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Allopregnanolone treatment improves plasma metabolomic profile associated with GABA metabolism in Fragile X-associated Tremor/Ataxia Syndrome: a pilot study

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Abstract

Currently there is no effective treatment for the Fragile X-associated Tremor/Ataxia Syndrome (FXTAS), a late-onset neurodegenerative disorder. In this pilot study, we evaluated whether allopregnanolone, a natural neurosteroid that exerts beneficial effects in neurodegenerative diseases, nervous system injury and peripheral neuropathies, could improve lymphocytic bioenergetics and plasma pharmacometabolomics in six males with FXTAS (68 ± 3 y old; *FMR1* CGG repeats 94 ± 4 ; FXTAS stages ranging from 3 to 5) enrolled in a 12-week open-label intervention study conducted at the University of California Davis from December 2015 through July 2016. Plasma pharmacometabolomics and lymphocytic mitochondria function were assessed at baseline (on the day of the first infusion) and at follow-up (at the end of the infusion within 48

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Author contribution

EN assessed most of the mitochondrial outcomes, helped drafting, edited and approved the final version of the manuscript; AS carried out cognitive and psychological testing on the patients, revised the manuscript and approved the final manuscript; JW acquired neuroimaging data, performed statistical analysis, and interpreted the results, reviewed and approved the final version of the manuscript; AT and NRC analyzed neuropsychiatric tests, reviewed and approved the final version of the final version of the manuscript; FT provided lymphocytes, performed the genotyping, revised and approved the manuscript; MR provided expertise in intravenous injection of allopregnanolone, reviewed the manuscript and approved its final version; RH carried out clinical assessment of these subjects, wrote clinical findings, revised and approved the final version of the manuscript; CG conceptualized the study, designed the experiments, analyzed the metabolomics data, and wrote the manuscript.

Conflict of interest

RH has received funding from Novartis, Roche/Genentech, Alcobra, and Neuren for treatment trials in fragile X syndrome, autism and Down syndrome. She has also consulted with Novartis, Fulcrum, Zynerva and Roche/Genentech regarding treatment for fragile X syndrome. The other authors have no conflicts of financial interest relevant to this article to disclose.

h). In parallel, quantitative measurements of tremor and ataxia, and neuropsychological evaluations of mental state, executive function, learning, memory and psychological symptoms were evaluated at the same time points. Allopregnanolone treatment impacted significantly GABA metabolism, oxidative stress and some of the mitochondria-related outcomes. Notably, the magnitude of the individual metabolic response, as well as the correlation with some of the behavioral tests, was overwhelmingly carrier-specific.

Based on this pilot study, allopregnanolone treatment has the potential for improving cognitive and GABA metabolism in FXTAS aligned with the concept of precision medicine.

Keywords

Allopregnanolone; bioenergetics; FXTAS; GABA; lymphocytes; pharmacometabolomics

Introduction

Allopregnanolone (5 α -pregnane-3 α -ol-20-one) is a neurosteroid synthesized from progesterone through sequential reduction by the enzymes 5 α -reductase and 3 α -hydroxysteroid oxidoreductase [1]. “Neurosteroids” are brain metabolites of circulating steroid hormones—including progesterone, deoxycorticosterone and testosterone—but also products of *de novo* synthesis from cholesterol [2]. Localization of enzymes involved in their synthesis has been reported in glutamatergic and GABAergic neurons and glial cells of cortex, hippocampus and amygdala [3,4]. When produced endogenously, allopregnanolone’s mode of action seems to preclude the interaction with classical steroid hormone receptors, unless it is converted enzymatically to classical steroids [5]. Allopregnanolone binds to GABA_A receptors and acts as a positive allosteric modulator of GABAergic neurotransmission [6]. When administered exogenously, allopregnanolone exerts anxiolytic, sedative and anticonvulsant effects, beneficial actions related to enhanced GABA_A receptor-mediated inhibitory network activity [7,8]. Allopregnanolone has also been shown to interact with the pregnane X receptor, a ligand-activated transcription factor [9], and with G-protein-coupled membrane progesterone receptor in breast cancer cells *in vitro* [10].

At the molecular level, allopregnanolone has been implicated in neural progenitor cell proliferation and neurogenesis, both *in vitro* [11–13] and *in vivo* (embryonic and adult brains [14–16]). It stimulates myelination, reduces injury-induced inflammation and exerts protective and trophic effects on neurons during normal development and under pathological conditions [17,18]. Interestingly, allopregnanolone was able to reverse hippocampal-dependent learning and memory deficits [16] and reduce amyloid deposition and prevent neuronal loss in mouse models of Alzheimer’s disease [15,19]. It has been proposed that allopregnanolone may confer neuroprotection by decreasing apoptosis through the reduction of caspase-3 protein expression [20] and inhibiting the mitochondrial permeability transition pore [21].

Altered GABAergic metabolism has been observed in carriers of *FMR1* premutation alleles (55 to 200 CGG repeats) [22–24] as well as in murine models of the disease [25]. Some of these carriers develop FXTAS, a neurodegenerative disorder characterized by progressive

kinetic tremor, gait ataxia, executive function and memory deficits, peripheral neuropathy, Parkinsonism, cognitive decline into dementia, as well as psychiatric problems including depression and dysautonomia [26,27]. In the context of this study, *in vitro* abnormal bursting activity of hippocampal neurons from a murine model of the premutation was hampered by the neurosteroid allopregnanolone, providing a mechanism for neuroprotection [28].

Considering that currently only palliative therapies [29] are available for FXTAS, but no specific and effective treatments that will either halt or slow neurodegeneration, and based on the above reports, here we tested the hypothesis that allopregnanolone improves GABA metabolism, oxidative stress and bioenergetics, thereby delaying or counteracting some of the psychiatric deficits of FXTAS.

To this end, we utilized pharmacometabolomics [30,31] to capture the circulating metabolic signature of allopregnanolone. Furthermore, we characterized the bioenergetics of circulating lymphocytes and relate the putative improvements to cognitive and behavioral outcomes previously reported by us as a result of the open-label allopregnanolone treatment [32].

Materials and Methods

Study Design and Patients

A 12-week open-label nonrandomized preliminary trial of allopregnanolone treatment was conducted at the University of California Davis MIND Institute's Fragile X Research and Treatment Center. Six males with FXTAS received weekly infusions of allopregnanolone. Recruitment occurred from December 2015 through March 2016 with the last participant completing treatment in July 2016. Notification of the study was provided on [ClinicalTrials.gov](https://clinicaltrials.gov) (Identifier: NCT02603926). Written informed consent was obtained from all the participants. Inclusion criteria encompassed men ages 50–85 with premutation carrier status and a diagnosis of FXTAS (Table 1). Exclusion criteria involved other serious systemic disease, alcohol or other drug abuse, and current use of phenytoin. More details can be found in our previous study [32].

Intervention

The weekly infusions and follow-up observations were performed at the UC Davis Clinical and Translational Science Center, Clinical Research Infusion Center. Allopregnanolone (2 mg) was infused at a concentration of 0.5 mg/ml in 6% sulfobutylether- β -cyclodextrin with 0.9% NaCl (dispensed by the UC Davis Investigational Drug Pharmacy) over 30 min followed by a flush of 30 min; the 2nd week of treatment the infusion increased to 4 mg, and in the 3rd week to 6 mg. This latter dose was well tolerated by all carriers and continued for the rest of the 12 weeks. Other infusion protocol details were described before [32].

Assessments and Follow-Up

Clinical Assessment—Baseline clinical examination and neuropsychological assessments were performed 48 h before the first infusion and follow-up assessment was carried out within 48 h after the last infusion. A detailed physical and neurological

examination was performed and videotaped as previously described [33], emphasizing the major features of FXTAS: cerebellar ataxia, intention tremor and neuropathy findings such as vibration sense and pinprick sensation.

Neuropsychological and Emotional Assessment—These assessments were described in detail before [32] and summarized as following: Learning and memory were evaluated using the California Verbal Learning Test 2 (CVLT2). Working memory was assessed using Wechsler Memory Scale-IV (WMS-IV). Other cognitive outcomes included Mini Mental Status Exam (MMSE), Behavior Dyscontrol Scale (BDS-2), Cambridge Neuropsychological Test Automated Battery (CANTAB) and Controlled Oral Word Association Test (COWAT). For the assessment of neuropsychological symptoms the Symptom Checklist-90-Revised (SCL-90-R) and Beck Anxiety Inventory (BAI) were used. CATSYS provided information on the degree of tremor and sway by measuring improvements in hand tremor and balance.

Genotyping

Genomic DNA was isolated from 3 ml of peripheral blood using Gentra Puregene Blood Kit (Qiagen) following standard procedure. CGG repeat sizing was performed by PCR and Southern blot analysis as previously described [34].

Lymphocyte collection

Blood samples were obtained at baseline (on the day of the first infusion but before starting the infusion) and at follow-up (within 48 h after the last allopregnanolone infusion) in the morning and typically before lunch. Lymphocytes were collected in BD Vacutainer CPT Tube with sodium heparin and subsequently re-suspended in freezing medium (RPMI-1640, 10% fetal calf serum, 10% dimethyl sulfoxide) and stored at -80°C, as previously described [35]. For analysis of mitochondrial outcomes, thawed lymphocytes suspensions were centrifuged at 200 g for 5 min at 4°C, resuspended in 20 mM HEPES (pH 7.4) with the supplementation of protease and phosphatase inhibitors (Sigma), hand-homogenized (30 strokes on ice), followed by two freezethawing cycles. Lymphocytes collected from control individuals were used to obtain reference values (see below). Protein concentration was determined with a Pierce BCA protein assay kit (Life Technologies) according to the manufacturer's instructions.

Mitochondrial outcomes

Evaluation of mitochondrial Complexes was carried out spectrophotometrically in a Tecan Infinite M200 microplate reader equipped with the Magellan software as described previously in detail [36,37]. The activity of NADH-quinone oxidoreductase (NQR, Complex I) was evaluated at 340 nm following the oxidation of NADH [36]. Absorbance changes were followed for 10 min at 37°C. Rotenone-sensitive activities were calculated from the linear part of A versus time and using an extinction coefficient of $6.22 \text{ (mM} \times \text{cm)}^{-1}$. Succinate-cytochrome *c* reductase (SCCR), which evaluates Complex II-III activity, was measured using 1–6 µg protein/well at 30°C following the reduction of oxidized cytochrome *c* at 550 nm, as described before [37]. The rate sensitive to the inhibition by 2-thenyltrifluoroacetone (1 mM) was taken as Complex II-III-specific activity. The activity of

Complex IV was measured as previously described [36,37] following the oxidation of reduced cytochrome *c* at 550 nm and 37°C in the presence of 5–20 µg of protein, with or without 240 µM (freshly prepared) KCN. Activity was calculated from the linear part of LN absorbance versus time plots and using an extinction coefficient of 21 (mM × cm)⁻¹. Complex V was evaluated by following ATPase activity [37] in 1–6 µg protein/well. Rates sensitive to the ATPase-specific inhibitor oligomycin (4 mM) were taken as Complex V specific activity. The activity of citrate synthase (CS) was evaluated at 30°C and 412 nm following the reduction of 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of 2–8 µg of cell lysate [36]. The reaction was started by adding 0.5 mM oxalacetic acid. Rates were calculated from the linear part of A/min and expressed by mg protein, using an extinction coefficient of 11,400 (M × cm)⁻¹. All activities were expressed as nmol × (min × mg protein)⁻¹. Reference values for each individual activities were obtained from lymphocytes isolated from control individuals (*n* = 13 for Complex I; *n* = 11 for Complex II-III; *n* = 6 for Complex IV; *n* = 9 for Complex V; *n* = 8 for citrate synthase).

Pharmacometabolomics

Label-free and untargeted metabolomics of plasma was performed at the UC Davis West Coast Metabolomics Center. Pharmacometabolite levels were expressed as the relative ratio of spectral counts for each individual metabolite normalized by the total metabolite counts (excluding unknowns) per subject. After data quality control and normalization of data, we tested for significant differences in the levels of each compound between pre- and post-allopregnanolone groups using two-tailed Wilcoxon matched-pairs signed rank test. More detailed information can be found in our previous reports [24,23]. Subsequent pathway analysis was carried out using the MetaboAnalyst package 2.0 [38]. Reference values were obtained with plasma obtained from non-carrier subjects (*n* = 16; [24]).

Statistical Analysis

All statistical analyses were conducted with the GraphPad Prism software (version 6.1). Due to the small sample size, the false discovery rate was set at 10% and, as such, the statistical significance was set at adjusted *p* values > 0.100. For each outcome, the 95% CI was calculated with values obtained from control individuals. Based on controls reference values, pre- and post-treatment outcomes were counted and grouped in the following 4 categories: (i) outcomes outside of the reference values at baseline and within reference values at follow-up; (ii) outside of the reference values both at baseline and follow-up; (iii) within reference values at baseline and outside at follow-up; (iv) within reference values at both baseline and follow up. These categories were then used to perform the McNemar's test with the continuity correction. As predictive indicator of the association between allopregnanolone treatment and changes in metabolic outcomes the odds ratios (OR), and associated *p* value, for paired proportions was calculated for each carrier. In our study, the OR represents the odds that changes in molecular outcomes occur as a result of allopregnanolone exposure, compared to the odds of the same changes occurring in the absence of exposure. OR >1 indicates higher odds of association between molecular outcomes variations and allopregnanolone treatment; OR < 1 indicates lower odds of association; OR = 1 indicates lack of association [39].

Results

Six carriers of the *FMRI* premutation, all affected with FXTAS, were recruited for this pilot study. Their average age was 68 ± 3 y (mean $\pm$ SEM) and the mean *FMRI* CGG repeats in blood were 94 ± 4 with FXTAS stages ranging from 3 to 5 (Table 1). Plasma and lymphocyte samples were obtained from FXTAS-affected carriers to evaluate pharmacometabolomics and bioenergetics, respectively, on the day of the first allopregnanolone infusion and 48–72 h after the last. The choice of using untargeted pharmacometabolomics vs. selecting a number of peripheral parameters was based on the suitability of capturing the broad response to a pharmacological intervention with a pleiotropic drug such as allopregnanolone. Behavioral and psychiatric tests, reported in detail in our previous study [32], were performed two days before the first infusion and 2–3 days after the last.

Pharmacometabolomics of plasma samples from 6 FXTAS-affected carriers pre- and post-allopregnanolone treatment resulted in the detection of 322 metabolites, from which 9 were significantly different pre- and post-treatment based on the two-tailed Wilcoxon matched-pairs signed rank test; Table 2). Pathway analysis revealed that these metabolites were part of the following pathways: neurotransmission (GABA, serotonin), oxidative stress, Parkinsonism or involuntary movements, and mitochondrial function.

Pharmacometabolomics of allopregnanolone treatment showed improved GABA metabolism

Plasma from patients with FXTAS treated with allopregnanolone showed lower levels of both 3,4-dihydroxybutyric and 4,5-dihydroxyhexanoic acids ($p < 0.031$; Table 2) with higher values of Glu/Gln (1.34-fold) and a trend toward higher (1.4-fold) contents of succinate. Taken together, these results suggested an improvement in the GABA metabolism (Fig. 1). This is illustrated by the deficiency in succinic semialdehyde dehydrogenase (SSADH) which results in a shift of the GABA transamination product succinic semialdehyde (SSA) from succinate towards the formation of γ -hydroxybutyric acid (GHB; [40]), 3,4-dihydroxybutyric acid (β oxidation product of GHB; [41]) and 4,5-dihydroxyhexanoic acid (likely product of the reaction of SSA with acetylCoA [42]; Fig. 1).

Remarkably, GHB is also a neuropharmacologically active compound and may be a primary neurotoxic agent [43] acting mainly as an inhibitor of presynaptic dopamine release [44]. In this respect, while no homovanillic or 5-hydroxy-indolacetic acids (products of dopamine and serotonin metabolism, respectively) were detected by our mass spectrometry analyses, higher levels of tryptophan (precursor of the brain neurotransmitter serotonin; $p < 0.094$; Table 2), accompanied by lower concentrations of indolacetic acid (IAA; potentially toxic uremic tryptophan metabolite produced by the gut-microflora and associated with ROS formation [45]; $p < 0.063$) suggested a decreased serotonin turnover as a result of allopregnanolone treatment.

Consistent with these molecular outcomes associated with GABA metabolism and the role of GABA and GABA receptors in cognition, memory formation and maintenance, learning, stress, depression and anxiety [46,47], improvements in mental state (MMSE), cognition and

executive function (BDS-2, COWAT, CANTAB One Touch Stockings, OTS, and CANTAB paired association test, PAL), working and episodic memory (WMS-IV and CANTAB Paired Associates Learning, PAL) and anxiety (SCL-90-R) were reported for the majority of the carriers [32].

Allopregnanolone impacts oxidative stress, anxiety and depression

Overall, allopregnanolone was effective at lowering the levels of the three markers of oxidative stress, i.e. IAA (see above), methionine sulfoxide (i.e. oxidation product of methionine) and 2-hydroxybutyric acid (Table 2). The latter is as an early marker for both insulin resistance and impaired glucose regulation resulting from increased lipid peroxidation and oxidative stress [48] and the production of its precursor 2-oxobutyrate is enhanced under conditions of increased oxidative stress, excess glutathione demand and disrupted mitochondrial energy metabolism [49]. No other metabolite related to oxidative stress damage or response (i.e., cysteine disulfide over cysteine, alpha-tocopherol, vitamin C, taurine) was different between treatments.

Increased inflammation and oxidative stress [50,51] have been reported in samples from carriers of the *FMR1* premutation with and without FXTAS [24,52,36] in association with psychiatric and neurological disorders [53]. Considering that after allopregnanolone treatment, lower levels of markers of oxidative stress were recorded and that levels of IAA have been reported to be correlated with anxiety and depression [45], it is possible that a decreased oxidative stress status is linked to a lower incidence of psychiatric problems. Consistent with this premise, carriers showed in average a reduction in overall psychological distress indicated by the Global Severity Index from SCL-90R, specifically depression, somatization and positive symptoms, but not anxiety *per se* [32].

Tremor, Parkinsonism, dyskinesia and N-acetylornithine after allopregnanolone treatment

After receiving allopregnanolone treatment, the levels of NAO were significantly higher than those at baseline ($p = 0.031$; Table 2). NAO is a minor component of mammalian blood after proteins were removed [54] and it is an intermediate in arginine metabolism. While there is no characterization of the role of this compound in the CNS, only one study has linked (not causally) decreased NAO to the use of the antipsychotic agent haloperidol [55]. Among the side effects of haloperidol, the risk for extrapyramidal symptoms (EPS) is perhaps the most salient [56]. EPS are a complex and potentially heterogeneous set of symptoms, characterized by involuntary movement and loss of motor control. These include parkinsonism, akathisia and tardive dyskinesia, which can sometimes persist long after drug treatment has ended [57]. Then, it is possible that the higher levels of NAO observed after allopregnanolone treatment are linked to decreased Parkinsonism or involuntary movements since haloperidol potentiates its occurrence. Contrary to this expectation, allopregnanolone treatment showed no significant improvements in tremor or ataxia as judged by the CATSYS analyses, except for one carrier after this trial [32].

Allopregnanolone treatment and bioenergetics

Plasma glyceric levels were lower after allopregnanolone treatment in carriers (by 20%; $p = 0.031$; Table 2). This change may result from lower level of triacylglyceride mobilization,

probably linked to an allopregnanolone-mediated improved management of the energy supply and demand. In agreement with this hypothesis, subjects affected with schizophrenia show high levels of glycerate and other compounds (eicosenoic acid, β -hydroxybutyrate, pyruvate and cystine), all linked to insufficient energy supply in the brain [58]. When comparing pre- and post-allopregnanolone levels of markers usually employed for energy management assessment (i.e., lactate-to-pyruvate ratios, 3-hydroxybutyrate, 2-ketoisocaproic acid and 2-hydroxyvaleric are markers of mitochondrial dysfunction) no significant differences were observed (Table 2).

We reasoned that if allopregnanolone treatment improved bioenergetics, either mitochondrial complex activities or their ratios would show differences to increase ATP output or accommodate the type of fuel oxidized. None of the five mitochondrial Complex or citrate synthase activities (as a marker for mitochondrial matrix), tested in lymphocytes from the carriers pre- and post-allopregnanolone treatment, reached statistical significance (Table 2). Taken together these findings show that allopregnanolone had a minor effect on bioenergetics, not comparable to that reached by the effects on GABA metabolism. Based on the heavy reliance of mitochondrial ATP for brain and the relatively milder effect of allopregnanolone on bioenergetics, we expected some improvements in tests related to cognition after allopregnanolone treatment (BDS-2, MMSE and CANTAB one-touch stockings mean latency and problem solved on first choice and paired association test, except spatial working memory). Indeed, only two showed statistically significant improvements (BDS-2 and CANTAB PAL Total Errors Adj. [32]) with a broad range of responses among carriers.

Carrier-specific responses to allopregnanolone treatment

Based on our previous study on the carrier-specificity in the response of fibroblasts from premutation carriers to antioxidants [59], we wanted to test whether the levels of statistical significance obtained for the molecular outcomes could be driven by one or more carriers. To account for inter-carrier variability, we computed the odds ratio (OR) for each individual as a predictive tool for the occurrence of changes in molecular outcomes in response to allopregnanolone treatment (Table 3). Overall, combining all responses from all carriers, the OR was 2.42 ($p = 0.06$) indicative of an association between allopregnanolone treatment and the occurrence of molecular outcome changes in this cohort of carriers (Table 3). Of the two carriers with the highest OR (most favorable post-treatment outcome), one (JN) showed statistical significance at $p = 0.062$, followed by JH ($p = 0.109$). In terms of functional outcomes, both carriers showed some degree of improvements in both CANTAB PAL Total Errors and BDS-2, with JN having the highest BDS-2 score among the 6 carriers. Conversely, the two carriers with the lowest OR (JP and GS, OR = 1 and 1.7 respectively) were also the ones with the lowest number of errors in the CANTAB PAL Total Errors Adj. test. While GS had the longest CGG repeats, JP was the oldest carrier and the only one whose MRI scan showed confluent FLAIR hyperintensities in both cerebral and cerebellar white matter with values higher than the rest of the carriers before and after allopregnanolone treatment [32]. Of note, the apparent discrepancies between molecular and behavioral/psychiatric outcomes could be bridged by considering a placebo-dependent behavioral response, of which the molecular outcomes are voided.

A linear regression model was applied to clarify the contribution of the three variables CGG, age and FXTAS stage to the OR values. The best model was represented by the following equation: $OR = 42.5 - 0.69 * CGG + 10.98 * FXTAS \text{ stage} - 0.072 * Age * FXTAS \text{ stage}$ (Supplementary Information; p -value of the F statistics computed by ANOVA < 0.001). Based on the Type III sum of squares and the r^2 value of 1.0, the variables CGG repeats, FXTAS stage and age*FXTAS stage contributed significantly to 100% of the variability of the OR, with FXTAS stage being the most influential. Based on this model, the individual combination of these variables explains the carrier-specific response to allopregnanolone treatment.

Discussion

In this study, we tested whether allopregnanolone elicited therapeutic effects for carriers of the *FMR1* premutation affected with FXTAS by adding and extending novel and important pharmacometabolomic and bioenergetics data to our recently published study [32]. This pilot report is highly relevant, for there is no treatment for this disease except symptomatic treatment, and the benefit of potentially extrapolating these results to other neurodegenerative diseases and/or triplet-nucleotide repeat diseases is currently being assessed [60,61].

Plasma pharmacometabolomic profile from allopregnanolone-treated carriers with FXTAS showed differences pointing mainly at improvements in GABA metabolism. The allopregnanolone-mediated molecular improvements were carrier-specific (Table 3), aligned with the concept of precision medicine as we previously highlighted in the context of antioxidants as a possible therapy for this genetic disease [36].

The improvements in GABA metabolism are aligned with findings in murine models of the premutation such as reduced expression of vesicular GABA and Glu transporters in hippocampal neurons [28] and several subunits of the GABA_A receptor [25], and in human carriers with the detection of ubiquitin-positive intranuclear inclusions in neurons, including GABAergic ones, dysregulation of neurotransmitter systems (including mGluR1/5 and GABA_A) [62] and the altered plasma Glu and GABA levels [24]. Our previous work on allopregnanolone treatment carried out on the same individuals showed a relatively higher incidence of carriers displaying improvements in cognition, working memory, and other psychiatric problems (depression, somatization, and positive symptoms) [32]. Notably, four of the carriers after allopregnanolone treatment exhibited improvements in memory skills as evidenced by the auditory N400 component of event-related potentials [32]. In this regard, a connection between memory (e.g., for location of objects, short or long term memories) and GABA neurotransmission has been shown in several murine models [63,64]; moreover, it has been proposed that pharmacological amelioration of deficits in GABA neurotransmission in schizophrenia may be particularly effective in normalizing the neural network activity required for working memory function [65]. The beneficial effects exerted by allopregnanolone seem in line with a GABA_A receptor-dependent signaling [11–13] (and not through allopregnanolone-mediated up-regulation of cholesterol homeostasis [19,9]), particularly at restoring GABA levels and/or eliminating GHB in excess, resembling the phenotype of SSADH deficiency. Indeed, some of the clinical features of SSADH deficiency

overlap with those observed in the carriers of the premutation (e.g., ataxia, seizures, sleep disturbances [66,67]) and allopregnanolone improved GABA metabolism, sleep deprivation-induced anxiety-related stress and oxidative damage in a murine model of SSADH deficiency [68,69]. While the functional significance of the effect of allopregnanolone in FXTAS is not fully elucidated, the question that arises is why carriers respond to this treatment as if they were SSADH deficient. Considering that human mitochondrial SSADH is regulated via a redox-switch modulation [70], and that mitochondrial ROS and markers of oxidative stress-mediated damage are higher in biological samples from carriers with and without FXTAS (this study and [24,52,36]) as well as in a number of neurodegenerative disorders including Alzheimer's disease [71,72], it is possible that carriers exhibit a ROS-mediated SSADH loss-of-function ensuing in a SSADH biochemical phenocopy. Alternatively, the mitochondrial targeting of SSADH may be impaired due to a compromised mitochondrial import/processing of nuclear-encoded mitochondrial proteins, as we reported previously for fibroblasts from carriers of the premutation [73]. Thus, increased oxidative stress, exacerbated by SSADH deficiency, may elicit altered GABA neurotransmission and accumulation of ROS-mediated damaged biomolecules in brain, possibly contributing to the onset and/or progression of FXTAS and ameliorated by allopregnanolone via modulation of GABA metabolism.

Although further studies are needed to replicate and extend these findings (see **Limitations of this Study** Section), the analyses on this relatively small cohort provides the basis to identify predictive biomarkers for pharmacological interventions in carriers. Moreover, the findings of disease-type-derived alterations in metabolic profiles may provide useful clues for etiological investigations. The fact that metabolic and psychological tests revealed changes pre- and post-treatment grants the need for additional studies. This is especially relevant in light of recent studies showing that structural analogs of allopregnanolone showed higher anti-apoptotic and proliferation-promoting activities *in vitro* than the natural neurosteroid and that stereoisomers of the analogs have distinct profiles of activity, suggesting the possibility of exploiting their neuroprotective properties with or without simultaneously stimulating neurogenesis [14].

Limitations of this study

This open label study was conducted without allopregnanolone-treated non-carriers of the premutation, included only six premutation male carriers with FXTAS, participants had varying ages and FXTAS stages. Moreover, the majority of subjects were obtained from a single clinic and no placebo effect was studied. It is possible that the power of the study and/or the relatively short intervention period were insufficient to detect all of the allopregnanolone-associated long-term behavioral and metabolite changes. However, it is unlikely that the changes reported here and in our previous study are more dependent on the progression of the disease over the 12-week treatment than the pharmacological intervention *per se* because the progression of this and any other neurodegenerative disease would not be significantly noticeable in this short period. Although it could be argued that changes in plasma or lymphocytes do not necessarily correspond to those in CNS, several studies have shown a correlation between blood levels of specific metabolites and those in CSF in different pathophysiological states [74] including Alzheimer's [75]. However, the degree to

which non-CNS metabolomes (e.g., plasma, serum, saliva, and urine) reflect CNS neurobiology remains uncertain due to the limitation of metabolite passage through the blood-brain barrier [76]. While the influence of prescription drugs on any of the outcomes evaluated in this study cannot be excluded, levels of naproxen, salicylate and acetaminophen were not different across carriers either at pre- or post-allopregnanolone treatment and all carriers were receiving their regular medications during the allopregnanolone study. Our experience with longitudinal studies in humans shows that repeat measures provide reliable metabolic patterns within individuals so that common patterns of metabolic change can be identified even with smaller groups, e.g., 8–10 subjects. Finally, omics could be affected by several factors, such as eating habits or diurnal variation, which are unknown or not addressed in the current design. However, taking the blood samples in the morning and with non-fasting carriers attempted to minimize them.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Allopregnanolone-mediated modulation of the GABA pathway in FXTAS-affected carriers

Diagram of GABA metabolism in humans. In this study, lower levels of both 3,4-dihydroxybutyric and 4,5-dihydroxyhexanoic acids associated with higher contents of succinate and increased levels of glutamate were observed in FXTAS-affected carriers upon allopregnanolone treatment (red arrows), suggesting an improvement in the γ -aminobutyric acid (GABA) metabolism, via activation of the succinic semialdehyde dehydrogenase (SSADH)-mediated pathway. In agreement with this, impairments of SSADH activity results in a shift of the GABA transamination product succinic semialdehyde (SSA) from succinate towards the formation of γ -hydroxybutyric acid (GHB) as well as of 3,4-dihydroxybutyric acid (β -oxidation product of GHB) and 4,5-dihydroxyhexanoic acid (likely product of the reaction of SSA with acetylCoA).

Table 1.

Demographic information on the cohort of subjects that underwent allopregnanolone treatment

Carrier number (name initials)	Age (y) at study	CGG repeats in lymphocytes	FXTAS Stage
1 (SL)	68	85	3
2 (JN)	68	88	4
3 (JH)	57	104	5
4 (JP)	79	83	3
5 (SG)	64	105	4
6 (GS)	74	98	5

Table 2.

Metabolomics and mitochondrial outcomes in carriers in response to allopregnanolone treatment

Outcome	Pre-infusion (median [range])	Post-infusion (median [range])	Paired Wilcoxon two-tailed test (p-value)
<i>Pharmacometabolites</i>			
GABA Pathway			
Glu/Gln	7.7 [4.3–17]	10.3 [5.3–19.8]	0.063
Succinic acid	0.15 [0.11–0.19]	0.21 [0.15–0.23]	0.125
4,5-dihydroxyhexanoic acid	0.12 [0.08–0.15]	0.08 [0.07–0.09]	0.031
3,4-dihydroxybutanoic acid	0.22 [0.11–0.28]	0.17 [0.10–0.20]	0.031
<i>Serotonin pathway</i>			
Tryptophan	6.7 [3.1–9.6]	8.2 [6.5–12.3]	0.094
Indole-3-acetate	0.5 [0.4–0.6]	0.4 [0.2–0.6]	0.063
<i>Oxidative stress</i>			
Methionine sulfoxide	0.73 [0.65–0.79]	0.6 [0.5–0.8]	0.094
2-Hydroxybutyric acid	9.1 [6.5–17.3]	8.0 [4.6–8.2]	0.094
Indole-3-acetate	0.5 [0.4–0.6]	0.4 [0.2–0.6]	0.063
<i>Parkinsonism and involuntary movements</i>			
N-acetylmethionine	0.9 [0.6–1.2]	2.2 [1.0–3.4]	0.031
<i>Metabolites related to mitochondrial function</i>			
Glyceric acid	1.0 [0.7–1.8]	0.8 [0.6–1.3]	0.031
2-Hydroxyvaleric acid	0.5 [0.4–0.8]	0.5 [0.3–0.6]	0.156
Lactate-to-pyruvate ratio (L/P)	16.3 [4.3–26.8]	18.9 [13.5–25.3]	0.438
3-Hydroxybutyric acid	2.1 [1.1–5.3]	1.5 [0.5–4.4]	0.563
2-Ketoisocaproic acid	5.1 [3.5–6.7]	4.4 [3.6–5.6]	0.438
<i>Lymphocytic mitochondrial outcomes</i>			
Complex I	197 [130–319]	200 [110–331]	0.844
Complex II-III*	35 [11–61]	34 [23–47]	>0.999
Complex IV	23 [17–46]	20 [13–30]	0.219
Complex V	76 [31–90]	47 [28–81]	0.156
Citrate synthase	125 [93–160]	141 [110–165]	0.219

All outcomes with $n = 6$ except (*) with $n = 5$. Statistical significance (in Italics) was set with an FDR of 10%. Pharmacometabolite levels were expressed as the relative ratio of spectral counts for each individual metabolite normalized by the total metabolite counts per subject (see Methods) and multiplied by 1,000 to avoid scientific notation. Lymphocytic mitochondrial activities are expressed as $[\text{nmol} \times (\text{min} \times \text{mg protein})^{-1}]$.

Table 3.

Carrier-specific pharmacometabolites and lymphocytic mitochondrial outcomes pre- and post-allopregnanolone treatment

Outcomes		Carrier number (initials)												Controls	
		1 (SL)		2 (JN)		3 (JH)		4 (JP)		5 (SG)		6 (GS)			
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Reference values	
Pharmacometabolites*															
GABA pathway															
Glu/Gln		5.6	9.0	6.5	7.2	14.6	14.4	17.0	19.8	4.3	5.3	8.9	11.6	7.2–13.1	
Succinic acid		0.14	0.18	0.15	0.15	0.17	0.24	0.15	0.22	0.11	0.23	0.19	0.19	0.15–0.23	
4,5-dihydroxyhexanoic acid		0.15	0.09	0.12	0.09	0.08	0.08	0.10	0.07	0.12	0.09	0.12	0.09	<0.11	
3,4-dihydroxybutanoic acid		0.20	0.11	0.22	0.20	0.11	0.10	0.24	0.19	0.28	0.18	0.22	0.16	<0.20	
Serotonin pathway															
Tryptophan		9.6	12.2	8.0	8.0	6.2	8.1	3.1	10.4	7.1	6.5	4.4	7.0	7.0–8.1	
Indole-3-acetate		0.64	0.57	0.45	0.47	0.36	0.26	0.55	0.42	0.35	0.22	0.62	0.40	<0.52	
Oxidative stress															
Methionine sulfoxide		0.74	0.68	0.79	0.78	0.74	0.54	0.70	0.57	0.72	0.75	0.65	0.49	<0.74	
2-Hydroxybutyric acid		8.5	8.2	11.5	8.2	7.5	4.6	6.5	7.0	17.3	8.0	9.8	8.1	<10.4	
Parkinsonism and involuntary movements															
N-acetylmornithine		0.78	0.97	1.19	3.28	0.65	3.44	0.87	2.31	0.88	2.02	0.86	1.18	1.23–1.84	
Metabolites related to mitochondrial function															
Glyceric acid		1.05	0.73	0.65	0.55	1.22	0.84	1.77	1.28	0.90	0.77	0.91	0.85	0.83–1.09	
2-hydroxyvaleric acid		0.50	0.57	0.50	0.45	0.41	0.29	0.39	0.36	0.67	0.49	0.80	0.62	<0.62	
Lactate-to-pyruvate ratio		14	19	4	19	27	17	16	25	16	14	20	25	8–20	
3-hydroxybutyric acid		1.2	1.5	5.3	4.4	2.4	1.1	1.7	3.0	4.6	4.9	1.1	1.4	<2.8	
2-ketoisocaproic acid		6.7	5.6	4.6	4.4	6.7	4.4	3.8	4.4	5.5	3.6	3.5	4.6	<6.4	
Lymphocytic mitochondrial activities															
Complex I		220	268	290	110	174	224	319	176	130	160	150	332	93–268	
Complex II-III		31	ND	39	38	31	33	38	23	61	47	11	34	26–61	
Complex IV		36	13	22	20	17	19	24	21	46	30	18	21	14–60	
Complex V		74	68	31	28	78	38	83	44	90	50	70	81	31–93	

The 95% CIs were calculated with values obtained with either plasma or PBMCs of control individuals (see Methods). Outcomes outside reference values were highlighted in black (pre-treatment) or bolded (post-treatment). Outcomes outside the 95%CI at baseline were highlighted in gray. Outcomes within reference values at pre- and post-treatments were neither highlighted nor bolded. ND, not determined due to limited sample availability. Odds ratios (OR) were calculated as a predictive indicator of the association between allopregnanolone treatment and molecular outcomes changes for each carrier. P values were computed with the McNemar's test (in Italics are *p* values which reached statistical significance). OR >1 indicates higher odds of improved molecular outcomes association with allopregnanolone treatment; OR < 1 indicates lower odds of association; OR = 1 indicates lack of association. Behavioral tests are shown as score difference between post- and pre-infusion. Other details are included in Table 1 legend.