androgen) and 1,4,6-androstatriene-3,17-dione (ATD) (steroidal aromatase inhibitor), or [4] flutamide (FLUT; androgen antagonist) and ATD (n = 5/group). These treatments enable isolation of androgen and estrogen activities. In the dorsal raphe, MAO-A and MAO-B expressions were determined with *in situ* hybridization (ISH) and protein expression of aromatase was determined with immunohisto-chemistry (IHC). CSF concentrations of 5HIAA, 3-methoxy-4-hydroxyphenylglycol (MHPG), and homovanillic acid (HVA) were determined with liquid chromatography/mass spectrometry (LC/MS). From the same animals, previously published data on serotonin axon density were used as a proxy for CNS serotonin. Aromatase conversion of T to estrogen (E) suppressed MAO-A (positive pixel area, $p = 0.0045$), but androgens increased MAO-B (positive pixel area, $p = 0.014$). CSF 5HIAA was suppressed by conversion of T to E (Cohen's $d = 0.6$). CSF 5HIAA was positively correlated with MAO-A-positive pixel area ($r^2 = 0.78$). CSF 5HIAA was inversely correlated with serotonin axon-positive pixel area ($r^2 = 0.69$). In summary,

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DISCLOSURE

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Aaron Kim, BS, has nothing to disclose.

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CSF 5HIAA reflects MAO-A activity rather than global serotonin. Low CSF 5HIAA may, in this paradigm, reflect higher serotonin activity. Androgens lower MAO-A activity via metabolism to E, thus elevating CNS serotonin and decreasing CSF 5HIAA. Since androgens increase certain types of aggression, these data are consistent with studies demonstrating that lower MAO-A activity is associated with elevated serotonin and increased aggression.

Keywords

male; serotonin; MAO-A; MAO-B; aromatase; CSF 5HIAA

INTRODUCTION

Androgen administration to animals has been used to model aggression in humans (Bonson et al., 1994; Melloni and Ferris, 1996; Mehlman et al., 1997; McGinnis, 2004). An early hypothesis in the neurobiology of behavior was that androgens decrease serotonin, and low serotonin precipitates impulsive or provoked aggression (Popova, 2006; Yanowitch and Coccaro, 2011). However, meta-analysis of earlier studies and the interpretation of their results, cast considerable doubt on this hypothesis in humans (Berman et al., 1997; Olivier, 2004; Duke et al., 2013).

Another line of investigation indicated that elevated serotonin increased aggression. Two seminal studies showed childhood maltreatment combined with a low-activity monoamine oxidase A (MAO-A) polymorphism correlated with later antisocial problems (Caspi et al., 2002; Huang et al., 2004). These studies were confirmed in a meta-analysis of 27 studies (Byrd and Manuck, 2014). Furthermore, a PET study showed that in adult men, the activity of brain MAO-A inversely correlated with the Multi-Personality Question test trait measure of aggression (but not with other personality traits). That is, the lower the MAO-A activity in cortical and subcortical brain regions, the higher the self-reported aggression (Alia-Klein et al., 2008). The low-activity allele also caused elevated aggression in mother-reared rhesus macaques (Newman et al., 2005). In addition, MAO-A knockout mice have elevated brain levels of serotonin, norepinephrine (NE) and dopamine (DA) and manifest increased aggressive behavior. In contrast, monoamine oxidase B (MAO-B) knock-out mice did not exhibit aggression and only levels of phenyl-ethylamine were increased (Shih et al., 1999).

In nonhuman primates (NHPs), we showed that androgen (testosterone, T or dihydrotestosterone, DHT) administration to castrated male macaques *increased* TPH2 mRNA, but the aromatization of T to estradiol (E) underlay serotonin axon density in a terminal field (Bethea et al., 2014). Moreover, serotonin in axons and fenfluramine-induced serotonin/prolactin release positively correlated with each other and both were *higher* in T-treated castrated male macaques compared to DHT-treated males supplemented with an aromatase inhibitor (Bethea et al., 2013, 2014). Hence, androgen treatment could have increased serotonin synthesis via TPH2 mRNA expression, but E-dependent serotonin axonal content appears to determine fenfluramine-induced serotonin/prolactin release.

Part of the heuristic hypothesis regarding low serotonin and impulsive aggression has been the implication that the concentration of 5-hydroxyindole acetic acid (5HIAA) in

cerebrospinal fluid (CSF) is a direct indication of central serotonin or serotonin turnover in the brain of human and NHPs (Linnoila et al., 1983). However, an early study in rats suggested that 5HIAA concentration was due largely to monoamine oxidase (MAO; EC1.4.3.4) activity rather than serotonin release (Wolf et al., 1985). This observation has been born out in humans with mutant or deleted MAO-A (Caspi et al., 2002; Huang et al., 2004; Alia-Klein et al., 2008; Byrd and Manuck, 2014). Association of low-activity MAO-A with increased serotonin and impulsive aggression needs to be reconciled with consistent observations of low CSF 5HIAA and increased impulsive aggression in male macaques (Mehlman et al., 1997).

MAO-A and MAO-B are the principle enzymes that deaminate biogenic amines in both the brain and peripheral tissues (Von Korff, 1979). In NHPs, MAO-A prefers serotonin and MAO-B prefers NE over serotonin (Garriek and Murphy, 1982). MAO isoform protein localization was consistent with mRNA expression (Richards et al., 1992; Luque et al., 1996) and both MAO-A and MAO-B were present in serotonin neurons (Westlund et al., 1988; Richards et al., 1992; Luque et al., 1996; Saura et al., 1996; Gundlach et al., 2001).

MAO-A gene expression in the raphe and several hypothalamic areas was significantly suppressed in E-treated ovariectomized female macaques (Gundlach et al., 2001). Other studies indicated that elevated T during mating season was associated with relatively low levels of platelet MAO activity in male macaques although the isoform was not specified (Redmond et al., 1976). Unfortunately, these studies do not clarify the question of how T affects MAO-A and -B in the brain of macaques. Thus, a study of MAO-A and -B in NHPs with a direct examination of brain serotonin and 5HIAA in the same animals was lacking. We hypothesized that the concentration of 5HIAA in CSF would be related to MAO-A activity, which in turn inversely correlates with CNS serotonin.

In this study, we examined the effects of androgens and aromatase block on MAO-A and MAO-B mRNA expression with *in situ* hybridization (ISH) as well as aromatase protein expression with immunohistochemistry (IHC) in the dorsal raphe of male macaques. In addition, we measured the CSF concentrations of 5HIAA, 3-methoxy-4-hydroxyphenylglycol (MHPG) and homovanillic acid (HVA), metabolites of serotonin, NE and DA, respectively, in the same animals. The density of serotonergic axons coursing into the locus coeruleus (LC) was previously quantified with an antibody directed against serotonin per se in the same animals (Bethea et al., 2014), and the measurements were used as a proxy for brain serotonin. Correlations between enzyme mRNA expression, 5HIAA and serotonin were sought using group averages of each variable.

EXPERIMENTAL PROCEDURES

This experiment was approved by the IACUC of the Oregon National Primate Research Center (ONPRC) and conducted in accordance with the 2011 Eight Edition of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Japanese macaques (*Macaca fuscata*) were utilized for the study. This study was conducted on brain tissue and CSF fluid from the same animals used in (1) previous studies on behavior and fenfluramine challenges (Bethea et al., 2013); (2) serotonin-related gene expression and

innervation of the LC (Bethea et al., 2014), and (3) characterization of reproductive steroid receptors in the dorsal raphe (Bethea et al., 2015).

Troop

The Japanese macaques were born and raised in a 2-acre outdoor corral at ONPRC with approximately 300 individuals. The troop has been the subject of extensive behavioral studies since it arrived at ONPRC in 1965 (Eaton, 1974; Eaton et al., 1990). The troop composition is relatively stable and the age structure is comparable to that of a natural troop (Maruhashi, 1982). Like other macaque species, the hierarchical organization of the troop is along matriarchal lineages. The dominance hierarchies of the matriarchal lines are well documented, and have remained stable for the past 50 years. In the wild, males normally leave the natal troop and so their dominance is less a function of their mother's status and more a function of their age, size and social skills. Males cannot leave our troop on their own so in that respect, there are more males than a natural troop although they are removed for research and sales with attention to troop stability.

Study animals and treatments

Twenty adult male Japanese macaques were assigned to this project, castrated and socially housed as previously described (Bethea et al., 2013). The experimental period was conducted for 3 months during the mating season when aggression is highest among males in the troop. Although the animals were housed indoors, their annual rhythms continue to be manifested (Rostal et al., 1986). The animals used in this study were the same animals used in previous studies (Bethea et al., 2013, 2014, 2015).

Treatment groups were established to disassociate activation of androgen receptors (AR) from estrogen receptors (ER). In year 1, half of the animals were treated with placebo or testosterone (T) ($n = 5/\text{group}$) and then euthanized. In year 2, the remaining animals were treated with DHT + aromatase inhibitor (1,4,6-androstatriene-3,17-dione (ATD)) or an androgen antagonist (flutamide; FLUT) + ATD ($n = 5/\text{group}$) and then euthanized. The T treatment achieved serum T concentrations previously reported in intact Japanese macaques (Eaton and Resko, 1974; Rostal et al., 1986). Dosing and inhibition of brain aromatase with ATD administration were previously reported in macaques (Ellinwood et al., 1984). The dose of DHT significantly elevated DHT ~10-fold over the normal concentration observed in intact or T-treated macaques (Bethea et al., 2013).

The rationale for the different treatment groups has been explained in detail (Bethea et al., 2014). Briefly, T treatment exposed the brain to DHT via 5 α reductase metabolism and to E via aromatase metabolism [=high AR and high ER activation]. The placebo group would have little or no activity at AR, but independent de novo production of neural E from cholesterol could remain [=low AR and reduced ER activation] (Mukai et al., 2006; Tsutsui, 2012). The DHT + ATD group would have high activity at AR, and ~90% inhibition of aromatase (Ellinwood et al., 1984), which would lead to virtually no ER activation [=high AR and no ER activation]. It was also previously reported that ATD activated AR in castrated macaques (Resko et al., 1993). Therefore, the FLUT + ADT group was expected to have no androgen activity and inhibition of most aromatase, which in turn significantly

reduced activation of both AR and ER [=no AR and no ER activation]. Please see Bethea et al. (2014) for a diagrammatic summary of the treatments and their effects.

Blood samples were obtained via saphenous venipuncture under ketamine sedation for immunoassay of T, DHT, and E to verify treatment efficacy and the results were previously reported (Bethea et al., 2013, 2014). Briefly, in the T-treated group, serum T equaled 7.14 ± 0.98 ng/ml and DHT equaled 2.93 ± 0.83 ng/ml. In the DHT-treated group, serum T equaled 0.88 ± 0.59 ng/ml and DHT equaled 20.52 ± 5.32 ng/ml. The castrate + placebo group had 0.347 ± 0.25 ng/ml of serum T and 0.15 ± 0.03 ng/ml of DHT. The FLUT + ATD group had castrate concentrations of T and DHT. However, the presence of the androgen antagonist and aromatase inhibitor made serum concentrations irrelevant. Serum E was undetectable in all animals at all time points. As evidenced from the standard error of the means, the variance in hormone levels within the groups was very small.

Weights, ages, ranks, and behavior

Each animal was weighed before treatment and at intervals during treatment when blood samples were obtained, implants were placed, prior to surgery and prior to necropsy. The ages were known from exact birth dates obtained from daily observations of the corral by ONPRC technicians. The dominance ranks were determined from win-loss recordings during focal observations after the animals were placed into social housing [supplement, (Bethea et al., 2013)] and dominance did not correlate with T or any other endpoint previously measured. Focal observations using Observer Software were routinely conducted on all animals prior to a fenfluramine challenge and the results have been published (Bethea et al., 2013, 2014).

Euthanasia

Monkeys were euthanized at the end of the treatment periods (3 months) with no discontinuation of treatment prior to necropsy. Euthanasia, collection of the brain and other tissues was performed according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association and performed by an expert veterinary pathologist. Monkeys were transported to the necropsy suite under ketamine sedation (100 mg in 1 ml; Ketathesia, Butler Schein, Dublin, OH, USA). They were then administered pentobarbital (30 mg/kg, i.v., Hospira, Lake Forest, IL, USA) and exsanguinated with severance of the descending aorta.

Tissue preparation

Perfusion and preparation of the brain have been previously described in detail (Bethea et al., 2014). The pontine midbrain blocks were cut on a sliding microtome at 25 μ m, and half of the sections were mounted on Superfrost Plus Slides (Fisher Scientific, Pittsburgh, PA, USA), dehydrated under vacuum overnight and then stored at -80°C until processing for ISH assays. The rest of the sections were stored at -20°C in cryoprotectant for subsequent immunohistochemical (IHC) assays.

Riboprobes

The construction, synthesis and linearization methods of the MAO-A and MAO-B riboprobes were described in Gundlach et al. (2001).

Digoxigenin ISH raphe nucleus

Digoxigenin ISH was applied to determine if MAO-A and MAO-B mRNA abundance differed between treatment groups. The non-isotopic ISH procedure, described in detail elsewhere (Berg-von der Emde et al., 1995; Lima et al., 2009; Bethea et al., 2012), utilized digoxigenin-labeled MAO-A and MAO-B cRNAs. Three levels of the dorsal raphe were analyzed at 500- μ m intervals. The sections were matched anatomically and rostral, medial and caudal sections from all animals were processed together. The negative control slide was processed identically except that T7 RNA polymerase drove transcription to produce a sense probe instead of an antisense probe. Nonspecific staining was very sparse.

Densitometric analysis of digoxigenin ISH

Sections (three levels/animal) were video-captured with the Marianas Stereology Workstation (Intelligent Imaging Innovations, Denver, CO, USA) and Slidebook 5.0 (Olympus Imaging Systems, Center Valley, PA, USA), which created a montage of the dorsal raphe at each level. The image was exported as a Tiff file and Image J (NIH freeware) was used to perform the image analysis. For each anatomical level, the largest representation of the dorsal raphe was chosen from among all the animals. A square outline was placed over the chosen dorsal raphe and the exact dimensions were recorded. The size of the square changed at each morphological level, but the same size square was used for all of the animals at each particular anatomical level. Image J enabled segmentation of the image into positive (stained) and negative (unstained) pixels with the same saturation parameters. The program then provided the area of positive pixels. The same procedure was applied with each gene at each anatomical level. The outlined area was also measured to ensure equal areas were analyzed across all animals. Therefore, for each animal the following data were obtained from all sections: average positive pixel area and average fraction of the total area that was positive. Average data were chosen due to rare missing data points that precluded comparison of the total positive pixel area.

Aromatase IHC

Four midbrain sections containing the dorsal raphe were removed from -20°C storage in cryoprotectant buffer and washed in KPBS buffer four times for 15 min each (rinsed), immersed in 1% hydrogen peroxide for 30 min, rinsed and then incubated with the following blocking solutions: Vector normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) for 60 min; Vector avidin for 20 min; Vector biotin for 20 min. Sections were then incubated for 24 h at 4°C in primary antibody to aromatase (Rabbit anti-human aromatase; polyclonal aa400–502; LS-B521; LifeSpan BioSciences, Inc., Seattle, WA, USA). This antibody was characterized across a range of titers with positive and negative controls. The primary antibody to aromatase was diluted in 2% NGS, 0.02 M KPBS and 0.4% Triton at 1/500. Sections were then rinsed, incubated in Vector biotinylated goat anti-rabbit serum for 60 min, rinsed, incubated with Vector ABC reagent for 60 min, rinsed, incubated with

0.05% diaminobenzidine (DAB kit, Vector Laboratories) containing 3% hydrogen peroxide for 1–10 min, and finally rinsed. Sections were mounted on Superfrost Plus slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) and dried overnight under vacuum. The sections were further dehydrated through a graded series of ethanols and xylene. The sections were finally mounted under glass with DPX.

Densitometric analysis of aromatase

Four sections at different morphological levels of the raphe were immunostained for aromatase and analyzed for each animal. The sections were morphologically matched between animals using anatomical reference points. Image capture and analysis with Image J were the same as described above for digoxigenin-ISH signals. Data were then averaged across the four sections from each individual animal.

CSF sample collection

CSF samples were obtained immediately after the start of exsanguination by puncture of the cisterna magna and withdrawal of CSF into a 3-cc syringe by the veterinary pathologist. The ~1 ml sample was placed on ice and transported to storage at –80 °C until assay. Samples were accepted if they were clear of hemolysis.

Liquid chromatography/mass spectrometry (LC/MS) for 5HIAA, MHPG and HVA

CSF samples were assayed by LC/MS in the Bioanalytical Shared Resource/ Pharmacokinetics Core (BSR/PKCore) at the Oregon Health and Sciences University. Three different dilutions of the samples were assayed to get all values in a linear range below the maximum detectable concentration of the instrument. Concentrations of 5HIAA, MHPG and HVA, the metabolites of serotonin, NE and DA, respectively, were determined with an Applied BioSystems 4000 Q TRAP instrumentation. Dr. Dennis Koop, Department of Physiology and Pharmacology, Oregon Health and Sciences University, directs the core.

Statistical analysis

All morphological measurements were averaged across the sections, generating one overall value for each animal. CSF samples were assayed in triplicate and averaged, generating one value per animal. Therefore, for each type of assay the variance around the mean of each group reflects the difference between animals. All data were compared with analysis of variance (ANOVA) followed by Newman-Keuls post hoc pairwise comparison. Data were tested for unequal variance and if present, a non-parametric ANOVA was applied (Kruskal-Wallis). Combined group data were compared with a *t*-test. Data were additionally compared to determine effect size using Cohen's *d* analysis (<http://www.uccs.edu/~lbecker/>). In addition, regression analysis was applied to gene expression and CSF 5HIAA. Serotonin axon density in the LC was previously determined in the same animals with an antibody to serotonin (Bethea et al., 2014). Therefore, quantitation of serotonin axon density from the previous study was correlated with 5HIAA. Each variable was represented with the group means. All statistical analyses were conducted using the Prism Statistic Program 5.0 (GraphPad, San Diego, CA, USA). A confidence level of $p < 0.05$ was considered significant.

RESULTS

ISH and IHC signals

As illustrated in Fig. 1, the dig-ISH signal for MAO-A and MAO-B, as well as the IHC staining for aromatase were specific and robust in the dorsal raphe. MAO-A and MAO-B (top and middle panels) were localized in the typical large- and medium-sized serotonin-like neurons within the dorsal raphe, the medial fascicularis and the median raphe. Aromatase (bottom panels) was localized to the large serotonin-like neurons and a variety of other sized neurons. Aromatase was widely distributed within and outside the dorsal raphe. It appeared largely neuronal, and not glial, with the antibody utilized.

Anatomy of the dorsal raphe

The morphology of the dorsal raphe is illustrated in Fig. 2. Aromatase immunostaining was used for the illustration because the staining was robust. Morphological levels 1, 2 and 3 were used for MAO-A and -B ISH. All four levels were used for aromatase IHC. Subdivisions of the dorsal raphe were not analyzed independently.

Quantitative analysis

The regulation of MAO-A mRNA expression is illustrated in Fig. 3. There was a significant difference between the groups in positive pixel area ($F [3,14] = 7.8$; $p = 0.0027$). Post hoc analysis showed that individually, the placebo-, DHT + ADT- and FLUT + ADT-treated groups were significantly higher than the T-treated group ($p < 0.05$, each comparison). To further distinguish the roles of the androgenic and estrogenic metabolites of T, the groups with no metabolism to E (ADT-treated groups combined) were compared to the groups with metabolism to E and de novo production of E (T and placebo groups combined). There was a significant difference in the positive pixel area representing MAO-A expression in the presence and absence of E ($t [16] = 3.299$; $p < 0.0045$). MAO-A expression was suppressed in the presence of E. However, there was no difference in the positive pixel area between combined groups with androgens (T- and DHT-treated) compared to groups with no androgen activity (placebo- and FLUT-treated).

The regulation of MAO-B mRNA expression is illustrated in Fig. 4. There was no difference in positive pixel area between the groups by ANOVA. To further distinguish the roles of the androgenic and estrogenic metabolites of T, the groups with no metabolism to E (ADT-treated groups) were compared to the groups with metabolism to E and de novo production of E (T and placebo groups). There was no difference in MAO-B-positive pixel area in the presence or absence of E. However, there was a significant difference in the positive pixel area between combined groups with androgens (T- and DHT-treated) compared to combined groups with no androgen activity (placebo- and FLUT-treated). MAO-B was higher in the presence of androgens ($t [15] = 2.82$; $p = 0.014$).

Although aromatase was prominent in neurons of the dorsal raphe, it did not change with treatment (Fig. 5).

CSF amines

CSF 5HIAA, the MAO-A generated metabolite of serotonin, was not statistically different between the groups as shown in Fig. 6, top. There is a great deal of individual variation in this measurement (within-group differences) and the small number of animals made it impossible to detect between group differences with ANOVA or *t*-test. However, there appeared to be a trend toward lower 5HIAA in the presence of E, but no effect of T (Fig. 6, bottom).

Effect size

These data were further examined with Cohen's *d* analysis for effect size (Table 1). "*Effect size* is a name given to a family of indices that measure the magnitude of a treatment effect. Unlike significance tests, these indices are independent of sample size. Cohen defined effect sizes as small, $d = 0.2$; medium, $d = 0.5$; and large, $d = 0.8$ " (from Lee A. Becker, PhD, <http://www.uccs.edu/lbecker/effect-size.html>). Cohen's *d* for the presence or absence of E on MAO-A-positive pixel area equaled ~1.66, which was large. In contrast, Cohen's *d* for the presence or absence of androgens on MAO-A-positive pixel area was ~0.3, which is small. Comparison of MAO-B-positive pixel area in the presence or absence of E indicated that Cohen's *d* was ~0.07, which is less than small, whereas the effect size in the presence or absence of T was ~1.6, which was a large effect (Table 1).

Cohen's *d* for 'some E' versus 'no E' on 5HIAA was ~0.5, which lies between small and medium. Cohen's *d* for 'androgen' versus 'no androgen' on 5HIAA was ~0.06, which is an order of magnitude less than small. Thus, it is probably safe to say that E affected 5HIAA and androgens did not. The 5HIAA data parallels the effect size analysis of MAO-A ISH assays. It should be noted that the T-treated group was included in both 'the presence of E' and 'the presence of androgen' analyses (Table 1).

CSF MHPG and CSF HVA were not different in the presence or absence of either E or T. Neither of these metabolites reflected MAO-A or MAO-B gene expression (data not shown).

Correlations

To examine the relationships between MAO-A, 5HIAA and serotonin, a series of linear regressions were performed using the group means ($n = 4$) for each variable (Fig. 7). In the top panel, there was a positive correlation between 5HIAA and MAO-A-positive pixel area ($r^2 = 0.78$). Thus, as MAO-A went down, so did 5HIAA. In the bottom panel, quantitative measurements of serotonin-positive pixel area in the LC were extracted from a previous study of the same animals (Bethea et al., 2014), and used as a numerical reflection of the amount of serotonin reaching terminal fields. Serotonin axon-positive pixel area was inversely correlated with 5HIAA with $r^2 = 0.69$, indicating that as 5HIAA decreased, serotonin increased.

DISCUSSION

Human relevance

Violence is “the intentional use of physical force or power, threatened or actual, against oneself, another person, or against a group or community, which either results in, or has a high likelihood of resulting in injury, death, psychological harm, mal-development, or deprivation” (Krug et al., 2002). Violent aggression is a serious burden on human society. In 2010 worldwide, all forms of violence caused approximately 1.34 million deaths compared to about 1 million in 1990 (Lozano et al., 2012). For every death due to violence, there are numerous non-fatal injuries resulting from violence. In 2008, medical attention was needed for over 16 million cases of serious non-fatal violence-related injuries. Other forms of violence such as elder maltreatment, intimate partner violence and child maltreatment are highly prevalent (http://www.who.int/violence_injury_prevention/violence/global_campaign/gcwp_plan_of_action.pdf). CDC estimates the cost of violent deaths in the US at \$47.2 billion in 2005 (<http://www.cdc.gov/ViolencePrevention/violentdeaths/index.html>).

In addition, the prevalence of aggression in persons with dementia or intellectual disabilities is estimated in the 30–50% range and poses a significant threat to caregivers. Treatment with antipsychotic medications is often initiated at the onset of agitated/aggressive behaviors during emergency room visits (Lindenmayer, 2000), whereas selective serotonin reuptake inhibitors (SSRIs) have little efficacy in the treatment of these agitated/aggressive pathologies (Fava, 1997; Goedhard et al., 2006; Sohanpal et al., 2007). Moreover, other sedating or antipsychotic medications are often the last resort for long-term care.

Androgens and aggression

Androgen administration, including anabolic steroids, to rodents (mice, rats, or hamsters) increases dominance, competitive inter-male aggression, aggression in response to provocation and aggression in resident intruder paradigms (Bonson and Winter, 1992; Melloni and Ferris, 1996; McGinnis et al., 2002; Clark and Henderson, 2003; McGinnis, 2004). In NHPs, T increases prior to and during mating season and correlates with an increase in aggression (Rostal et al., 1986). In addition, a significant positive correlation existed between aggression and plasma T in a group of 34 male monkeys (Rose et al., 1971). A correlation was also found between competitive aggression and T in CSF (Higley et al., 1996; Mehlman et al., 1997). In humans, there are data to suggest that in populations predisposed to antisocial behavior, the concentration of T in saliva (free T) may be a factor in instigation of more aggressive crimes (Kreuz and Rose, 1972; Dabbs et al., 1987). It has also been hypothesized that T is associated with competition in males, and that prison inmates have poorer control of competitive urges (Archer, 1991). Since androgens increase competitive aggression in macaques (Higley et al., 1996), it is possible to determine the effect of androgens on various neural systems and then postulate their involvement in aggressive behavior.

Androgens, serotonin synthesis and axonal serotonin

The ability of this laboratory to examine different components of serotonin regulation in the same monkeys suggested that elevated competitive aggression, elevated CSF concentrations of T and decreased CSF 5HIAA in macaques likely co-occur with increased serotonin and low activity of MAO-A. Indeed, T (via metabolism to E) suppressed MAO-A thereby decreasing CSF 5HIAA. T and DHT also increased TPH2 gene expression, which is likely accompanied with increased TPH enzyme expression, and increased TPH enzyme should result in an increase in serotonin production (Bethea et al., 2000, 2014; Sanchez et al., 2013).

However, E from aromatization of T, was also necessary for optimum axonal serotonin content (Bethea et al., 2014). Serotonin axon density was greatest in T-treated animals, modestly reduced in castrated males and severely reduced by aromatase block. TPH2 gene expression (potentially serotonin production) can be dissociated from serotonin in terminal fields with DHT + ATD. That is, DHT increased TPH2 gene expression, but ATD reduced detection of serotonin axons in a terminal field. Thus, when DHT + ATD-treated animals were exposed to a releasing agent like fenfluramine, there was little serotonin available for release. This may mean that in animals treated with non-aromatizable anabolic steroids, TPH2 enzyme may be elevated, but it does not necessarily lead to elevated serotonin in terminal fields where many serotonin measurements have been made. Indeed, Grimes and Melloni found a significant reduction in serotonin varicosities of anabolic steroid-treated hamsters in the hypothalamus and amygdala (Grimes and Melloni, 2002). However, this observation may not reflect the overall function of the serotonin system. The mechanism whereby E promotes axonal content of serotonin is not resolved.

Serotonin regulation by MAO-A

An important aspect of serotonin regulation or availability is its degradation. Serotonin is predominately degraded by MAO-A (Fowler and Tipton, 1984; Youdim and Finberg, 1991), which was suppressed by E in both male and female macaques. In female macaques, the ovaries produce E; and E therapy suppressed MAO-A expression (Gundlach et al., 2001). In males, E is produced by the aromatization of T and by de novo synthesis in the brain (Roselli and Resko, 1984; Roselli et al., 1987; Fester et al., 2011). Our data show that inhibition of aromatase, and absence of E production, significantly elevated MAO-A. The suppressive action of E on MAO-A would lead to lower production of CSF 5HIAA as observed herein. Our data are also consistent with previous observations that T administration to castrated male rats decreased MAO-A activity in the cortex (Meyers et al., 2010). Another study in neuroblastoma and glioblastoma cells in culture showed that a specific androgen, R1881, increased the MAO-A promoter and catalytic activities through ARs (Ou et al., 2006). However, we found that serotonin neurons in male macaques lack AR (Bethea et al., 2015). Thus, the lack of an effect of androgens on MAO-A in male serotonin neurons *in vivo* may be due to the absence of AR.

MAO-B

We observed an increase in raphe MAO-B in the presence of T and DHT. Inhibition of aromatase by ATD had no effect, implying that androgens increase MAO-B via AR, which

do not co-localize in serotonin neurons (Bethea et al., 2015). In the raphe, increased MAO-B could degrade incoming NE from the LC (Samuels and Szabadi, 2008). LC NE plays a major role in vigilance in NHPs (Aston-Jones et al., 1996) and anxiety in humans (Coccaro et al., 1991). Therefore, elevated MAO-B might decrease vigilance and/or attention to socially provocative stimuli (Haller et al., 1998).

Inverse relationship of CSF 5HIAA and CNS serotonin

The CSF 5HIAA samples exhibited variation within each group. This was caused by one sample in each group exhibiting a concentration of 5HIAA approximately 1/10 of the other samples in the same group. It has been reported that CSF 5HIAA was not correlated with animal weight, elapsed time from capture to sample or time of day (Mehlman et al., 1997). Blood contamination can increase detectable serotonin so any sample with visual hemolysis was discarded. Statistically, the low samples were within two standard deviations of the mean of the remaining samples so they were included in the analysis. We found that the absolute levels of 5HIAA exhibited a trend toward suppression in the presence of E, but not androgens, with a medium effect size. However, for the Cohen's *d* analysis, the T group was included in both the 'presence of E' combination and in the 'presence of androgen' combination. Therefore, these analyses were informative, but not entirely definitive. Hindsight regarding experimental design always conjures better approaches. Future studies are planned in which more definitive treatments will be applied and samples of CSF will be rapidly collected after ketamine sedation. Nonetheless, the trend in our data is the same as that reported with statistical significance (and many more samples) in several other studies of humans and NHPs (Linnoila et al., 1983; Tuinier et al., 1995; Higley et al., 1996; Mehlman et al., 1997).

This study suggests how CSF 5HIAA reflects central serotonin. There was a positive correlation between 5HIAA and MAO-A gene expression, both of which were significantly suppressed by E and unaffected by T. Thus, when MAO-A expression decreased, 5HIAA also decreased and vice versa. In addition, 5HIAA was *inversely* correlated to axonal serotonin indicating that serotonin was higher when MAO-A activity was lower [degradation was decreased]. Therefore, decreased serotonin degradation and decreased CSF 5HIAA probably mean an increase in available serotonin if all else is equal. NE, DA, arginine vasopressin (AVP), tachykinin, and glutamate have also been implicated in aggression in a number of rodent studies (Ferris and Potegal, 1988; Levine et al., 1990; Haller et al., 1998; Giegling et al., 2007; Asahina et al., 2014), but how the entire circuit functions in the presence and absence of androgens, or provocation, in primates needs further investigation.

Aromatase

Aromatase was expressed in large serotonin-like neurons of the dorsal raphe, medial fascicularis, and median raphe, as well as in many other neurons suggesting that neuronal aromatase is widespread in the male midbrain. Our treatments did not alter aromatase protein detection in the dorsal raphe, and the protein expression of aromatase was not affected by inhibition of aromatase activity with ATD. A previous study demonstrated that aromatase mRNA expression increased in the hypothalamic preoptic area with T and DHT

treatment, and in the medial basal hypothalamus with T only. However, aromatase mRNA was not regulated by androgens in any of the 12 other brain areas examined (Resko et al., 2000). In addition, T increased aromatase *activity* in hypothalamic areas that did not show regulation of aromatase *mRNA* (Roselli and Resko, 1989). Thus, these observations indicate that aromatase was constitutively expressed in macaque midbrain and that inhibition of activity with ATD did not significantly impact aromatase protein detection.

CONCLUSION

Our data reconcile studies of elevated T, elevated aggression, and lower CSF 5HIAA in NHPs with studies that show low-activity MAO-A produced elevated serotonin and aggression in humans and rodents (Mehlman et al., 1994; Higley et al., 1996; Caspi et al., 2002; Huang et al., 2004; Newman et al., 2005; Alia-Klein et al., 2008; Wobber et al., 2013; Byrd and Manuck, 2014; Goetz et al., 2014). T or DHT increased TPH2 mRNA, which promotes increased serotonin synthesis; and the metabolism of T to E decreased MAO-A, decreased CSF 5HIAA, increased serotonin in axons of terminal fields and increased fenfluramine-stimulated serotonin/prolactin release (Bethea et al., 2013, 2014, 2015). Therefore, the combined androgenic and estrogenic activities of T likely increase CNS serotonin and decrease serotonin degradation to 5HIAA that in turn, correlates with increased overall aggression depending on environmental context.

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Abbreviations

5HIAA	5-hydroxyindoleacetic acid
ANOVA	analysis of variance
AR	androgen receptors
CSF	cerebrospinal fluid
DHT	dihydrotestosterone
ER	estrogen receptors
FLUT	flutamide
HVA	homovanillic acid
IHC	immunohistochemistry
ISH	<i>in situ</i> hybridization
LC	locus coeruleus

LC/MS	liquid chromatography/mass spectrometry
MAO-A	monoamine oxidase-A
MAO-B	monoamine oxidase B
MHPG	3-methoxy-4-hydroxyphenylglycol
NE	norepinephrine
NGS	normal goat serum
NHPs	nonhuman primates
ONPRC	Oregon National Primate Research Center

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Fig. 1. Photographs of MAO-A and MAO-B *in situ* hybridization (ISH) signals and aromatase immunohistochemistry (IHC) signal (indicated in the left margin). The left column contains low-power montage photographs of the dorsal raphe region that were obtained on the Marianas workstation with Slidebook 5.0 (panels A, D, G). The middle and right columns contain photographs obtained with a Zeiss Axioplan brightfield microscope and a Zeiss AxioCam digital camera (panels B, C, E, F, H, I). The MAO-A and -B signals were largely localized to neurons in the dorsal raphe. Aromatase staining was more widespread, occurring in multiple cell types in the dorsal raphe and in the large acetylcholine neurons of the trochlear nucleus (nucleus of the IVth facial nerve) located under the arches of the dorsal raphe. Aromatase-positive neurons were also widespread outside of the photographed area.

Fig. 2.

Photographs of four different morphological levels of the dorsal raphe nucleus. The sections were immunostained for aromatase, which provided robust staining and illumination of the morphology of the dorsal raphe. In anterior levels 1 and 2, the neurons underneath the arches of the dorsal raphe are aromatase-positive neurons of the trochlear nucleus (nucleus of the IVth facial nerve).

MAO-A

Fig. 3. Histograms illustrating the positive pixel area covered by MAO-A ISH staining. The *top* panel illustrates the significant difference in MAO-A-positive pixel area across the four groups. In the *bottom* panels, groups were combined and compared with a *t*-test. *Left*: the combined group with some E present in the brain (T and placebo) was suppressed compared to the combined group with no E present in the brain (DHT + ATD and FLUT + ATD). *Right*: the combined group with some androgen present in the brain (T and DHT + ATD) was not different compared to the combined group with no androgen present in the brain

(placebo and FLUT + ATD). *Top panel - different from T-treated group, Newman-Keuls post hoc analysis. *Bottom panels – difference between groups with *t*-test, $p < 0.05$.

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MAO-B

Pixels

Pixels ± Estrogen

Pixels ± Androgen

Fig. 4.

Histograms illustrating the positive pixel area covered by MAO-B ISH staining. The top panel shows MAO-B-positive pixel area across the four groups (not different, ANOVA). In the bottom panels, groups were combined and compared with a *t*-test. *Left*: the combined group with some E present in the brain (T and placebo) was not different compared to the combined group with no E present in the brain (DHT + ATD and FLUT + ATD). *Right*: the combined group with some androgen present in the brain (T and DHT + ATD) was higher

compared to the combined group with no androgen present in the brain (placebo and FLUT + ATD). *Bottom panels – difference between groups, with t -test $p < 0.05$.

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Fig. 5.

Histogram illustrating the aromatase-positive pixel area in the dorsal raphe. There was no difference in the aromatase-positive pixel area in the dorsal raphe.

CSF 5HIAA

Fig. 6. Histograms illustrating the concentration of 5HIAA in CSF of the four treatment groups. There was no difference in CSF 5HIAA across the four groups by ANOVA. In the bottom panels, groups were combined and compared with a *t*-test. *Left*: the combined group with some E present in the brain (T and placebo) appeared slightly, but not significantly, lower compared to the combined group with no E present in the brain (DHT + ATD and FLUT + ATD). *Right*: the combined group with some androgen present in the brain (T and DHT +

ATD) was not different compared to the combined group with no androgen present in the brain (placebo and FLUT + ATD).

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Fig. 7. Illustration of pertinent regression analyses of 5HIAA versus MAO-A mRNA expression (top) and 5HIAA versus serotonin (5HT) immunostaining in the axons innervating the LC (bottom). *Top*: there was a positive correlation between MAO-A-positive pixels and 5HIAA. *Bottom*: there was an inverse correlation between 5HT-positive pixels and 5HIAA.

Table 1Comparison of endpoints with Cohen's *d* analysis for effect size

Groups*	Cohen's <i>d</i>
MAO-A pixels ± ER	1.649
MAO-A pixels ± AR	0.332
MAO-B pixels ± ER	0.091
MAO-B pixels ± AR	1.836
5HIAA ± ER	0.548
5HIAA ± AR	0.117

* Individual treatment groups were combined that had activation of ER or activation of AR. Therefore, T- and placebo-treated groups had ER activation to different degrees, whereas ATD-treated groups had no ER activation (DHT + ATD and FLUT + ATD). T- and DHT-treated groups had AR activation, whereas placebo- and FLUT-treated groups had no AR activation. Cohen's *d* 0.8 is considered a large effect.