Acute tryptophan depletion in humans: a review of theoretical, practical and ethical aspects

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The acute tryptophan depletion (ATD) technique has been used extensively to study the effect of low serotonin in the human brain. This review assesses the validity of a number of published criticisms of the technique and a number of previously unpublished potential criticisms. The conclusion is that ATD can provide useful information when results are assessed in conjunction with results obtained using other techniques. The best-established conclusion is that low serotonin function after tryptophan depletion lowers mood in some people. However, this does not mean that other variables, altered after tryptophan depletion, are necessarily related to low serotonin. Each aspect of brain function has to be assessed separately. Furthermore, a negative tryptophan depletion study does not mean that low serotonin cannot influence the variable studied. This review suggests gaps in knowledge that need to be filled and guidelines for carrying out ATD studies.

Introduction

The acute tryptophan depletion (ATD) technique has been in use for more than a quarter-century now, and it continues to be a popular method to study the effects of low serotonin in humans. However, there remains some controversy about the extent to which serotonin function may be altered and the possible role of other psychoactive compounds in inducing changes. There is also disagreement about the best methodology to use. Finally, although the technique is designed to lower serotonin, and low serotonin is associated with suicide,[1] there has been little discussion of the ethical aspects of the technique. The purpose of this review is to discuss the evidence concerning these issues.

Rationale for the ATD method

Traditionally, studying the role of serotonin in psychiatric disorders relied on 2 main methods: looking at measures related to serotonin in patients with psychiatric disorders and investigating the mechanism of action of drugs used to treat disorders. However, low levels of a serotonin-related compound in, for example, depressed patients could be due to low serotonin causing depression, depression causing low serotonin, or some unknown factor causing both. Furthermore, levels of 5-hydroxyindoleacetic acid (5-HIAA) in the

cerebrospinal fluid (CSF), an indication of brain serotonin synthesis, are associated more with suicide than depression.[2] There is considerable overlap between 5-HIAA levels in the CSF of depressed patients and controls. Many depressed patients have normal or even high 5-HIAA levels in the CSF, while some nondepressed people have low levels. Also, if antidepressants can work by increasing serotonin function, that does not necessarily mean that low serotonin was involved in the etiology of depression. A more direct way to study the effects of low serotonin on mood is to lower human brain serotonin synthesis and look at mood changes. The first direct approach used to study the effects of lowering serotonin in humans was that of Shopsin and colleagues,[3,4] who showed that parachlorophenylalanine (PCPA), an inhibitor of tryptophan hydroxylase, reversed the effect of both imipramine and tranylcypromine, presumably by lowering brain serotonin. To reverse the effect of antidepressants, PCPA was given at a dose of 0.5–2.5 g per day. The extent to which this might lower brain serotonin synthesis is uncertain, as a dose of 1 g per day for 6 days failed to lower baseline 5-HIAA in the CSF, although it did lower the increase of 5-HIAA in the CSF after administration of probenecid to inhibit efflux of 5-HIAA from the CSF.[5] However, concerns about the possible toxicity of PCPA at doses needed to cause significant inhibition of tryptophan hydroxylase have limited its experimental use.

The technique to lower brain serotonin using dietary components was developed in rats by Biggio and colleagues.[6] Normally when rats are put on a diet with any amino acid imbalance, there is a rapid and marked decline in food intake,[7] which would limit any decline in brain tryptophan and serotonin if the food was deficient in tryptophan. Therefore, Biggio and colleagues[6] trained their rats to eat all their daily food ration in only 2 hours. Then their normal diet was replaced by one that was similar, except for the absence of tryptophan. This resulted in a rapid and substantial lowering of plasma and brain tryptophan, and of brain serotonin and 5-HIAA. Gessa and colleagues[8] studied the mechanism of the reduction of brain serotonin by administering different mixtures of amino acids orally to rats. When they gave rats a mixture of all the essential amino acids except for tryptophan, there was a substantial decrease in brain tryptophan and serotonin. However, when they gave the same dose of a mixture of all the other large neutral amino acids (LNAA), which compete with tryptophan for entry into the brain,[9] the decline in brain tryptophan was much smaller, and the decline in brain serotonin was not statistically significant. Given that the mixture of LNAA was missing 6 of the essential amino acids, they concluded that the tryptophan deficient diet induced protein synthesis and that tryptophan and serotonin were depleted because tryptophan was incorporated into protein. On the other hand, inhibition of tryptophan uptake into the brain was not as important as the lowering of tryptophan levels. This conclusion was confirmed by Moja and colleagues,[10] who demonstrated that the depletion of tryptophan could be inhibited by the protein synthesis inhibitor cycloheximide.

The ATD technique was first applied in humans by Concu and colleagues[11] in 1977. They gave an 18.2 g tryptophan-free mixture of 9 amino acids to healthy men. Serum tryptophan declined by 42%, and those given the active mixture showed increased anxiety relative to controls who received a mixture containing tryptophan. In 1984, Moja and colleagues[12] used a similar mixture that omitted glycine and weighed 12.2 g. They studied the effect on sleep in healthy volunteers and found a decrease in stage 4 sleep latency and an increase in stage 4 sleep duration during the first 3 hours of sleep. In 1985, my colleagues and I[13] reported that a 100 g mixture of 15 amino acids lowered plasma tryptophan in healthy men by 76% and caused a lowering of mood. Subsequently Delgado and colleagues[14] performed a study that was conceptually similar to those of Shopsin and colleagues.[3,4] The differences were that the depressed patients were being treated with a greater variety of antidepressants and that serotonin was lowered by the same ATD mixtures used in our study.[13] The results were similar to those of Shopsin and colleagues,[3,4] in that lowering serotonin caused a temporary reappearance of the symptoms of depression in most patients. This result led to the widespread use of ATD to study the effects of low serotonin in humans.

Validity of the ATD method for examining the effects of low human brain serotonin function

The original idea behind the ATD method was to mimic the low serotonin synthesis thought to be involved in lowered mood. Low serotonin synthesis would presumably result in low serotonin function. To date, the experimental evidence in humans is related only to the effect on serotonin synthesis, not function. Numerous studies have shown that ATD causes a substantial lowering of human plasma tryptophan and the ratio in plasma of tryptophan to the sum of the other LNAA that compete with tryptophan for entry into the brain (trp/ΣLNAA). After ATD, there is a correlation between plasma trp/ΣLNAA, but not plasma tryptophan, and the level of the serotonin metabolite 5-HIAA in the CSF.[15] Nishizawa and colleagues[16] used PET, with α-[^{11}C]methyl-L-tryptophan as a tracer, to study the rate of serotonin synthesis after ATD in healthy volunteers. In all brain regions measured, the decline in serotonin synthesis was greater than 85% and was greater in women than in men. The lowering of serotonin synthesis after ATD had been confirmed in several studies that demonstrated a lowering of 5-HIAA in the CSF of volunteers undergoing ATD.[15,17–20]

Recently, van Donkelaar and colleagues[21] suggested that "direct evidence that ATD decreases extracellular [serotonin] concentrations is lacking" and that "several studies provide support for alternative underlying mechanisms of ATD." While this has been challenged by Crockett and colleagues,[22] these are important issues that need to be discussed further, as all relevant issues were not discussed in these 2 papers.

Does ATD lower serotonin function?

The challenge to the ATD method by van Donkelaar and colleagues[21] and the response by Crockett and colleagues[22] focus in part on rat studies looking at serotonin release in the brain, and tend to focus on whether or not ATD alters serotonin release. There are advantages in reframing the discussion by asking if there are particular circumstances in which ATD lowers serotonin release in particular brain areas. The factors that control serotonin release are very complex. A recent paper reporting an attempt to model mathematically what happens at the serotonergic synapse concluded in part that "many difficult questions remain in order to fully understand how serotonin biochemistry affects serotonin electrophysiology and vice versa."[23] Furthermore, rat brains are very different from human brains, and the environment of rats undergoing serotonin release studies is very different from that of humans undergoing ATD studies. In addition, serotonin release is not regulated the same way in all brain areas, so trying to extrapolate results from a single area of rat brain to the human brain is unlikely to provide useful insight.

If the regulation of serotonin function in humans has any similarities to that in experimental animals, ATD may have different effects depending on the environment and the mental state of the human being studied. In experimental animals the rate of firing of serotonin neurons is increased during periods of behavioural arousal and/or motor activity, with subgroups of neurons firing at higher rates during rhythmic motor activities, such as feeding, grooming and increased respiration.[24] Serotonin release, measured by brain microdialysis, is, as expected, increased by factors that increase the firing of serotonin neurons. If ATD decreases release of serotonin, it probably

does so by depleting the reserve of serotonin in the pool used for release during neuronal firing. If the firing rate is greater, then there is likely to be greater depletion of the releasable pool. Therefore, a plausible hypothesis is that any effect of ATD on serotonin release is likely to be greater when the participants are in a greater state of arousal. For example, arousal associated with being in a functional magnetic resonance imaging scanner, compared with being in a quiet room filling in rating scales, may cause changes in the effect of ATD on serotonin release.

If the reserve of serotonin in the releasable pool is a factor in determining the magnitude of any effect of ATD, then effects may be different in different brain areas. Nishizawa and colleagues[16] estimated rates of serotonin synthesis in human brain areas using PET with α-[11C]methyl-L-tryptophan as a tracer. Combining those data with published data on the concentration of serotonin in different brain regions determined postmortem, the study determined that, for example, the time needed to synthesize the amount of serotonin found in the putamen was in the range of 31–48 minutes, but in the cortex the time needed was 0.8–1.3 minutes.

The discussion of these findings indicates that we are far from being able to give definitive answers about the circumstances in which ATD might lower serotonin release and the areas of the brain in which it might occur. This is why the interpretation of ATD studies needs to take into account results from other studies using different techniques. As mentioned, the ATD technique was originally developed to see whether the association between low serotonin and low mood was causal. The older results suggesting an association between low serotonin and low mood support the idea that when ATD lowers mood it does so by decreasing release and function of serotonin. However, the effect of ATD on mood depends on the characteristics of the individuals being studied, with effects ranging from nothing, to a dramatic lowering of mood in newly discovered depressed patients who are treated with serotonergic antidepressants.[25] The lack of mood lowering could be due to adaptive processes in the brain preventing any significant decrease in serotonin function. Alternatively, given that nothing in the brain is controlled by a single neurotransmitter, it could be that other neuronal systems involved in mood regulation have a homeostatic effect that prevents lowered serotonin function from lowering mood. However, what little experimental evidence there is raises the possibility that the difference between individuals who show a lowering of mood and those who do not may be related to the regulation of the serotonergic system.

Price and colleagues[26] studied the hormonal responses of untreated depressed patients when they received an intravenous infusion of tryptophan after either ATD or a control amino acid mixture. The cortisol response to tryptophan was significantly greater after ATD than after the control treatment, suggesting that ATD upregulated postsynaptic receptors. When the authors performed a similar study with an infusion of m-chlorophenylpiperazine instead of tryptophan, the results were similar, suggesting that serotonin 2A/2C receptors were upregulated.[27] Yatham and colleagues[28] followed this up by investigating whether alterations of sero-

tonin 2 receptors might explain differences in response to ATD. They measured serotonin 2 receptors after ATD and control amino acid mixtures in newly recovered depressed patients who were being treated with selective serotonin reuptake inhibitors. In the 9 patients who did not become temporarily depressed after ATD, serotonin 2 receptors were significantly reduced after ATD compared with after the control mixture. There was no significant difference for the 8 patients who did become depressed. While this result shows that rapid changes can occur in the brain in response to ATD, how the change in serotonin 2 receptors might be related to changes in serotonin release from neurons and serotonin function is not known.

Because of the lack of direct information on how ATD influences serotonin release, negative results also have to be interpreted in light of other data. For example, although there is some evidence that the loudness dependency of auditory event-related potentials (ERPs) is related to brain serotonin function, this measure is not altered by ATD.[29–31] While this raises the possibility that the loudness dependency of auditory ERPs is not related to serotonin function, the evidence is by no means definitive. Although there is increasing evidence, summarized in the paragraphs that follow, that ATD lowers serotonin function in brain areas related to the regulation of mood, this does not necessarily mean that it lowers serotonin function in brain areas related to the loudness dependency of auditory ERPs. For negative ATD results, as for positive results, interpretations have to take into account the broader evidence on the topic.

There is good, although not definitive, evidence that ATD can alter serotonin function in some circumstances.

Does ATD alter aspects of brain function other than serotonin?

Van Donkelaar and colleagues[21] pointed out a number of possible changes, other than serotonin synthesis, that might be responsible for the effects of ATD. These include changes in brain nitric oxide (NO), cerebrovascular changes, decreased brain-derived neurotrophic factor (BDNF) and decreased kynurenine pathway metabolites. Other possibilities not mentioned by van Donkelaar and colleagues[21] are effects of amino acid imbalance, direct effects of tryptophan on protein synthesis and on the organic cation transporter 2 (OCT2), effects on melatonin, and side effects and unblinding. These possibilities are discussed in the paragraphs that follow.

Citrulline and nitric oxide
Two rat studies using a gelatin-based tryptophan-deficient amino acid mixture, which has a somewhat different amino acid composition from the mixture commonly used in humans, raised brain levels of citrulline relative to a similar mixture containing tryptophan.[32,33] Because arginine is converted into citrulline and NO, van Donkelaar and colleagues[21] suggested that the increase in citrulline might indicate increased NO production. Given the lack of any information on the mechanism of the increase in citrulline levels and the fact that this finding was in rats and used a different amino acid

formulation, the relevance of this for human ATD studies is uncertain.

Cerebrovascular changes

Van Donkelaar and colleagues[21] pointed out that serotonin is a vasoconstrictor and that ATD could potentially alter cerebral blood flow. However, the possibility that peripheral serotonin might alter brain blood flow after ATD is unlikely. Acute tryptophan depletion in humans does not lower blood serotonin levels.[34] Most of the serotonin in the blood is stored in platelets, and presumably the 5- to 7-hour duration of an ATD study is not long enough to deplete platelet serotonin stores. Whereas a decreased vasoconstrictive effect due to lowered serotonin might increase blood flow, van Donkelaar and colleagues[21] mention a rat study in which ATD decreased blood flow in several rat brain regions.[33] In that study, a tryptophan-deficient amino acid mixture lowered the plasma trp/ΣLNAA ratio by 40%, but, for unknown reasons, the decline in the trp/ΣLNAA ratio did not result in an alteration in brain tryptophan, serotonin or 5-HIAA. Therefore, the changes in blood flow were not due to alterations in brain serotonin function. The significance of this study is difficult to discern, given that it was carried out in rats and that brain tryptophan and serotonin were not lowered, as might be expected. Alterations in blood flow after ATD, due to a nonserotonergic mechanism, are a theoretical possibility that could be explored.

Brain-derived neurotrophic factor

Van Donkelaar and colleagues[21] cited studies demonstrating ATD does "not have a direct effect on peripheral and central BDNF levels" but nonetheless suggested that BDNF might be involved in the effects of ATD as "there are coregulating mechanisms between BDNF and the [serotonin] system in general." Serotonin influences many different brain functions because the small number of serotonin neurons project to all areas of the brain, and may have secondary effects on many different neuronal systems.[35] Effects of ATD that are due to altered serotonin function must be mediated by other neuronal systems, of which BDNF may well be one. However, the issue of importance is whether altered serotonin function is the primary factor in the effect of ATD, not whether altered serotonin function may have secondary effects on other neuronal systems. Given the interconnectedness of neuronal systems, large changes in the synthesis of one neurotransmitter would be expected to change other systems.

Decrease in metabolites along the kynurenine pathway

Synthesis of serotonin is a minor pathway of tryptophan metabolism. More than 90% of tryptophan is metabolized by tryptophan-2,3-dioxygenase and indoleamine-2,3-dioxygenase, the first enzymes on the kynurenine pathway.[36] Two metabolites on that pathway, kynurenic acid and quinolinic acid, can potentially have effects on brain function.[37] Kynurenic acid is a competitive antagonist at all 3 ionotropic glutamate receptors, whereas quinolinic acid is an agonist at the *N*-methyl-D-aspartate (NMDA) receptor. However, the normal concentrations of kynurenic acid and quinolinic acid

in the brain are probably low compared with the concentrations needed to act at NMDA receptors.[38] Furthermore, as discussed by Heyes and colleagues,[39] the concentration of quinolinic acid in the human brain and CSF is 1–3 orders of magnitude above control levels in inflammatory disorders that are associated with neurologic deficits, suggesting that a putative decline from normal quinolinic acid levels after ATD is unlikely to have any important functional significance. Kynurenic acid also has an antagonistic action at nicotinic receptors,[40] and reducing rat brain kynurenic acid with an inhibitor of kynurenine aminotransferase, the enzyme that converts kynurenine to kynurenic acid, improves performance in the Morris water maze, suggesting that kynurenic acid is normally present in physiologically relevant concentrations in the brain. However, ATD does not lower human plasma kynurenic acid levels.[41] Quinolinic acid has not been measured after ATD. Nonetheless, both kynurenic acid and quinolinic acid are derived from kynurenine, and kynurenine crosses the blood–brain barrier. If regulation of the conversion of kynurenine to kynurenic acid is different in the brain and periphery, then levels of kynurenic acid in the brain and periphery may not be related. Measurements of kynurenic and quinolinic acids in human CSF after ATD are needed to see if they are changed.

Amino acid imbalance

Extensive animal literature exists on the effect of amino acid–imbalanced diets,[7] of which the ATD mixture is an example. In rats, imbalanced diets decrease food intake. The initial sensor of this effect, which can occur within 30 minutes, seems to be altered essential amino acid levels in the anterior piriform cortex, and the effect is mediated by serotonin 3 receptors.[42-44] Although the decline in food intake is mediated by serotonin receptors, the effect is not specific to amino acid imbalances involving tryptophan, so any effect might also apply to studies with phenylalanine/tyrosine deficient mixtures. The significance for human ATD studies of the ingestion of amino acid mixtures with an unphysiological imbalance of amino acids is not known. However, ATD does not decrease food intake in humans,[45-47] so if the human brain contains a system for detection of amino acid imbalance that is similar to that in the rat brain, such a system is unlikely to alter other aspects of brain function. However, this does not rule out effects of amino acid imbalance acting through different mechanisms in different brain areas.

Protein synthesis

Tryptophan is unique among the amino acids in that it can alter protein synthesis. Tryptophan supplements can increase protein synthesis in both the rodent liver[48] and brain,[49,50] an effect that seems to be mediated by tryptophan binding on cell nuclei.[51] This raises the possibility that human brain protein synthesis is different after the ingestion of tryptophan-containing and tryptophan-deficient amino acid mixtures. The implications of this are not known.

The organic cation transporter 2

OCT2 is present primarily in the kidneys, and is involved in

the elimination of cations. In a recent metabolomic study in humans, urinary tryptophan levels varied with different polymorphisms of *OCT2*. This, together with other experimental approaches, such as the demonstration that tryptophan inhibits the transport of known OCT2 substrates, led the authors to suggest that tryptophan is an endogenous substrate for OCT2.[52] Oct2 is also expressed in the brain, and recently Bacq and colleagues[53] tested the idea that Oct2 may be the low-affinity transporter that acts to supplement the high-affinity serotonin and noradrenaline transporters in the removal of serotonin and noradrenaline from the synaptic cleft. *Oct2*-knockout mice showed behavioural differences from control animals in models of depression. For example, *Oct2*-knockout mice showed a marked increase in immobility time compared with wild-type mice in the forced swim and the tail suspension tests. Furthermore, clearance of noradrenaline and serotonin in the brain was diminished, supporting the hypothesis of Bacq and colleagues.[53] Whether tryptophan inhibits the transport of noradrenaline and serotonin by OCT2 is not known. However, if it does, a decline in brain tryptophan after ATD might increase the reuptake of noradrenaline and serotonin, thereby decreasing their function. Animal research is required to determine whether lowered brain tryptophan might alter noradrenaline and serotonin function via OCT2.

Melatonin

The rate of synthesis of melatonin depends on tryptophan availability and, as might be expected, ATD lowers nighttime plasma melatonin[54] and urinary 6-hydroxymelatonin sulphate.[55,56] This may contribute to the effects of ATD on sleep.[12,57] Presumably ATD also lowers the already low levels of melatonin in the daytime, but this is unlikely to be of functional significance.

Side effects and unblinding

In our first study, my colleagues and I[13] reported that some participants experienced nausea. Because of evidence that both misattribution of physiologic cues induced by the setting and information given to the participants can influence emotional state,[58] in a second study we investigated whether these factors might contribute to the lowering of mood.[59] This was done by adding an instructional manipulation and an environmental manipulation. In the instructional manipulation, participants were either told or not told about various sensations, such as nausea or lethargy, they might experience after ingesting the amino acid mixtures. In the environmental manipulation, participants were maintained in either a supportive and comfortable setting or in an unrewarding and unstimulating environment for the duration of the study. In the control group that received the amino acid mixture containing tryptophan, the environmental manipulation had the expected effect on mood. However, neither manipulation influenced the ability of ATD to lower mood. This decreases the possibility that side effects or environment can contribute to the lowering of mood after ATD, but does not rule out that possibility given the problems in disproving a phenomenon.

The amino acid mixtures used in ATD studies are rather unpalatable depite various strategies used to diminish their unpalatability. These strategies include adding strong flavours, such as chocolate, to the amino acid suspension, putting the worst tasting amino acids (arginine, cysteine and methionine) in capsules, or, in the case of lysine, using the monohydrochloride instead of the free base.[14,59] Administering the mixture very cold will also help as it will decrease the taste of the amino acids by lowering the amount that dissolves in the water, and it will decrease the amount that volatilizes thereby diminishing the smell. However, whatever strategies are used, side effects will occur. Ingestion of the mixture results in up to 100 g of crystalline amino acids sitting in the stomach until the acid environment of the stomach dissolves them. As might be expected in these circumstances, both nausea and vomiting can occur. However, side effects have not often been studied. When they are, often there is no difference in side effects between ATD and control conditions, as found, for example, by Klaassen and colleagues.[60] However, aan het Rot and colleagues[61] found that dizziness, headache and nausea were all worse after ATD than after the placebo treatment. Furthermore, bright light, which prevented the lowering of mood in the ATD group, also diminished dizziness and nausea in that group. Serotonin receptors involved in emesis are present in both the gut and brain,[62] and the effect of bright light suggests that brain receptors contribute to the nausea and dizziness.

More research is needed on the side effects of ATD. If side effects are different in ATD and control groups, this might contribute to unblinding. If not, side effects could still potentiate any lowering of mood. In the study by aan het Rot and colleagues,[61] the measurement of side effects occurred at the same time as the measurement of mood. Normally, side effects, such as nausea, are worst in the first 2 hours after administration of the amino acid mixtures, and research is needed on whether side effects early on can predict later changes in mood or other measures. Unblinding will definitely occur in some circumstances, such as the temporary reappearance of the symptoms of clinical depression after ATD.[14] Assessment of the blinding should be routine in ATD studies in circumstances when less dramatic changes are expected in order to assess whether unblinding could contribute to the results. However, given the evidence to date, and the fact that many participants in ATD studies experience minimal side effects, unblinding owing to side effects is not likely to contribute significantly to the effects of ATD.

The need for positive control treatments and other strategies to rule out nonserotonergic mechanisms

Parachlorophenylalanine, an inhibitor of tryptophan hydroxylase, would not be expected to cause an amino acid imbalance or changes in the levels of tryptophan or tryptophan metabolites along the kynurenine pathway. Like ATD,[14] PCPA can cause a temporary reversal of the effects of antidepressants in recovered depressed patients.[3,4] Given that the only action that PCPA and ATD seem to have in common is a lowering of serotonin and that both can lower mood, the most likely mechanism for the mood lowering effect of both compounds

is lowered serotonin levels. Studies using PCPA and other outcome measures, such as food intake and cognition, would reveal whether PCPA and ATD have the same effect on those measures, thus strengthening the idea that ATD does alter other measures through lowered serotonin. However, because of the potential toxicity of PCPA, it is unlikely to be used in further studies investigating the effects of low serotonin levels in humans. However, various positive control treatments could be used to rule out some of the alternative explanations when investigating measures other than mood.

Klaassen and colleagues[60] used lysine depletion as a positive control when studying the effects of ATD on mood in healthy participants. Acute tryptophan depletion, but not a lysine-deficient amino acid mixture or a normal control amino acid mixture, caused a modest lowering of mood. While the depletion of lysine was somewhat less than that of tryptophan, this study suggested that an amino acid imbalance cannot explain the mood change.

Another positive control for ATD is acute phenylalanine/tyrosine depletion (APTD), which is used to study the effect of lowering catecholamine levels. When ATD has effects that are not seen with APTD or with a combination of tryptophan and phenylalanine depletion, this rules out any effect of an amino acid imbalance or decline in protein synthesis. A small number of studies fit this criterion. Leyton and colleagues[63] compared the effects of ATD and APTD with a control treatment on mood after a stress in healthy women. Both depletions lowered mood. However, ATD but not APTD had other significant effects, such as increasing hostility. Harrison and colleagues[64] demonstrated that ATD but not APTD impaired declarative memory in healthy women. Scholes and colleagues[65] found that ATD and APTD, but not the combined depletion, decreased Stroop interference in healthy men, indicating increased attentional control. Finally, Mann and colleagues[66] studied healthy men who showed disruption of pre-pulse inhibition after ATD, but not after APTD. These studies suggest that an amino acid imbalance does not explain the effects of ATD on a fairly wide range of measures.

Because amino acid imbalance affects food intake in rodents,[7] positive control treatments should be included in any ATD study looking at ingestive behaviours in humans. So far this has not been done.[45,67]

There is good, although not definitive, evidence that effects of ATD act through depletion of serotonin, not other mechanisms. However, this has to be assessed for each outcome measure used in ATD studies.

Theory and practice in depleting tryptophan: What is the best ATD mixture?

An important objective of most ATD studies is to lower tryptophan levels as much as possible. Evidence to date suggests that the decline in plasma tryptophan has to be around 60% or greater to see any effect on mood.[68] Presumably, with a smaller lowering of tryptophan levels any effect on serotonin function is below the threshold needed to alter mood. The large decline in tryptophan levels that is needed may be related to the regulation of serotonin release. A recent study

looking at release of serotonin and dopamine (DA) in the rat brain using voltammetry concluded "that [serotonin] transmission is mostly sensitive to uptake and metabolic degradation mechanisms," while "[DA] transmission is constrained by synthesis and repackaging."[69] The fact that DA transmission is constrained in part by synthesis explains why the APTD method works. Tyrosine hydroxylase is close to saturated with tyrosine under normal conditions[70] and the enzyme is subject to feedback inhibition by catecholamines,[71] unlike tryptophan hydroxylase, which is only half saturated with tryptophan[72] (i.e., the concentration of tryptophan in the human brain is about equal to the Km of the enzyme for tryptophan) and is not subject to feedback inhibition by serotonin. These facts, taken in isolation, suggest that APTD depletion would not work to the same extent as ATD. However, DA release is constrained in part by DA synthesis, unlike serotonin release, which is not constrained primarily by serotonin synthesis. Therefore, presumably, the decline in DA synthesis has to be less for APTD to alter DA function than the decline in serotonin synthesis has to be for ATD to alter serotonin function. This may explain why a large decline in tryptophan levels is needed to see effect of ATD on mood.[68]

In addition to lowering plasma tryptophan levels significantly, secondary objectives of a suitable amino acid mixture are to minimize the problems associated with ingestion of an unpalatable mixture of amino acids and the subsequent nausea. Tryptophan levels are depleted by incorporation of tryptophan into protein, so the ideal mixture should be optimized to stimulate protein synthesis. This is why the original mixture used by my colleagues and I[13] was based on the proportions of amino acids in human milk, which is presumably optimal for humans. Glutamate and aspartate were omitted from the mixture because of concerns about the toxicity of the free amino acids when ingested by humans.[73] Given that those 2 amino acids comprise more than 25% of the amino acids in milk, by weight,[74] omitting them had the additional advantage of reducing the amount that participants needed to ingest.

Omitting nonessential amino acids from the amino acid mixture will not alter its ability to lower tryptophan levels only if the nonessential amino acids are present in the body at levels that will not limit the rate of protein synthesis. This could occur if the pool of nonessential amino acids is sufficiently large, or if their rate of synthesis keeps up with their rate of incorporation into protein. This raises the question whether any nonessential amino acids are needed in the tryptophan amino acid mixture. This was tested by Wolfe and colleagues,[75] who used a 31.5 g tryptophan-free amino acid mixture. The amino acids were in similar proportions, but half the amounts, as in the original 100 g mixture that my colleagues and I used,[13] so there was a decrease of 68.5% in the total weight of amino acids from the original mixture and of 37% from a half-strength mixture. This treatment caused a 79% decrease in plasma tryptophan in healthy women. Unfortunately, the mixture omitted an essential amino acid, histidine. While there is some controversy on this point, histidine is generally considered an essential amino acid.[76] In

rodents, histidine influences histamine levels to a greater extent than tryptophan influences serotonin levels. As discussed, tryptophan hydroxylase is normally about half saturated with tryptophan levels, and administering tryptophan can increase serotonin 2-fold.[72] However, in rats, histidine administration can increase brain histamine levels as much as 7-fold.[77,78] This suggests that the normal histidine levels in the brain are well below the Km of histidine decarboxylase for histidine. If this is so, then a decline in histidine levels should cause a greater decline in histamine synthesis than the decline of serotonin after an equivalent lowering of tryptophan. In a human study, a histidine-deficient amino acid mixture lowered plasma histidine and changed responses in some laboratory measures of behaviour.[79] Therefore, a tryptophan- and histidine-deficient amino acid mixture might influence the function of both serotonin and histamine. The absence of histidine from the control mixture would mean that histamine might be low in both control participants and in those receiving the deficient mixture. As pointed out by Hayward and colleagues,[80] in these circumstances the tryptophan- and histidine-deficient mixture might be probing the interaction between serotonin depletion and histamine depletion. The same criticism, a lack of histidine, applies to the original mixture used by Moja and colleagues,[12,81] which was subsequently changed somewhat by Stadler and colleagues,[82] and used in several subsequent studies (e.g., the study by Dingerkus and colleagues[83]). A needed area of further research would be to study whether adding nonessential amino acids to a tryptophan-deficient amino acid mixture containing only essential amino acids would alter the lowering of plasma tryptophan levels and side effects.

Another tryptophan-deficient amino acid mixture that has been used is collagen-based.[84] Collagen is a protein that is naturally free of tryptophan. Compared with the formula based on human milk, it has much higher levels of glycine and much lower levels of methionine, and it includes hydroxyproline. It lowered human plasma tryptophan levels by 74%. As with the mixture lacking non-essential amino acids, a direct comparison is needed of the ability of this mixture to lower plasma tryptophan and minimize side effects relative to the other mixtures discussed here.

Badawy and colleagues[85] have criticized the ATD mixture formulation used by my colleagues and I[13] on the grounds that it may decrease the synthesis of catecholamines. They concluded this by looking at the ratio of the plasma level of phenylalanine plus tyrosine to the sum of the plasma level of the branched chain amino acids. With the ATD mixture, this ratio decreased by about 50%, suggesting that catecholamine synthesis was lowered. The rationale for this ratio is that all LNAAs are transported into the brain by the same transporter and compete with each other for uptake into the brain.[36] However, although phenylalanine is a substrate for tyrosine hydroxylase, it is not hydroxylated as efficiently as tyrosine, which is why untreated phenylketonuria is associated with large decreases, rather than increases, in catecholamine synthesis.[86] When using the more appropriate ratio of plasma tyrosine to the sum of all the other LNAAs, ATD causes the ratio to decrease slightly[63] or not at all.[87] Small

changes in the tyrosine ratio are not a concern. Human plasma amino acids vary throughout the day by as much as 50% from minimum to maximum, due to diurnal rhythms and, more important, to protein intake.[88] Ratios of tyrosine or tryptophan to other LNAAs also vary, although to a lesser extent than the individual amino acids. The important issue is that large changes are needed in tryptophan (or tyrosine) to cause changes in biogenic amine function. Therefore, changes in the availability to the brain of the biogenic amine precursors that are not much greater than their normal physiologic variation are probably not functionally significant.

Another important issue is the dose of mixture to use in ATD studies. My colleagues and I[89] found that 25 g, 50 g, 75 g and 100 g mixtures reduced human plasma tryptophan by 42%, 60%, 65% and 64%, respectively. Dougherty and colleagues[90] also found a more robust depletion with a 100 g mixture than a 50 g mixture. However, the 2 mixtures caused similar lowing of mood — the 100 g mixture caused greater attrition — even though ratings of somatic symptoms were similar in the 2 groups. The widespread use of higher doses is probably prompted by the normal concern of researchers to maximize the chances of seeing an effect. However, in some circumstances a lower dose may be better. For example, Hayward and colleagues,[80] using a 31 g ATD mixture, found changes in cognitive process with no change in mood in recovered depressed patients. This showed that the cognitive changes were not mediated by lowered mood. If a higher dose had been used, mood changes might have occurred, eliminating the possibility of concluding that mood changes did not mediate the cognitive changes.

Delgado and colleagues[14] introduced what they described as a low-tryptophan diet given the day before an ATD active or control test day. A more salient description would be a low-protein diet. Whether this additional intervention enhanced tryptophan depletion on a test day is not known, but it is a study that could usefully be done. However, there are 2 potential rationales for the use of the low-protein diet. The first is that it standardizes the diet the day before the test. The second is that if the low-protein diet causes negative nitrogen balance it may enhance protein synthesis and, therefore, the depletion of tryptophan when the tryptophan-deficient amino acid mixture is given. Until the degree of tryptophan depletion is tested with and without the low-protein diet the day before a test, some researchers will probably continue to use it for its possible benefits.

The variability in the decline in plasma tryptophan within ATD studies and between studies using the same mixture is due to unknown factors. While some studies use a mixture adjusted for body weight (e.g., the study by Dingerkus and colleagues[83]), some studies have adopted the strategy of Ellenbogen and colleagues[91] of giving both men and women a fixed dose, with the dose for women adjusted for their lower average body weight. However, as discussed, the mechanism of action of ATD mixtures is to induce protein synthesis; therefore, presumably the correct adjustment would not be for body weight but for the mass of tissues (presumably mainly liver and muscle) responsible for the increase in protein synthesis. Therefore, the potential advantage of adjusting

for body weight is not clear. Other factors may be more important in regulating protein synthesis. For example, in elderly people there is diminished muscle protein synthesis in response to ingestion of an amino acid load.[92]

Theory and practice in depleting tryptophan: What is the best control mixture?

The appropriate control tryptophan-containing mixture has, like the ATD mixture, been a matter of some controversy. Weltzin and colleagues[93] pointed out that the control mixture used by my colleagues and I[13] caused a decline in tryptophan availability, as indicated by the plasma ratio of tryptophan to the sum of the other LNAAs and that this could be corrected by increasing the amount of tryptophan in the mixture from 2.3 g to 4.6 g. However, this adjustment is probably not needed for several reasons. The decline in the plasma ratio of tryptophan to the sum of the other LNAAs is well below the threshold needed to see any effect on mood.[68] Any protein meal will lower the plasma tryptophan ratio, and yet a protein meal causes little or no decline in levels of the tryptophan or the serotonin metabolite 5-HIAA in human CSF.[94] In addition, the amino acid mixture based on the amino acid composition of a protein has some physiologic plausibility and, furthermore, will be a conservative control treatment. Therefore, there seems to be no good rationale for adding extra tryptophan to the mixture.

Given that the standard control treatment lowers the tryptophan ratio, a pertinent question is whether using a lower-strength depletion mixture would be a plausible control treatment. This question arose when tryptophan became unavailable in various countries after tryptophan from a single manufacturer that contained a toxic trace impurity caused a number of deaths and disabilities due to a disorder that came to be called eosinophilia myalgia syndrome.[95] Krahn and colleagues[55] compared the effect of 100 g and 25 g tryptophan-deficient amino acid mixtures. They confirmed the finding of my colleagues and I[89] that the 25 g mixture caused a much smaller decline in tryptophan availability and reported that the 8 participants who received both mixtures could not distinguish between them. However, the 2 treatments were given 4 weeks apart, so the participants' lack of ability to distinguish between them may have been due to incomplete memory of the sensations they experienced. The 25 g mixture will obviously provide only 25% of the caloric load of the 100 g mixture, so greater feelings of hunger would be expected 5–7 hours after the mixture ingestion, which is when measures are usually obtained. Furthermore, when humans ingest a meal, it enhances memory,[96] which raises the possibility that memory will be enhanced by the 100 g mixture relative to the 25 g mixture 5 hours later. For these reasons, the use of a 25 g control mixture is not recommended, and results obtained using this methodology should be accepted with caution.

Ethical aspects of ATD

Given that low serotonin levels are associated with suicide[1,2] and that ATD can lower mood and increase impulsivity,[25]

ethical considerations are of great importance in ATD studies. Miller and Rosenstein[97] discussed the ethics of symptom provocation studies, including ATD, in psychiatric patients. They emphasized that a symptom provocation study must have a favourable risk:benefit ratio in terms of the risk and the knowledge expected to result, and also the anticipated distress of the participants, in relation to the distress typical of the disorder being studied. They quote the experience of a recovered depressed patient from the ATD study of Delgado and colleagues:[14]

She began to cry inconsolably and described her emotions as being "out of control." She said that she did not know why she was crying but could not stop. She also described psychic anxiety, difficulty concentrating, loss of energy, loss of self-confidence, and a sense that nothing was worthwhile. She felt as if all the gains she had made over the past few weeks had "evaporated."

Delgado and colleagues[14] go on to mention that the next morning she felt "back to herself," and that, although she would not want to repeat the experience, it had been worthwhile because of what she had learned about her illness. Miller and Rosenstein[97] suggest that a potential indicator of the severity of distress experienced by patients in challenge studies is the need to treat the symptoms caused by the provocation. I am not aware of any ATD studies in which medication was needed to treat side effects. Booij and colleagues[98] administered a questionnaire among recovered depressed patients who completed ATD about the quality of the informed consent procedure and their experiences during the study. They concluded that "participants were quite satisfied with the informed consent procedure. They had understood that this was a fundamental research project and personal benefits were not expected. However, some participants still found it a positive experience."

Miller and Rosenstein[97] emphasized the importance of excluding patients with certain clinical vulnerabilities from symptom-provocation studies. This would exclude any patient with previous suicide attempts or significant suicidal ideation from ATD studies. They also mention the importance of follow-up of participants. To minimize risk, researchers carrying out ATD studies have an obligation to reverse the biochemical changes as quickly as possible at the end of a study. In most studies there is no mention that this is done by any means other than allowing participants to start eating normally at the end of the study. This is in spite of the fact that administering 1 g of tryptophan at the end of a study will cause plasma tryptophan to rise to a level about twice as high as the pre-ATD baseline levels within 1 hour.[99] This should normalize serotonin levels in all brain areas in less than an hour, given the known rates of synthesis of serotonin and its content in different areas of the human brain.[16] Even in ATD studies of healthy people there should be careful follow-up. They should be given tryptophan after ATD (and placebo after the control mixture to maintain the blinding). Given that ATD can increase aggression and impulsivity[25] as well as lower mood, participants should not be allowed to drive home and should preferably be contacted that evening

and the next day to ensure that there were no adverse effects.

While the number of ATD studies carried out to date with no important adverse effects is reassuring, this is probably owing to the short duration of the metabolic change. Longer depletions present important additional problems. In a double-blind study, Applebaum and colleagues[100] gave a 100 g ATD mixture every day for a week to 9 manic patients who were newly admitted to hospital. Eight patients received a placebo drink. Acute tryptophan depletion caused a greater decline in ratings of mania. As pointed out,[101] the possible implications for a long-term decline in protein synthesis due to low tryptophan levels are not known. Also, a diet with an amino acid imbalance causes deposition of fat in the rat liver.[7] If the risk:benefit ratio of a longer-term ATD study is considered favourable, such a study should be done with great caution and careful monitoring, which would include the diet and metabolism of the participants.

Recommendations for future research

The purpose of this section is to highlight some of the studies that need to be done to answer important questions concerning ATD studies and to make recommendations about the design of ATD studies. To help rule out (or possibly to help strengthen) explanations other than lowered serotonin function for the effects of ATD, probably the most important need is to measure kynurenic and quinolinic acids in human CSF after ATD. An alteration of these metabolites, thereby changing their action on nicotinic receptors and altering aspects of cognition is possibly the least implausible explanation for a nonserotonergic effect of ATD on any measure.

The main insight into the variable mood response after ATD is in the study of Yatham and colleagues.[28] In recovered depressed patients who showed no lowering of mood after ATD, there was an ATD-induced reduction of serotonin 2 receptors. This change did not occur in patients who showed a lowering of mood. If this finding is replicated, further studies should investigate whether lack of response to ATD in other situations is associated with a reduction of serotonin 2 receptors. Studies investigating what factors mediate the reduction in serotonin 2 receptors may be relevant to the regulation of mood in patients not undergoing ATD studies.

Another factor that has hardly been studied that may explain some of the variability in response to ATD is the interaction between different effects of ATD. For example, in studies on cognition, a lowering of mood may have effects on some aspects of cognition. A recent article points out that altered tryptophan alters human social interaction along the agonistic–affiliative axis. Given that more positive social interactions tend to improve mood and that quarrelsome social behaviour may lower mood, the exact nature of a participant's social interactions during an ATD study may influence the mood response.[102] The implications for these types of interactions should be studied, and the environment of the participants during an ATD study should be controlled as much as possible.

As discussed, another important research need is a comparison of the abilities of some of the different ATD mixtures to lower plasma tryptophan levels and cause side effects. A test

of whether a low-protein diet the day before ATD does in fact enhance the lowering of tryptophan levels is also needed. The results of such studies would allow a more rational choice of what procedure to use in ATD studies. If most of the research community settles on a specific protocol to deplete tryptophan, this would help in comparisons of different studies.

The issues raised in this review lead to a number of specific recommendations about the conduct of ATD studies.

- Side effects of ingesting the mixture should be measured soon after ingestion of the mixtures and at later time points. The relationship between the side effects and the main outcome measures should be studied.
- The success of blinding should be assessed by asking participants to guess at the end of the study whether (or when for crossover studies) they were given an ATD mixture.
- Social interactions of participants should be standardized as much as possible.
- Consideration should always be given to the inclusion of a positive control group.
- Studies should include measures of all potential ATD-induced changes that may alter the response of the main outcome measure.
- Participants who receive a tryptophan-deficient amino acid mixture should be given 1 g of tryptophan at the end of a study day to restore their serotonin levels as rapidly as possible. In crossover studies they can be given a placebo instead of tryptophan to maintain the blinding. Participants should not be allowed to leave until 1 hour after the tryptophan supplement is administered and their mood is within the normal range.

Conclusion

The best evidence that ATD can lower serotonin function comes from studies on the lowering of mood. As discussed in this review, a lowering of mood can occur after the administration of PCPA, an inhibitor of tryptophan hydroxylase. Also, in circumstances when ATD causes a lowering of mood, it does not occur when a positive control, acute lysine depletion, is used in the same participants. These 2 findings rule out, with a high degree of probability, explanations other than a decline of serotonin function for the lowering of mood. Alternative explanations have not been tested in all circumstances in which a lowering of mood occurs. However, the idea that ATD may lower mood by decreasing serotonin in some circumstances and via another mechanism in other circumstances is unlikely. The statement that lowered serotonin function can lower mood in some circumstances, while not 100% proven, is probably one of the better-established causal mechanisms in biological psychiatry.

In many circumstances, ATD does not lower mood. The mechanisms responsible for the differential effect in different people are currently unknown. One possibility is that a lack of any change in mood is due to adaptive responses in the serotonergic system, and, as discussed, alterations in serotonin 2 receptors are a possible candidate for such an adaptive response. However, in those who do not show a lowering of mood after ATD, other neuronal systems may act

homeostatically to prevent alterations in serotonin function from lowering mood. Possibly both mechanisms may act together. The bottom line is that while a decline in mood does seem to be associated with lowered serotonin function, a lack of change in mood cannot be taken to imply necessarily that there is not a change in serotonin function. This applies to other aspects of brain function too. A lack of change in a variable after ATD is certainly not proof that serotonin is not involved in regulating that variable.

While the evidence that ATD lowers mood through a decline in serotonin function is strong, that does not imply that a decline in serotonin function is responsible for all the effects of ATD. Each outcome has to be assessed separately. An increase in aggressive responding induced by ATD[25] is likely to be related to lowered serotonin function, given the large body of animal research, which includes rodents and monkeys, showing that lowering serotonin levels can increase aggression[103] and the human data showing an association between low serotonin-related measures and aggression.[104] This conclusion illustrates how the relevant data has to be assessed carefully for each outcome. While animal models do not add much weight to the conclusion about the role of low serotonin in the regulation of mood, given that animal models cannot faithfully model mood in humans, aggression in animals and humans is similar enough to use animal data in arriving at a conclusion. While no positive control treatments have been used in ATD aggression studies, the other evidence is strong enough to suggest that other mechanisms related to ATD are unlikely to be involved. Nonetheless, studies using a positive control treatment in ATD aggression studies would strengthen the conclusion that low serotonin function can promote aggression.

The action of ATD that is most likely to be related to a nonserotonergic mechanism is that on aspects of cognitive function, which, as discussed, could be related to altered action of kynurenic acid on nicotinic receptors. However, if ATD is found not to lower human CSF kynurenic acid, a possible role of kynurenic acid in the effect of ATD on cognition would be unlikely.

The examples given above illustrate how results from ATD studies on aspects of brain function must be interpreted in conjunction with data from other types of studies measuring biochemical variables or from related studies not employing ATD. However, for each variable the nature of those other studies may be different. Different areas of the brain are involved in different functions of serotonin. In different areas of the brain, projections of serotonin neurons may have different presynaptic receptors and be regulated differently. Care needs to be taken to assess what other studies need to be done or taken into account when looking at the effects of ATD on different functions.

Some of the criticisms that have been raised against the ATD method are not valid, and some have not been posed in the right way. For example, one of the important issues is not whether ATD does or does not lower serotonin function, but whether it does so in particular circumstances in humans. This review also discusses some potential criticisms that have not been dealt with adequately or that have not been raised

at all. In spite of the all the complexities in interpreting the results of a technique that is very simple in concept, ATD studies have provided, and should continue to provide, useful information on the implications of low human brain serotonin function for different aspects of brain function.

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Correction

Are addictions diseases or choices?

There was an error in the affiliations listed for Marco Leyton in the July 2013 editorial (*J Psychiatry Neurosci* 2013;38(4):219-21). Dr. Leyton is from the Department of Psychiatry, McGill University, Montréal, and the Center for Studies in Behavioral Neurology, Department of Psychology, Concordia University, Montréal, Que., Canada.

We apologize for this error.