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Standardization of formulations for the acute amino acid depletion and loading test

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Abstract

The acute tryptophan (Trp) depletion (ATD) and loading (ATL) and the acute tyrosine (Tyr) plus phenylalanine (Phe) depletion (ATPD) tests are powerful tools for studying the roles of cerebral monoamines in behaviour and symptoms related to various disorders. The tests use either amino acid mixtures or proteins. Current amino acid mixtures lack specificity in humans, but not in rodents, because of the faster disposal of branched-chain amino acids (BCAA) by the latter. The high content of BCAA (30-60%) is responsible for the poor specificity in humans and we recommend, in a 50g dose, the control formulation of Young et al. (1985) with a lowered BCAA content (18%) and minor modifications as a common control for the above tests. With protein-based formulations, α -lactalbumin is specific for ATL, whereas gelatine is only partially effective for ATD. We recommend the use of the whey protein fraction glycomacropeptide (GMP) as an alternative protein. Its BCAA content is ideal for specificity and the absence of Trp, Tyr and Phe render it suitable as a template for 7 formulations (separate and combined depletion or loading and a truly balanced control). We invite the research community to participate in standardization of the depletion and loading methodologies by using our recommended amino acid formulation and developing those based on GMP.

Keywords

Acute tryptophan depletion and loading; Acute tyrosine and phenylalanine depletion; Anxiety; Behaviour; Branched-chain amino acids; Glutamatergic function; Glycomacropeptide; Serotonin; Tryptophan; Tyrosine

Introduction

The acute tryptophan (Trp) depletion (ATD) and loading (ATL) and the acute tyrosine (Tyr) and phenylalanine (Phe) depletion (ATPD) tests are powerful tools for investigating the roles of the cerebral indolylamine serotonin (5-hydroxytryptamine; 5-HT) and the catecholamines

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dopamine (DA) and noradrenaline (NA) in behaviour and symptoms expressed in certain disorders. Typically, measures of appetitive, aggressive and impulsive behaviours and of anxiety, mood, movement, reward, sleep and cognition (including declarative memory, associative conditioning and executive function) are studied in these behavioural conditions (Reilly et al., 1997; Moore et al., 2000; Robbins and Murphy, 2006; Richard et al., 2009; Mendelsohn et al., 2009).

Recently, Sobczak and Schruers (2014) raised a range of important points concerning the ATD test and the composition of its formulations, which could have significant impact on interpretation of behavioural, biochemical and clinical observations, and called for standardization of this methodology. Standardisation is a highly desirable goal, as it can facilitate comparability of behavioural and biochemical data across studies and disciplines, and ensure a more accurate interpretation of findings. We have raised and addressed some of the points discussed by Sobczak and Schruers (2014) elsewhere and this seems an opportune moment to reiterate these, extend the discussion of the other points raised and propose formulations that can help standardization of the methodology.

A successful depletion or loading formulation must at least meet the criteria listed in Table 1. Establishing a range of ideal formulations for these tests will be a challenging task for the psychopharmacological research community, given the paucity of studies on monoamine research methodology and its validation. Much experimental effort is therefore required and, in an attempt to stimulate research on this area, we shall consider in the following discussion how the criteria listed in Table 1 are met in existing formulations and what improvements could be introduced.

Side effects of amino acid formulations

As well as from the different paradigms referred to by Sobczak and Schruers (2014) in panic studies, side effects can also arise from the ATD amino acid formulation itself, as referenced in Sobczak and Schruers (2014) and Dougherty et al. (2008). We addressed this latter issue in healthy volunteers by halving the traditional 100g dose of the original formulation of Young et al. (1985) and found (Dougherty et al., 2008) that attrition from somatic side effects was confined to females receiving the 100g formulations for ATD and ATL (acute Trp loading), with a drop-out rate of 20% in each group (4/20), thus reflecting the greater sensitivity of females to the side effects of the 100g dose described by Sobczak and Schruers (2014). The somatic symptoms were fewer in the 50g, than the 100g, loading formulation, whereas no significant somatic symptom differences were observed between the 50g or 100g ATD doses.

These findings suggest that: (1) attrition by females may be related to amino acids other than Trp, possibly BCAA, and is not due to females receiving the full 100g dose as males, rather than a smaller dose based on the average 16.7% lower body weight of females (Ellenbogen et al., 1996); (2) somatic side effects may involve both Trp and other amino acids. The precise basis of the greater sensitivity of females to amino acid mixtures is not clearly understood. BCAA may be involved. However, although females exhibit a lower rate of Leu oxidation than males, especially after exercise (Phillips et al., 1993), calculation of plasma

[Leu] during ATD or ATL from our previous study (Dougherty et al., 2008) revealed no significant gender differences (data not shown). Studies in Canada on healthy volunteers of both genders (Benkelfat et al., 1994; Ellenbogen et al., 1996, 1999) implicate family history of depression and prior depressive episodes, though no clear-cut conclusions could be drawn. Further work is therefore required to establish the basis of the greater female sensitivity.

To minimize side effects, we recommend the 50g dose for ATD, especially as its lowering of mood and of the plasma [total Trp] to [competing amino acids] (CAA) ratio (an expression of Trp availability to the brain) is similar to that of the 100g dose (Dougherty et al., 2008). Also, there are only small differences in the decrease in the [free Trp]/[CAA] ratio from baseline between the 2 ATD doses: 89% with the 50g, and 96% with the 100g, dose, at 5h. An additional advantage of the 50g dose is the lower content of branched-chain amino acids (BCAA), whose metabolic effects could impact behaviour in a number of ways (see below).

Specificity of amino acid formulations

That plasma Trp depletion is specifically induced by the absence of Trp from the ATD formulation is suggested by the finding (Klaassen et al., 1999a) that depletion of another essential amino acid, lysine, does not lower plasma [Trp]. In discussing the biochemical basis of the ATD test and its specificity, we (Badawy et al., 2010a) concluded that the ATD formulation of Young et al. (1985) may involve not only serotonin depletion, but also that of DA and possibly also NA, because it also induces a decrease in the [Phe + Tyr]/[BCAA + Trp] ratio, thereby decreasing Tyr availability to the brain. The decrease in this latter ratio is even greater with ATL. This could impact catecholamine-specific behaviours and those influenced by catecholamines, particularly because of the well documented interaction of 5-HT with DA function, exemplified among others by data from PET studies with ¹¹C-raclopride (Smith et al., 1997; Cox et al., 2011), and the differences in effects of these 2 monoamines on cognition (Booij et al., 2003; Matrenza et al., 2004). Thus, whereas ATD selectively impairs memory encoding and consolidation, ATPD selectively impairs spatial working and recognition memory without influencing declarative memory performance. However, combined ATD/ATPD does not affect the cognitive measures sensitive to either depletion alone (Matrenza et al., 2004). Even, in situations in which ATD and ATPD separately exert similar effects on a specific behaviour, e.g., decreasing Stroop interference (corresponding to improved attentional control), their combined effect is only slightly higher than in the placebo controls (Scholes et al., 2007). Taken together, 5-HT-DA interactions can undermine interpretation of behavioural measures and this could be compounded further by the ability of current ATD (or ATPD; see below) formulations to undermine cerebral monoamine synthesis by decreasing precursor availability. Studies using separate and combined ATD and ATPD with amino acid or protein-based formulations with improved specificity are more likely to elucidate further the behavioural effects resulting from 5-HT-DA interactions.

It can also be seen from previous studies that, even the control formulation for ATD is associated with decreases in the above ratio and those of [free Trp]/[CAA] and [total Trp]/[CAA] (Badawy et al., 2010a). The reason for the poor specificity is the presence of BCAA

at relatively high concentrations (~ 30%) in the Young et al. (1985) control and test formulations, based on the composition of human milk in the context of safety. We addressed the specificity experimentally in healthy volunteers given the control formulation with different contents of BCAA: the traditional (30% BCAA) and 3 others containing 20%, 30% and 40% less BCAA and found (Badawy et al., 2010b) that normalisation of the 2 Trp ratios and the Tyr ratio was achieved by the latter formulation (with an 18% BCAA content) and recommend this latter new control formulation for ATD and ATL.

The control and test formulations of Sheehan et al. (1996) for acute tyrosine plus phenylalanine depletion (ATPD) may not only be limited to depletion of DA or NA, but also that of serotonin, as they also decrease the [total Trp]/[CAA] ratio by up to 62% and 96% respectively (Badawy et al., 2010a). This is also not surprising, as the BCAA content of these formulations is even higher (48-61%) than that (30%) of the above ATD formulations. We believe that, for future studies using amino acid mixtures (as opposed to protein-based formulations), our new control formulation (Table 2) can serve as a common control for both ATD and ATPD and also ATL and ATPL (acute Tyr and Phe loading) with the appropriate deletions for the depletion studies and suitable additions for Trp loading as recommended by Young et al. (1985). ATPL has not been attempted before using amino acid mixtures; only loading with Tyr alone has been performed, but for different purposes.

What is a suitable control amino acid formulation?

Investigators have used various versions of the original control formulation of Young et al. (1985). The Trp content of the original formulation (2.3%) was increased in some studies to 2.9%, 3.1%, 4.1% and 4.6% (Klaassen et al., 1998; Reidel et al., 1999; see also other references in Mendelsohn et al., 2009 and Badawy et al., 2010a). The reason for increasing the Trp content is to avoid Trp depletion by the control formulation. With Trp additions of 2.3%-3.1%, the Trp]/[CAA] ratio was still decreased, whereas the larger Trp additions increased this ratio as would be expected with Trp loading. Sobczak and Schruers (2014) rightly imply that adding Trp to the control formulation at 3.0% represents Trp loading, quoting data from Schruers et al. (2000). While such loading affects hepatic Trp metabolism (see below) its effect on Trp availability to the brain depends on level of competition from CAA. Schruers et al. (2000), however, measured only plasma [Trp], but not the [Trp]/[CAA] ratio, which, as stated above, is decreased in the control formulation containing this amount of Trp. This further emphasizes the ability of an excess of BCAA to lower Trp availability to the brain even in control formulations with increased Trp content. Thus, in the absence of the recommended level of BCAA suggested by us, increasing the Trp content of the control formulation from the original 2.3% does not serve any useful purpose, but is likely to complicate interpretation of results, as it could either exaggerate or minimize differences between tests and controls (Booij et al., 2005).

Other investigators used a low ATD formulation dose as a “negative” or “conservative” control in studies in controls and depressed patients. While this may be a useful paradigm to study dose responses (see, e.g., Booij et al., 2005), it cannot substitute for an appropriate control, not only for being deficient in Trp, but also in BCAA. Any impact of the full BCAA dose on behaviour (see further below) will be minimized in such a control group. In the

above study in remitted depressed patients, the low-dose ATD formulation also improved attention to neutral stimuli in the Stroop interference test, thereby minimizing the effect of the larger dose. Other studies showed that, whereas low-dose ATD did not influence mood, it impaired other behaviours, e.g., emotional and cognitive processing, sleep and REM latency and autobiographical memory (Moore et al., 1998; Hayward et al., 2005; Al-Haj et al., 2012). Thus, the full impact of the behavioural effects of the full ATD dose can only be minimized by using a low-dose ATD as control. Even in a number of other studies, what was assumed to be a low ATD dose induced a strong Trp depletion approaching that of the full dose (see references in Merens and van der Does, 2007), thus further emphasizing the need to standardise the methodology.

Any amount of tryptophan added is a tryptophan load

Any amount of Trp added to the control preparation is a Trp loading dose, the consequences of which should be considered in ATD or ATL studies. Sobczak and Schruers (2014) allude to the potential role of kynurenine metabolites in behavioural outcomes. This has been a largely neglected aspect in ATD studies. Production of kynurenines may be important not only in relation to the Trp load, but also to the leucine (Leu) component of the control and test formulations. The potential role of Leu is considered separately below. Over 95% of dietary Trp is oxidized in the hepatic kynurenine pathway, the rate-limiting enzyme of which is the first, Trp 2,3-dioxygenase (TDO: formerly Trp pyrrolase) (Bender, 1982; Badawy, 2002). The flux of Trp down the pathway is enhanced by 2 mechanisms: simple substrate mass action and net activation of TDO. Doses of Trp of 50 mg/kg (or less) do not activate rat liver TDO (Badawy and Evans, 1975), but undergo simple flux down the pathway. Thus, a 25 mg/kg body wt dose of Trp (equivalent to a ~ 1.75g dose in a 70 kg human) administered alone to rats significantly increases kynurenine and 3-hydroxykynurenine concentrations in plasma by 100% and 39% respectively and in brain by 46% and 29% respectively, at 1h (Gál et al., 1978). Smaller, but still significant, increases are seen with a 10 mg/kg Trp dose. Cerebral uptake of kynurenine is not limited, unlike that of its metabolites kynurenic and quinolinic acids (Fukui et al., 1991). Elevation of brain kynurenine bypasses the negligible activity of indol-3-ylamine 2,3-dioxygenase and can thus result in kynurenic acid formation. However, kynurenine has also to compete with Trp and its 5 uptake competitors for entry into the brain. Thus, in the presence of the relatively huge amounts in plasma of the 5 Trp competitors from the control and test formulations and the generally > 50-fold higher plasma [Trp] in the control formulation relative to that of kynurenine, entry of the latter into the brain will be inhibited. Studies in rats (Coppola et al., 2013) indeed show that BCAA decrease brain kynurenic acid levels (see further below).

Effects of leucine

Leu can exert important effects during ATD (or ATPD) tests at several levels, which could impact behavioural and biochemical changes. These effects, outlined in Table 3, are discussed below.

The incidence of pellagra in populations existing largely on Trp-poor staples, such as maize or sorghum, is caused in part by the presence of high levels of Leu (for review, see Badawy,

2014). The pellagragenic effect of Leu involves changes in a number of enzymes of the kynurenine pathway, notably inhibition of kynureninase and activation of TDO. The flux of Trp through TDO is enhanced in hepatocytes isolated from high Leu-fed rats (Salter et al., 1985) and we found (Badawy et al., 2014) that Trp flux down the pathway in normal subjects is enhanced dose-dependently by BCAA in the control ATD formulation, with Leu being the responsible component. We therefore suggest that, in addition to stimulation of protein synthesis, increased hepatic Trp uptake and metabolism during ATD is an additional mechanism of action of Leu contributing to the depletion of plasma Trp and the decrease in its availability to the brain.

BCAA are metabolized first by BCAA aminotransferase (BCAT) to their corresponding keto acids (BCKA), converting in the process 2-oxoglutarate to glutamate. BCAA are therefore nitrogen donors for the synthesis of this excitatory amino acid and the inhibitory neurotransmitter GABA with Leu playing a major role (for references, see Badawy, 2013a), and it is of interest that the brain cytosolic isoenzyme of BCAT is located in GABAergic and glutamatergic neurons (Garcia-Espinosa et al., 2007). A high BCAA content, as that in the ATD formulation of Young et al. (1985) can therefore enhance glutamatergic activity resulting in an excitatory environment that can induce anxiety (Tsapakakis and Travis, 2002). This may partly explain the increased panic in studies using the above formulation, as opposed to the low BCAA gelatine-based formulation used by Colasanti et al. (2011). Another potential mechanism of increased anxiety is that of decreased brain kynurenic acid demonstrated in rats treated with BCAA (Coppola et al., 2013). The absence of enhanced fear response after ATD in the 2 studies by Töru et al. (2006) and Hood et al. (2010) in antidepressant-remitted patients cannot be explained by a glutamatergic mechanism, as subjects received the high BCAA dose, but may involve normalisation of the serotonin status through liver TDO inhibition and decreased plasma cortisol (Badawy, 2013c) by the SSRIs (specific serotonin reuptake inhibitors) used.

As well as inducing an excitatory environment and anxiety, glutamatergic activation by BCAA may influence many behaviours at multiple levels. Thus, glutamate receptors play a pivotal role in cognitive function (Robbins and Murphy, 2006) and influence significantly both DA and serotonin function (Tsapakakis and Travis, 2002; Laruelle et al. 2003). These potential interactions should be considered in future behavioural studies using ATD/ATPD.

Plasma tyrosine and phenylalanine as precursors of catecholamines

A decrease in Tyr and Phe availability to the brain during ATD is likely to lower brain dopamine synthesis, which could impair its release and function, including modulation of function of metabotropic NMDA (*N*-methyl-*D*-aspartate) glutamate receptors and GABA activity (Laruelle et al., 2003), thereby influencing behavioural measures in ATD. Although Sobczak and Schruers (2014) state that catecholamines enhance fear conditioning, we suggest that the enhanced fear response observed by Klaassen et al. (1998) and Schruers et al. (2000) does not involve catecholamines for the following reason. Although the amount of Phe and Tyr present in the Young et al. (1985) formulation is not inconsiderable (12.6%), the competition from the greater BCAA content (30%) causes a decrease in the [Tyr + Phe]/[BCAA + Trp] ratio of ~50% (Badawy et al., 2010a). Phe and Tyr entry into the brain is

therefore decreased and disadvantaged only by BCAA, even-though Phe has the greatest affinity for the carrier system (see Badawy, 2013b and references cited therein). It is therefore most likely that the above enhanced fear response is mediated by a combination of serotonin depletion and BCAA influencing glutamatergic and/or GABAergic activity.

Whereas ATD may decrease Tyr availability to the brain in humans, this may not be the case in experimental animals. In the study by Ardis et al (2009) in which ATD induced the expected depletion of plasma Trp and brain serotonin, no significant changes in concentrations of NA, DA or its metabolites in the brain regions examined were observed, and the authors concluded that the effects of ATD are limited to serotonin depletion. This specificity was established further recently in a comparative study in mice (Sánchez et al., 2014) demonstrating specificity of ATD for serotonin and ATPD for DA. This is almost certainly the case for the following reasons. BCAA are metabolized differently in rodents and humans. For example, the rat metabolizes BCAA faster than man (Suryawan et al., 1998), hence the use of 2 ATD or ATPD doses 30 or 90 min apart, in the above 2 studies. BCAT activity in human tissues is only 10% of that in the corresponding rat tissues (Suryawan et al., 1998). The similarly higher BCKA dehydrogenase in rat tissues will additionally promote further transamination of BCAA. Accordingly, the rapid transamination of BCAA in the rat (and possibly also the mouse) is almost certain to prevent accumulation of unchanged BCAA in plasma in amounts sufficient to compete with Trp or Phe and Tyr for cerebral uptake. For example, whereas plasma levels of the 3 BCAA are elevated by 147-244% (Dougherty et al., 2008; Badawy et al., 2010b) at 3h after administration of 50g of the ATD formulation of Young et al. (1985), the increases at 3h in rats treated with a similar dose of BCAA (200 mg/kg) are very modest (10-36%) (Agharanya and Wurtman, 1982). Future ATD and ATPD studies in rodents should include measurements of plasma BCAA levels, preferably individually, rather than as a group. Taken together, while rodents metabolize BCAA differently from humans, their rapid disposal of BCAA could confer specificity on existing ATD or ATPD formulations than is currently the case in humans. Monoamine-related behaviour could therefore be studied in rats and mice with assured specificity and without significant interference from glutamatergic/GABAergic stimulation.

Insulin, high-fat diets and plasma free tryptophan

Sobczak and Schruers (2014) pointed out that in the studies by Klaassen et al. (1998) and Schruers et al. (2000), the ATD mixture included 33g of vegetable fat and 63g of carbohydrates and emphasized the effects of insulin release on plasma Trp binding and peripheral BCAA uptake. These additions are clearly undesirable and should not be made. As only plasma total Trp was measured in both studies, and not free Trp or CAA, the extent to which carbohydrate or fat influenced Trp availability to the brain remains unknown. For example, what is the level of decrease in plasma free Trp and BCAA caused by insulin inhibiting lipolysis and increasing muscle BCAA uptake and metabolism? Inhibition of liver TDO activity by acute or chronic carbohydrate (glucose, sucrose or starch) administration and by chronic administration of high-fat diets, demonstrated in rats (Badawy and Evans, 1976; Badawy et al., 1984), leads to increased plasma free and total [Trp] and its availability to the brain. Additionally, fatty acids released from triacylglycerols (triglycerides) can

further increase plasma free Trp by displacement from albumin-binding sites (Badawy, 2010). These potential effects could confound interpretation of the desired biochemical changes during ATD tests and should therefore be avoided. Vegetable fats and carbohydrates are not solvents for amino acid mixtures, but can act as diluents, emulsifiers and palatability tools. In our ATD studies (Badawy et al., 2010b), the amino acid mixtures are dissolved and/or suspended in water sweetened with a Sweet and Low® sugar substitute and flavoured with an 1/8th of a teaspoon each of raspberry and lemonade Kool-Aid®.

Most ATD studies do not include measurement of plasma free Trp or its ratio to CAA. While the assumption is that free Trp will also be decreased, there will be instances wherein the changes in plasma Trp disposition (see Badawy, 2010) do not follow the expected direction, e.g. if lipolysis is enhanced by stressful release of adrenal medullary catecholamines, which could occur in depression (Badawy, 2013c) and potentially also anxiety states, and it is of interest that plasma free Trp was indeed measured in the studies by Tõru et al. (2006) and Hood et al. (2010) in panic and generalized anxiety disorders patients. The baseline plasma free [Trp] values reported by Tõru et al. (2006) in panic patients (1.6-2.5 µM or 0.32-0.51 µg/ml) were, however, considerably lower than normal values (~ 6.36 µM or 1.3µg/ml) (Dougherty et al., 2008), almost likely because of increased albumin binding due to frozen storage of plasma prior to ultrafiltration (Badawy, 2010). Ultrafiltration must therefore be made with freshly isolated plasma. Also, although equilibration between the free and albumin-bound Trp is rapid, it is the free fraction that is immediately available for cerebral and other tissue uptake. Plasma free Trp is a major determinant of Trp flux through TDO and consequent production of neuroactive kynurenines. Inclusion of plasma free Trp, whenever possible, could not only help interpretation of ATD data, but also establish the baseline Trp disposition status and identify its physiological determinants (Badawy, 2010).

Time-point for ratio determination

Sobczak and Schruers (2014) emphasized the importance of the time point(s) of biochemical (and behavioural) testing during ATD with the aim of standardizing the methodology. In our previous studies (Dougherty et al., 2008; Badawy et al., 2010b), we performed a full 7h time- course analysis of biochemical parameters during the ATD tests to enable investigators to select the appropriate time-interval(s) for biochemical measurements and in relation to behavioural measures. As shown in Table 4, for the traditional control formulation of Young et al. (1985) at a 50g dose, concentrations of all plasma 6 CAA are maximally increased at 2h, with all ratios decreasing maximally at 2-3h. With ATD at 50g or 100g, the free and total [Trp] are decreased maximally at 5-6h and their ratios to CAA at 4h. With ATL, free and total [Trp] are increased maximally at 3-5h and their ratios to CAA at 3-5h. In either case (ATD or ATL), the increases in the 5 Trp competitors are maximal at 2-5h. Thus ratios should be measured at the time of their maximum changes. As stated above, the decreases in the ratios in the control (serotonin-unspecific) formulation with the 30% BCAA content occur maximally 2h earlier than those with the ATD formulation. This further underscores the need to use our proposed (modified) control formulation with the 18% BCAA content, in which all ratios are normalized (Table 4), not only to improve specificity, but also to avoid or eliminate incorrect comparisons of ratios between test and control formulations at different time-intervals.

The question of choice of the time point after maximum ATD is observed at which behavioural measures are best assessed is also important. We have discussed this issue at some length (Dougherty et al., 2008 and references cited therein). Briefly, based on imaging and cerebrospinal fluid studies, central measures of serotonin synthesis and function occur ~ 2h after maximal decreases in the plasma [Trp]/[CAA] ratios. Accordingly, behavioural measures could be extended beyond the usual 5h point to establish the duration of the central changes.

For obvious reasons, the behavioural and biochemical effects of the depletion or loading tests could not be extended well beyond ~8-10 h without further food intake, hence the paucity of longer-time studies. Only the study by Delgado et al. (1990) in remitted depressed patients was conducted without food intake over a 24h period following the ATD drink. Lowered mood was maximal at 7h, 2h after maximal plasma Trp depletion, but was still present at 24h. In healthy first degree relatives of patients with affective disorders, Klaassen et al. (1999b) found that mood is lowered at 6 h after ATD, but not at 24h. The [Trp]/[CAA] ratio was decreased maximally at 4-6h, and was still below baseline values at 16-24h, but this was due to intake of an additional ATD dose at 14h. Subjects in this latter study also received dietary carbohydrate and lipid during the 24h study. If observations are required beyond the 10h time-point, account must be taken of any potential effect of concurrent food intake.

Protein-based formulations

Protein-based formulations for ATD (gelatine) and ATL (α -lactalbumin or whey protein hydrolysate) have been used (see references in Badawy, 2013a). With the whey protein hydrolysate and α -lactalbumin, Trp loading has been successful in increasing plasma Trp concentration and availability to the brain. However, as shown in the study by Colasanti et al. (2011) and previously by Nieuwenhuizen et al. (2009), gelatine is only partially effective in inducing ATD, with a decrease in the [Trp]/[CAA] ratio (~ 60%) not dissimilar to that (61%) observed by us (see Badawy et al., 2010a) with the unmodified control formulation of Young et al. (1985). As shown in Table 3 by Sobczak and Schruers (2014), gelatine is deficient not only in Trp, but also in BCAA (6%: well below our recommended 18% level) and Phe + Tyr (2.2% vs 12.6% in the Young et al., 1985 formulation). Thus, the low BCAA content of gelatine is likely responsible for the sub-optimal (60%) Trp depletion and the low Phe and Tyr content is bound to lead to ATPD in both test and control formulations. Taken together, this may explain the inability of gelatine to enhance panic behaviour and suggests that this protein may not be the best protein-based formulation for ATD. For ATL, α -lactalbumin, with a 5.9% Trp content, is potentially specific for 5-HT enhancement, as its BCAA content (20.9%) (Heine et al., 1991) is close to that (18%) recommended by us and so is unlikely to undermine significantly Tyr availability to the brain. This notion is supported by the observation (Booij et al., 2006) that it induces only a small (10%) decrease in the [Tyr]/[CAA] ratio.

Palatability and freedom from the side effects of amino acid mixtures are important requirements in protein-based formulations. While gelatine can be suitably flavoured, its moderate Trp depleting potential limits its use as a robust tool in these studies. Another

palatable protein already used by patients with phenylketonuria is the whey protein fraction glycomacropeptide (GMP). This 64-amino acid peptide is unique in several respects: (1) acceptable palatability; (2) absence of not only Phe, but also Tyr and Trp; (3) its BCAA content is the closest (~ 19%) to that (18%) recommended by us to ensure specificity. The potential use of GMP as a template for 7 formulations for ATD, ATPD, combined depletion, ATL, ATPL and combined loading and a placebo control has been suggested, with inclusion of the appropriate amounts of Trp and/or Phe and Tyr specified (Table 5; for details, see Badawy, 2013a) The psychopharmacological research community may wish to consider exploring this novel idea as a way of standardizing the depletion and loading methodologies. Also, as it has been proposed (Badawy, 2013a) that GMP alone or supplemented with Trp may form the basis of a nutritional therapy of manic and psychotic disorders by virtue of its Phe- and Tyr-free composition, development of these formulations can lead to their use in schizophrenia and other dopamine-related conditions and as unique tools for imaging and other neuroscience research on cerebral monoamines.

In relation to our recommended use of BCAA in amino acid formulations at an 18% concentration and the similar content in GMP, little information is available on the effect on behaviour or monoamine function *in vivo* of BCAA doses smaller than those in current amino acid formulations. In the dose-finding study by Gijsman et al. (2002), a 10g dose of a BCAA mixture (of Val, Ile and Leu in a 3:3:4 proportion) was effective in decreasing significantly the [Trp]/[BCAA] and [Tyr + Phe]/[BCAA] ratios by 68% and 78% respectively at 5h. The potential effects of this dose on serum prolactin or neuropsychological measures could not be discerned, because of the absence of a control group.

Conclusions and recommendations

We hope that the issues raised by Sobczak and Schruers (2014) and discussed further here will stimulate the psychopharmacological research community to consider our proposals for standardization of the depletion and loading methodologies using either amino acid mixtures or protein (α -lactalbumin and GMP)-based formulations. Developmental studies with GMP along lines previously published (Badawy, 2013a) are required and we invite researchers to contribute to this development. We propose the following recommendations for amino acid- or protein-based formulations.

Amino acid-based formulations

1. Existing ATD and ATPD formulations appear to be specific for the intended targets in studies in rats and mice, because of the rapid BCAA disposal in these species. However, future studies in these species could also explore our recommended amino acid formulation.
2. For ATD, ATL, ATPD and ATPL studies in humans, we recommend the use of a 50g dose of the formulation in Table 1 as a common control ensuring specificity.
3. For ATD and ATPD, the relevant amino acid(s) should be deleted from the control formulation, whereas for ATL, Trp should be added in the amounts stated by Young et al. (1985).

4. As ATPL has not been attempted before using amino acid mixtures, Tyr and Phe should be added to our control formulation in greater amounts to be titrated experimentally.

Protein-based formulations

1. α -Lactalbumin appears to be suitable and specific for Trp loading.
2. Gelatine is not suitable for Trp depletion, because of its moderate efficacy due to its low BCAA content.
3. GMP offers a more likely alternative to gelatine and also as a template for standardization of the methodology with 7 different formulations (Table 5).
4. Used alone, GMP can serve as a formulation for combined ATD and ATPD. However, as stated above, effects on certain behaviours similarly influenced by ATD and ATPD separately could be abolished by their combination. This possibility can be assessed in future studies.
5. Doses of GMP of up to 60g should first be tested for combined depletion and consequent behavioural changes and a suitable dose inducing robust changes should be used for optimization of the following 6 formulations, as recommended (Badawy, 2013a).
6. For separate ATD and ATPD, GMP should be supplemented with Phe + Tyr or with Trp respectively in the amounts suggested.
7. Supplemented with Trp, Phe and Tyr in the amounts suggested, GMP can provide an appropriate control with a similar BCAA content as all other formulations.
8. For ATL, ATPL and combined loading, larger amounts of Trp or Phe + Tyr should be added as suggested.

Standardization of this methodology will be a challenging task requiring a systematic effort in developing and assessing the biochemical and behavioural properties of our proposed amino acid and protein-based formulations in normal subjects and those with behavioural disorders, and taking into consideration a variety of factors that could impact the study parameters, including experimental conditions, gender, 5-HT-DA interactions and glutamatergic stimulation by BCAA. We hope that our proposals will provide an initial contribution towards, and a catalyst for, standardization of this methodology, which will ultimately lead to development and validation of an ideal set of formulations for these tests.

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Table 1
Criteria for the ideal amino acid depletion and loading formulations

1	Palatability
2	Freedom from, or acceptable (tolerable), side effects not leading to attrition
3	Robust and reproducible changes in the study parameters
4	A control formulation of a similar composition to the test formulation, but including a moderate amount of the amino acid(s) to be depleted or loaded, and which does not alter the study parameters
5	Specificity for the intended target

Table 2
Composition of the recommended 16-amino acid control formulation for depletion and loading studies (51.25g dose)

Amino acid	Amount (g)	Amino acid	Amount (g)
Tryptophan	1.15	Cysteine	1.64
Phenylalanine	2.85	Glycine	1.94
Tyrosine	3.45	Histidine	1.94
Leucine	4.05	Lysine	5.41
Isoleucine	2.40	Methionine	1.82
Valine	2.73	Proline	7.41
Alanine	3.34	Serine	4.19
Arginine	2.98	Threonine	3.95

The original control formulation by Young et al. (1985) has been modified to include 40% less of each BCAA and proportionate increases in the 10 non-competitors.

Table 3
Effects of leucine (BCAA) during ATD and ATPD tests

1	Stimulation of protein synthesis → decreased plasma Trp
2	Activation of liver Trp 2,3-dioxygenase → decreased plasma Trp
3	Competition with Trp, Tyr and Phe for cerebral uptake → decreased 5-HT and DA synthesis
4	Competition with kynurenine for cerebral uptake → decreased brain kynurenic acid → increased anxiety
5	Enhancement of glutamatergic function (including activation of NMDA receptors) → a hyperexcitability state causing anxiety and other behavioural, including cognitive, deficits
6	Modulation of GABAergic function secondarily to glutamate stimulation

Table 4
Time points of maximal changes in study parameters in control and test formulations of Young et al. (1985) for ATD and ATL

Parameter	Time after intake (h)				Modified Control*	
	ATD 50 g	ATD 100 g	ATL 50 g	ATL 100 g	Control 50 g	Control 50 g
Free [Trp]	5 (↓)	5 (↓)	3 (↑)	4 (↑)	2 (↑)	2 (↑)
Total [Trp]	5 (↓)	6 (↓)	3 (↑)	5 (↑)	2 (↑)	2 (↑)
[CAA]	3 (↑)	3 (↑)	2 (↑)	5 (↑)	2 (↑)	2 (↑)
[Free Trp]/[CAA]	4 (↓)	4 (↓)	3 (↑)	4 (↑)	3 (↓)	-
[Total Trp]/[CAA]	4 (↓)	4 (↓)	5 (↑)	3 (↑)	2 (↓)	-
[Tyr + Phe]	3 (↑)	3 (↑)	5 (↑)	4 (↑)	2 (↑)	2 (↑)
[BCAA]	3 (↑)	3 (↑)	2 (↑)	4 (↑)	2 (↑)	2 (↑)
[BCAA + Trp]	2 (↑)	3 (↑)	2 (↑)	5 (↑)	2 (↑)	2 (↑)
[Tyr + Phe]/ [BCAA + Trp]	2 (↓)	3 (↓)	2 (↓)	5 (↓)	2 (↓)	-

Values are based on data from Dougherty et al. (2008) and Badawy et al. (2010b). Amino acid mixtures were consumed over a 20-min period and measurements were made before and hourly for 7h after consumption of drinks. All formulations were those of Young et al. (1985), except the modified control* in which BCAA were decreased by 40% as shown in Table 2. Symbols: ↓ (decrease); ↑ (increase); - (no change: normalisation).

Table 5

Proposed composition of the 7 GMP-based formulations for the acute tryptophan and tyrosine plus phenylalanine depletion and loading tests (for a 100 g total amount)

Number	Formulation	GMP	Trp	Tyr	Phe
1	Combined depletion:	100 g	–	–	–
2	ATPD:				
	Level 1	97.7 g	2.3 g	–	–
	Level 2	97.0–97.9 g	2.1–3.0 g	–	–
	Level 3	96.4–98.0 g	2.0–3.6 g	–	–
	Level 4	98.0–100.0 g	0.0–2.0 g	–	–
3	ATD:				
	Level 1	89.0 g	–	6.0 g	5.0 g
	Level 2	83.5–89.0 g	–	6.0–9.0 g	5.0–7.5 g
4	Placebo control:				
	Level 1	84.7–86.7 g	2.3 g	6.0–7.0 g	5.0–6.0 g
	Level 2	84.0–86.9 g	2.1–3.0 g	6.0–7.0 g	5.0–6.0 g
	Level 3	79.9–87.0 g	2.0–3.6 g	6.0–9.0 g	5.0–7.5 g
5	ATL:				
	Level 1	77.0 g	10.3 g	6.9 g	5.8 g
	Level 2	76.0–78.2 g	10.1–11.0 g	9.4–7.4 g	5.3–5.6 g
	Level 3	69.2–80.0 g	9.0–12.3 g	6.0–10.0 g	5.0–8.5 g
6	ATPL:				
	Level 1	77.6 g	3.6 g	10.2 g	8.6 g
	Level 2	76.1–79.1 g	3.1–4.1 g	9.7–10.7 g	8.1–9.1 g
	Level 3	68.0–80.5 g	3.0–6.0 g	9.0–15.0 g	7.5–11.0 g
7	Combined loading:				
	Level 1	70.9 g	10.3 g	10.2 g	8.6 g
	Level 2	69.4–72.4 g	9.8–10.8 g	9.7–10.7 g	8.1–9.1 g
	Level 3	60.0–74.5 g	9.0–14.0 g	9.0–15.0 g	7.5–11.0 g

Definitions of levels: 1 (most preferable), 2 (preferable), 3 (less preferable, but possible), 4 (least preferable, but still possible). Reproduced here with permission from Springer-Verlag, Berlin, from Badawy (2013a) [Badawy AA-B (2013) Review: Novel nutritional treatment for manic and psychotic disorders: a review of tryptophan and tyrosine depletion studies and the potential of protein-based formulations

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using glycomacropetide. *Psychopharmacol* 228: 347-358). Data are based on a published international patent application (Badawy 2012), protection of which was abandoned and the research community is free to develop this technology without any legal or commercial restraints.