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ORIGINAL RESEARCH

Specificity of the Acute Tryptophan and Tyrosine Plus Phenylalanine Depletion and Loading Tests Part II: *Normalisation of the Tryptophan and the Tyrosine Plus Phenylalanine to Competing Amino Acid Ratios in a New Control Formulation*

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Abstract: Current formulations for acute tryptophan (Trp) or tyrosine (Tyr) plus phenylalanine (Phe) depletion and loading cause undesirable decreases in ratios of Trp or Tyr + Phe to competing amino acids (CAA), thus undermining the specificities of these tests. Branched-chain amino acids (BCAA) cause these unintended decreases, and lowering their content in a new balanced control formulation in the present study led to normalization of all ratios. Four groups (n = 12 each) of adults each received one of four 50 g control formulations, with 0% (traditional), 20%, 30%, or 40% less of the BCAA. The free and total [Trp]/[CAA] and [Phe + Tyr]/[BCAA + Trp] ratios all decreased significantly during the first 5 h following the traditional formulation, but were fully normalized by the formulation containing 40% less of the BCAA. We recommend the latter as a balanced control formulation and propose adjustments in the depletion and loading formulations to enhance their specificities for 5-HT and the catecholamines.

Keywords: acute tryptophan depletion and loading, acute tyrosine depletion test, amino acid formulations, branched-chain amino acids, catecholamines, competing amino acids, dopamine, isoleucine, leucine, noradrenaline, phenylalanine, tryptophan, tyrosine, valine

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Introduction

The acute tryptophan (Trp) (ATD) and tyrosine (Tyr) plus phenylalanine (Phe) (ATPD) depletion and loading (ATL, ATPL) tests[1,2] are powerful tools for studying the role of the cerebral monoamines serotonin (5-hydroxytryptamine or 5-HT), dopamine (DA) and noradrenaline (NA) in healthy volunteers and in those with psychiatric and behavioural disorders. In the preceding article,[3] we described the general mechanisms of action of these depletion and loading tests, one of which is competition for transport across the blood-brain barrier between six competing amino acids (CAA), namely Trp, Tyr and Phe, plus the 3 branched-chain amino acids (BCAA) Val, Leu and Ile. We[3] also reviewed the many applications of these tests and the biochemical mechanisms underpinning their use, and presented evidence for their poor specificities for the intended monoamine changes. In the present paper, we have addressed the question of specificity experimentally using the control formulation as a starting point, to be followed subsequently by applying the outcome to the depletion and loading formulations, because the control formulation: (1) is common to both the ATD and ATPD tests and their loading counterparts, as it shares the same amino acid components, except when depletion and loading are required; (2) suffers undesirable decreases in both the [Trp]/[CAA] and the [Phe + Tyr]/[BCAA + Trp] ratios, which predict the rates of entry into the brain of Trp and Tyr + Phe respectively. It is, however important first to describe briefly here evidence for the poor specificity of the different formulations in current use and the rationale for the present study.

In their review, Reilly et al (1997)[4] noted that many investigators using the ATD or its control formulation did not determine the free or total [Trp]/[CAA] ratio and only a few measured peripheral levels of Tyr or its ratio to large neutral amino acids. While it must be assumed that the [Trp]/[CAA] ratio is decreased after ATD and increased after ATL, a decrease in this ratio has also been observed with a 100 g "balanced" control formulation for ATD containing the usual 2.3 g of Trp.[5] This ratio was also decreased in the control formulation if the Trp content was increased to 3.0 g,[6] whereas further increases to 4.1 g[7] or 4.6 g[8] of Trp led to elevations of this ratio. From data extracted from our previous detailed pharmacokinetic and behavioural study[9] comparing a 50 g with the traditional 100 g dose of the amino acid formulations for the ATD and ATL tests, we reported[10] that intake of 50 g of the control formulation containing 1.15 g of Trp decreased the [Free Trp]/[CAA] ratio maximally by 61%.

We also found[10] that the [Phe + Tyr]/[BCAA + Trp] ratio was decreased in the ATD, ATL and also in the control formulation by about 50%. Broadly similar decreases (40%–60%) in this latter ratio have been reported previously[11–13] in control formulations used in the ATPD test, which are essentially similar to the control formulation for the ATD or ATL test as far as the CAA competitors are concerned. Furthermore, in the ATPD test, both the control and the Phe plus Tyr-deficient formulations are associated with decreases in the [Trp]/[CAA] ratio, of 30%–62% with the control formulation, and of 34%–96% with the depleting formulation.[2,11,13–16]

As discussed in the preceding article,[3] the reason for the above undesirable decreases in the [Phe + Tyr]/[BCAA + Trp] and [Trp]/[CAA] ratios in the control or the corresponding relevant depletion (or loading) formulations is the relatively larger contents of the three BCAA (i.e. Leu, Val and Ile), compared with those of Phe, Tyr and/or Trp, in the original Trp[1] or Tyr + Phe[2] formulation [see the traditional control formulation (F0) in Table 2]. The above ratio decreases suggest that 5-HT, DA and/or possibly NA synthesis could be inhibited by the control formulation for the ATD and ATPD tests and also by the corresponding depletion or loading formulation, an effect that could confound interpretation of behavioural changes (or lack of them).

The rationale of the present study is that, based on the above observations and on theoretical graphs designed to maintain normal Trp and Tyr ratios under the depletion, loading, or balanced condition, if the contents of the [BCAA] or of [Phe + Tyr] were to be altered independently, we proposed[10] two strategies for normalizing these ratios: (1) decreasing the contents of the three BCAA by ~ 30%; or (2) increasing those of Phe and Tyr by ~ 50%. Of these, the first is the preferred strategy, because it avoids the metabolic consequences of Phe and Tyr loading likely to be associated with the latter strategy, because, if adopted, it could contribute to a further lowering of both the free and total [Trp]/[CAA] ratios in the control formulation. A third strategy, applicable only to the control

formulation, is to increase the Trp content.[17] However, while this may improve the [Trp]/[CAA] ratio, it can only further decrease the [Phe + Tyr]/[BCAA + Trp] ratio and thus lead to a greater depletion of brain catecholamines. In the present paper, we report the results of experiments testing the first of these strategies with regard to the control formulation, successfully demonstrate the normalization of the Trp and Tyr + Phe ratios by decreasing the contents of the BCAA in the control formulation, and propose new common formulations more specific for manipulations of central 5-HT and catecholamine synthesis. A summary of part of this work has appeared in abstract form.[18]

Materials and Methods

Participants

Participants were recruited through various media advertisements and those who met the necessary criteria by telephone interview were invited to undergo a rigorous psychiatric and medical screen. Those who passed the screening tests were invited to participate. Screening involved the Structured Clinical Interview for DSM-IV,[19] Axis I Disorders, a health history and physical examination, urine drug screen (for amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine metabolites and opiates), breath alcohol screen, and urine pregnancy screen. Exclusion criteria included: (1) past or present psychiatric diagnosis; (2) past or present medical condition that would interfere with results, including head injury causing loss of consciousness for >20 min; all medical screens were stringent and, if there was any question or doubt, the person was excluded; (3) smoking >1 packet of cigarettes per day; (4) positive breath alcohol; (5) positive urine drug or pregnancy screen. In total, 48 participants were recruited and were randomly assigned to one of four groups (n = 12 each). The participants gave their written informed consents to participate in the study, which was approved by the Institutional Review Board of Wake Forest University Health Sciences Center, NC, USA (where the study was performed) and conducted in accordance with the Declaration of Helsinki.

Design

Participants between groups were matched for age, gender, and ethnicity (Table 1). A between-, rather than a within-group design was chosen for formula administration to reduce participant burden and attrition, and, as can be seen in the Results section, significant baseline differences were observed in only 5 out of 60 biochemical parameters across the 4 study groups. Although female subjects were included, the phase of the menstrual cycle was not considered, which may represent a weakness of this study (see also the Discussion section). However, all 4 study groups included female subjects in roughly similar numbers, and analysis of the vital biochemical parameters in Table 3 revealed no significant gender differences. In view of this and of these small numbers, any likely effect on results will have been equally distributed.

General procedures

Participants were instructed to fast overnight, which was verified by self-report upon arrival at the laboratory at 07.30. Expired-air and urine samples were collected and tested for alcohol and metabolites of illicit drugs, as stated above. Participants were then transported from the laboratory to the General Clinical Research Center (GCRC) where an intravenous catheter was inserted into an antecubital vein at 08.15. The viability of the catheter was maintained throughout the day with a slow, steady saline drip. The study was double blind. To standardize drink administration and maintain its double blind, a GCRC staff member mixed each of the amino-acid formulations with 8 oz of cold

Table 1. Participant Characteristics.

	Formulation			
Parameter	**F0**	**F1**	**F2**	**F3**
N	12	12	12	12
Male/Female	5/7	6/6	6/6	6/6
Caucasian/African American	6/6	6/6	6/6	7/5
Age range (years)	21–36	21–38	21–36	18–38
Mean age ± SD	27.3 ± 5.4	26.3 ± 3.9	26.3 ± 5.3	26.7 ± 5.9

water, 1 packet of Sweet and Low® sugar substitute, and flavoured the beverage with 1/8th teaspoon each of raspberry and lemonade Kool-Aid.® Because of their aversive taste and odour, cysteine and methionine were administered in capsules (2 each) and swallowed with water. Neither the study staff members nor the participants were aware of the contents of the drinks being administered. Between 09.00 and 09.15, a study staff member administered one of the four 50-g drinks containing the traditional amounts of the balanced control formulation (F0), or a formulation containing 20% less (F1), 30% less (F2), or 40% less (F3) of the BCAA Leu, Val, and Ile. The differences were made up by proportionate (and hence small) increases (i.e. 20%, 30%, or 40% increases) divided proportionately across all the other amino acids, as can be seen in Table 2. The contents of Trp, Phe and Tyr of the experimental formulations remained the same as those of the traditional formulation (F0). Blood sampling was conducted by research nurses. A fasting baseline blood sample (10 ml) was drawn at 08.45, prior to drink administration. Research nurses withdrew 10 ml blood samples at 1-hour intervals for 7 h after participants consumed the amino-acid drink. During all procedures, participants relaxed in a reclining position and were allowed to read or watch television, but were not allowed to sleep. Participants continued fasting, receiving water only, until a Trp balanced meal was provided by the GCRC at 16.30. Fasting eliminates the short-term effects of food intake on plasma amino acids. Although levels of the plasma amino acids exhibit diurnal variations, the ratios of Trp, Tyr and Phe to the other CAA are not altered between the morning and afternoon hours (for references, see[20]), i.e. during the entire 7 h time-course of our study.

Biochemical laboratory procedures

Plasma was isolated in EDTA tubes and frozen at –80 °C until transported in the frozen state to Cardiff, Wales for analysis. Ultrafiltrates (at least 0.3 ml each) were prepared from fresh plasma, before freezing, using the Amicon (Millipore) MPS-1 partition microassembly (Amicon Bioseparations, Beverly, MA, USA; Millipore, Burlington, MA, USA) and were then stored along with the plasmas. Plasma free [Trp] was determined by a modification[21] of a standard fluorimetric procedure[22] as described in detail previously[23] in duplicate 0.1 ml portions of ultrafiltrates. Total [Trp] and those of the 5 Trp competitors (CAA) were determined by our recently developed rapid gas-chromatographic method[24] using norvaline as internal standard. GC data processing and handling were performed by the associated Total Chrome software (Perkin-Elmer) and results did not require correction, as recovery of amino acids was excellent (~100%).[24] Plasma free [Trp] was determined more economically by fluorimetry in preference to GC, as the former method can process ~ 60 samples within ~ 2 h, as opposed to >8 h by GC and both methods show a very high correlation ($r = 0.9774$).[24]

Statistical analysis

Results (expressed in μM or as ratios) were analysed statistically mainly by one-way analysis of variance (ANOVA) for between- and within-group differences, and, additionally for within-group differences (time factor versus baseline values), by paired *t*-tests, using Sigma Plot (Systat, UK) version 11, with which graphics were prepared. For multiple group comparisons using this programme, the Holm-Sidak test is recommended as the first line procedure, as it is more powerful

Table 2. Composition of the four amino-acid formulations.

Amino acids	Formulation (% change in the three BCAA)			
	F0 (0%)	F1 (–20%)	F2 (–30%)	F3 (–40%)
Tryptophan	1.15	1.15	1.15	1.15
Phenylalanine	2.85	2.85	2.85	2.85
Tyrosine	3.45	3.45	3.45	3.45
Leucine*	6.75	5.40	4.73	4.05
Valine*	4.55	3.64	3.19	2.73
Isoleucine*	4.00	3.20	2.80	2.40
Alanine†	2.75	3.05	3.19	3.34
Arginine†	2.45	2.71	2.84	2.98
Cysteine†	1.35	1.49	1.57	1.64
Glycine†	1.60	1.77	1.86	1.94
Histidine†	1.60	1.77	1.86	1.94
Lysine†	4.45	4.93	5.17	5.41
Methionine†	1.50	1.66	1.74	1.82
Proline†	6.10	6.75	7.08	7.41
Serine†	3.45	3.82	4.01	4.19
Threonine†	3.25	3.60	3.77	3.95
Total	51.25	51.24	51.25	51.25

Notes: *Branched-chain amino acids, the decreases in which were compensated for proportionately across the remaining amino acids. †In each formulation.

than the Tukey or Benferroni tests and can be used for both pairwise comparisons and those versus a control group. Because there were two outliers in the F1 group (see the Results section), the number of subjects in this group was only 10. Where group sizes differ, Dunn's test is used for pairwise comparisons and those versus a control group. Where the data failed the normality (Shapiro-Wilk) test, Kruskal-Wallis one-way ANOVA on ranks was performed. A two-tailed level of significance (P) was set at 0.05. All significant within- group differences from baseline are indicated by an asterisk on the left-hand-side of each column value at the 1–7 h time points in Table 3 and by an asterisk above the relevant graph points in Fig. 1. Significant group differences between values in the F0 group on the one hand and those in the F1, F2 or F3 group on the other at the 0–7 h time-intervals are indicated by various symbols on the right-hand-side of the relevant column values in Table 3. Other comparisons of group differences between the F1, F2 and F3 groups are described in the relevant parts of the text of the Results section.

Results

The amino acid drinks were all tolerable, with no reported side effects other than expected: namely drowsiness, a slight nausea that could also be attributed to fasting, and no emesis. There was no attrition in any group following drink consumption.

The full set of biochemical results is presented in Table 3 as a reference guide to enable investigators to assess likely changes in the various parameters of interest following consumption of amino acid formulations with varying composition of the BCAA. The significant changes in each of the 15 parameters listed with time from baseline are expressed by an asterisk on the left-hand-side of each column value (from 1–7 h) and will not be described further here, except for the ratios. Results in the F1 group are given as means of only 10 subjects, as there were two outliers (subjects numbers 1 and 2), who showed greatly elevated baseline values for the 5 Trp competitors (CAA) (by 97%–129%) than the average of the other 10 subjects), but a smaller elevation of baseline total [Trp] (of 58%–60%), suggestive of having had a high protein meal before the test. In this section, the effects of decreasing the contents of the 3 BCAA on their plasma concentrations and on those of the other

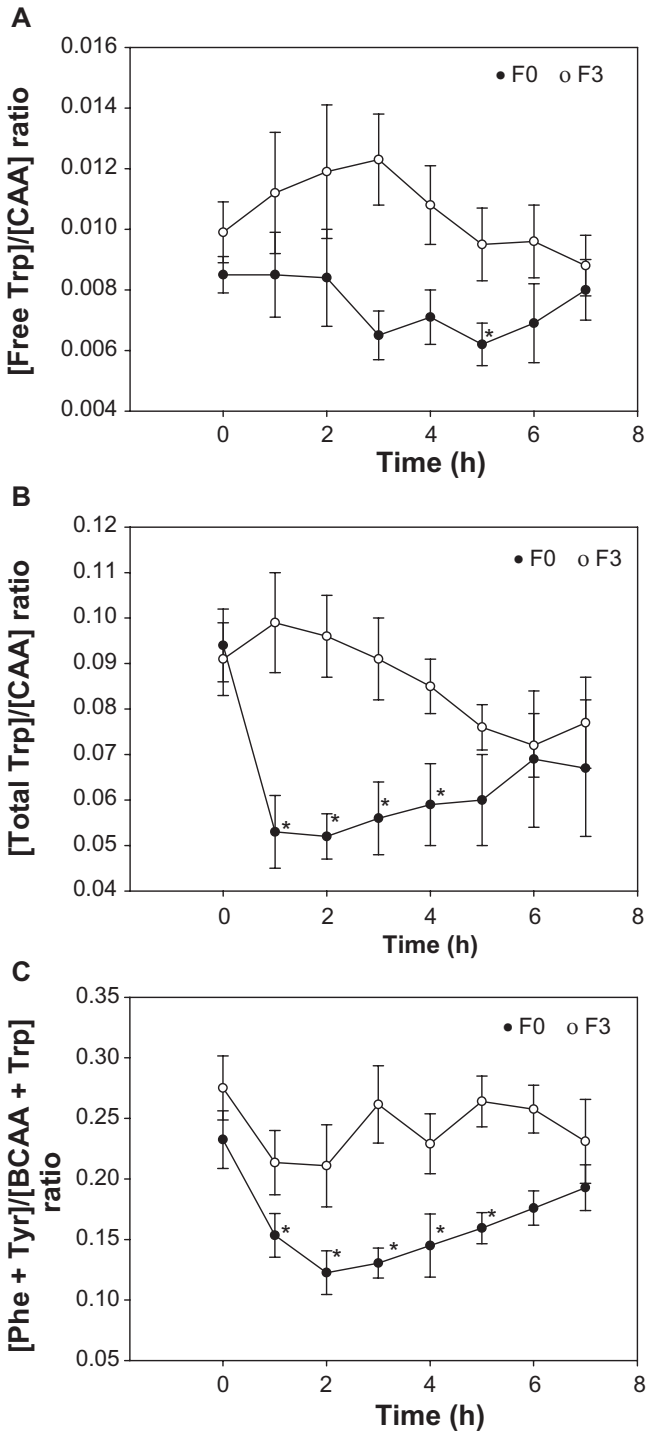

Figure 1. Time-course of changes in the ratios of free tryptophan (Trp) to competing amino acids (CAA; panel a), total Trp to CAA (panel b), and phenylalanine (Phe) plus tyrosine (Tyr) to the BCAA plus total Trp (panel c) as a function of time after intake of 50 g of the original balanced-control formulation (F0) and the formulation (F3) with 40% less of the branched-chain amino acids (isoleucine, leucine, and valine; BCAA). In this latter formulation, the contents of Phe, Tyr and Trp were held constant. Error bars represent SEM for $n = 12$ participants per group, except for F1, where a mean of 10 subjects is presented. The asterisk denotes a significant difference from the baseline value ($P = 0.05–0.0009$). For other statistical comparisons, see Table 3 and the relevant text in the Results section.

Table 3. Time-course of plasma amino-acid concentrations and ratios following consumption of four different 50 g amino-acid formulations with varying composition of branched-chain amino acids.

Parameter		–0.25 h	1 h	2 h	3 h	4 h	5 h	6 h	7 h
[BCAA]	F0	446 ± 30	*1480 ± 154	*1686 ± 191	*1526 ± 113	*1076 ± 113	*903 ± 88	730 ± 88	590 ± 66
	F1	512 ± 35	*1316 ± 119	*1558 ± 159	*1396 ± 112	*1099 ± 106	*795 ± 67	*705 ± 63	599 ± 61
	F2	415 ± 25	*1086 ± 79§	*1167 ± 70§	*1119 ± 42§	*941 ± 66	732 ± 50§	614 ± 45	507 ± 38
	F3	471 ± 38	*1001 ± 88¥	*1132 ± 121¥	*917 ± 89¥	*810 ± 79	*686 ± 41¥	544 ± 39	541 ± 48
[Val]	F0	258 ± 20	*797 ± 83	*875 ± 88	*772 ± 62	*593 ± 50	510 ± 48	411 ± 50	345 ± 40
	F1	289 ± 20	*714 ± 64	*800 ± 89	*723 ± 62	*582 ± 59	*448 ± 44	*403 ± 31	344 ± 42
	F2	223 ± 17	*567 ± 38§	*599 ± 27§	*576 ± 20§	*499 ± 35	*425 ± 31	*368 ± 27	297 ± 28
	F3	253 ± 20	*514 ± 47¥	*572 ± 56¥	*499 ± 55¥	*441 ± 44¥	*397 ± 24	324 ± 25	324 ± 32
[Leu]	F0	121 ± 9	*389 ± 48	*486 ± 76	*476 ± 63	*324 ± 49	*275 ± 32	224 ± 30	177 ± 23
	F1	148 ± 12	*378 ± 38	*490 ± 49	*444 ± 34	*350 ± 31	*249 ± 19	*220 ± 25	*185 ± 16
	F2	123 ± 8	*319 ± 28	*359 ± 30	*353 ± 20	*299 ± 22	209 ± 16	172 ± 14	153 ± 12
	F3	137 ± 11	*300 ± 25	*355 ± 42	*272 ± 25¥	*254 ± 28	200 ± 19¥	151 ± 12¥	155 ± 20
[Ile]	F0	67 ± 5	*294 ± 37	*325 ± 43	*278 ± 28	*195 ± 20	118 ± 12	95 ± 12	68 ± 7
	F1	75 ± 6	*224 ± 22	*268 ± 27	*229 ± 22	*167 ± 18	*98 ± 9	82 ± 10	70 ± 8
	F2	69 ± 6	*200 ± 18§	*209 ± 15§	*190 ± 11§	*143 ± 12	*98 ± 9	74 ± 7	57 ± 5
	F3	81 ± 10	*187 ± 17¥	*205 ± 25¥	*146 ± 15¥	115 ± 12	89 ± 5¥	69 ± 7	62 ± 8
[Free Trp]	F0	4.8 ± 0.2	*14.7 ± 1.8	*16.1 ± 1.8	*11.3 ± 1.0	*8.8 ± 0.6	*6.6 ± 0.3	6.0 ± 0.4	5.7 ± 0.3
	F1	6.2 ± 0.6¶	*17.1 ± 1.1	*14.4 ± 1.2	*11.4 ± 0.8	*8.6 ± 0.8	6.6 ± 0.5	6.0 ± 0.3	5.4 ± 0.5
	F2	5.8 ± 0.5§	*16.3 ± 2.1	*15.2 ± 1.6	*13.1 ± 1.6	*11.3 ± 1.3	9.4 ± 1.1	7.7 ± 0.8	6.5 ± 0.6
	F3	6.1 ± 0.4¥	*13.9 ± 1.1	*16.6 ± 1.5	*14.6 ± 0.7	*11.0 ± 1.1	8.4 ± 0.9	6.7 ± 0.5	6.0 ± 0.4
[Total Trp]	F0	53 ± 4	*91 ± 13	*100 ± 10	*98 ± 10	74 ± 6	63 ± 7	60 ± 10	48 ± 7
	F1	65 ± 8	*128 ± 7	*132 ± 17	*121 ± 12	*97 ± 8	70 ± 5	66 ± 7	54 ± 5
	F2	48 ± 4	*114 ± 13	*120 ± 8	*113 ± 12	*86 ± 13	77 ± 9	60 ± 5	47 ± 5
	F3	56 ± 5	*123 ± 13	*134 ± 17	*108 ± 10	*86 ± 7	67 ± 6	50 ± 4	52 ± 7
[Phe+ Tyr]	F0	116 ± 11	*241 ± 35	*219 ± 34	*212 ± 29	167 ± 16	154 ± 16	139 ± 15	123 ± 11
	F1	143 ± 21	*302 ± 35	*354 ± 47¶	*337 ± 25¶	*277 ± 26¶	219 ± 18	*201 ± 17	179 ± 24
	F2	132 ± 11	*255 ± 27	*215 ± 18	*258 ± 30	183 ± 19	187 ± 20	146 ± 18	125 ± 20
	F3	145 ± 13	*240 ± 23	*267 ± 30	*268 ± 38	205 ± 28	199 ± 26	153 ± 14	137 ± 20
[Phe]	F0	55 ± 7	*121 ± 20	*122 ± 20	*104 ± 15	71 ± 6	66 ± 9	64 ± 7	56 ± 7
	F1	69 ± 8	*167 ± 22	*192 ± 23¶	*164 ± 15¶	*119 ± 14¶	*96 ± 10	78 ± 10	74 ± 11
	F2	57 ± 6	*129 ± 12	*118 ± 11	*133 ± 14	*89 ± 12	80 ± 9	67 ± 8	53 ± 6
	F3	61 ± 9	*135 ± 15	*130 ± 19	*114 ± 15	92 ± 10	96 ± 10¥	65 ± 6	65 ± 10
[Tyr]	F0	61 ± 7	*120 ± 18	*97 ± 15	*108 ± 19	96 ± 14	88 ± 13	75 ± 10	67 ± 6
	F1	73 ± 14	*135 ± 18	*162 ± 29¶	*173 ± 19¶	*158 ± 20	*123 ± 12	*123 ± 13	105 ± 17
	F2	75 ± 9	*126 ± 18	97 ± 13	*125 ± 21	94 ± 13	107 ± 16	79 ± 12	72 ± 15
	F3	84 ± 7¥	105 ± 11	*137 ± 17	*154 ± 32	113 ± 15	103 ± 18	88 ± 9	72 ± 12
[BCAA + Trp]	F0	499 ± 31	*1571 ± 59	*1786 ± 192	*1624 ± 139	*1150 ± 113	*966 ± 86	790 ± 90	638 ± 67
	F1	577 ± 39	*1444 ± 126	*1690 ± 170	*1517 ± 119	*1196 ± 110	*865 ± 68	*771 ± 61	653 ± 61
	F2	486 ± 28	*1231 ± 87§	*1314 ± 73	*1251 ± 43§	*1046 ± 70	805 ± 52	683 ± 46	566 ± 40
	F3	527 ± 40	*1124 ± 94¥	*1266 ± 131	*1025 ± 92¥	*896 ± 84	*753 ± 45	594 ± 40	593 ± 51
[CAA]	F0	562 ± 36	*1721 ± 174	*1907 ± 209	*1738 ± 153	*1243 ± 109	*1075 ± 98	869 ± 99	713 ± 74
	F1	655 ± 49	*1618 ± 148	*1912 ± 200	*1733 ± 129	*1376 ± 117	*1014 ± 78	*906 ± 69	778 ± 76
	F2	547 ± 28	*1341 ± 96§	*1382 ± 68§	*1377 ± 56§	*1124 ± 79	*919 ± 61	760 ± 58	632 ± 53
	F3	616 ± 42	*1241 ± 99¥	*1399 ± 136¥	*1185 ± 105¥	1051 ± 101	885 ± 62	697 ± 49	678 ± 61

[6CAA]	F0	615 ± 37	*1812 ± 180	*2007 ± 216	*1836 ± 154	*1317 ± 108	*1120 ± 97	929 ± 101	761 ± 75
	F1	720 ± 54¶	*1746 ± 154	*2044 ± 212	*1854 ± 136	*1473 ± 121	*1084 ± 80	*972 ± 67	832 ± 77
	F2	595 ± 30	*1455 ± 105	*1502 ± 71	*1490 ± 54	*1210 ± 83	*996 ± 63	820 ± 59	679 ± 56
	F3	672 ± 99	*1364 ± 106¥	*1533 ± 147	*1293 ± 108¥	*1101 ± 106	952 ± 67	747 ± 51	730 ± 63
Free [Trp/ CAA]	F0	0.0085 ± 0.0006	0.0085 ± 0.0015	0.0084 ± 0.0016	0.0065 ± 0.0008	0.0071 ± 0.0009	*0.0062 ± 0.0007	0.0069 ± 0.0012	0.0080 ± 0.0010
	F1	0.0095 ± 0.0017	0.0098 ± 0.0015¶	0.0070 ± 0.0014	*0.0061 ± 0.0006	*0.0058 ± 0.0008	*0.0065 ± 0.0007	*0.0062 ± 0.0008	0.0065 ± 0.0013
	F2	0.0106 ± 0.0011	0.0122 ± 0.0021	0.0110 ± 0.0014	0.0095 ± 0.0010§	0.0101 ± 0.0013§	0.0102 ± 0.0016§	0.0101 ± 0.0018	0103 ± 0.0017
	F3	0.0099 ± 0.0010	0.0122 ± 0.0020	0.0119 ± 0.0022	0.0123 ± 0.0015¥	0.0108 ± 0.0013¥	0095 ± 0.0012¥	0.0096 ± 0.0012	0.0088 ± 0.0010
Total [Trp/CAA]	F0	0.094 ± 0.008	*0.053 ± 0.008	*0.052 ± 0.005	*0.056 ± 0.008	*0.059 ± 0.009	*0.060 ± 0.010	0.069 ± 0.015	0.067 ± 0.015
	F1	0.099 ± 0.008	0.079 ± 0.008¶	0.069 ± 0.007	*0.070 ± 0.006	*0.070 ± 0.007	*0.069 ± 0.006	0.073 ± 0.033	*0.069 ± 0.008
	F2	0.088 ± 0.007	0.085 ± 0.007§	0.087 ± 0.006§	0.082 ± 0.009	0.077 ± 0.011	0.084 ± 0.011	0.079 ± 0.009	0.074 ± 0.008
	F3	0.091 ± 0.008	0.099 ± 0.011¥	0.096 ± 0.009¥	0.091 ± 0.009¥	0.085 ± 0.006¥	0.076 ± 0.006	0.072 ± 0.007	0.077 ± 0.011
[Phe + Tyr]/ [BCAA + Trp]	F0	0.232 ± 0.024	*0.153 ± 0.018	*0.123 ± 0.018	*0.130 ± 0.012	*0.145 ± 0.026	*0.159 ± 0.013	0.176 ± 0.014	0.193 ± 0.019
	F1	0.248 ± 0.030	0.209 ± 0.014¶	0.209 ± 0.012¶	0.224 ± 0.014¶	0.232 ± 0.022¶	0.254 ± 0.018¶	0.262 ± 0.028¶	0.274 ± 0.030
	F2	0.272 ± 0.028	*0.207 ± 0.017§	*0.164 ± 0.017	*0.206 ± 0.064§	*0.175 ± 0.015	*0.232 ± 0.023§	*0.214 ± 0.020	*0.221 ± 0.022
	F3	0.275 ± 0.026	0.214 ± 0.026¥	0.211 ± 0.034¥	0.261 ± 0.032¥	0.229 ± 0.025¥	0.264 ± 0.021¥	0.258 ± 0.020¥	0.231 ± 0.035

Values (in µM or as ratios) are means ± *SEM* for each group of 12 participants at each time-interval, except those of the F1 group, which are means for 10 subjects. The asterisk denotes a significant difference from the baseline values (one-way ANOVA; $P = 0.025–0.001$), whereas the following symbols denote significant differences at the relevant time intervals between values in the F0 group and those in the F1 (¶), F2 (§) and F3 (¥) groups ($P = 0.05–0.001$).

3 competitors will be described first, to be followed by a description of changes in the ratios.

Effects of decreasing the contents of the BCAA on their plasma concentrations

As shown in Table 3, there was the generally expected dose-dependent decrease in the sum of plasma concentrations of Val, Leu, and Ile from the traditional formulation (F0) with decreasing contents of the 3 BCAA (–20%, –30% and –40% in the F1, F2 and F3 groups respectively). At baseline, [BCAA] did not differ significantly between any of the 4 groups ($P = 0.183$).

The data in Table 3 show that, following consumption of the formulation drinks, levels of BCAA did not differ significantly at any of the 7 hourly time points between the participants who consumed the traditional formulation (F0) and those who consumed the F1 formulation. By contrast, compared with the F0 formulation, [BCAA] were significantly lower in subjects who consumed the F2 or F3 at 1, 2, 3 and 5 h after drink consumption ($P = 0.034–0.001$). Compared with [BCAA] in the F1 group, significantly lower values were observed in the F2 and F3 groups at 3 h ($P = 0.017–0.002$) after formula consumption. There were no significant differences in [BCAA] between F2 and F3 participants at any time interval after formula intake.

An inspection of the data in Table 3 for the individual BCAA shows similar dose-dependent decreases in their concentrations as for their sum, with decreasing content of BCAA in the formulations. For Val, Leu and Ile, baseline values did not differ significantly between groups. For Val, there were significant differences between F1 and F3 at 1, 2, 3 and 4 h ($P = 0.037–0.005$). With Leu, significant differences were observed between F1 and F2 at 4 h ($P = 0.001$), F1 and F3 at 3, 4 and 6 h ($P = 0.049–0.001$), and F2 and F3 at 3 and 4 h ($P = 0.006–0.001$). Finally, with Ile, only the difference between F1 and F3 at 3 h was significant ($P = 0.009$).

Effects of decreasing the contents of branched-chain amino acids on plasma tryptophan, phenylalanine and tyrosine concentrations

As the contents of Trp, Phe and Tyr were not altered in the different formulations, it was assumed that

their plasma concentration-time profiles should be similar in all 4 groups of participants. The data in Table 3 show that this was largely the case. With both free and total [Trp], no significant group differences were observed, except those in [free Trp] at baseline between the F0 and the other groups (P = 0.05). Because of the absence of significant group differences in free and total [Trp], the percentage free Trp (an expression of Trp binding to albumin) showed no significant group differences before or during the 7 h time course of the experiment (data not shown). With Phe and Tyr, their sum was unexpectedly higher in the F1, compared with the other three groups. The only significant differences were observed with the F1 group, when compared with F0 at 2–4 h (P = 0.002–0.003), with F2 at 2 and 4 h (P = 0.009) and with F3 at 2 and 4 h (P = 0.071–0.038). As the results in Table 3 show, the higher [Phe + Tyr] in the F1 group was due to the greater rises in both [Phe] and [Tyr], with the rises in [Phe] immediately preceding those in [Tyr], as would be expected in a precursor-product relationship. Plasma [Phe + Tyr] values did not differ among any of the 4 formulation groups at baseline. There were also no differences found between the F0 and F2, the F0 and F3, and the F2 and F3 formulations at any of the 7 hourly time intervals after consumption of the drinks ($P > 0.10$).

Effects of decreasing the contents of branched-chain amino acids on the sum of their concentrations plus tryptophan ([BCAA + Trp]) and on the sum of the 5 Trp competitors ([CAA])

Whereas the sum of the 5 Trp competitors Val, Leu, Ile, Phe and Tyr ([CAA]) is used to calculate the [Free Trp]/[CAA] and [Total Trp]/[CAA] ratios, that of the [BCAA + Trp] is required to calculate the [Phe + Tyr]/[BCAA + Trp] ratio. As shown in Table 3, the sums of [BCAA + Trp] and of the 5 [CAA] were dose-dependently decreased from F0 to F3. For [BCAA + Trp], significant differences were observed between the F0 and F2 and F0 and F3 groups at 1 and 3 h (P = 0.036–0.001), and between F1 and F2 or F3 at 3 h (P = 0.05–0.001). With [CAA], all significant differences were observed at 1–3 h. These were between F0 and F2, F0 and F3, F1 and F2 and F1 and F3 (P = 0.05–0.001). The sum of all [6CAA] is also shown in Table 3 for completeness.

Effects of decreasing the contents of branched-chain amino acids on the [Free Trp]/[CAA], [Total Trp]/[CAA] and [Phe + Tyr]/[BCAA + Trp] ratios

As shown in Table 3, decreasing the contents of BCAA from the usual level in the traditional F0 group met with varying degrees of success in restoring the above three ratios to normal baseline levels.

Thus, the [Free Trp]/[CAA] ratio was decreased in F0 maximally by 27% at 5 h (P = 0.013; paired t-test) and F1 did not correct this decrease, which was 26% at 2 h, rising to 39% at 4 h and remaining at 32%–35% till 6 h (P = 0.034–0.004). In fact, none of the free Trp ratio values in the F1 group differed significantly from the corresponding values in the F0 group, except for that at 1 h. By contrast, no significant decreases in this ratio from baseline were observed in participants in the F2 or F3 groups at any time interval after formula intake. Both the F2 and F3 groups showed significant differences from the F0 and F1 groups at 3–5 h (P = 0.013–0.001).

The [Total Trp]/[CAA] ratio was consistently decreased from the baseline value in the traditional F0 group over the entire 7 h time course, with the decreases at 1–5 h (37%–45%) being significant (P = 0.0101–0.0019). Here again, F1 did not correct this decrease, which was also significant at all time intervals (P = 0.05–0.0124), except at 1 and 6 h. By contrast, no decreases in this ratio from baseline were observed in participants receiving the F2 and F3 formulae. Significant differences in this ratio were also observed between F1 and F3 at 2 and 3 h (P = 0.071–0.038) in addition to the differences between the F0 and other groups shown in Table 3.

Finally, the [Phe + Tyr]/[BCAA + Trp] ratio was also significantly decreased from baseline in the traditional F0 group by 32%–47% during the first 5 h (P = 0.0220–0.0009). After intake of F1, no significant decreases from baseline were observed and, in fact, all ratio values in F1 differed significantly from the corresponding values in F0 at the 1–6 h time points (P = 0.048–0.001). With F2, however, decreases in this ratio of between 15 and 40% occurred at 1–7 h, all of which were significant (P = 0.05–0.001). With

F3, however, no significant differences from baseline were observed at subsequent time intervals.

Thus, the [Free Trp]/[CAA] and [Total Trp]/[CAA] ratios were restored to normal in both the F2 and F3 formulations, whereas the [Phe + Tyr]/[BCAA + Trp] ratio was restored in F1 and F3, but not in F2. The reason for the effectiveness of F1 here is due to the unexpectedly higher [Phe + Tyr] in this formulation group. It would therefore seem reasonable and prudent to suggest that F3 would be the group of choice for maintaining normal ratios, as can be seen graphically in Fig. 1 comparing the decreased ratios in F0 with the normalized ones in F3.

Discussion

Specificity of existing formulations for the ATD and ATPD tests and their loading counterparts

We have expressed doubt about the specificity of the current formulations used in the acute Trp or Tyr depletion/loading tests to manipulate 5-HT and catecholamine synthesis. Initially, it was assumed that the absence of Trp from the ATD formulation could place Tyr and Phe at a greater advantage for entry into the brain, resulting in enhanced central DA and/or NA synthesis.[25] However, our subsequent studies[9,10] demonstrated the opposite, namely that the entry of Tyr and Phe into the brain is likely to be decreased due to the relatively larger contents of branched-chain amino acids (BCAA), compared with those of Tyr and Phe in the various formulations, which was reflected in the relative differences in their plasma concentration profiles (for details, see also[3]). As a consequence, the [Phe + Tyr]/[BCAA + Trp] ratio is significantly decreased in the ATD and more so in the ATL test, and even after the balanced control formulation.[10] This is almost certain to cause inhibition of dopamine synthesis. In fact, administration of a mixture of the 3 BCAA, Val, Leu, and Ile, either alone[14] or supplemented with Trp[16,26] is known to decrease the [Phe + Tyr]/[BCAA] ratio and to inhibit dopamine function, as expressed by an increased latency to respond to the spatial recognition memory task (in humans) or by amphetamine-evoked striatal dopamine release and amphetamine-induced hyperactivity (in rats). A Phe + Tyr-depleted amino acid mixture reduces CSF levels of catecholamine metabolites in vervet monkeys.[27] Other evidence for inhibition of central DA synthesis after Tyr depletion has already been described in the accompanying review.[3]

The need for a truly balanced control formulation

This need has already been emphasized.[4] Most investigators using the ATD or ATL test would agree that a "balanced" formulation should ensure that the control treatment maintains baseline values without altering the biochemical or behavioural parameters being studied, which would further enhance accurate interpretation of results. As BCAA play a pivotal role in the ATD test, the use of a balanced control formulation, rather than an amino-acid-free "neutral" placebo, is even-more important, particularly in relation to behavioural studies, because, apart from inducing a central 5-HT deficiency through Trp depletion and a central catecholamine deficiency through Phe and Tyr depletion, the BCAA have the potential to exert other equally important metabolic changes which could also further impact behaviour (for commentary, see[28]). Thus, in the human brain, BCAA are transaminated by branched-chain amino acid aminotransferase (BCAT) to branched-chain keto acids, converting in the process 2-oxoglutarate to glutamate.[29] BCAA are thus nitrogen donors for the synthesis of the excitatory amino acid glutamate and the inhibitory neurotransmitter γ-aminobutyric acid (GABA), with Leu playing a particularly prominent role[30–32] and it is noteworthy that the brain cytosolic isoenzyme of BCAT is located in GABAergic and glutamatergic neurons.[33] Since in humans, the brain normally accounts for 10%–20% of their total body metabolism,[34] a significant increase could be expected after loading with BCAA, as in the present work, resulting in enhanced synthesis of glutamate and GABA. Changes in glutamatergic and/or GABAergic neurotransmission are therefore expected under these conditions, which could impact on 5-HT and dopamine functions, with the potential to modulate behaviours associated with these cerebral monoamines.

The idea of improving the neutrality of the control formulation is not new, as previous attempts have been made. Thus, Weltzin et al (1994)[17] succeeded in maintaining the baseline [Trp]/[CAA] ratio by increasing the Trp content to 4.6 g/100 g of the traditional amino acid mixture. However, they did not measure

the [Phe + Tyr]/[BCAA + Trp] ratio and it is almost certain that, with this level of Trp loading (which is 45% of that of the ATL dose), or even without it, this latter ratio will have been decreased. A low dose ATD (25% of the normal one) has been used[35] as a control, based on a previous design.[36] However, although this low-dose mixture did not alter the [Tyr]/[CAA] ratio, it still decreased the [Trp]/[CAA] ratio by 42%, against a 92% decrease by the full dose. Surprisingly, the high ATD dose elevated the [Tyr]/[CAA] ratio by about 50%. However, interpretation of some, or all, of these biochemical changes is difficult because the subjects consumed a lunch during the test procedure. Still, while the use of a low-dose mixture may be useful in studies examining the effects of sub-optimal depletion of Trp and 5-HT, it cannot be considered an appropriate control for the ATD test dose.

In the light of this and the preceding discussion, we suggest that the most appropriate approach to enhancing the specificity of these formulations is by modulating the contents of the components which appear to be responsible for the lack of specificity, namely the 3 BCAA, and consider it important to begin with the balanced control formulation. Before discussing the normalization of the above ratios by our modified control formulation, the pharmacokinetic data of the different formulations tested are worthy of brief comment.

Pharmacokinetic changes across the different control formulations

As expected, concentrations of all three BCAA showed a broadly dose-dependent decrease (Table 3) with decreasing order of their contents in the different formulations. It is known[37] that the two major BCAA-metabolising enzymes, the aminotransferase and the keto acid dehydrogenase, exhibit similar kinetic properties towards their substrates and their corresponding intermediate products with no obvious preferences. The observation (Table 3) that the individual concentration-time profile of each of the 3 BCAA (i.e. Val, Leu, and Ile) followed a similar pattern to each other and as their combined sum provides further support to this concept.

Whereas the observed kinetics of Trp were expected, those of Phe and Tyr were not, given also their unchanged contents in the 4 formulations. The higher plasma [Phe] in F1 cannot be explained at present, but factors associated with Phe transport or Phe hydroxylase and/or Tyr hydroxylase activity may be involved. The similarly higher plasma [Tyr] in F1 may simply be a consequence of the high Phe being converted to Tyr by Phe hydroxylase or by Tyr hydroxylase,[38,39] rather than an independent behaviour of this Tyr dose.

Normalization of the free and Total [Trp]/[CAA] and [Phe + Tyr]/[BCAA + Trp] ratios by modulation of the contents of BCAA

As stated in the Introduction, our preferred strategy for normalisation of the Trp and Tyr ratios was to lower the contents of BCAA. In the present study, we examined this strategy in the control (traditional) formulation (F0) by reducing the contents of BCAA by 20% (F1), 30% (F2) and 40% (F3) and the results in Table 3 and Fig. 1 show that a 40% reduction of BCAA (in F3) led to complete normalization of all three ratios. We should point out that the lower [Phe + Tyr]/[BCAA + Trp] ratio observed after intake of the traditional formulation in the present work has been a robust and consistent finding in our previous studies[9,10] and those by others using the control formulation for the ATD[5,6] or the ATPD[11–13] test, whereas the extent and duration of the decrease in the [Free Trp]/[CAA] ratio after the traditional control formulation (F0) was somewhat variable, varying at the usual time of behavioural testing (5 h) between 28% (Fig. 1 in the present work) and 76%.[9] This may be explained by plasma free Trp being a labile parameter easily influenced by many physiological and other factors (for a review, see[40]). On the other hand, the [Total Trp]/[CAA] ratio in the traditional formulation was more strongly decreased in the present work, but not in our previous study.[10] As both the free and total [Trp]/[CAA] ratios are important predictors of changes in central [5-HT][3] both ratios must be determined and a decrease in either justifies rectification of the formulation. We particularly recommend the formulation with 40% less BCAA for the following reasons: (1) F3 normalized all 3 ratios, whereas F2 did not normalise the [Phe + Tyr]/[BCAA + Trp] ratio and F1 did not normalise the free or total [Trp]/[CAA] ratio; (2) a 20% less [BCAA] in F1 did not show a pharmacokinetic profile different from the traditional F0 and was

associated with abnormally high [Phe + Tyr], hence the normalisation of the [Phe + Tyr]/[BCAA + Trp] ratio by the F1 formulation.

Conclusions and Comments

A possible criticism of our study is that we did not control for the menstrual cycle in female subjects. It has been reported[41] that oestradiol-17β decreases the activity of branched-chain keto acid dehydrogenase complex in female rats only in the evening, by acting on this complex-specific kinase (BDK). While theoretically this may influence BCAA levels, this effect is more related to feeding behaviour in rats and may therefore be irrelevant in humans. However, it would be prudent in future human studies to establish if the phase of the menstrual cycle influences circulating [BCAA] and whether ATD and related tests should be conducted at a specific phase of this cycle.

We believe that our new control formulation containing 40% less BCAA should be adopted as the truly balanced control in studies employing the ATD and ATPD tests and their loading counterparts and should like to name it the acute balanced-control test (ABCT) formulation. Using a single unified control formulation for manipulation of Trp and Tyr plus Phe should contribute to uniformity in studies with the control condition and allow a better control reference in studies of combined or individual 5-HT, DA and NA depletion or loading. Investigators should no longer ignore the decrease in the [Phe + Tyr]/[BCAA + Trp] ratio in the ATD, ATL, or balanced-control formulations, nor those in the [Trp]/[CAA] ratios in the latter formulation and the ATPD or loading tests and their likely impacts on behaviour, particularly in patient populations. An important question arising from this work is whether the decreased content of BCAA in the proposed new ABCT formulation can also ensure that only the desired changes in ratios occur during the ATD, ATL, ATPD and ATPL tests. With the ATD and ATL tests, a 40% less BCAA formulation should result respectively in the expected decrease or increase in the [Trp]/[CAA] ratios. In fact, on theoretical grounds,[10] changing the content of BCAA should make little difference to the Trp ratios in the ATD test, whereas the 40% decrease can only enhance the desirable increases associated with ATL. As regards the [Phe + Tyr]/[BCAA + Trp] ratio, some provisional calculations were made from the data in Table 3 of the present work and from those reported by us previously[9] for the 50 g depletion and loading formulations. Calculation of this ratio revealed small fluctuations of between +7% and –15% from baseline in the ATD test and a near normal ratio around about 0.2 during the entire 7 h time course of the ATL test. With regard to the ATPD, a major decrease in the [Phe + Tyr]/[BCAA + Trp] ratio is guaranteed on the same theoretical grounds as for ATD, and calculations from previously reported data by the Canadian group[11] derived from the same traditional formulation as in the present work, but without Phe or Tyr, show that the free and total [Trp]/[CAA] ratios are moderately decreased (by 15 and 27% respectively); such modest decreases can easily be reversed by using our F3 with 40% less BCAA. The reason why the above decreases in the [Trp]/[CAA] ratios are moderate, in comparison with the greater decreases in other studies by the Oxford group, is almost certainly because the former group[11] used less BCAA (29% of the total dose) than did the above group (47%–48% of the total dose) (see, e.g. refs[2,12]). As for the ATPL, while the increase in the [Phe + Tyr]/[BCAA + Trp] is also guaranteed with our F3 formulation, it is difficult to predict with greater certainty whether the free and total [Trp]/[CAA] ratios will remain unaltered or undergo a decrease. Perhaps the contents of Phe and Tyr in the ATPL formulation need not be as high as could be assumed (perhaps only moderately higher than in the control formulation), specially as the lower content of BCAA should ensure that the [Phe + Tyr]/[BCAA + Trp] ratio will not be low. Another reason for having a moderately higher Phe and Tyr content in the ATPL formulation is to ensure that brain [Tyr] is not elevated excessively and thus avoid the substrate inhibition of Tyr hydroxylase activity.[42] Confirmation of all these provisional values and predictions will clearly require further work with the ATD, ATL, ATPD and ATPL test formulations comparing the traditional F0 with the 40% less BCAA F3 formulation under the same experimental conditions. We hope that by proposing a new balanced control formulation associated with normal Trp and Tyr ratios, we have made a useful contribution to the acute depletion and loading methodology that will serve to enhance interpretation of behavioural and biochemical data in future research.

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While the authors Dougherty and Richard were affiliated with Wake Forest University Health Sciences Center, NC, USA during the data collection for this study, they have since relocated to The University of Texas Health Science Center at San Antonio.

Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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