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Microstructural and metabolic alterations in the zebrafish brain induced by toll-like receptor 2 deficiency: insights from ultra-high field magnetic resonance imaging and spectroscopy

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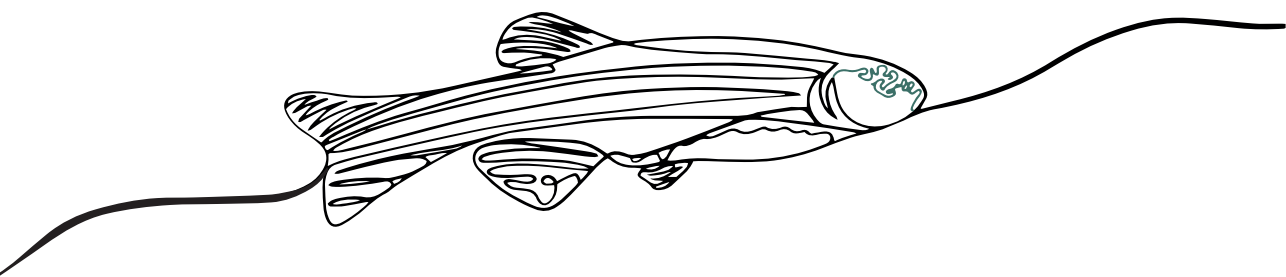
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LOCALIZED MRS AT 28.2 T REVEALS ALTERATION OF CEREBRAL METABOLITES IN TOLL-LIKE RECEPTOR 2 DEFICIENT ZEBRAFISH*

* Manuscript in preparation for publication

ABSTRACT

Toll-like receptor 2 (TLR2), a member of the TLR protein family, is crucial for immune and inflammatory responses. Although TLR2 is mainly found in immune cells, it is also present in the brain, particularly in microglia and astrocytes. In our recent work using diffusion MRI, we showed that a deficiency in the TLR2 gene causes specific changes in the brain microstructure, potentially indicative of compromised white matter integrity and axonal degeneration. However, it remains largely unclear if genomic deletion of TLR2 also has a direct effect on the cerebral metabolism. In the current work, we utilize non-invasive nuclear magnetic resonance spectroscopy techniques, namely high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) and localised ^1H magnetic resonance spectroscopy (MRS) to study metabolic changes in the brain of adult TLR2 deficient (*tlr2*^{-/-}) zebrafish. HR-MAS NMR at 14.1 T (600 MHz) provided highly resolved metabolic spectra of whole intact brain samples, showing significant changes in the levels of several neurotransmitters, energy-, and cell-specific metabolites in the *tlr2*^{-/-} group compared to a control group. To compare metabolic profile from very specific brain regions, we applied localized ^1H MRS at the state-of-the-art magnetic field strength of 28.2 T. Our results show a brain-wide increase in lactate and reduced levels of N-acetyl aspartate (NAA) in areas with specific white matter structures in the *tlr2*^{-/-} group compared to a control group, indicating reduced white matter integrity. Furthermore, we observed an alteration in the balance of excitatory and inhibitory neurotransmitters, as well as shifts in cell-specific metabolites indicative of neuroinflammation. These results suggest that TLR2 deficiency directly affects the cerebral metabolic profile, supporting previous findings of microstructural changes in the brains of *tlr2*^{-/-} zebrafish.



1. INTRODUCTION

Toll-like receptors (TLRs) have a vital role in the innate immune system as part of the pattern recognition receptors (PPR) ¹. The N-terminal ectodomain located on the outside of the TLR membrane recognises and binds to specific pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), originating from potentially harmful microorganisms and damaged host tissue, respectively². Activation of TLRs by PAMPs or DAMPs initiates events leading to the syntheses of antimicrobial peptides and pro-inflammatory cytokines³. Specific TLRs can distinguish specific DAMPs and PAMPs ^{4,5}. Among the TLRs, TLR2 stands out as a focal point of research. First identified in 1998^{6,7}, TLR2 exhibits a remarkable captivity to recognise a diverse array of PAMPs and DAMPs¹, with particular interest for its pivotal role in the immune response to *Mycobacterium tuberculosis*⁸ – an infection responsible for developing tuberculosis. TLR2 is expressed on the cell surface of various cell types, including macrophages, dendritic cells and monocytes⁹, where it is expressed as heterodimers with TLR1 or TLR6¹. Additionally, expression of TLR2 is conserved across species including humans, mice, and zebrafish. Although inflammatory responses mediated by TLR2 play a pivotal role in effective bacterial clearance, excessive or misguided TLR2 signalling can unintentionally lead to tissue damage^{10,11}. Particularly in the brain, TLR2 activation is targeted as a contributor to neuroinflammatory and neurodegenerative conditions¹². More recently, the role of TLR2 in maintaining normal brain function has gained attention. Genomic deletion of TLR2 causes significant neurobehavioral changes in TLR2-deficient models ^{13,14}. Furthermore, utilizing state-of-the-art magnetic resonance imaging (MRI) methods at ultra-high magnetic fields revealed TLR2 deficiency causes significant cerebral microstructural changes in the zebrafish (*Danio rerio*) (Chapter 3) and mouse models ¹³, including changes in the cortical thickness, restricted diffusion, and compromised white matter integrity. Moreover, transcriptomics and metabolomics on TLR2 deficient zebrafish larvae revealed the involvement of TLR2 in the transcription level of glycolysis genes and the role of TLR2 in controlling metabolism homeostasis¹⁵. Deficiency of TLR2 leads to downregulation of the glycolysis and gluconeogenesis pathways and significant metabolic changes in zebrafish larvae¹⁵. In addition, Bajorat *et al.*, showed TLR2 deficiency significantly elevates glucose uptake in all brain regions by 34.6%¹⁶. Their results suggest TLR2 has a role in controlling the cerebral amino acid, neurotransmitter, and energy metabolism, even in the absence of infection. However, the precise role of TLR2 in cerebral metabolism and its correlation to observed microstructural changes in the brain remains largely unclear.

In the current study, we utilize TLR2 knockout (*tlr2*^{-/-}) zebrafish as a model to study the effect of TLR2 deficiency on the cerebral metabolism. Nuclear magnetic resonance spectroscopy techniques, including high-resolution magic angle spinning (HR-MAS) and localized magnetic resonance spectroscopy (MRS), are well-established techniques for monitoring (cerebral)

metabolic changes. Monitoring metabolic changes through HR-MAS or MRS provides sensitive indicators of various (pathological) processes. In recent years, robust HR-MAS methods have been developed for monitoring metabolic changes in intact zebrafish embryos, successfully applied in toxicology studies of larvae exposed to polymethoxy-1-alkenes¹⁷, nano plastics¹⁸, zearalenone¹⁹, and brevetoxins²⁰ to reveal the toxicological mechanism. Furthermore, we utilized HR-MAS to analyse metabolic changes in the suprachiasmatic nucleus (SCN) in an Alzheimer's disease mouse model²¹, showing the potential of the technique to specifically monitor metabolic changes in brain tissue. Beyond HR-MAS Localized MRS is a spectroscopic technique utilizing magnetic gradient systems to facilitate precisely select specific tissue areas, allowing for *in vivo* monitoring of metabolites directly within their natural environment. Furthermore, in contrast to many other approaches in metabolomics, localized MRS eliminates the necessity for time-consuming extraction or surgical techniques. Previously, we utilized localized MRS to acquire the cerebral metabolic profile of zebrafish *in vivo*, allowing for the quantification of several metabolites²². However, despite its potential, the confined sensitivity of localized MRS and the tiny size of the zebrafish brain remained challenging. Very recently, we were able to utilize the full potential of recent developments in ultra-high field systems equipped with powerful gradient systems to obtain highly resolved ¹H localized MRS spectra from the zebrafish brain from a PRESS voxel with a volume as small as 125 nL (Chapter 4). Here, we apply optimized ¹H point-resolved spectroscopy (PRESS) sequences to compare local metabolic profiles from various brain regions of *tlr2*^{-/-} zebrafish to a control group.

We utilize HR-MAS at 14.1 T and ultra-high field localized ¹H MRS at 28.2 T to obtain cerebral metabolic spectra of zebrafish. Our results reveal genomic deletion of TLR2 significantly changes the cerebral metabolic profile. We observe an increase in *myo*-inositol (m-Ins) and decrease of levels of N-acetyl aspartate (NAA), markers for glial and neuronal cells, respectively. In addition we observe increased levels of the neurotransmitters glutamate (Glu) and γ -aminobutyric acid (GABA) and the amino acid alanine (Ala). Furthermore, we observe increased levels of lactate (Lac), indicating elevated energy requirement in the brain of TLR2-deficient zebrafish. Based on our findings and previous observations, we propose a model of the mechanism of TLR2 deficiency and its influence on cerebral metabolic and microstructural changes.

2. METHODS

Zebrafish husbandry

This study utilized the wild type AB/TL strain and the *tlr2*^{sa19423} zebrafish line from the Sanger Institute Zebrafish Mutation Resource (Hinxton, Cambridge, UK). *Tlr2*^{-/-} (mutant) and *tlr2*^{+/+} (control) zebrafish were obtained by subjecting zebrafish with homozygous TLR2 mutations

and their wild type counterparts to five rounds of outcrossing, respectively. Screening and breeding of zebrafish was carried out following previously described protocols^{23,24}, in compliance with university animal welfare committee guidelines (Licence numbers AVD1060020171767 and AVD10600202216175), EU animal protection directive international guidelines (2010/63/EU), and standard protocols (www.zfin.org). For HR-MAS, 28-months-old female *tlr2*^{-/-} and control zebrafish were euthanized by immersion in ice water (0 – 4 °C) for approximately 15 minutes, causing cessation of opercular movement. All specimens were euthanized during the same daytime period to minimize potential metabolic variation arising from circadian rhythms. After euthanizing, the brain was dissected, rapidly frozen in liquid nitrogen, and stored at -80 °C until usage. For MRI and MRS analysis, three-month-old male *tlr2*^{-/-} and control zebrafish were euthanized as described for HR-MAS analysis and kept on ice until usage. MRI and MRS analysis were performed on the same day as sacrifice.

¹H High-resolution magic angle spectroscopy

Metabolic profiling of the brain of *tlr2*^{-/-} and control zebrafish by ¹H HR-MAS was performed with a Bruker 14.1 T (600 MHz) NMR spectrometer (Bruker Biospin GmbH, Germany), equipped with a 4 mm HR-MAS ¹H/¹³C inverse probe, a magic angle gradient system, and a Bruker BVT30 temperature control unit. Dissected zebrafish brains (*n* = 3 for the control group, *n* = 4 for the *tlr2*^{-/-} group) were transferred to 4-mm zirconium oxide rotors (Bruker BioSpin AG, Switzerland) and 10 µL of deuterated phosphate buffer (100 mM, pH 7.0) containing 0.1 % (w/v) 3-trimetylsilyl-2, 2, 3, 3-tetradeuteropropionic acid (TSP) was added as a reference with its ¹H chemical shift δ = 0 ppm. All spectra were acquired at a spinning frequency of 6 kHz.

One-dimensional ¹H HR-MAS NMR spectra were recorded using a CPMG pulse sequence from Bruker's standard pulse program library with water suppression. Spectra were recorded using a spectral width of 8000 Hz and 16k data points. The data was averaged with *ns* = 512 and without dummy scans. A constant receiver gain of 2050, and an acquisition time of 0.17 s were used. The relaxation delay was set to a small value of 2 s to exclude high molecular weight molecules with short *T*₂ times, such as lipids. All spectra were processed by an exponential window function corresponding to a line broadening of 1 Hz and zero-filled before Fourier transformation. All spectra underwent processing involving an exponential window function corresponding to a line broadening of 1 Hz and zero-filling before Fourier transformation. Manual and automatic phasing, as well as baseline correction were performed using Topspin version 4.0.6.

Two-dimensional ¹H-¹H homonuclear correlation spectroscopy (COSY) HR-MAS spectra were recorded using a magnitude-mode 2D COSY with presaturation (cosyprqf) sequence from Bruker's standard pulse program library with water suppression. COSY spectra were acquired

with a spectral width of 9 kHz, corresponding to 15 ppm at 14.1 T, employing 1024 data points in the t_2 domain and 512 data points in the t_1 domain. This configuration resulted in a FID resolution of 8.80 Hz for t_2 and 17.58 Hz for t_1 . COSY data were recorded with $ns = 4$, 16 dummy scans, a relaxation delay of 2 s, and an acquisition time of 114 and 56 ms for t_2 and t_1 , respectively. The data were zero-filled to 2048 data points, providing a square matrix. Spectra were symmetrised to reduce noise and obtain well defined cross-signals.

Magnetic resonance imaging and spectroscopy

MRI and MRS experiments were conducted using a Bruker (Bruker Biospin GmbH, Germany) vertical bore system operating at 28.2 T (1200 MHz), configured with a Micro5 iProbe gradient system. The system was water-cooled, provided maximum gradient strengths ($G_{\max}$) of 3 T/m, and was equipped with a 5 mm inner diameter birdcage resonator, and a GREAT60 gradient power supply. Data acquisition and processing was performed with a Linux workstation, running Bruker imaging software ParaVision 360 v3.3 (Bruker Biospin GmbH, Germany). During analysis, zebrafish ($n = 3$ for the control group, $n = 5$ for the *tlr2^{-/-}* group) were embedded in perfluoropolyether (Fomblin Y, Solvay Solexis S.P.A.).

Three T_1 -weighted gradient echo images, oriented perpendicularly relative to each other to obtain axial, coronal, and sagittal slices, were simultaneously acquired using a fast low angle shot (FLASH) pulse sequence. This facilitated the precise selection of the volume of interest for localized ^1H MRS. FLASH measurements were performed with an echo time $TE = 4$ ms, a repetition time $TR = 100$ ms, at a flip angle of 30° , and $ns = 1$. The field of view (FOV) was 8×8 mm, at a matrix size of 128×128 voxels, obtaining a resolution of 63×63 μm , at a slice thickness of 300 μm , making the total scan time 13 seconds.

T_2 -weighted spin echo images were acquired by the multi-slice rapid acquisition with relaxation enhancement (RARE) pulse sequence to show the position of selected MRS voxels at a high resolution. RARE measurements were performed with $TE = 5.6$ ms, $TR = 3000$ ms, using 4 refocussing pulses per excitation, and $ns = 16$. The FOV was 8×8 mm, at a matrix size of 256×256 voxels, obtaining a resolution of 31×31 μm , at a slice thickness of 200 μm , making the total scan time 51 minutes.

For one-dimensional ^1H localized MRS, we utilized a PRESS pulse sequence²⁵. The PRESS sequence incorporates three slice-selective RF pulses (90° - 180° - 180°), applied simultaneous with z , y , and x gradients to target the specified voxel. PRESS measurements were performed with $TE = 15$ ms, $TR = 2000$ ms. The PRESS sequence used 2048 complex points and an acquisition bandwidth of 12 kHz / 10 ppm. Three voxels were selected in the zebrafish brain; (1) in the forebrain, covering part of the pallium, (2) in the midbrain, covering part of the

mesencephalon, cerebellum, and diencephalon, and (3) in the hindbrain, covering part of the cerebellum and rhombencephalon. In the midbrain, voxels with a size of $0.8 \times 0.8 \times 0.8 \text{ mm}^3$ and $V_{\text{voxel}} = 512 \text{ nL}$ were selected. Data were acquired with $ns = 512$, with a total scan time of 17 minutes. In the forebrain and hindbrain, voxels of $0.6 \times 0.6 \times 0.6 \text{ mm}^3$ and $V_{\text{voxel}} = 216 \text{ nL}$ were selected, and data were acquired with $ns = 1024$, with a total scan time of 34 minutes. To limit the effects of chemical shift displacement, the excitation and refocusing RF pulses had a transmitter bandwidth $tBW = 15 \text{ kHz}$ as previously reported (Chapter 4), resulting in a spatial fat-water shift of $224 \mu\text{m}$ along the z , x , and y axes. The excitation frequency was applied at $\nu_1 = 3.0 \text{ ppm}$, minimizing the chemical shift displacement for signals emerging from our target metabolites within the range of $\delta = 4.5 - 2.0 \text{ ppm}$. For suppression of the water signal, a variable pulse power and optimized relaxation delays (VAPOR) sequence²⁶ was applied prior to the PRESS pulses. Outer volume suppression (OVS) was integrated in an interleaved manner with the VAPOR sequence. The OVS consists of six suppression slices, each repeated three times and aligned parallel to the PRESS voxel, reducing the PRESS gradient spoilers. Magnetic field homogeneity within each voxel was achieved by automatic local second-order shimming prior to data acquisition.

Data processing

Metabolites identification was performed by comparison of obtained ^1H spectra to references as described in Chapter 4. Metabolite quantification was performed through Chenomx NMR suite 8.2 (Chenomx Inc., Edmonton, Alberta, Canada). The tCr at $\delta = 3.03 \text{ ppm}$ was used to estimate the relative metabolic concentrations. A one-way analysis of variance (ANOVA) was utilized for statistical analysis by OriginPro v.8 (Originlab, Northampton, MA, USA), with $p < 0.05$ considered to indicate significant differences.

3. RESULTS

HR-MAS based metabolic profile of TLR2 deficient zebrafish brain

Figure 5.1 shows a representative one-dimensional ^1H HR-MAS spectrum acquired from an adult zebrafish brain. The spectrum exhibits high resolution, enabling the identification and quantification of multiple brain metabolites. Metabolic signal assignment was based on reference ^1H spectra^{22,27-29}. The tCr signal showed strong signals at $\delta = 3.03 \text{ ppm}$ and $\delta = 3.94 \text{ ppm}$, allowing the relative quantification of metabolites. Creatine normalization is commonly used for the quantification of cerebral metabolites for its strong ^1H NMR signal and low variability³⁰.

Figure 5.2 shows a two-dimensional ^1H - ^1H COSY HR-MAS spectrum acquired from the adult zebrafish brain. The 2D COSY spectrum clearly reveals metabolites which are severely

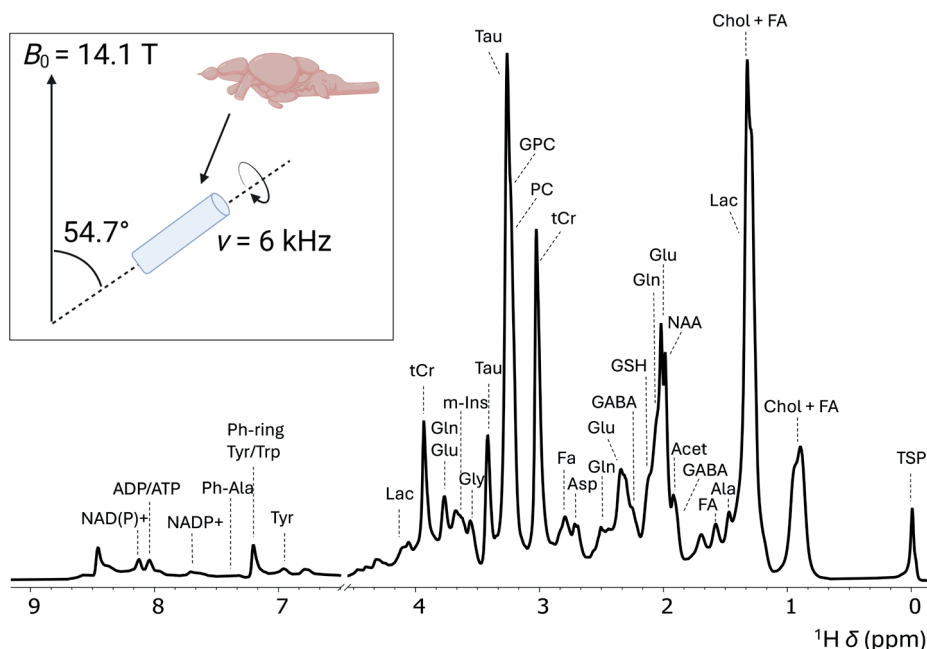


Figure 5.1. Representative ^1H HR-MAS spectrum acquired from the dissected brain of an adult female zebrafish.

HR-MAS measurements were performed with a Bruker 14.1 T (600 MHz) NMR spectrometer at a temperature of 277 K (4 °C), at a rotation frequency $\nu = 6$ kHz. For the acquisition of ^1H HR-MAS spectra, a CPMG pulse sequence was utilized with water suppression, $ns = 512$, and a relaxation delay of 2 s. TSP was used to calibrate the ^1H chemical shift (at $0 \delta = \text{ppm}$). Acet, acetate; ADP, adenosine diphosphate; Ala, alanine; Asp, aspartate; ATP, adenosine triphosphate; Chol, cholesterol; Fa, fatty acids; GABA, γ -aminobutyric acid; Gln, glutamine; Glu, glutamate; Gly, glycine; GPC, glycerophosphocholine; GSH, glutathione; Lac, Lactate; m-Ins, myo-inositol; NAA, N-acetyl aspartate; NAD(P)+, nicotinamide adenine dinucleotide phosphate; PC, phosphocholine; Ph-Ala, Phenylalanine; Ph-ring, Phenyl ring; Tau, taurine; tCr, total creatine; TSP, 3-trimethylsilyl-2, 3, 3-tetradeuteriopropionic acid; Trp, tryptophan; Tyr, tyrosine;

overlapping in 1D ^1H NMR spectra, based on their cross-peaks. Prominent correlations include the cross-peak for Lac at $\delta(4.11, 1.32)$ ppm, GABA at $\delta(1.88, 2.28)$ ppm, NAA at $\delta(2.49, 2.68)$ ppm, m-Ins at $\delta(3.51, 4.06)$ ppm, and Ala at $\delta(1.56, 3.76)$ ppm. Furthermore, the cross-peaks of Glu and glutamine (Gln) were found well resolved at $\delta(2.08, 2.33)$ ppm and $\delta(2.12, 2.43)$ ppm, respectively. In addition, we observed the cross-peak signals of macromolecules previously assigned by Behar and Ogino ³¹, labelled M1 $\rightarrow$ 5 and M4 $\rightarrow$ 7 at $\delta(0.95, 2.05)$ ppm and $\delta(1.67, 2.98)$ ppm, respectively. A detailed overview of correlations assigned from metabolites found in the zebrafish brain is shown in Table 5.1.

Figure 5.3A shows 1D ^1H HR-MAS spectra from *tlr2*^{-/-} and control adult zebrafish brain. Cerebral metabolic profiles indicated increased levels of various metabolites, including the neurotransmitter Glu and GABA. In addition, elevated signals of Ala, acetate, NAA, aspartate, taurine, m-Ins, adenosine diphosphate/adenosine triphosphate (ADP/ATP), and nicotinamide adenine dinucleotide phosphate (NADP⁺) were observed in the *tlr2*^{-/-} group compared to the control group. Quantitative analysis of metabolites in *tlr2*^{-/-} and control zebrafish brain

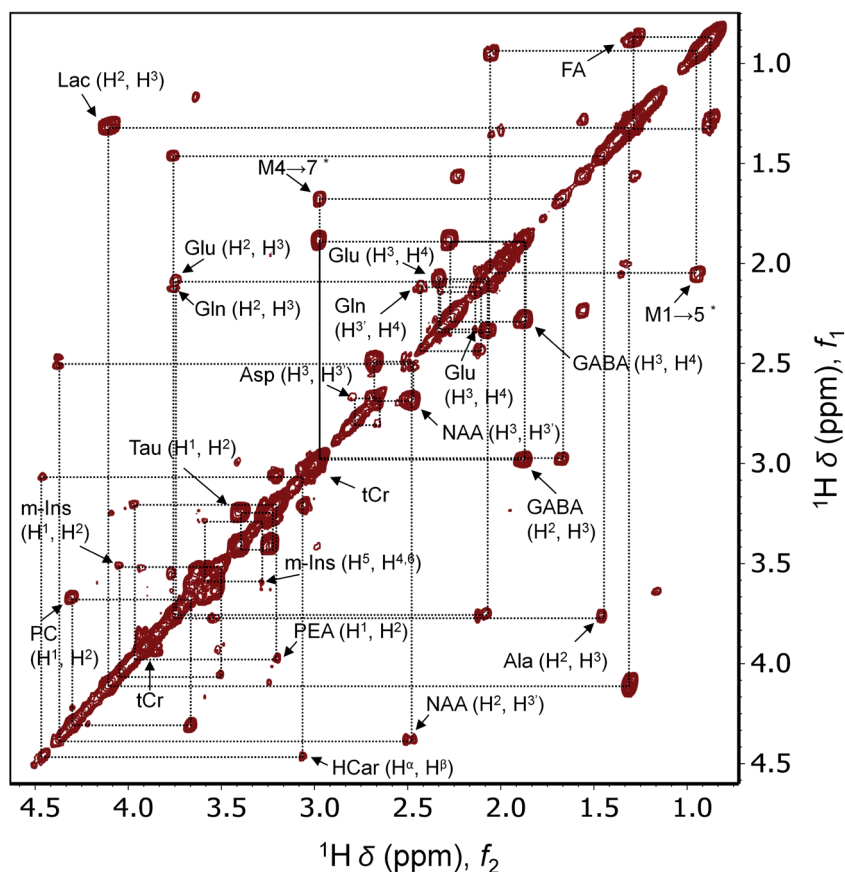


Figure 5.2. Representative ^1H - ^1H homonuclear correlated spectroscopy (COSY) HR-MAS spectrum acquired from the dissected brain of an adult female zebrafish. HR-MAS measurements were performed with a Bruker 14.1 T (600 MHz) NMR spectrometer at a temperature of 277 K (4 °C), at a rotation frequency of 6 kHz. TSP was used to calibrate the ^1H chemical shift (at 0 ppm). Ala, alanine; Asp, aspartate; Fa, fatty acids; GABA, γ -aminobutyric acid; Gln, glutamine; Glu, glutamate; HCar, homocarnosine; Lac, Lactate; m-Ins, myo-inositol; NAA, N-acetyl aspartate; PC, phosphocholine; PEA, phosphoryl ethanolamine; Tau, taurine; tCr, total creatine. * M1 $\rightarrow$ 5 and M4 $\rightarrow$ 7 arise from macromolecules as assigned by Behar and Ogino³¹.

revealed statistically significant changes in the cerebral metabolite level of *tlr2*^{-/-} zebrafish compared to the control group (Figure 5.3B). Significantly elevated levels of Glu (0.987 *tlr2*^{-/-} versus 0.8767 control; *p* = 0.040), Gln (0.3454 *tlr2*^{-/-} versus 0.2743 control; *p* = 0.049), and GABA (1.033 *tlr2*^{-/-} versus 0.7997 control; *p* = 0.031) were observed, metabolites involved in the glutamate/GABA-glutamine cycle.

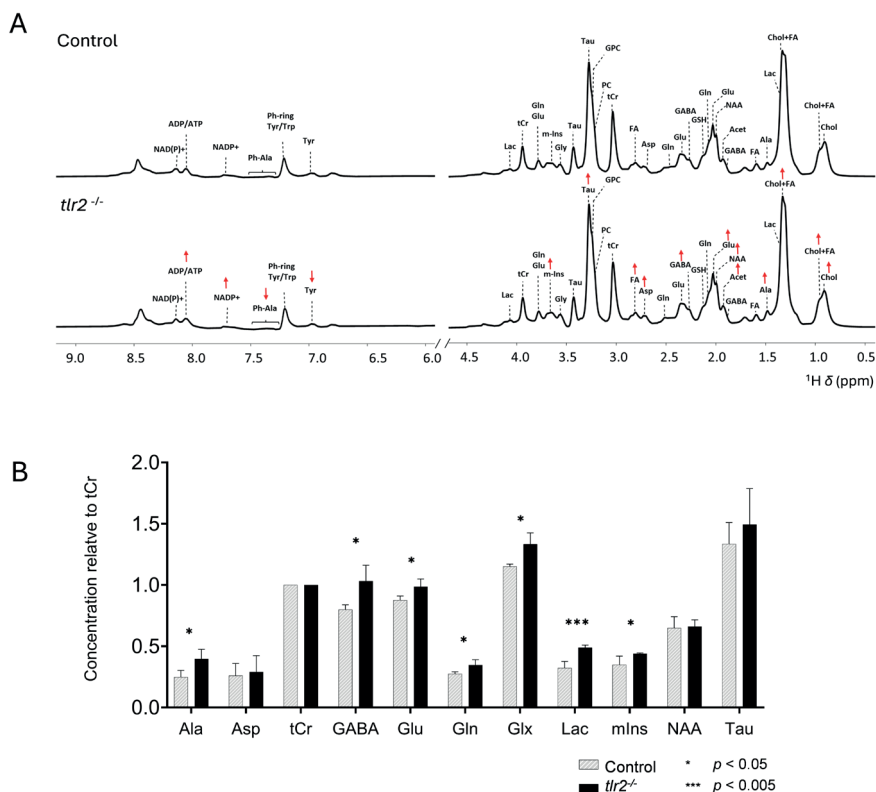


Figure 5.3. [A] One-dimensional ¹H HR-MAS NMR spectra acquired from the dissected brain of adult control and *tlr2*^{-/-} zebrafish (*n* = 3 for the control group, *n* = 4 for the *tlr2*^{-/-} group). Spectra were normalized to the intensity of the total creatine (tCr) signal at δ = 3.03 ppm for comparison. Upregulated metabolites are indicated by upward facing red arrows (↑), downregulated metabolites by downward facing red arrows (↓). Calibration of the ¹H chemical shift was performed utilizing TSP (at δ = 0 ppm). **[B]** Metabolic profile of whole brain HR-MAS analysis of control and *tlr2*^{-/-} adult zebrafish. Concentrations are reported relative to the total creatine (tCr) signal. Statistical analysis was performed by the t-test, and significance was established with a *p* < 0.05. Acet, acetate; ADP, adenosine diphosphate; Ala, alanine; Asp, aspartate; ATP, adenosine triphosphate; Chol, cholesterol; Fa, fatty acids; GABA, γ-aminobutyric acid; Gln, glutamine; Glu, glutamate; Glx, ; Glu + Gln; Gly, glycine; GPC, glycerophosphocholine; GSH, glutathione; Lac, Lactate; m-Inos, myo-inositol; NAA, N-acetyl aspartate; NAD(P)+, nicotinamide adenine dinucleotide phosphate; PC, phosphocholine; Ph-Ala, Phenylalanine; Ph-ring, Phenyl ring; Tau, taurine; tCr, total creatine; TSP, 3-trimethylsilyl-2, 2, 3, 3-tetradeuteriopropionic acid; Trp, tryptophan; Tyr, tyrosine;

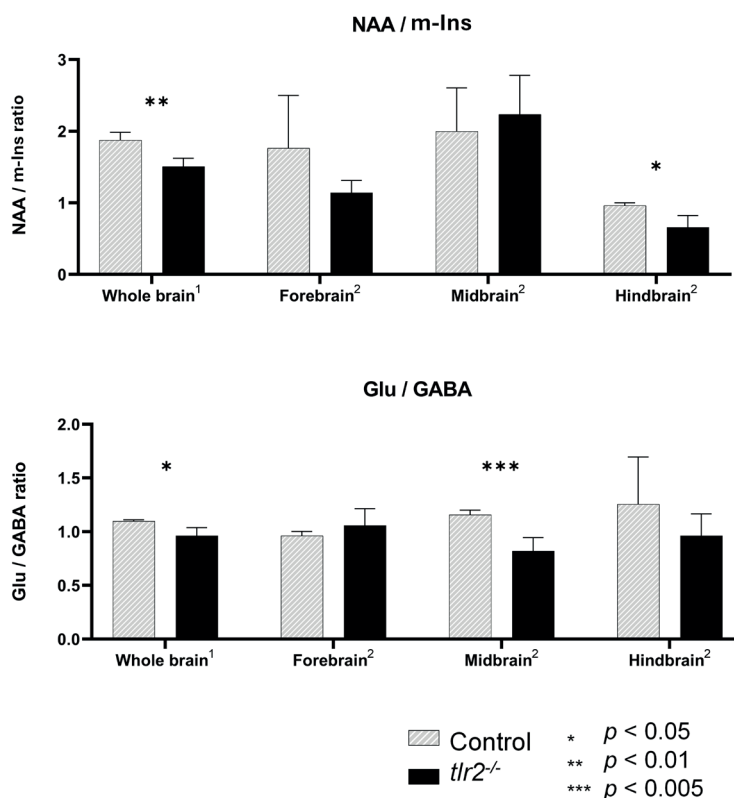


Figure 5.4. [Top] NAA / m-Ins ratio and [bottom] Glu/GABA ratio found in whole brain analysis and in localized regions. For localized spectroscopy, voxels were selected in the pallium (forebrain), the mesencephalon, cerebellum, and diencephalon (midbrain), and in the cerebellum and rhombencephalon (hindbrain). Statistical analysis was performed by the t-test, and significance was established for $p < 0.05$. ¹ Whole brain analysis was performed utilizing HR-MAS at 14.1 T. ² Regional brain analysis was performed utilizing localized MRS at 28.2 T.

Additionally, a significantly lower Glu/GABA ratio was observed for *tlr2*^{-/-} zebrafish compared to the control group (0.9622 *tlr2*^{-/-} versus 1.097 control; $p = 0.030$). This indicated a relative shift towards increased GABA levels (see Figure 5.4). Furthermore, increased levels of Ala (0.3970 *tlr2*^{-/-} versus 0.2486 control; $p = 0.038$) and Lac (0.4893 *tlr2*^{-/-} versus 0.3229 control; $p = 0.002$) were identified, both integral constituents of the “astrocyte-neuron lactate shuttle” mechanism. Moreover, elevations of m-Ins were found for *tlr2*^{-/-} zebrafish compared to the control group, as well as a significantly reduced NAA/m-Ins ratio (1.508 *tlr2*^{-/-} versus 1.877 control; $p = 0.008$), indicative of a relative increase of m-Ins (see Figure 5.4).

Table 5.1. ^1H -chemical shift and connectivity assignment of metabolites observed in ^1H - ^1H COSY HR-MAS spectra of the adult zebrafish brain. Assignment and annotation based on reference ^1H spectra^{22,27-29}. Ala, alanine; Asp, aspartate; Fa, fatty acids; GABA, γ -aminobutyric acid; Gln, glutamine; Glu, glutamate; HCar, homocarnosine; Lac, Lactate; m-Ins, myo-inositol; NAA, N-acetyl aspartate; PC, phosphocholine; PEA, phosphoryl ethanolamine; Tau, taurine

Metabolite	Group	δ (ppm)	Cross-peak
Ala	^2CH	2.77	2 – 3
	$^3\text{CH}_3$	1.47	3 – 2
Asp	$^3\text{CH}_2$	2.81	3 – 3'
		2.67	3' – 3
GABA	$^2\text{CH}_2$	3.00	2 – 3
	$^3\text{CH}_2$	1.89	3 – 2, 3 – 4
	$^4\text{CH}_2$	2.29	4 – 3
Gln	^2CH	3.77	2 – 3
	$^3\text{CH}_2$	2.13	3 – 2, 3 – 4
	$^4\text{CH}_2$	2.44	4 – 3
Glu	^2CH	3.75	2 – 3
	$^3\text{CH}_2$	2.08	3 – 2, 3 – 4
		2.15	3' – 4
	$^4\text{CH}_2$	2.35	4 – 3, 4 – 3'
HCar	$^\alpha\text{CH}$	4.48	α – β
	$^\beta\text{CH}_2$	3.07	β – α
Lac	^2CH	4.12	2 – 3
	$^3\text{CH}_3$	1.32	3 – 2
m-Ins	^1CH	3.52	1 – 2
	^2CH	4.07	2 – 1
	^4CH	3.60	4 – 5
	^5CH	3.29	5 – 4, 5 – 6
	^6CH	3.60	6 – 5
NAA	^2CH	4.39	2 – 3'
	$^3\text{CH}_2$	2.69	3 – 3'
		2.52	3' – 2, 3' – 3
PC	$^1\text{CH}_2$	4.32	1 – 2
	$^2\text{CH}_2$	3.68	2 – 1
PEA	$^1\text{CH}_2$	3.99	1 – 2
	^2CH	3.21	2 – 1
Tau	$^1\text{CH}_2$	3.41	1 – 2
	$^2\text{CH}_2$	3.24	2 – 1

Localized ^1H MRS spectra of zebrafish brain regions at 28.2 T

Single voxel localized ^1H MRS at 28.2 T was conducted across three distinct regions in the zebrafish brain. Given the irregular morphology of the zebrafish brain, voxel volumes were tailored to accommodate the shape and volume of selected brain regions, aiming to mitigate extracerebral lipid or metabolite signal interference. PRESS voxels were selected in the forebrain, midbrain, and hindbrain of the zebrafish as illustrated in Figure 5.5A. The midbrain permitted a PRESS voxel of $0.8 \times 0.8 \times 0.8 \text{ mm}^3$, corresponding to $V_{\text{voxel}} = 512 \text{ nL}$. The selected voxel covered parts of the mesencephalon, cerebellum, and diencephalon. For the forebrain and hindbrain regions, a voxel size of $0.6 \times 0.6 \times 0.6 \text{ mm}^3$ was used, corresponding to $V_{\text{voxel}} = 216 \text{ nL}$. In the forebrain, the selected voxel was precisely positioned in the pallium. In the hindbrain, the selected region overlapped with the cerebellum and rhombencephalon. To address the smaller voxel sizes utilized in the forebrain and hindbrain, the total number of scans was doubled compared to those conducted in midbrain analysis. For all brain regions, highly resolved spectra were obtained from control and *tlr2*^{-/-} adult zebrafish (Figure 5.5 B – D), allowing for the identification and quantification of brain metabolites. To ensure accurate quantification of cerebral metabolites in localized MRS data, consistent use of metabolite signals, *i.e.* chemical shift, is required due to chemical shift displacement effects. Table 5.2 shows the metabolite signals utilized for quantification, in alignment with our previous report (see Chapter 4). In the forebrain (Figure 5.5B) we observed a significant increase in Lac level ($0.6938 \text{ } tlr2^{-/-}$ versus 0.4922 control; $p = 0.034$). In the midbrain (Figure 5.5C), a significant increase in Ala relative to tCr ($0.0804 \text{ } tlr2^{-/-}$ versus 0.0417 control; $p = 0.001$), GABA relative to tCr ($0.3689 \text{ } tlr2^{-/-}$ versus 0.3122 control; $p = 0.005$), and Lac relative to tCr ($0.9036 \text{ } tlr2^{-/-}$ versus 0.6551 control; $p = 0.030$) was observed. Additionally, a significant decrease in the Glu / GABA ratio was observed in the midbrain of the *tlr2*^{-/-} group compared to the control group ($0.8201 \text{ } tlr2^{-/-}$ versus 1.158 control; $p = 0.0047$), indicating a relative shift to GABA (see Figure 5.4). In the hindbrain (Figure 5.5D), a significant increase in Lac relative to tCr ($1.123 \text{ } tlr2^{-/-}$ versus 0.5412 control; $p = 0.0004$) and significant decrease in NAA relative to tCr ($0.1467 \text{ } tlr2^{-/-}$ versus 0.2502 control; $p = 0.0006$) were observed. Additionally, the hindbrain showed a significant decrease in the NAA / m-Ins ratio ($0.6594 \text{ } tlr2^{-/-}$ versus 0.9626 control; $p = 0.022$), indicating a relative shift towards m-Ins (see Figure 5.4).

4. DISCUSSION

In this study, we utilized HR-MAS and localized MRS to conduct a comprehensive metabolic analysis of intact adult zebrafish brain. One-dimensional ^1H HR-MAS provided high-resolution metabolic spectra of intact adult zebrafish brain, allowing for quantitative analysis based on peak area. Additionally, highly resolved two-dimensional homonuclear ^1H - ^1H COSY HR-MAS was utilized to identify relevant metabolites based on their cross-peaks. To acquire regional

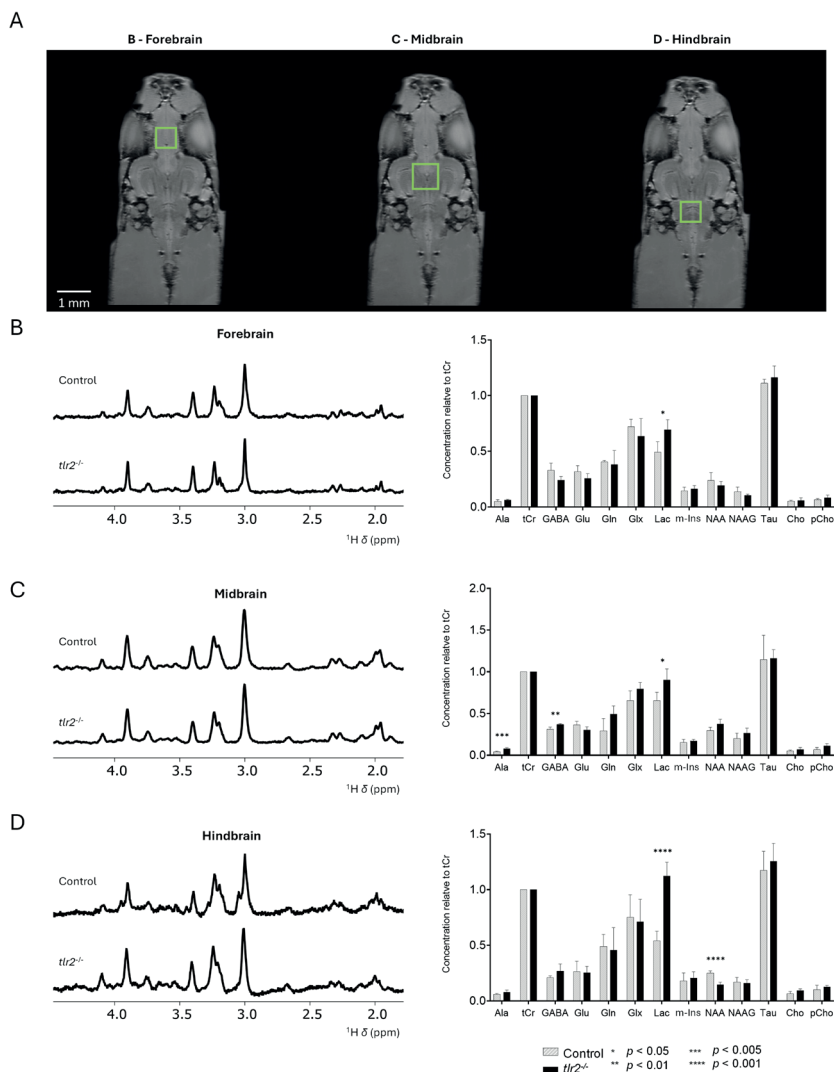


Figure 5.5. Localized ^1H MRS analysis of brain metabolites from *tlr2^{-/-}* adult male zebrafish compared to a control group. [A] Anatomical RARE image, coronal slice, of a zebrafish brain highlighting selected PRESS voxels in the forebrain, midbrain, and hindbrain. In the forebrain and hindbrain, PRESS voxels of $0.6 \times 0.6 \times 0.6 \text{ mm}^3$ with $V_{\text{voxel}} = 216 \text{ nL}$ were selected. In the midbrain a PRESS voxel of $0.8 \times 0.8 \times 0.8 \text{ mm}^3$ with $V_{\text{voxel}} = 512 \text{ nL}$ was selected. Highly resolved one-dimensional localized ^1H MRS of the control and *tlr2^{-/-}* group were obtained from the [B] forebrain, [C] midbrain, and [D] hindbrain used for the quantification of cerebral metabolites. The localized ^1H MRS spectra are all normalized to the intensity of the tCr signal at $\delta = 3.03 \text{ ppm}$. Quantification of cerebral metabolites is performed relative to the tCr signal at $\delta = 3.03 \text{ ppm}$, utilizing specific ^1H signals for each metabolite as indicated in Table 5.2. Statistical analysis was performed by the t-test, and significance was established for $p < 0.05$. Ala – alanine, tCr – total creatine, GABA – γ -aminobutyric acid, Glu – glutamate, Gln – glutamine, Lac – Lactate, m-Ins – myo-inositol, NAA – N-acetylaspartate, NAAG – N-acetylaspartyl-glutamate, Tau – taurine, Cho – choline, pCho – phosphocholine

Table 5.2 Concentrations of cerebral metabolites detected in the forebrain, midbrain, and hindbrain of TLR2 deficient (tlr2^{-/-}) and control zebrafish, utilizing localized ¹H magnetic resonance spectroscopy at 28.2 T. Functional groups and their chemical shift (in ppm) used for quantification per metabolite is indicated. Quantification performed relative to the total creatine (tCr) signal at 3.03 ppm. Statistical analysis was performed by the Tukey test, and significance was established with a p-value of < 0.05. * p < 0.05; ** p < 0.01; *** p < 0.005; **** p < 0.001. Ala – alanine, tCr – total creatine, GABA – γ-aminobutyric acid, Glu – glutamate, Gln – glutamine, Lac – Lactate, m-Ins – myo-inositol, NAA – N-acetylaspartate, NAAAG – N-acetylaspartyl-glutamate, Tau – taurine, Cho – choline, pCho – phosphocholine

Metabolite	Group used for quantification	Forebrain		Midbrain		Hindbrain	
		Control	tlr2 ^{-/-}	Control	tlr2 ^{-/-}	Control	tlr2 ^{-/-}
Ala	³ CH ₃ (δ = 1.47 ppm)	0.049 ± 0.008	0.063 ± 0.003	0.042 ± 0.004	0.076 ± 0.002 ***	0.059 ± 0.003	0.078 ± 0.01
GABA	³ CH ₂ (δ = 1.89 ppm)	0.331 ± 0.029	0.242 ± 0.017	0.312 ± 0.013	0.369 ± 0.007 **	0.21 ± 0.007	0.27 ± 0.033
Glu	⁴ CH ₂ (δ = 2.45 ppm)	0.317 ± 0.025	0.255 ± 0.022	0.362 ± 0.021	0.311 ± 0.019	0.263 ± 0.044	0.255 ± 0.029
Gln	² CH (δ = 3.75 ppm)	0.405 ± 0.006	0.38 ± 0.063	0.293 ± 0.07	0.49 ± 0.057	0.489 ± 0.051	0.456 ± 0.105
Lac	² CH (δ = 4.09 ppm)	0.492 ± 0.044	0.694 ± 0.045 *	0.655 ± 0.047	0.89 ± 0.073 *	0.541 ± 0.04	1.123 ± 0.063 ****
m-Ins	⁴ CH (δ = 3.61 ppm)	0.144 ± 0.016	0.169 ± 0.009	0.155 ± 0.017	0.169 ± 0.011	0.18 ± 0.034	0.227 ± 0.013 *
NAA	² CH ₃ (δ = 2.01 ppm)	0.239 ± 0.034	0.193 ± 0.018	0.297 ± 0.018	0.391 ± 0.025	0.25 ± 0.009	0.147 ± 0.011 ****
NAAAG	² CH ₃ (δ = 2.04 ppm)	0.136 ± 0.02	0.102 ± 0.006	0.203 ± 0.03	0.29 ± 0.014	0.17 ± 0.019	0.159 ± 0.016
Tau	² CH ₂ (δ = 3.25 ppm)	1.111 ± 0.017	1.162 ± 0.052	1.195 ± 0.103	1.171 ± 0.059	1.174 ± 0.081	1.254 ± 0.084
Cho	N(CH ₃) ₃ (δ = 3.19 ppm)	0.052 ± 0.004	0.058 ± 0.012	0.05 ± 0.006	0.076 ± 0.01	0.064 ± 0.01	0.093 ± 0.007
pCho	N(CH ₃) ₃ (δ = 3.21 ppm)	0.065 ± 0.004	0.082 ± 0.012	0.067 ± 0.012	0.123 ± 0.009	0.101 ± 0.019	0.127 ± 0.007

insights, one-dimensional ^1H localized MRS was performed to study potential variation in the metabolic changes in different region of the zebrafish brain. Building upon our previous discovery of TLR2's involvement in metabolic regulation in zebrafish larvae¹⁵, our current findings shed light on significant alterations in cerebral metabolites attributed to TLR2 deficiency.

Elevated astrogliosis and diminished neuronal integrity in TLR2 deficient zebrafish

A significant increase in m-Ins concentration was found in the brain of *tlr2*^{-/-} zebrafish compared to the control group. Of the nine inositol stereoisomers, m-Ins is the primary form found in all tissue types of vertebrates³². M-Ins is found in membrane phosphatidylinositol (phospholipids) and mediates volume and osmosis regulation³³. In the brain, m-Ins is primarily found in glial cells, e.g. astrocytes³³. Elevated levels of m-Ins are consistently observed in neurodegenerative and neurological conditions characterised by reactive glial cells, i.e. astrogliosis. For example, heightened levels of cerebral m-Ins were found in Alzheimer's disease^{21,34}, multiple sclerosis³⁵, and gliomatosis cerebri³⁶. Furthermore, we observed significantly lower levels of NAA in the hindbrain of *tlr2*^{-/-} male zebrafish. NAA is found predominantly in the cell body and axons of neurons. Reduced levels of NAA correlated to neuronal integrity loss in neurodegenerative disorders, including Alzheimer's disease¹³. Elevated cerebral m-Ins levels in TLR2 deficient zebrafish may signify an upsurge in reactive glial cells, while diminished NAA potentially reflects a loss of neuronal integrity, thus reflecting the environment of cell populations. Previously, upregulated astrogliosis was reported for a TLR2-knockout mouse model with Alzheimer's disease, indicating TLR2 deficiency increases sensitivity for astrocytic reactivity¹³. Additionally, TLR2 deficiency exhibits compromised blood-brain barrier (BBB) integrity, attributed to diminished levels of tight junction proteins¹⁴. Reduced BBB integrity may partially account for increased sensitivity to astrogliosis and loss of neuronal integrity, as the infiltration of blood-borne factors and/or pathogens into the central nervous system, facilitated by BBB breaches, is recognised a contributing factor to initiate reactive astrogliosis³⁷. Furthermore, diminished BBB integrity can lead to neuronal degeneration³⁸ and is targeted as a contributing factor in the pathology of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), MS, and Huntington's disease³⁹. In addition, our findings of reduced NAA in the brain of the *tlr2*^{-/-} zebrafish compared to control is in line with expectations based on previous reports of neuronal integrity loss caused by TLR2 deficiency. Previously (Chapter 3), we reported significant changes in diffusion MRI tensor and kurtosis metrics indicative of compromised white matter integrity and degeneration of axons in the brain of *tlr2*^{-/-} adult zebrafish. Furthermore, Zhou *et al.* found genomic deletion causes reduced cortical thickness, diminished neuronal density, and white matter damage¹³. Beyond the individual levels of m-Ins and NAA, we found a significant shift in the NAA / m-Ins ratio towards m-Ins in the *tlr2*^{-/-} zebrafish brain. Previously, a similar shift in the NAA / m-Ins ratio

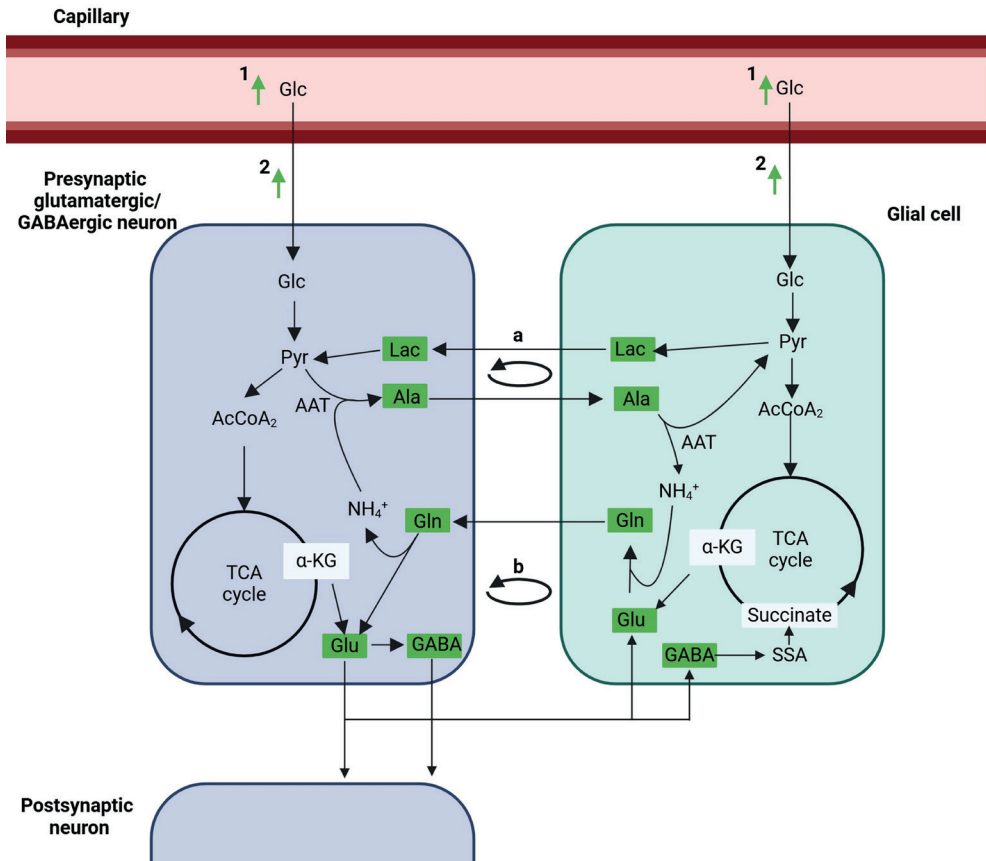


Figure 5.6. Overview of observed metabolic changes in the brain of TLR2 deficient zebrafish, highlighting disrupted neurotransmitter homeostasis. This simplified illustration depicts two crucial metabolic cycles occurring between neuronal and glial cells; (a) the lactate-alanine cycle⁵⁷ and (b) the glutamate/GABA-glutamine cycle⁵⁷. These cycles are interconnected through alanine transaminase (AAT) and ammonia (NH_4^+). Metabolites highlighted in green (Lactate, alanine, glutamine, glutamate, and GABA) were significantly upregulated in the brain of *tlr2*^{-/-} zebrafish compared to a control group. Furthermore, green arrows indicate (1) elevated glucose levels¹⁵ and (2) increased cerebral glucose uptake¹⁶ previously observed in TLR2-deficient models. Created with biorender.com.

correlated to the neurodegenerative progression of Alzheimer's disease⁴⁰. With localized MRS, we found a similar shift in the NAA / m-Ins ratio, specifically in the *tlr2*^{-/-} zebrafish hindbrain. In the zebrafish hindbrain, several major white-matter structures are situated, including the ventral rhombencephalic commissure (Cven), medial longitudinal fascicle (MLF), and lateral longitudinal fascicle (LLF). In a quantitative diffusion MRI analysis, we found significant changes in diffusivity and kurtosis metrics of these white-matter structures, indicative of compromised

white matter integrity and axonal degeneration, processes for which diminished levels of NAA are expected. Alternatively, elevated levels of m-Ins and diminished levels of NAA may stem from an augmented baseline of astrocyte cell numbers. TLR2 deficiency has an impact on neural progenitor cells (NPCs) development, altering cellular composition arising from NPCs⁴¹. Specifically, deficiency in TLR2 compromised neurogenesis, leads to a diminished population of neurons and an increased abundance of astrocytes derived from NPCs⁴¹. As m-Ins is primarily found in glial cells and NAA in neurons, this shift in cellular population caused by genomic deletion of TLR2 could lead to elevated levels of m-Ins and lower NAA concentration.

Beyond m-Ins and NAA, we found significantly elevated Lac levels in the *tlr2*^{-/-} brain compared to the control group. In the brain, Lac is involved in several processes. Beyond used to meet the energetic needs of neurons, Lac acts as a signalling molecule, exerting influence over excitability, plasticity, and the consolidation of memory. Furthermore, Lac participates in regulating essential homeostatic functions⁴². Cerebral Lac is primarily generated in glial cells, with 85% of the glucose consumed by glial cells released in the form of Lac under normal resting conditions⁴³. In glial cells, Lac is produced through aerobic glycolysis. This metabolic pathway generates energy from glucose (or alternatively glycogen) via pyruvate, rather than undergoing conversion through the tricarboxylic acid (TCA) cycle. From glial cells, Lac is transported to neurons via the astrocyte-neuron lactate shuttle mechanism, where it is converted to pyruvate to produce energy via the TCA-cycle (see Figure 5.6). In the context of cerebral energetics, neurons are the predominant energy consumers, accounting for approximately 80% of the energy expenditure in the brain⁴⁴. Intriguingly, despite the substantial variation in energy demand between neurons and glial cells, the total cerebral glucose uptake by these cell types is similar⁴⁵. Additionally, elevated cerebral glucose uptake during intensified neuronal activity is primarily accounted for by glial cells, with neurons maintaining stability⁴⁶. Furthermore, significantly elevated glucose uptake is indicative of increased metabolic activity within the astrocytic network⁴⁶. Previously, Hu *et al.* found a TLR2 deficiency significantly elevates glucose levels in *tlr2*^{-/-} zebrafish larvae, possibly caused by downregulated glycolysis and gluconeogenesis. Furthermore, Bajorat *et al.* reported brain wide increased glucose consumption as a consequence of TLR2 deficiency¹⁶. Here, we observe that TLR2 deficiency causes a significant increase of Lac in three distinct locations of the zebrafish brain; in the forebrain, midbrain, and hindbrain. Previously, significantly elevated Lac levels were also observed in *tlr2*^{-/-} zebrafish larvae¹⁵. Several neurological processes could account for changes in cerebral Lac levels. Elevated Lac was correlated to cerebral inflammation across various conditions⁴⁷⁻⁵³, a process of which astrocyte activation is a fundamental process⁵⁴. Thus, elevated Lac levels in combination with an increased m-Ins level is a strong indication of elevated numbers of reactive astrocytes in TLR2 deficient zebrafish. However, cerebral cortical hypoperfusion, observed in TLR2 deficient mice¹⁴, could also

account for increased Lac levels. Under normal oxygen tension conditions, neurons produce energy through glycolysis, forming 32 – 36 ATP molecules from each glucose molecule via the TCA cycle. Under hypoxic or anoxic conditions, the number of ATP molecules formed from each glucose molecule is reduced to 2 and the reduced availability of oxygen shifts glucose metabolism to anaerobic glycolysis, increasing Lac levels⁵⁵. Reactive glial cells as a direct consequence of hypoperfusion might also explain observed metabolic changes, as hypoperfusion potentially causes mild astrogliosis⁵⁶.

TLR2 deficiency causes disrupted neurotransmitter balance

The majority of the cerebral energy consumption is linked to glutamatergic and GABAergic neurotransmission⁵⁸. Therefore, changes in cerebral glucose uptake and the adjustments in glycolysis and gluconeogenesis observed in TLR2 deficient models likely impact, or are connected to, neurotransmission homeostasis. Maintaining the equilibrium between neuronal excitation and inhibition is important as disruptions in this balance can result in various brain related pathological conditions⁵⁹. In previous work, a notable increase in Glu was found utilizing whole-body *tlr2*^{-/-} zebrafish larvae analysis¹⁵. These changes in Glu levels, the primary excitatory neurotransmitter, might indicate TLR2 has a role in the metabolism of the central nerve system¹⁵. Building upon this, our current investigation delves deeper, affirming and expanding upon these earlier results. Specifically, we demonstrate that the elevated Glu level extends beyond the larval stage, persisting specifically within the adult *tlr2*^{-/-} zebrafish brain. Glu plays a pivot role in various neuroplasticity mechanisms and is important for areas relying on neuroplasticity to adapt to changes in environmental conditions, including cognition, learning, and mood⁶⁰. In zebrafish, Glu is found omnipresent in the brain and spinal cord⁶¹, although higher concentrations of glutamatergic neurons are reported in specific brain regions, including the olfactory bulb, the stratum periventricular of the optic tectum, and the habenula⁶². Following exocytosis release, there is a substantial increase in Glu levels within the extracellular fluid. Maintaining balance of the extracellular Glu level is crucial for neurological health. Glu disbalance disrupts brain homeostasis, potentially leading to adverse effects. Insufficient extracellular Glu can lead to a depletion in the available energy, affecting cellular functioning. Conversely, an excess of extracellular Glu can result in overexcitement of nerve cells, potentially leading to cell death in a process known as excitotoxicity⁶³. Elevated Glu levels have been observed in various neurodegenerative and neurological disorders, including ALS and Parkinson's disease^{64,65}. Astrocytes play a pivot role in the removal of excess Glu. In the glutamate-glutamine cycle, extracellular Glu is up taken by astrocytes and converted to Gln in an enzymatic reaction of involving glutamine synthetase (GS) and ammonia. Subsequently, Gln is released by astrocytes and taken up by neurons and synthesized to Glu, releasing ammonia.

Besides a significant increase in Glu, we observed a significant increase in Gln and Ala. Ala accounts for approximately 25% of the conversion of Gln to Glu in glutamatergic neurons⁶⁶. The ammonia released in neurons in the conversion of Glu to Gln is (partially) used in the production of Ala utilizing alanine transaminase (AAT). The Ala is transported to astrocytes as part of the lactate-alanine cycle, where it converts to ammonia for the synthesis of Gln in the glutamate-glutamine cycle⁵⁷. In this way, the glutamate-glutamine cycle and lactate-alanine cycle are connected and changes in molecular levels involving one metabolic pathway may influence the other pathway. For example, disrupted energy metabolism and decreased glucose levels cause decreased levels of both Glu and Ala in Huntington's disease⁶⁷. Contrary, TLR2 deficiency causes increased cerebral glucose uptake¹⁶ and increased glucose levels¹⁵, which may explain increased Glu, Gln, and Ala levels presented in the current work. Another possible explanation for elevated cerebral Glu and Ala levels could arise from cerebral ammonia intoxication as a consequence of diminished BBB integrity observed in *tlr2*^{-/-} model¹⁴. Under normal conditions, ionic ammonia (NH_4^+) is unable to freely pass through the BBB⁶⁸. Although gaseous ammonia (NH_3) may diffuse through the BBB, this only forms a minor fraction of the total ammonia in the blood⁶⁸. Cerebral hyperammonemia can result in excessive extracellular Glu and increased levels of Ala and Gln⁶⁹⁻⁷². Furthermore, hyperammonemia can induce neuroinflammation⁷³. However, to the best of our knowledge, no studies have been conducted to monitor whether the diminished BBB integrity in TLR2 deficient models results in cerebral hyperammonemia. Thus, at present, no evidence is available to substantiate this hypothesis.

Alongside increased levels of Glu, we found elevated GABA in the brain of TLR2 deficient zebrafish. GABA is the major inhibitory neurotransmitter and synthesised from Glu. In the zebrafish brain, GABA is mainly expressed in the olfactory bulb, preoptic nuclei, retina, telencephalon, ventral thalamus, subpallium, pretectum, hypothalamus, and posterior tubercular nuclei of the zebrafish brain^{62,74}. In these regions, GABA mediates processes including memory and learning, motor functions, and neural development⁶¹. A disruption GABA regulation can profoundly affect the brain, with altered GABA reported in neurological disorders, including attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), epilepsy, depression, anxiety, and neurodegenerative diseases including Parkinson's disease and Alzheimer's disease (reviewed by Yoo Sung Kim and Bo-Eun Yoon⁷⁵). Specifically, elevated levels of GABA, as well as a decreased glutamate/GABA ratio, are observed in ASD^{59,76} and during stress⁷⁷. Alternatively, hypoperfusion, previously observed as a consequence of TLR2 deficiency¹⁴, may also lead to long lasting increase in GABA levels⁷⁸.

Metabolic changes in TLR2 deficient zebrafish mimic schizophrenia

Previously, TLR2-deficient models revealed genomic deletion of TLR2 causes significant changes in their behaviour^{13,79}. Noteworthy, TLR2 deficient mice showed parallels with

schizophrenia⁸⁰, with behavioural changes including symptoms reminiscent of psychosis, including increased movement, withdrawal from social interactions, cognitive impairments and anxiety (negative schizophrenia symptoms). Additionally, TLR2 deficiency caused ventricle enlargement, another hallmark of schizophrenia⁸⁰. Remarkably, we report metabolic changes similar to those observed in schizophrenia patients, including elevated Glu^{81,82}, Gln⁸¹, m-Ins⁸³, and Lac⁸¹. GABA levels in schizophrenia report conflicting results, although most studies reported GABA levels are affected in schizophrenia⁸⁴⁻⁸⁶. In addition, significantly elevated cerebral glucose uptake is reported in both TLR2 deficiency^{16,87}, 70% of schizophrenia patients exhibited reactive astrogliosis^{87,88}, and both hypoperfusion and hyperperfusion was observed in the brain of schizophrenia patients, although hypoperfusion is more commonly associated with negative schizophrenia symptoms, e.g. blunted affect, alogia, and anhedonia, while hyperperfusion is associated with positive symptoms, e.g. hallucination, thought disorder, and delusions⁸⁹. While our study may not specifically investigate the origin of the striking parallels observed between schizophrenia and TLR2 deficiency, our findings shed light on intriguing metabolic alterations within the brain that seem to mirror each other.

5. CONCLUSION AND FUTURE OUTLOOK

In conclusion, we report TLR2 plays a role in maintaining the cerebral metabolic homeostasis even in the absence of infections. Genomic deletion of TLR2 causes significant changes in the level of several metabolites in the zebrafish brain. This includes changes in the excitatory and inhibitory neurotransmitter balance, potentially explaining neurobehavioral and cerebral microstructural changes observed in TLR2 deficient models. Furthermore, we observed changes in cell-specific metabolites, indicative of neuroinflammation and neurodegeneration, also in line with previous observations. In future work, advanced MRI and MRS techniques could provide further insight in the effect of TLR2 deficiency on metabolic changes in the zebrafish brain. Amongst these techniques, chemical exchange saturation transfer (CEST) for glucose (glucoCEST), glutamate (gluCEST), and *myo*-inositol (MICEST) could provide detailed spatial information regarding the distribution of these metabolites over the brain of *tlr2*^{-/-} zebrafish.

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