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Published in:
Neurobiology of Disease

DOI:
10.1016/j.nbd.2020.105198

Publication date:
2021

Document version
Publisher's PDF, also known as Version of record

Document license:
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Citation for published version (APA):
Andersen, J. V., Christensen, S. K., Westi, E. W., Diaz-delcastillo, M., Tanila, H., Schousboe, A., Aldana, B. I., & Waagepetersen, H. S. (2021). Deficient astrocyte metabolism impairs glutamine synthesis and neurotransmitter homeostasis in a mouse model of Alzheimer's disease. Neurobiology of Disease, 148, [105198]. https://doi.org/10.1016/j.nbd.2020.105198
Deficient astrocyte metabolism impairs glutamine synthesis and neurotransmitter homeostasis in a mouse model of Alzheimer’s disease

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ARTICLE INFO

Keywords: Glutamate/GABA-glutamine cycle GABA metabolism Anaplerosis Neurotransmitter

ABSTRACT

Alzheimer’s disease (AD) leads to cerebral accumulation of insoluble amyloid-β plaques causing synaptic dysfunction and neuronal death. Neurons rely on astrocyte-derived glutamine for replenishment of the amino acid neurotransmitter pools. Perturbations of astrocyte glutamine synthesis have been described in AD, but whether this functionally affects neuronal neurotransmitter synthesis is not known. Since the synthesis and recycling of neurotransmitter glutamate and GABA are intimately coupled to cellular metabolism, the aim of this study was to provide a functional investigation of neuronal and astrocytic energy and neurotransmitter metabolism in AD. To achieve this, we incubated acutely isolated cerebral cortical and hippocampal slices from 8-month-old female 5xFAD mice, in the presence of 13C isotopically enriched substrates, with subsequent gas chromatography–mass spectrometry (GC-MS) analysis. A prominent neuronal hypometabolism of [U-13C]glucose was observed in the hippocampal slices of the 5xFAD mice. Investigating astrocyte metabolism, using [1,2-13C]acetate, revealed a marked reduction in glutamine synthesis, which directly hampered neuronal synthesis of GABA. This was supported by an increased metabolism of exogenously supplied [U-13C]glutamine, suggesting a neuronal metabolic compensation of the reduced astrocytic glutamine supply. In contrast, astrocytic metabolism of [U-13C]GABA was reduced, whereas [U-13C]glutamate metabolism was unaffected. Finally, astrocyte de novo synthesis of glutamate and glutamine was hampered, whereas the enzymatic capacity of glutamine synthetase for ammonia fixation was maintained. Collectively, we demonstrate that deficient astrocyte metabolism leads to reduced glutamine synthesis, directly impairing neuronal GABA synthesis in the 5xFAD brain. These findings suggest that astrocyte metabolic dysfunction may be fundamental for the imbalances of synaptic excitation and inhibition in the AD brain.

1. Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized by gradual cognitive decline and dementia (Masters et al., 2015; Querfurth and LaFerla, 2010). A central pathological feature of AD is the accumulation of insoluble amyloid-β (Aβ) aggregates, predominantly manifesting in the cerebral cortex and hippocampus (Masters et al., 2015). Cerebral accumulation of Aβ leads to a complex cascade of cellular responses arising decades before clinical symptoms (De Strooper and Karran, 2016). Synapses are particularly vulnerable in AD leading to pronounced synaptic dysfunction (Reiddy and Beal, 2008; Spires-Jones and Hyman, 2014). The most abundant glial cell type, the astrocyte, reacts strongly to Aβ pathology. Astrocytes near Aβ plaques become hypertrophic and proliferative, termed reactive astrogliosis (Acosta et al., 2017; Rodriguez et al., 2009; Liddelow et al., 2017). Since astrocytes are crucial for synaptic formation and signaling processes, it has been hypothesized that malfunctioning astrocytes may facilitate or accelerate synaptic dysfunction and neurodegeneration in AD (De Strooper and Karran, 2016; Oksanen et al., 2019; Carter et al., 2019; Walton and Dodd, 2007; Steele and Robinson, 2012).

In the mammalian brain, glutamate is the primary excitatory neurotransmitter, whereas γ-aminobutyric acid (GABA) serves as the
main inhibitory transmitter. Synaptic transmission is maintained by bidirectional collaboration between neurons and astrocytes (Barros et al., 2018). Astrocytes enmesh most synapses and are essential for removal of released neurotransmitter glutamate and GABA from the synaptic cleft. Glutamate synthesized in the astrocytes is released and taken up by neurons for replenishment of the respective neurotransmitter pools (Bak et al., 2006; Sonnewald et al., 1993). The synthesis of glutamate is catalyzed by glutamine synthetase (GS, EC: 6.3.1.2) which is exclusively localized in astrocytes (Norenberg and Martinez-Hernandez, 1979). The shutting of neurotransmitters from neurons and glutamine from astrocytes is collectively known as the glutamate/GABA-glutamine cycle and is essential for normal cerebral function (Bak et al., 2006; Hertz, 2013; Waagepetersen et al., 2007). The synthesis of glutamate, GABA and glutamine is directly linked to cellular energy metabolism. The tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate serves as the direct precursor of glutamate synthesis, and thereby both GABA and glutamine synthesis (Mckenna, 2013; Westergaard et al., 1996). It is essential to counterbalance reactions that consume TCA cycle intermediates. Such reactions, that replenish the amounts of TCA cycle intermediates, are termed anaplerotic (Sonnewald, 2014). In this regard astrocytes are essential, as they express the main anaplerotic enzyme in the brain, pyruvate carboxylase (PC, EC: 6.4.1.1) (Schousboe et al., 2019), needed for de novo synthesis of glutamate and glutamine. Astrocyte glutamine synthesis is crucial for neurotransmitter homeostasis and reduced expression and activity of GS has been described in both AD patients and mouse models of AD (Walton and Dodd, 2007; Robinson, 2000; Kulijewicz-Nawrot et al., 2013; Olabarria et al., 2011; Smith et al., 1991). However, whether changes in glutamine synthesis functionally affect neuronal neurotransmitter synthesis or glutamate/GABA cycling in AD is not clear.

The aim of this study was to provide a better understanding of how Aβ pathology functionally affects neuronal and astrocytic energy and neurotransmitter metabolism. To achieve this, we incubated acutely isolated cerebral cortical and hippocampal slices from 8-month-old female 5xFAD mice, in the presence of [13C]acetate (282014, sodium salt, 99%) and left overnight at 4°C, before transfer to a solution containing mouse brain freeze solution (0.05 M sodium phosphate buffer, 20% sucrose, 40% cold 70% ethanol. The tissue was subsequently sonicated and left overnight at 4°C, before sectioning on a microtome into 35 μm thick coronal sections (Leica SM 2000R). The sections were preheated for 30 min at 80 °C in 0.05 M citrate solution (pH 6), rinsed with TBS-T and incubated with the primary antibody (mouse anti-Aβ, 1:1000) overnight. Sections were subsequently incubated with the secondary antibody (goat anti-mouse, 1:400) for 2 h at 20 °C, before transfer to a solution containing mouse StreptAvidin (1:1000) for 2 h. Finally, sections were incubated for 3 min in Ni-enhanced DAB solution. Sections were automatically scanned (Hamamatsu NanoZoomer XR) and analyzed using Adobe Photoshop. Aβ plaques were measured using the colour range command. The final values were obtained by dividing the Aβ plaques area by the total area of the section.

2.2. Animals and ethical approval

Transgene male 5xFAD mice (TG(APPSwF1Lon,PSEN1*M146L-286V/+6799Vas, Jax strain: 006554) and wild-type females (Jax strain: 100012), both on B6/SJLF1J background, were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and a colony was bred and maintained at Department of Drug Design and Pharmacology, University of Copenhagen. The 5xFAD mice express five mutations of familial AD in the amyloid precursor protein (APP) and presenilin 1 (PSEN1) genes under the neuron-specific Thy1 promoter, leading to cerebral amyloid deposition (Oakley et al., 2006). The mice were housed together in individually ventilated cages in a specific pathogen-free, humidity and temperature-controlled facility with 12 h light/dark cycle and free access to water and chow. Heterozygote 5xFAD mice were all used at 8 months of age, corresponding to a relatively advanced disease stage (Oakley et al., 2006), and wild-type littermates were used as controls (referred to as controls). Since 5xFAD mice display sex-specific variations in the development of brain amyloid pathology (Oakley et al., 2006), only female mice were included in this study. Mice were genotyped from ear clippings by a standard PCR protocol (Jax protocol: 23370) for the APP gene using the following primers: transgene forward: AGG ACT GAC CAC TCG ACC AG (olMR3610), transgene reverse: CGG GGG TCT AGT TCT GCA T (olMR3611), internal positive control forward: CTA GGC CAC AGA ATT GAA AGA TCT (olMR7338), internal positive control reverse: GTA GGT GGA AAT TCT AGC ATC ATC C (olMR7339). All experiments were approved by the Danish National Ethics Committee and performed according to the European Convention (ETC 123 of 1986).

2.3. Quantification of amyloid-β (Aβ) by immunohistochemistry

To quantify the regional cerebral Aβ load, 5xFAD and control mice were deeply anesthetized and transcardially perfused with ice-cold saline solution for 5 min, followed by perfusion with 4% PFA solution (0.4 M sodium phosphate buffer, 4% PFA, pH 7.4) for 9 min at 10 mL/min. The brains were carefully transferred to 4% PFA solution, post-fixated for 4 h at 4 °C and subsequently transferred to a 30% sucrose solution and left overnight at 4 °C. The tissue was then transferred to an anti-freeze solution (0.05 M sodium phosphate buffer, 20% sucrose, 40% ethylene glycol) and stored at −20 °C. Tissue was frozen on dry-ice before sectioning on a microtome into 35 μm thick coronal sections (Leica SM 2000R). The sections were preheated for 30 min at 80 °C in 0.05 M citrate solution (pH 6), rinsed with TBS-T and incubated with the primary antibody (mouse anti-Aβ, 1:1000) overnight. Sections were subsequently incubated with the secondary antibody (goat anti-mouse, 1:400) for 2 h at 20 °C, before transfer to a solution containing mouse StreptAvidin (1:1000) for 2 h. Finally, sections were incubated for approx. 3 min in Ni-enhanced DAB solution. Sections were automatically scanned (Hamamatsu NanoZoomer XR) and analyzed using Adobe Photoshop. Aβ plaques were measured using the colour range command. The final values were obtained by dividing the Aβ plaques area by the total area of the section.

2.4. Microwave fixation of brain tissue

To instantly halt all cerebral metabolic activity, 5xFAD and control mice were euthanized by focused beam microwave irradiation to the head (Gerling Applied Engineering). The mice were decapitated, the cerebral cortical and hippocampal areas dissected and transferred to ice-cold 70% ethanol. The tissue was subsequently sonicated and
centrifuged (4000 g × 20 min) and the supernatant was removed and lyophilized before HPLC analysis.

2.5. Brain slice incubations

Incubation of acutely isolated cerebral cortical and hippocampal mouse brain slices were performed as previously described (McNair et al., 2017). Experiments were performed on one mouse at a time. Briefly, a 5xFAD or control mouse, was euthanized by cervical dislocation, decapitated and the brain transferred to ice-cold artificial cerebrospinal fluid (ACSF) containing in mM: NaCl 128, NaHCO3 25, d-glucose 10, KCl 3, CaCl2 2, MgSO4 1.2, KH2PO4 0.4, pH = 7.4. The cerebral cortex (mainly occipital and temporal areas) and hippocampus were dissected and sliced (350 μm, coronal plane) on a McIlwain tissue chopper (The Vibratome Company, O’Fallon, MO, USA). The slices were kept just below the surface of 10 ml 37 ºC oxygenated (5% CO2/95% O2) ACSF and pre-incubated for 60 min. Subsequently, the media were exchanged for ACSF containing the stable isotopes: 5 mM [U-13C]glucose, 5 mM [1,2,13C]acetate, 0.2 mM [U-13C]glutamate, 0.2 mM [U-13C]glutamine, 0.2 mM [U-13C]GABA, 1 mM [1,2-13N]H2O or 5 mM [3,13C]glucose (all conditions except [U-13C]glucose and [3,13C]glucose, further contained 5 mM unlabeled α-glucose), and incubated for additional 60 min. Incubations were terminated by transferring slices into ice-cold 70% ethanol. The slices were sonicated and centrifuged (4000 g × 20 min) and the supernatant was removed and lyophilized before GC-MS or HPLC analysis. Pellets were saved for protein determination by Pierce protein assay.

2.6. Metabolic mapping using gas chromatography-mass spectrometry (GC-MS) analysis

The 13C and 15N enrichment of TCA cycle intermediates and amino acids in brain slice extracts was determined by GC-MS analyses as previously described (Walls et al., 2014). Briefly, slice extracts were reconstituted in water, acidified, extracted twice with ethanol and the metabolites were derivatized using N-tert-butylidemethylsilyl-N-methyl-trifluoroacetamide. Samples were analyzed by GC (Agilent Technologies, 7820A, J&W GC column HP-5 MS) coupled to MS (Agilent Technologies, 5977E). The isotopic enrichment was corrected for the natural abundance of 13C and 15N by analyzing standards containing the unlabeled metabolites of interest. Data is either presented as the molecular carbon labeling (MCL), which is the weighted average of 13C accumulation (Fig. 3), or as M + X, where M is the molecular ion and X is the number of 13C or 15N atoms in the molecule (Fig. 4-7). The MCL can be calculated for substrates entering cellular metabolism as acetylCoA leading to subsequent 13C labeling accumulation and is calculated as:

\[
\text{MCL} = \frac{(M + 1^*) + (M + 2^*) + (M + 3^*) \ldots (M + X^*)}{\text{Total number of } 13^C \text{ atoms in molecule}}
\]

2.7. Determination of amino acid amounts by high-performance liquid chromatography (HPLC) analysis

Aqueous extracts were analyzed by reverse-phase HPLC (Agilent Technologies, 1260 Infinity, Agilent ZORBAX Eclipse Plus C18 column) to quantitatively determine the amounts of amino acids (Andersen et al., 2017a). A pre-column derivatization with o-phthalaldehyde and fluorescent detection, λex = 338 nm, λem = 390 nm, was performed. Gradient elution with mobile phase A (10 mM NaH2PO4, 10 mM Na2B4O7, 0.5 mM NaN3, pH 8.2) and mobile phase B (acetonitrile 45%; methanol 45%; H2O 10%, V:V:V) was performed. The amounts of amino acids were determined from analysis of standards containing the amino acids of interest.

2.8. Experimental design and statistical analyses

Data is presented as means ± standard error of the mean (SEM), with individual data points presented. Each data point (represented by either a circle or a square in the graphs) represents biological replicates (i.e. from individual animals), which is denoted by ‘n’ in the figure legends. In most cases two independent groups were compared (5xFAD vs. controls) and Student’s unpaired t-test was applied corrected for multiple comparisons using the Benjamini-Hochberg procedure with a critical value for false discovery of 0.10 (Benjamini and Hochberg, 1995). The significance level was set at p < 0.05 and is indicated with a single asterisk.

3. Results

3.1. Cerebral amyloid-β deposition in 5xFAD mice

The extent of Aβ formation in the 5xFAD brain is dependent on the brain region (Oakley et al., 2006). We therefore started out by assessing the Aβ burden of the heterozygote 5xFAD mice in the two regions of interest: the cerebral cortex and the hippocampus, by immunohistochemistry (Fig. 1). Extracellular Aβ plaques were present in both the cerebral cortex (Fig. 1A) and hippocampus (Fig. 1B) of the 5xFAD mice. We found no Aβ immunoreactivity in the brain of wild-type control mice (Fig. S1). The area of Aβ deposition was more than twice as high in the cerebral cortex (9.8%) compared to the hippocampus (3.9%) of 5xFAD mice (Fig. 1C), which is in agreement with previous reports (Oakley et al., 2006; Jawhar et al., 2012). The stainings further confirmed a high load of cerebral Aβ, signifying that 8 months of age corresponds to a relatively advanced stage of brain amyloidosis in the 5xFAD mouse.

3.2. Reduced neurotransmitter amounts in 5xFAD cerebral cortex

We then investigated how the total amounts of amino acids might be affected in the 5xFAD brain. Brain tissue was fixated with a focused beam of microwaves, immediately terminating all metabolic activity. This is particularly important when assessing GABA amounts as this neurotransmitter rapidly accumulates post-mortem (Washek et al., 2018). Glutamate, glutamine and GABA amounts were all significantly reduced in cerebral cortical tissue of the 5xFAD mice when compared to controls (Fig. 2A). In contrast, we did not observe any differences in amino acid amounts in the 5xFAD hippocampus (Fig. 2B). The reduced amounts of all three constituents of the glutamate/GABA-glutamine cycle may indicate alterations in neurotransmitter homeostasis in the 5xFAD cerebral cortex.

3.3. Neuronal glucose hypometabolism and hampered astrocyte glutamine synthesis

To functionally investigate cellular energetics and neurotransmitter synthesis, we next incubated brain slices from 5xFAD and control mice in media containing [U-13C]glucose and analyzed slice extracts for 13C enrichment of cellular metabolites by GC-MS. Since the energy demand of neurons is much higher than that of astrocytes, the majority of glucose metabolism in the brain can be attributed to the neurons (Yu et al., 2018). [U-13C]Glucose is metabolized via glycolysis into pyruvate M + 3, giving rise to M + 3 labeling of lactate which was unchanged in the 5xFAD slices (Fig. S2A), indicating maintained glycolytic activity. Pyruvate M + 3 is converted to acetylCoA M + 2 which enters the TCA cycle, giving rise to 13C accumulation in cellular metabolites (here presented as the average 13C enrichment, molecular carbon labeling (MCL), Fig. 3; blue bars). In the 5xFAD hippocampus, a marked neuronal oxidative hypometabolism of [U-13C]glucose was observed, as the average 13C accumulation was reduced in all measured metabolites (citrate, malate, aspartate, glutamate and GABA). The reduced oxidative glucose metabolism was not as prominent in the cerebral cortical slices,
where only the \(^{13}\)C enrichment of citrate was found to be significantly reduced. Glucose is also metabolized in astrocytes leading to \(^{13}\)C enrichment in glutamine, which is exclusively synthesized in astrocytes (Norenberg and Martinez-Hernandez, 1979). We found a clear reduction in the \(^{13}\)C labeling in glutamine from metabolism of [U-\(^{13}\)C]glucose in both cerebral cortical and hippocampal slices of 5xFAD mice. Next, we provided the slices with [1,2-\(^{13}\)C]acetate, a substrate entering the TCA cycle as acetylCoA M + 2 and primarily being metabolized in astrocytes (Sonnewald et al., 1993) (Fig. 3, green bars). The \(^{13}\)C enrichment of aspartate from [1,2-\(^{13}\)C]acetate metabolism was reduced in hippocampal slices of the 5xFAD mice. Furthermore, the \(^{13}\)C labeling in glutamine was found to be reduced in both regions, again clearly demonstrating hampered astrocyte glutamine synthesis in the 5xFAD slices. Strikingly, this was also directly reflected in the \(^{13}\)C enrichment of GABA derived from [1,2-\(^{13}\)C]acetate metabolism, which was likewise reduced in both cerebral cortical and hippocampal slices of the 5xFAD mice. We did not find any differences in intracellular amino acid amounts, measured by HPLC, between slices of control and 5xFAD mice (Table S1). The results above show region-specific changes in neuronal glucose oxidation and demonstrate that diminished astrocyte glutamine synthesis directly hampers neuronal GABA synthesis in the 5xFAD brain.

Fig. 1. Larger amyloid-β (Aβ) deposition in cerebral cortex than hippocampus of heterozygote 5xFAD mice. Representative Aβ stainings of the cerebral cortex (A) and the hippocampus (B) of 8-month-old female heterozygote 5xFAD mice. Scale bars: A: 100 μm, B: 250 μm. (C) Quantification of regional Aβ immunoreactive areas in the cerebral cortex and hippocampus of the 5xFAD mice. See Fig. S1 for representative Aβ stainings of wild-type (control) mice. Mean ± SEM, n = 3, Student’s paired t-test, * < 0.05.

Fig. 2. Reduced glutamate, glutamine and GABA amounts indicate dysfunctional neurotransmitter cycling in 5xFAD cerebral cortex. Amino acid amounts of microwave-fixated cerebral cortical (A) and hippocampal (B) tissue of 5xFAD mice. Mean ± SEM, n = 6–7, Student’s unpaired t-test with Benjamini-Hochberg correction, * < 0.05.
Fig. 3. Hampered astrocyte glutamine synthesis impairs neuronal GABA synthesis.

Molecular carbon labeling (MCL) from [U-13C]glucose (left, neuron, blue bars) and [1,2-13C]acetate (right, astrocyte, green bars) metabolism in acutely isolated cerebral cortical and hippocampal brain slices of 5xFAD mice. Metabolism of [U-13C]glucose can primarily be attributed to neurons, whereas [1,2-13C]acetate is predominantly metabolized in astrocytes (investigated separately). The glutamate/GABA-glutamine cycle consists of astrocyte uptake of glutamate and GABA, astrocyte glutamine synthesis via the enzyme glutamine synthetase (GS), astrocyte release of glutamine and neuronal glutamine uptake. Neurons utilize glutamine for replenishment of both the glutamate and GABA pools. The glutamate/GABA-glutamine cycle is linked to cellular metabolism by the two enzymes aspartate aminotransferase (AAT) and glutamate dehydrogenase (GDH) in both neurons and astrocytes. Note that glutamate and GABA are released from different subpopulations of neurons, but is displayed here together for simplicity. AAT: aspartate aminotransferase, GAD: glutamate decarboxylase, GDH: glutamate dehydrogenase, GS: glutamine synthetase, MCL: molecular carbon labeling, PAG: phosphate-activated glutaminase. Mean ± SEM, n = 6–7, Student’s unpaired t-test with Benjamini-Hochberg correction, * < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.4. Dysregulations of cerebral glutamine and GABA metabolism

Next, we sought to unravel how uptake and metabolism of each individual part of the glutamate/GABA-glutamine cycle may contribute to the dysfunctional neurotransmitter homeostasis in 5xFAD brain. To explore this, brain slices were incubated in the presence of $^{13}$C-enriched glutamate, glutamine or GABA. $[^{13}C]$Glutamate is predominantly taken up and metabolized by astrocytes, although a minor fraction will also enter presynaptic neurons (McKenna, 2013; McNair et al., 2019). The M + 5 labeling in glutamate can be used as an indicator of $[^{13}C]$glutamate uptake, which was unchanged in both cerebral cortical and hippocampal slices of the 5xFAD mice (Fig. 4). In the astrocyte, glutamate can either be used for glutamine synthesis or enter oxidative metabolism as $\alpha$-ketoglutarate and give rise to M + 4 labeling in all subsequent TCA cycle intermediates. We found no changes in the $^{13}$C enrichment of glutamine M + 5 suggesting maintained glutamine synthesis from exogenously applied glutamate. In the TCA cycle, we likewise observed that all measured TCA cycle intermediates was unchanged. Neuronal GABA synthesis from $[^{13}C]$glutamate, either directly from glutamate M + 5 or from astrocyte-derived glutamine M + 5, was likewise maintained in the 5xFAD slices.

Glutamine is exclusively synthesized in astrocytes and is released to be taken up by neurons, where it is serving as a crucial substrate for replenishment of the glutamate and GABA pools (Bak et al., 2006; Waagepetersen et al., 2007). The enzyme initiating metabolism of glutamine by catalyzing the conversion of glutamine into glutamate, phosphate-activated glutaminase (PAG), is primarily located in neurons (Hogstad et al., 1988; Kvamme et al., 2001). The metabolism of $[^{13}C]$glutamine can therefore be assumed to primarily take place in neurons. We found no change in glutamine M + 5 labeling from incubation with $[^{13}C]$glutamine suggesting unchanged glutamine uptake capacity in the 5xFAD slices (Fig. 5). Once taken up, glutamine can be converted into glutamate, which can be utilized for GABA synthesis or enter the TCA cycle as described above. Intriguingly, we observed increased $^{13}$C labeling in all derived metabolites, including glutamate and GABA, from metabolism of $[^{13}C]$glutamine in cerebral cortical slices of the 5xFAD mice. This was also reflected, albeit to a lesser degree, in the 5xFAD hippocampal slices where elevated $^{13}$C enrichment was observed in fumarate and succinate.

We have recently demonstrated that GABA is extensively metabolized in brain slices and that the astrocyte represents the cell type with the most active GABA metabolism (Andersen et al., 2020). When applying $[^{13}C]$GABA as metabolic substrate, we found a slight decrease in GABA M + 4 labelling in cerebral cortical slices of 5xFAD mice.

![Fig. 4. Maintained astrocyte glutamate uptake and metabolism.](image-url)
mice which could indicate reduced GABA uptake capacity (Fig. 6). GABA enters the TCA cycle as succinate and give rise to M + 4 or M + 3 labeling in TCA cycle intermediates and the derived amino acids glutamate and glutamine. A general reduction in oxidative metabolism of [U-13C]GABA was observed as several metabolites displayed reduced 13C labeling in both cerebral cortical and hippocampal slices of 5xFAD mice (malate, citrate and aspartate). Furthermore, 13C enrichment of glutamine derived from the oxidative metabolism of GABA was reduced in both brain regions of the 5xFAD mice.

Taken together, the results above demonstrate complex and cell-specific dysfunction of the glutamate/GABA-glutamine cycle in the 5xFAD brain. Uptake and oxidative metabolism of glutamate was unaffected and so was synthesis of glutamine and GABA from exogenous glutamate. Uptake of glutamine was likewise maintained in the 5xFAD slices. However, the neuronal capacity for glutamine metabolism was increased, which likely represents a metabolic compensation in response to the inadequate astrocyte supply of glutamine. Finally, a prominent decrease in astrocyte metabolism of GABA was observed, including deficient glutamine synthesis, establishing dysfunctional astrocyte GABA metabolism as a pathological trait in the 5xFAD brain.

3.5. Maintained glutamine synthetase (GS) capacity, but reduced astrocyte de novo glutamate synthesis

Our results so far indicate that particularly astrocyte metabolism and glutamine synthesis are impaired in the 5xFAD brain. Therefore, we
finally wanted to probe two specific astrocytic metabolic pathways. Ammonia is primarily fixated in the brain via glutamine synthesis (Brusilow et al., 2010) and we therefore applied excess $^{15}$N$\text{NH}_4^+$ to the brain slices (1 mM) to test the capacity of GS (Fig. 7A). If $^{15}$N$\text{NH}_4^+$ is fixated with a molecule of unlabeled glutamate by GS, it gives rise to M$^+$ labeling in glutamine, which was found to be unaltered in the 5xFAD slices. $^{15}$N$\text{NH}_4^+$ can also be fixated via glutamate dehydrogenase (GDH) activity, giving rise to M$^+$ labeled glutamate, which likewise was unchanged. This glutamate (M$^+$) can be used for further fixation of $^{15}$N$\text{NH}_4^+$ by GS, leading to M$^+$ labeling in glutamine which was also found to be unaltered. This unchanged level of ammonia fixation indicates a sustained enzymatic capacity of GS in the 5xFAD brain. The M$^+$ labeled glutamate can also be transaminated by aspartate aminotransferase (AAT) to aspartate M$^+$, the labeling of which was found unaltered. Finally, glutamate M$^+$ can also be decarboxylated into GABA M$^+$, in which a significant reduction was observed in the cerebral cortical slices of the 5xFAD mice and the same was observed for glutamine. These results demonstrate a maintained anaplerotic activity via PC, whereas astrocyte de novo synthesis of glutamate and glutamine is reduced in the 5xFAD cerebral cortical slices.

4. Discussion

Here we present a functional investigation of regional brain energy and neurotransmitter metabolism of the 5xFAD mouse model of AD (summarized in Fig. 8). We demonstrate that deficient astrocyte glutamine synthesis directly hampers neuronal synthesis of neurotransmitter
4.1. Brain metabolic dysfunction in AD

Cerebral hypometabolism of glucose, measured by uptake of the glucose analogue $[^{18}F]$FDG and visualized by positron emission tomography (PET), is a robust marker of AD development in both patients and mouse models (Mosconi et al., 2008; Bouter and Bouter, 2019; Gordon et al., 2018). These metabolic changes arise in the early preclinical phase and correlate well with dementia symptom severity and neurodegeneration (Mosconi et al., 2008). A hypometabolic phenotype was also recapitulated in the 5xFAD slices, as we observed a prominent reduction of [U-13C]glucose metabolism, particularly in the hippocampus. The 5xFAD hippocampus displayed larger metabolic deficits when compared to the cerebral cortex, in spite the fact that Aβ burden was lower in the former region. These findings are in line with mapping of cerebral Aβ load and metabolism in AD patients, as La Joie et al. (La Joie et al., 2012) recently demonstrated pronounced hippocampal hypometabolism with limited Aβ burden. These observations could suggest that the hippocampus is highly metabolically vulnerable in AD, possibly independent of Aβ accumulation. A recent large-scale proteomic study revealed a strong, and likely causative, correlation between expression of proteins related to glial metabolism and AD pathology (Johnson et al., 2020). Furthermore, several reports have described astrocyte metabolic adaptations to Aβ in-vitro, including alterations of glycolytic and mitochondrial activity (Allaman et al., 2010; van Gijsel-Bonnello et al., 2017; Oksanen et al., 2017; Abramov et al., 2004). However, here we found a largely maintained TCA cycle metabolism of [1,2-13C]acetate, indicating conserved functional astrocyte energy metabolism in the 5xFAD slices. It should be noted that a fraction of acetate will be metabolized in neurons (Andersen et al., 2017b). However, the largest 13C enrichment from metabolism of [1,2-13C]acetate in brain slices is recovered in glutamine, supporting that astrocytes are the main cell type responsible for acetate metabolism. It was recently reported that astrocytes can contribute to $[^{18}F]$FDG-PET signals (Zimmer et al., 2017), which forces a reevaluation of the altered brain metabolism in AD to include potential changes in astrocyte metabolism (Cartier et al., 2019). The exact mechanisms underlying the cerebral metabolic decline in AD are not yet fully understood, but Aβ has been shown to impair mitochondrial function (Querfurth and LaFerla, 2010; Reddy and Beal, 2008; Hirai et al., 2001). However, Aβ deposition leads to a highly complex cascade of cellular reactions, including inflammation, oxidative stress and calcium dysregulation (De Strooper and Karran, 2016). Investigating how these complex cellular reactions functionally affects both neuronal and...
astrocytic energy metabolism, will be important for understanding the metabolic aspect of AD pathology.

4.2. Neurotransmitter metabolism and recycling in AD

Synapses are vulnerable in AD, and synaptic loss is, together with metabolic decline, one of the strongest correlates with dementia symptoms (Spire-Jones and Hyman, 2014; Inglessen et al., 2004). In contrast to the decline in glucose metabolism in the 5xFAD brain, the observed changes in neurotransmitter homeostasis and metabolism were more prominent in the cerebral cortex than in the hippocampus. The overall reduction in glutamate, glutamine and GABA amounts in the 5xFAD cerebral cortex might indicate general neurodegeneration, which has been observed at around 9 months of age in this model (Oakley et al., 2006; Eimer and Vassar, 2013). Alterations in neurotransmitter recycling have been suggested to mediate synaptic dysfunction in AD, but have so far been sparsely investigated (Walton and Dodd, 2007; Steele and Robinson, 2012). Using [1,2-13C]acetate to probe astrocyte metabolism, we demonstrate that malfunctioning glutamine synthesis is leading to impaired GABA synthesis in both cerebral cortical and hippocampal slices from 5xFAD mice. To our knowledge, this is the first functional demonstration that dysfunctional astrocyte glutamine synthesis is directly affecting neurotransmitter homeostasis in AD. We have previously demonstrated functional glutamine transfer from astrocytes to neurons during brain slice incubations (Andersen et al., 2017b). Particularly, that experimental inhibition of astrocyte glutamine synthesis depletes the neuronal GABA pool (Andersen et al., 2017b), which supports our findings in the 5xFAD brain. The observation is further in line with the increased neuronal metabolism of [U-13C]glutamine, suggesting a neuronal metabolic compensation due to insufficient astrocytic supply of glutamine. GABAergic neurons have been described as more robust and less prone to degeneration in AD compared to glutamatergic neurons (Reinikainen et al., 1988). However, from our functional investigation of neurotransmitter metabolism, we observed the largest alterations in the GABAergic system (Fig. 8). No changes in glutamate uptake or metabolism were observed in the 5xFAD slices. On the other hand, we found an indication of reduced GABA uptake in the 5xFAD cerebral cortical slices. This could be explained by reduced GABA transporter expression, which has been described in brain tissue of AD patients (Fuhrer et al., 2017). Further, we found a marked reduction in oxidative GABA metabolism, which we recently identified to take place primarily in the astrocytes (Andersen et al., 2020). Oxidative metabolism of [U-13C]GABA led to the largest 13C enrichment in glutamine. It is tempting to speculate that the observed reductions in GABA metabolism may, in part, lead to the hampered glutamine synthesis in the 5xFAD brain, but further studies are needed to confirm this. Two recent reports have suggested that astrocytes might accumulate GABA in AD (Jo et al., 2014; Wu et al., 2014). The authors provided conflicting mechanisms of astrocytic GABA synthesis. However, our findings of hampered astrocyte GABA metabolism provide an alternative mechanism of astrocyte GABA accumulation in AD. This is in line with reports of reduced activity of the enzyme initiating GABA metabolism, GABA transaminase (GABA-T), in the AD cerebral cortex (Sherif et al., 1992). In AD research, the GABAergic system has received less attention that the glutamatergic (Calvo-Flores Guzman et al., 2018). However, our study demonstrates clear perturbations of the GABAergic system, which may underlie synaptic dysfunction. We found that the 5xFAD hippocampus displayed hampered GABA synthesis from metabolism of [U-13C]glutamate and [15N]NH3, which is not directly dependent on astrocyte provided glutamine. This inherent reduction in hippocampal GABA synthesis is likely exacerbated by the deficient astrocyte glutamine provision, which was also observed in this region. This could lead to a reduced GABAergic tone and hereby potentially contribute to the neuronal hyperactivity and epileptic phenotype commonly observed in both mouse models of AD and human AD patients (Busche et al., 2008; Busche et al., 2012; Minkeviciene et al., 2009; Born, 2015; Vossel et al., 2016). Manipulation of GABAergic signaling have been shown to be able to modulate brain metabolism (Nasrallah et al., 2009). Since altered GABA signaling and GABA receptor density have been demonstrated in the AD brain (Li et al., 2016) this could potentially impact both energy- and neurotransmitter metabolism. Finally, we recently demonstrated similar deficiencies in neuronal GABA synthesis from astrocyte-derived glutamine in the R6/2 mouse model of Huntington’s disease (Skotte et al., 2018), suggesting that dysfunctional glutamine and GABA cycling could be a common metabolic trait in several neurodegenerative diseases.

4.3. Astrocytes and glutamate synthesis in AD

The original amyloid cascade hypothesis revolves around neurons, but it is becoming clear that astrocytes are an integral part of the complex AD pathology (De Strooper and Karran, 2016; Acosta et al., 2017; Rodriguez et al., 2009; Carter et al., 2019; Johnson et al., 2020; de Majo et al., 2020). Astrocytes in vicinity of Aβ plaques become reactive, leading to a hypertrophic and proliferative phenotype, termed astrogliosis (Acosta et al., 2017; Rodriguez et al., 2009; Liddelow et al., 2017). Interestingly, cellular atrophy has been reported for astrocytes distal to Aβ plaques (Rodriguez et al., 2009; Kulijewicz-Nawrot et al., 2013; Olabarría et al., 2011). It has been speculated that these morphological alterations may cause astrocytes to provide less support to the synapse and thereby potentially drive synaptic dysfunction (De Strooper and Karran, 2016; Oksanen et al., 2019; Carter et al., 2019; Walton and Dodd, 2007; Steele and Robinson, 2012). Reduced expression and activity of GS in the AD brain has been described by several reports (Robinson, 2000; Kulijewicz-Nawrot et al., 2013; Olabarría et al., 2011; Smith et al., 1991). However, whether the reduced glutamine synthesis functionally affects neuronal function has not been elucidated before. Here we found clear evidence that deficient astrocyte glutamine synthesis is directly hampering neuronal GABA synthesis. Interestingly, we also found that the capacity of GS was maintained in the 5xFAD brain when challenged with excessive amounts of ammonia. This is also in accordance with the observation that glutamine synthesis was unaffected when exogenous [U-13C]glutamate was applied, suggesting that the decreased glutamine synthesis is not caused by a direct enzymatic dysfunction of GS. Glutamine synthesis was only hampered when the astrocytic TCA cycle and α-ketoglutarate was required as precursor. Astrocyte glutamate synthesis is primarily mediated by transamination activity of AAT, whereas glutamate metabolism is mainly catalyzed by GDH (McKenna, 2013; Westergaard et al., 1996). We did not observe compelling evidence of malfunctioning AAT activity in the 5xFAD brain. We did not find any differences in aspartate amounts of the microwave-fixed tissue and the aspartate labeling was further found to be unchanged for the majority of applied isotopically enriched substrates. However, we did observe a reduction in 13C aspartate labeling selectively from metabolism of [1,2-13C]acetate, which may reflect an astrocyte specific reduction in AAT activity. However, this was not observed from metabolism of [1-13C]glucose which also reflects astrocyte metabolism. This may, in part, be caused by the different metabolic entry points, as the 13C label from [1,2-13C]acetate enters the TCA cycle as acetylCoA, whereas [1-13C]glucose enters as oxaloacetate. [1-13C]Glucose entering via PC provides 1C labeling in the immediate precursor of aspartate, namely oxaloacetate, whereas labeling of aspartate from [1,2-13C]acetate metabolism relies on functional TCA cycle activity. The selective reduction in 13C labeling in aspartate from [1,2-13C]acetate may therefore be a subtle indication of perturbed astrocyte TCA cycle function. Elevated levels of brain ammonia have been described in AD patients (Seiler, 2002). This, in combination with decreased glutamine synthesis, could potentially increase the need for GDH-mediated glutamate synthesis for fixation of the excess ammonia. Intriguingly, Neuner et al. (Neuner et al., 2017) have described a correlation between reduced GDH expression and impaired memory function in 5xFAD mice, suggesting that GDH dysfunction could be driving
memory deficits. Reduced expression of DHG has also been reported in the brains of the 3xTG mouse model of AD (Ciavardelli et al., 2010). Furthermore, a recent study found reduced gene expression of AAT (GOT2) in AD patient brain samples (Mahajan et al., 2020). However, from the present study we cannot conclude whether the hampered astrocyte de novo glutamate synthesis is mediated by perturbed AAT or DHG activity and further studies are needed to clarify this matter. Astrocyte glutamine synthesis relies on sufficient anaplerosis mediated via PC activity. Two recent studies reported reduced in vivo PC activity in rodent models of AD (Nilsen et al., 2014; Tiwari and Patel, 2014). We observed no change in the entry of pyruvate by PC activity from metabolism of \( ^{13}\text{C}\)glucose in the 5xTAD slices. However, we did find that specifically astrocyte de novo glutamate and glutamine synthesis was diminished. Our study suggests that metabolic alterations in astrocytes may be fundamental for synaptic dysfunction and neurotransmitter imbalance in AD, particularly of the GABAergic system. The integration of astrocyte energy and neurotransmitter metabolism into the complex pathophysiology of AD will be essential for understanding the underlying mechanisms of the disease.

Data availability

All data of this study is available from the corresponding authors upon request.

Funding

This study was financially supported by Tømmerhandler Vilhelm Bangs Fond (JVA), Torben & Alice Frimodts Fond (JVA), Ludvig Tegners’ Legat (JVA), Grosserer L. F. Foghts Fond (JVA), Familien Hede Nielsen’s Fond (JVA), Augustinus Fonden (JVA, 19-2676), the Lundbeck Foundation (JVA, R333-2019-1244) and Horslev Fonden (HSW, 203866).

Author contributions

Conceptualization; JVA & HSW. Data curation; JVA. Formal analysis; JVA, SKC, MDC. Funding acquisition; JVA & HSW. Investigation; JVA, SKC. Methodology; all authors. Project administration; JVA & HSW. Writing - original draft; JVA. Writing - review & editing; all authors.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Acknowledgements

The Scholarship of Peter & Emma Thomsen is gratefuly acknowledged for personal financial support to JVA. We thank Heidi Nielsen for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2020.105198.

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