Annals of Psychiatry and Clinical Neuroscience

Research Article
Published: 09 Oct, 2020

Effects of Gender in Rat Models of Depression: Acute Tryptophan Loading in Chronically Tryptophan-Depleted Rats Some New Old Data

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Abstract

Study looks at effects of acute TRP loading on TRP metabolism in TRP depleted male and female rats. Sprague-Dawley rats were fed a low TRP diet for 0 (control), 6, 14 and 21 days. Animals were then loaded (ip) with TRP solution (80 mg/kg) one hour prior to being sacrificed. Procedures were carried out between 08.00 to 10.00 hours to reduce effects of estrus in females.

TRP depletion directly enhanced the effect of TRP loading on plasma TRP, brain TRP, 5-HT, 5-HIAA and DA in male rats. Enhancements increased synchronously with the length of dietary period. In females, only plasma and brain TRP were similarly enhanced but brain 5-HT and 5-HIAA remained unchanged or reduced. In females, brain DA was similar to control across dietary periods.

Results suggest an impairment of the TRP metabolic pathway in female rats only. Findings increase evidence differentiating male and female rats in terms of TRP metabolism and 5-HT neurotransmission and may suggest an area of potential treatment-resistance. Increases in DA may increase euphoria and decrease depression-like state in male rats only? Our recent evidence suggests that changes in inflammatory cytokines and HPA alter hepatic TRP/kynurenic metabolism which may indirectly affect central glutamate neurotransmission and end-point depression-like behavior in this model of depression. Our most recent results show that the multi-modal antidepressant, vortioxetine reversed TRP depletion-induced behavioral change in the FST and biomarkers of treatment resistance in female rats whereas paroxetine did not. Overall, alternative treatments may be more effective in women with treatment-resistant depression than in men.

Keywords: Depression; Tryptophan depletion model; Gender; Estradiol; Tryptophan metabolic pathway

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Received Date: *16 Sep 2020*
Accepted Date: *06 Oct 2020*
Published Date: *09 Oct 2020*

Citation:
Franklin M. Effects of Gender in Rat Models of Depression: Acute Tryptophan Loading in Chronically Tryptophan-Depleted Rats Some New Old Data. Ann Psychiatr Clin Neurosci. 2020; 3(3): 1035.

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Introduction

That the brain differs in make-up between males and females is not new. It is known that anatomists in the nineteenth century were able to establish that human brain weight differed across gender. Brain sex differences directly and particularly affect biochemical processes, can contribute to the susceptibility to specific diseases and influence certain defined behaviors. It is also recognized that genetic sex differences may lead to differences across genders in the origins and progression of disease and how differences in neural development could result in changes in cognition and behavior. Hence, it is important that gender is taken into account as a highly relevant contributing factor for the understanding of the pathogenesis of depression. Major depression is a common psychiatric disorder with complex and multifactor origins. Its pathogenesis is associated with deficits of monoamines, particularly serotonin (5-hydroxytryptamine, 5-HT) with consequential receptor functional alterations, Hypothalamic Pituitary Adrenal (HPA) axis dysfunction and abnormalities of the inflammatory/immune system. It happens more frequently in women than men and is frequently associated with treatment resistance.

Why is depression more prevalent among women? Increased prevalence of depression is directly with hormonal changes in women, more of tenduring puberty, prior to menstruation, following pregnancy [1] and at the perimenopause. Early studies of mood fluctuations postpartum suggested that reduced corticosteroid output may be causative [2]. Whilst a later study suggested that the massive hormonal flux changes that follow birth may be related to downward mood changes [3]. It follows that female hormonal fluctuations may be a trigger for depression. For instance in this study placental estriol and progesterone were present in extreme concentrations during pregnancy

only for them to crash to virtually zero levels within a very few days of parturition. Indeed, one recent animal model of depression in female rats utilizes progesterone withdrawal [4]. However, the greater majority of preclinical studies focus on male animals simply to avoid variability in behavior because this can be associated with hormonal cycles.

It is commonly recognized that estrogens may influence depressive symptoms including irritability, insomnia, appetite, diet and other general physical aspects [5,6]. A recent study has suggested that women who have used an oral contraceptive (particularly monophasic contraceptives), demonstrated reduced rates of major depression and anxiety compared with nonusers [7]. This suggests that moderating estradiol levels may be protective. Study results suggest that Hormone Replacement Therapy (HRT), particularly during the perimenopausal period, could be effective in the prevention of postmenopausal depression in women [8]. In essence, these studies suggest that estradiol could have a protective effect on the pathology that underpins depression and that reductions in estradiol may increase the risk for depression.

Previous studies of ours show that when TRP-depleted female ovariectomized rats are neuroendocrine challenged with serontonergic - acting drugs that the hormonal responses are significantly reduced (actually to almost no response) when compared to TRP-depleted intact female rats at diestrus (unpublished data). When TRP-depleted ovariectomized female rats received estrogen replacement treatment (with plasma estadiol levels in the diestrus range), hormonal responses to neuroendocrine challenge by serotonergic - acting drugs are restored and indeed significantly enhanced when compared to TRP-depleted intact female rats at diestrus. In castrated male rats hormone responses to neuroendocrine challenge by serotonergic-active drugs are reduced compared to intact males. However, in direct contrast to female rats, hormonal responses to such challenges in castrated male rats treated with testosterone replacement are only restored to that found in intact males and are not enhanced [9]. Such findings suggest to the author, that estradiol is more potent than testosterone at restoration of hormonal responses to neuroendocrine challenge by serotonergic drugs such as fenfluramine, mCPP and buspirone. Overall, such results appear to underpin the general notion that female rats are more sensitive/vulnerable to changing hormonal environments than their male counterparts.

Why then do men, who lack systemic estrogen, have lower rates of depression than women? It is certainly known that men have low levels of circulating estrogen due to a metabolic conversion of testosterone and this may presume some protective level of brain estrogen receptor function. However, it is more likely in men that the steadiness of testosterone secretion and its effect on androgen receptors in the brain may confer more protection than the fluctuating levels of estrogens in females [10]. These thoughts may be too simplistic in nature and that in reality it is much more complex, than simply down to differences in hormonal secretion rates.

Biochemical diversities additionally occur between male and female rats in terms of their neurotransmitter systems. Biochemical sex differences in the central monoamine systems of the rat have been reported [11]. Some studies describe sex differences in the serotonergic system, which at least on a pre-synaptic level, looks to be more obvious in females [11,12]. Behavioral studies indicate that the apparent biochemical enrichment of the brain 5-HT system of females can have functional effects, when the 5-HT system is pharmacologically challenged by administration of a monoamine oxidize inhibitor with L-TRP, the 5-HT syndrome (a specific behavior with characteristic actions e.g. fore-paw treading in the rat) and the change in body temperature produced are more pronounced in female than male rats [12-14]. Carlsson and Carlsson [15] conducted a regional study of the rat brain. They showed that the female rat generally contained significantly greater amounts of both 5-HT and 5-HIAA in most of the areas of the brain studied than did male rats. They suggested that the female brain 5-HT system had greater potential than the male rat brain. More recent studies with SSRIs have demonstrated gender preferential effects in rats. Longer term administration of fluoxetine reduced fear responses during extinction learning and extinction recall in female but not in male rats and this effect appears to be modulated by the estrous cycle [16]. Several studies have report that women respond more effectively to Selective 5-HT Re-uptake Inhibitors (SSRI) than men [5,17,18]. Such an understanding could help develop sex-specific therapies, which will be more helpful for women. For example, understanding how SSRIs may interplay with estrogens could help improve the efficacy of current treatments used for anxiety disorders in women. Also, gender differences in psychotropic drug responses can be related to pharmacokinetic sex differences and therefore this should additionally be taken into account when results from animal and human studies are considered and interpreted [19,20].

Generally speaking in preclinical pharmacological studies only male rats have been used to develop and validate models of psychiatric disorders [21]. Female behavior is frequently assumed to be similar to male behavior, but more variable. Hence, female animals are not usually utilized, since researchers believe that estrous cycles may confound expected results [22]. Few animal models of depression have tested female rats. However, the Chronic Mild Stress (CMS) model has been tested in both male and female rats. The model is focused on the idea that low level log-term and unpredictable stressors, similar to those that we as humans might have in our everyday lives, can induce depression in some vulnerable individuals [23]. As in other depression models, CMS produces a broad range of behavioral deficits and altered sleep patterns. Some behavioral alterations are reversed by chronic, but not acute, treatments with common antidepressants [24]. CMS disrupts the HPA axis and induces a depression-like phenotype in both male and female rats [25]. The CMS model is widely used and is generally recognized as an effective one, however it is known to be difficult to use and is time consuming to set up and run. Additionally, because the types of stressors and length of stress varies widely from lab to lab, resulting behavioral outcomes are often variable in nature.

TRP depletion reduces brain 5-HT synthesis and causes an up regulation of postsynaptic 5-HT receptors. It is of interest that male rats TRP depleted and castrated, demonstrate a loss of postsynaptic 5-HT receptor up regulation as demonstrated by enhanced plasma prolactin responses to the 5-HT2C agonist, mCPP9. However, prior treatment with testosterone propionate or estradiol benzoate respectively showed either restoration of the 5-HT receptor up regulation with testosterone or no effect in the case of estradiol. It was suggested that testosterone treatment enabled a reduction of 5-HT synthesis and thus restored 5-HT receptor up regulation and that estradiol treatment was unable to do this. Whereas in ovariectomized TRP depleted female rats again demonstrate a loss of postsynaptic 5-HT receptor up regulation as demonstrated by enhanced plasma prolactin responses to the 5-HT2C agonist, mCPP but that treatment

with estradiol reinstates postsynaptic 5-HT2C receptor up regulation and indeed enhances the response (Franklin, unpublished data). Treatment with testosterone did not restore 5-HT2C receptor up regulation. The findings overall suggests that estradiol perhaps has a more potent effect on 5-HT synthesis than testosterone. Hence, because estradiol is known to vary through the rat estrus cycle and indeed in the female menstrual cycle too, that this may induce changes in the level of 5-HT synthesis through those cycles. This may also be true in the female menopause when estradiol tends to zero output. It is therefore possible because of this that the female gender may be more vulnerable to depression than males.

Defining and basic findings from our previous studies researching the a etiology of eating disorders such as anorexia nervosa, suggested that longer Term Tryptophan (TRP) depletion by diet could form the basis for a unique animal model of depression [9,26,27]. We have presented depression models in both male [28,29] and female rats [30]. TRP depletion by diet induced changes in female rats which were generally larger and occurred earlier than in the males. Numerous defining time-course studies in these models demonstrated that 2 weeks of TRP depletion produced optimal effects on both biochemical and behavioral measures of depression. Beyond this time point, the model fails to demonstrate good construct validity due largely to the inception of adaptive changes in animals following extended TRP depletion [9].

Diet-induced TRP depletion in female rats resulted in a significant reduction of brain 5-HT and induction of depression-like behavior [29]. The depression-like state was associated with an increase in the stress hormone aldosterone, which has a potential relationship to depression [30-32]. In the female rat model of depression [29,33], aldosterone secretion increased after just 4 days of TRP depletion, prior to the rise in serum corticosterone and may potentially be an early marker for depression onset. TRP depletion led to cortical 5-HT2A receptor up regulation and paroxetine treatment normalized this effect. However, the TRP depletion-induced behavioral changes were not reversed by paroxetine thus suggesting a non-5-HT mediated mode of resistance [29]. Changes in the Kynurenine (K)/Kynurenic Acid (KA) ratio, magnesium and N-methyl-D-Aspartate (NMDA) receptor expression could not be reversed by paroxetine treatment and hence the K pathway and glutamate neurotransmission might be involved in the mechanisms of the development of resistance to SSRIs in this model [29]. It should be noted that similar effects in male rats were reversed by paroxetine treatment [28]. Interestingly, a recent study has shown that kynurenic acid is lower in female Caucasians than men, which may explain their lower incidence of schizophrenia and perhaps also their greater vulnerability to depression [34]. American and Hispanic women have a lower TDO and TRP oxidation relative to free TRP than the corresponding men. Authors suggested that future studies of the kynurenine pathway in relation to health and disease should focus on gender and ethnic differences.

Changes in corticosterone and aldosterone concentrations were significantly negatively correlated to weight gain in the male rats but not in females [28,29].

The involvement of the glutamate system in mood disorders such as depression was initially proposed based on preclinical studies of NMDA receptor blockers [35]. In addition, early clinical studies showed altered glutamate levels in serum and cerebrospinal fluid from patients with mood disorders [36,37]. There is also mounting evidence that inflammation is linked with dysregulation of the kynurenine pathway in at least some suicide patients which could possibly be the result of an imbalance of neuroactive metabolites such as quinolinic acid and KA found in such patients [38]. That TRP-depletion induced NMDA receptor expression up regulation was not reversible by SSRI treatment in our female animal model of depression may suggest that the glutamate system is compromised in treatment-resistant depression, at least in females.

We have used the chronic TRP-depletion model of depression to test the viability of potential new antidepressants. We evaluated the effects of vortioxetine and paroxetine on biomarkers associated with TRP depletion including serum aldosterone, corticosterone and IL-6 levels together with indirect indicators of glutamate neurotransmission in our female rat model [39]. The relatively new multimodal antidepressant, vortioxetine reduced TRP depletion-induced increases of serumal dosterone, corticosterone, IL-6 and Nmethyl-D-aspartate and α7-nicotinic acetylcholine receptor expression in the amygdala and hippocampus, respectively. Paroxetine showed little effect except a reduction of aldosterone. Vortioxetine but not paroxetine reversed TRP depletion-induced reductions of both serum and brain kynurenic acid. In conclusion, vortioxetine, but not paroxetine, enabled reversals of TRP depletion-induced changes of depression-like behavior and markers of glutamatergic activity. Vortioxetine reversed significantly TRP depletion-induced reductions of pineal melatonin and 5-HT and significantly increased pineal NA [40]. Paroxetine did none of these things.

Aims

A number of biochemical gender differences in the serotonergic and other systems have been described both in our depression models and indeed between depressed patient groups. The present described study was carried out twenty-five years ago. This work has not been published previously. The reasons for carrying out this study have been obscured by time passed. However, the resultant data from this study are entirely relevant to our more recent studies which have tried to disseminate some of the mysteries and possible causes of treatment resistance in depression across gender in our animal models. The aim was to study the effect of a low TRP diet over various time periods on exogenous TRP loading in both male and female rats. The TRP load described here was taken directly from the study of Wurtman et al. [41]. They reported brain TRP effects to dosing loads over the range 0 to 125 mg/kg TRP. Brain TRP was increased in a dose dependent manner in their study. The dose of 80 mg/kg chosen here was close to the maximum dose used by those authors and it was predicted that this would cause significant changes in brain TRP content in our animals.

Methods

Animals

In this study male and female Sprague-Dawley (SD) rats were purchased from Tuck (Battle Bridge, Essex, UK). Animals weighed around 200 g were housed five to a cage and were maintained on a 12-h light cycle (06.00 h to18.00 h). They had free access to food and water at all times. The low TRP diet and its equivalent control diet (RM-1) were purchased from Scientific Diet Supplies Ltd (Witham, Essex, UK). The basic make-up of the diets consisted of cereal product (maize) 34%, vegetable protein (soya meat) 13%, animal protein (gelatine) 5%, energy source (corn oil, caster sugar) 38% and supplement (vitamin/mineral mix) 10%. Diets were prepared in pellet form. Manufacturers calculated that the control diet contained 0.19%

Table 1: Shows the effect of a low TRP diet over the periods 0 to 21 days of exogenous TRP loading on plasma TRP, brain TRP, 5-HT, 5-HIAA and DA in male and female rats respectively. Each value represents the mean ± SEM from 5 animals.
*p<0.05; **p<0.01 v control (0)

	Male				Female			
Days	**0**	**7**	**14**	**21**	**0**	**7**	**14**	**21**
Plasma T.TRP	52.5 ± 9.8	78.3 ± 10.6	87.5 ± 15.5	88 ± 6.5**	50.5 ± 4.9	65.3 ± 4.7	88.5 ± 5.6**	68.5 ± 3.9*
µg/g	*19.7 ± 0.5*	*11.7 ± 0.7*	*9.9 ± 1.8*	*12.5 ± 0.9*	*21 ± 1.7*	*15.4 ± 1*	*16.5 ± 1.2*	*17.6 ± 1.2*
Plasma F.TRP	27.4 ± 0.9	46 ± 8.4	73.9 ± 15.9*	57.4 ± 9.9**	23.4 ± 2.6	28.6 ± 3	29.8 ± 1.9	28.4 ± 2
µg/g	*2.0 ± 0.3*	*1.5 ± 0.4*	*1.2 ± 0.3*	*1 ± 0.3*	*2.3 ± 0.4*	*1.8 ± 0.2*	*1.3 ± 0.1*	*1.4 ± 0.1*
Brain TRP	11.1 ± 3.3	23.6 ± 4.8	28.2 ± 3.6*	29.3 ± 1.5**	12 ± 0.9	13.2 ± 0.7	20.4 ± 2.2*	26.4 ± 1.8**
µg/g	*4.5 ± 0.5*	*3.5 ± 0.4*	*3.1 ± 0.8*	*2.4 ± 0.1*	*2.9 ± 0.3*	*2.2 ± 0.4*	*2 ± 0.2*	*1.5 ± 0.2*
Brain 5-HT	1.2 ± 0.4	2 ± 0.2	2.9 ± 0.6*	3.3 ± 0.6*	1.3 ± 0.1	1 ± 0.1	0.9 ± 0.1	0.8 ± 0.8
µg/g	*0.9 ± 0.1*	*0.6 ± 0.1*	*0.7 ± 0.1*	*0.5 ± 0.1*	*1.1 ± 0.2*	*0.8 ± 0.2*	*0.7 ± 0.2*	*0.6 ± 0.2*
Brain 5-HIAA	1.2 ± .1	1.6 ± 0.2	1.7 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	1 ± 0.1	0.5 ± 0.1*	0.6 ± 0.1*
µg/g	*1.1 ± 0.1*	*0.7 ± 0.1*	*0.7 ± 0.1*	*0.3 ± 0.2*	*1.5 ± 0.1*	*1.2 ± 0.2*	*1.0 ± 0.1*	*0.8 ± 0.2*
BCAAs	621 ± 18	642 ± 52	488 ± 33*	489 ± 40*	552 ± 28	559 ± 17	441 ± 25*	384 ± 26**
µg/g	*307 ± 15*	*256 ± 35*	*225 ± 46*	*285 ± 38*	*335 ± 43*	*307 ± 51*	*259 ± 61*	*358 ± 23*
Brain DA	1.8 ± 0.5	1.6 ± 0.3	3.7 ± 0.7*	3.2 ± 0.4*	1.6 ± 0.2	1.4 ± 0.2	1.8 ± 0.4	1.5 ± 0.3
µg/g	-	-	-	-	-	-	-	-

TRP and that the reduced TRP diet contained 0.06% TRP (it should be noted that this is higher than the low TRP diet used in our more recently described studies at 0.04% TRP e.g. Franklin et al. [28]). All animals were on the diet for a minimum period of 7 days prior to commencement of experiments.

Laboratory analysis

Plasma free and total TRP together with brain TRP were measured by High Performance Liquid Chromatography (HPLC) with fluorometric end-point detection. Brain 5-HT, 5-HIAA and Dopamine (DA) were measured by HPLC with coulometric end-point detection [42]. For total TRP, samples were deproteinized with methanol prior to injection into the HPLC system. For plasma free TRP levels, plasma was initially passed through a 10,000 molecular weight cut-off filter unit (Millipore, UK Ltd, Watford, UK) using centrifugation. This separates the free TRP from that bound to albumin. For monoamine analysis, whole brain tissue samples were homogenized and deproteinized with 5 volumes of 5% trichloro acetic acid, followed by centrifugation before to direct injection into the HPLC.

The intra- and inter-assay Coefficients of Variation (CVs) across all HPLC procedures described ranged 4.2% to 6.4% respectively.

Plasma Branch Chain Amino Acids (BCAA's) were measured by an enzymatic technique utilizing leucine dehydrogenase and UV end-point detection [43,44].

Plasma estradiol concentrations were measured by an in-house Radioimmunoassay (RIA) which utilized charcoal separation and for which both intra- and inter-assay CVs were respectively <5%.

Statistics

Differences between plasma TRP, brain TRP and brain amine data were analyzed by ANOVA with post-hoc t-tests for independent means.

Experimental

In the experiment four groups each of male and female rats (n=5) were fed the low TRP diet for 0 (the control diet only), 6, 14 and 21 days respectively. Experiments were carried between 08.00-10.00 hours. Blood was taken into pre-heparinized tubes *via* the trunk. Subsequently, the whole brain was removed and quick-frozen on dry ice and stored at -80°C until required for analysis.

The TRP loading solution was prepared by dissolving L-TRP (Sigma Ltd., Gillingham, UK) in glacial acetic acid and then was brought to a neutral pH with 5M potassium hydroxide. Lastly, the solution was made up to a final concentration of 80 mg/5 ml with 5% glucose in water. Animals were injected intra peritoneally (ip) with the TRP solution (80 mg/kg) one hour prior to them being sacrificed by decapitation after initial stunning. Studies have shown that 5-HT concentrations demonstrate distinct characteristic changing patterns in many hypothalamic, limbic and midbrain structures with a decrease during proestrus and an increase during estrus in synchrony with changes in estradiol [44]. To avoid the possibility of large variance in results due to the estrus cycle effects, all experiments in female rats including those in comparative studies, were done during the morning (08.00 to 10.00) when estradiol levels are known to be low [45] (Franklin, unpublished data).

Results

Plasma TRP (total and free) and BCAA, brain TRP, 5-HT and 5-HIAA content following TRP loading and periods on a low TRP diet in male and female rats are given in Table 1. Also shown are values for normal TRP depleted over the same periods of time for both male and female rats. These have been added for comparison only and have not been statistically compared since the studies were run approximately 18 months apart NB none of the data has been published previously.

Repeated measures ANOVA of the plasma total TRP data showed a significant effect of time (F=5.9; DF=3.24; p=0.004) and non-significant effects of sex (F=2.5; DF=1.8; p=0.5) and sex by time interaction (F=0.6; DF=3.24; p=0.64). Over the period of TRP dieting, TRP loading increased total TRP from 2.5-fold to 7-fold in male and 2.5-fold to 4-fold in female rats respectively as compared to those

TRP depleted only. The total TRP was significantly increased on day 21 (p=0.02) in the male rats and on days 14 and 21 (p<0.001 and 0.02 respectively) in female rats on the low TRP diet when compared to those on the control diet.

Repeated measures ANOVA of the plasma free TRP data shows significant effects of both sex (F=86.2; DF=1.8; p<0.0001) and time (F=3.5; DF=3.24; p=0.03) but non-significant effects of sex by time interaction (F=2.1; DF=3.24; p=0.13). Over the period of TRP dieting, TRP loading increased free TRP from 13-fold to 60-fold in male and 10-fold to 20-fold in female rats respectively as compared to those TRP depleted only. The plasma free TRP level in male rats increased significantly by up to 3-fold over the dieting period compared to control, the maximum occurring after 14 days (p=0.02 for both 14 and 21 days respectively). Whereas in the female rat, levels increased by only 20% over the dieting period compared to control, none of which reach significance.

Repeated measures ANOVA of brain TRP data shows significant effects of sex (F=152.4; DF=1.8; p<0.0001), time (F=20.4; DF=3.24; p<0.0001) and sex by time interaction (F=5.4; DF=3.24; p=0.006). Brain TRP content in the male rat was more than two-fold greater than normal following TRP loading than in animals on the control diet, whereas in female rats, brain TRP content was increased four-fold. Brain TRP content increased with the period of dieting in both male and females. Brain TRP content was slightly greater in males than females, but none were significant.

Repeated measures ANOVA of brain 5-HT data shows a significant effect of sex (F=17.2; DF=1.8; p=0.003), but non-significant effects of time (F=1.1; DF=3.24; p=0.36) and sex by time interaction (F=2.1; DF=3.24; p=0.14). The brain 5-HT content in male rats was only slightly increased in loaded animals on the control diet as compared to normal (105%). It then increased significantly relative to the period of dieting. Whereas in the female rat, brain 5-HT content was initially slightly raised compared to normal, in rats on the control diet, however it remained reduced when compared to control values throughout the period of dieting. The brain 5-HT content was significantly greater in the male than the female rats over the period of the diet (p=0.007, 0.05 and 0.03 for days 6, 14 and 21 of the low TRP diet respectively).

Repeated measures ANOVA of brain 5-HIAA data showed significant effects of sex (F=143.3; DF=1.8; p<0.0001), time (F=2.9; DF=3.24; p=0.05) and sex by time interaction (F=5.8; DF=3.24; p=0.004). Brain 5-HIAA in male rats on the control diet after TRP loading were similar to levels found in normal male rats, whereas in female rats levels were thirty percent below normal. There was no significant difference between male and female animals on the control diet. Over the period of the diet, brain 5-HIAA levels in the male rat rose to a peak on day 14, whereas in the female, levels fell steadily from the control day to a minimum on day 14. Content was decreased in female rats on days 14 (p=0.02) and 21 (p=0.04) verses control. The 5-HIAA content was significantly higher in the male rats than female rats on day 14 of the diet (p=0.003).

Repeated measures ANOVA of plasma BCAA data showed significant effects of sex (F=12.3; DF=8.1; p=0.01), time (F=13.4; DF=24.3; p<0.0001) but not sex by time (F=0.3; DF=24.3; p=0.84). Over the period of the diet the BCAA levels decreased in both male and female rats, being significant on days 14 and 21 (male p=0.02 and =0.05; female p=0.02 and 0.005 respectively). The BCAA concentrations in the female rats were generally lower than those in the males over the same time period. However the difference was not significant.

Repeated measures ANOVA of brain DA data showed significant effects of sex (F=19.9; DF=30.4; p=0.0001), time (F=4.9; DF=30.4; p<0.05) but not sex by time (F=0.06; DF=30.4; p=0.99). Over the period of the diet, brain DA levels in the male rat rose to a peak on day 14 whereas in the female levels were similar across the time periods. In male rats only, brain DA content was significantly greater than those in controls on days 14 (p=0.03) and 21 (p=0.05) respectively.

Discussion

In both the male and female rats brain TRP was significantly increased by the TRP load and in this sense confirms the findings of Wurtman et al. [41], although they only report results for the male rat. The diet-induced period of TRP depletion greatly facilitated the TRP loading effects on plasma TRP and brain TRP in both the male and female rats.

Specifically, plasma total TRP concentrations were increased similarly in both male and female rats and the TRP-loading induced increase appeared directly related to the length of the dieting period i.e. the longer the period of TRP depletion the greater the increase in TRP level. This was more obvious in the male rat and was significantly different from the female on day 21 (p=0.03). Plasma free TRP concentrations in the control groups were similar in both male and female rats. However, the percentage increase in the male rat was twice that in the female when compared to their normal control levels. The increase in the male rat as compared to the female was significantly greater on days 14 and 21 (p=0.03 and 0.02) respectively.

Brain TRP content was not significantly increased in male rats on the control diet (mainly due to a larger variance in this group), but did increase significantly in the female rat. However, the brain TRP content was found to increase relative to the length of the dieting period, it being greatest at 21 days in both male and females. In the present study, the transport of TRP across the blood-brain barrier appears to be normal, although it seems to be enhanced by the low TRP diet in both female and male rats. This apparent increase in TRP diffusion across the blood-brain barrier may be as a consequence of reduced TRP pools within the rat brain brought about by the TRP depleting diet and possibly also aided by a fall in plasma BCAAs over the same period. Plasma BCAA concentrations generally fell over the period of the diet in both male and female rats. The fall in BCAAs noted in both male and female rats perhaps suggests that this may be a small but significant compensatory reduction to allow for an increased flow of TRP back into the brain to make up the shortfall caused by the low TRP diet. These data are in direct contrast to BCAAs found in our more recent studies in our male and female rat depression model studies where levels were generally steady and not significantly affected by dietary TRP depletion for up to 28 days compared to those rats on a control diet [28,29].

In the male rat TRP loading appeared to have only a small yet positive effect on brain 5-HT content in animals on the control diet whereas in the female rat brain 5-HT was unchanged. There was no significant difference between the levels in the male and female controls. The brain 5-HT content in the male rat rose by more than three-fold as compared to those in the control group by the end of the dietary period on day 21 and compare directly with those changes reported by Wurtman et al. [41]. In direct contrast, the female rat brain 5-HT content appeared to reduce very gradually through the

period of TRP depletion to levels below the initial control value. The male rat 5-HT content was significantly greater than that in the female rat over the whole period of study. The fact that brain 5-HT content was unchanged and indeed reduced during the period of dieting in the female suggests that there may be some impairment in the female rat TRP-hydroxylase system that converts TRP to 5-HTP, or with the second enzyme system which converts 5-HTP to 5-HT (i.e. aromatic acid decarboxylase). However, Ashley et al. [45] found little alteration in the level of TRP hydroxylase in their study in male rats on a low TRP diet for 28 days.

Brain 5-HIAA content in the male rat increased by about 30% on days 6 and 14 of the treatment period compared to control and then fell back to this level again on day 21 of the diet. In contrast, the female rat brain 5-HIAA content decreased by up to 60% as compared to control over the dieting period and again suggests that there may be a problem with 5-HT metabolism. The difference between the male and female rat brain 5-HIAA content after dieting was significant on days 14 ($p<0.001$). In general our results reflect those found by Wurtman et al. [41] and only deviate from them with respect to the small effects of TRP loading on brain 5-HT metabolism in the female rat.

Brain DA increased with the length of TRP depletion in male rats after TRP loading. Whilst in females, brain DA was similar to control across all TRP depletion periods. We have not generally monitored DA content during TRP depletion studies. However, it is perhaps of interest to note that analysis of individual amino acid levels by HPLC has consistently shown that following chronic TRP depletion in brain areas such as the PFC that phenylalanine but not tyrosine is increased in comparison to control animals (i.e. those not TRP depleted), though this is not generally found to be significant. This usually occurs in male rats only. Both phenylalanine and tyrosine are precursors of DA. This perhaps, may suggest that phenylalanine but not tyrosine might preferentially cross the blood brain barrier during TRP depletion in male rats as opposed to females. Interestingly, DA is the neurotransmitter of "euphoria". Hence, it could be suggested that in male rats that increases in DA lead to increases of euphoria and perhaps a decreased depression-like state.

This study adds to the increasing evidence differentiating the male and female rats in terms of TRP metabolism and 5-HT neurotransmission. Additionally, the present findings might suggest yet another possible pointer to treatment-resistance in the direction of TRP metabolic change and 5-HT neurotransmission. This possibly may have some knock-on effect elsewhere in the brain, since our more recent evidence strongly suggests that 5-HT modulation is not directly involved in treatment resistance per sec. Treatment with the SSRI, paroxetine reduced 5-HT2 receptor binding in TRP depleted female rats but failed to reverse depression-like behavior in those same animals [29]. The opposite was true in male rats [28]. Additionally to those neurochemical and behavioral changes observed previously in male rats, TRP depletion in females was associated with a significant reduction of serum magnesium concentrations, increased serum interleukin-6, enhanced gene expression of orexin A in the frontal cortex and an induced rise in NMDA receptor Bmax in the amygdale [29]. Depression-like behavior, NMDA receptor up regulation, enhancement of the K/KA ratio and magnesium were resistant to paroxetine treatment. Hence, with respect to treatment resistance, the underlying mechanisms may involve pro-inflammatory cytokines, the kynurenine pathway, magnesium, glutamate neurotransmission and the orexin pathway. This model of treatment-resistant depression may be useful for the future development of new compounds with novel antidepressant properties. It is therefore interesting that the relatively new multimodal antidepressant, vortioxetine which has potent SERT action as well as other strong therapeutic actions is able to reverse TRP depletion-induced behavioral change in the FST and biomarkers of treatment resistance such as those previously mentioned in female rats whereas paroxetine was unable to do this [39,40]. This may further suggest that alternatively acting treatments other than directly acting SSRIs, may be more effective in women with treatment-resistant depression than in men e.g. vortioxetine [39,40].

Analysis of plasma concentrations of estradiol in female rats, showed that all were below 30 pg/ml, a level defined as being within diestrus according to Butcher et al. [44]. There were no differences across groups (i.e. by T-test, $p=0.28$ to 0.94 respectively). Hence, all data is directly comparable and without estrus cycle influence. For direct comparison with females, all male rat procedures were likewise carried out during the morning on all the experimental days. The above findings and our previous studies with animal models of depression taken together with numerous other reported studies [16,22] show how important it is to run such experiments across gender groups. It is additionally important to make sure that in all such studies carried out in female rats that the period of estrus cycle is known and defined. This is important because 5-HT is known to vary through the estrus cycle in synchrony with changes in estradiol [46].

Summary

Overall, the TRP depleting diet was found to directly enhance the effect of TRP loading on plasma TRP, brain TRP, 5-HT and 5-HIAA in the male rat. These enhancements increased synchronously with the time period of the TRP-depleting diet. Whereas in the female, only plasma and brain TRP were similarly enhanced, whilst brain 5-HT and 5-HIAA remained unaltered or at times reduced. It is suggested that there may be an impairment of the TRP to 5-HIAA metabolic pathway in female rats. This study adds to the increasing evidence differentiating male and female rats in terms of TRP metabolism and 5-HT neurotransmission. The findings might suggest yet another possible pointer to treatment-resistance in the direction of TRP metabolic change and subsequent 5-HT neurotransmission. However, more recent evidence of ours supports the idea that 5-HT modulation is not in itself directly involved in treatment resistance per sec. The studies suggest that changes in inflammatory cytokines and the HPA cause alterations in the hepatic TRP/kynurenic pathway which then indirectly affect central glutamate neurotransmission and end-point depression-like behavior in this TRP-depletion model of depression in rats. A most recent study of ours showed that the relatively new multimodal antidepressant, vortioxetine which has potent SERT action as well as other strong therapeutic actions is able to reverse TRP depletion-induced behavioral change in the FST and biomarkers of treatment resistance such as those previously mentioned in female rats whereas paroxetine was unable to do this40. This may further suggest that alternatively acting treatments other than directly acting SSRIs, may be more effective in women with treatment-resistant depression than in men e.g. vortioxetine. Brain DA increased with the length of TRP depletion in male rats after TRP loading. Whilst in females, brain DA was similar to control across all TRP depletion periods. In all studies we have measured plasma estradiol concentrations in female rats to define estrus status. We have also run all procedures during the early morning hours when estradiol is known to be at a minimum through the estrus cycle.

Hence, it is strongly recommended that these actions are engaged for all such similar experiments.

Suggested Further Studies

Although we have not done the experiment, it would be most interesting to find out if the same experiment were carried out i.e. the effect of acute TRP loading on TRP depleted rats across genders to look at behavioral change using the FST. From the present data one might predict that acute TRP loading would reverse TRP depletion-induced behavioral changes in the FST in male rats but not in females. We would also predict that other biomarkers which we have labeled as possible measures of depression treatment-resistance such as up regulation of N-Methyl-D-Aspartate (NMDA) receptors in the amyg dala, would additionally be irreversible in female rats as compared to males.

Funding

This research was funded internally.

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