

Title page

Voxel-based analysis of postnatal white matter microstructure in mice exposed to immune challenge in early or late pregnancy.

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Abstract

Maternal infection during prenatal life is a risk factor for neurodevelopmental disorders, including schizophrenia and autism, in the offspring. We and others have reported white matter microstructure abnormalities in prefrontal-striato-temporal networks in these disorders. In addition we have shown that early rather than late maternal immune challenge in the mouse model precipitates ventricular volume change and impairs sensorimotor gating similar to that found in schizophrenia. However, it is not known whether the timing of maternal infection has a differential impact upon white matter microstructural indices. Therefore this study directly tested the effect of early or late gestation maternal immune activation on post-natal white matter microstructure in the mouse. The viral mimic PolyI:C was administered on day 9 or day 17 of gestation. *In-vivo* diffusion tensor imaging (DTI) was carried out when the offspring reached adulthood. We describe a novel application of voxel-based analysis to evaluate fractional anisotropy (FA). In addition we conducted a preliminary immunohistochemical exploration of the oligodendrocyte marker, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), to determine whether differences in myelination might contribute to any changes in FA observed. Our results provide experimental evidence that prenatal exposure to inflammation elicits widespread differences in FA throughout fronto-striatal- limbic circuits compared to control saline exposure. Moreover, FA changes were more extensive in the group exposed earliest in gestation.

Keywords: DTI, VBM, FA, CNPase, PolyIC, prefrontal-striatal-limbic circuits.

Introduction

Evidence from multiple directions supports a consensus view that schizophrenia and related disorders including autism have onset early in neurodevelopment (Bailey et al., 1998; Beckmann, 1999; Bullmore et al., 1998; Bunney et al., 1995; McAlonan et al., 2005; Murray, 1994; Murray et al., 1992; Pilowsky et al., 1993). We have previously reported diffusion tensor imaging (DTI) data supporting white matter microstructure anomalies in these conditions (Cheung et al., 2009; Cheung et al., 2008). This likely contributes to the differences in functional connectivity consistently observed during higher-order cognitive processing in schizophrenia and autism compared to typically developing individuals (Demirci et al., 2009; Foucher et al., 2005; Just et al., 2007; Koshino et al., 2005; Schlosser et al., 2003; Zhou et al., 2007).

Epidemiological studies implicate maternal infection during prenatal life as a strong risk factor for neurodevelopmental disorders in the offspring (Brown, 2006; Chess, 1971; O'Callaghan et al., 1991; Sham et al., 1992; Takei et al., 1995). This has established a platform for the development of rodent models in which maternal infection precipitates a behavioural phenotype with features similar to those found in schizophrenia and/or autism spectrum (Fatemi et al., 2005; Fatemi et al., 2008a; Shi et al., 2003). It appears that non-specific activation of the maternal immune response (MIA) using viral analogues is sufficient to bring about behavioural phenotypic change in the offspring (Meyer et al., 2005, 2006; Meyer et al., 2007; Patterson, 2002; Watanabe et al., 2004) and that early gestational exposure triggers more extensive behavioural and neuroanatomical sequelae than later exposure (Li et al., 2009; Meyer et al., 2007).

There is preliminary data to support a link between prenatal inflammatory exposure in late gestation and changes in white matter markers in offspring (Fatemi et al., 2008b), however the latter study was restricted to pre-selected regions of interest (ROI) and examined only a late gestational exposure. Given the crucial role of timing in determining phenotypic outcome (Li et al., 2009; Meyer et al., 2007), we examined white matter FA in adult mice following exposure in early gestation, day 9 (GD9) and late pregnancy (GD17) time points. Schizophrenia and autism spectrum disorders are complex and unlikely to be explained by any single, well-circumscribed lesion. Therefore, in order not to restrict our analysis to predefined ROIs, we planned to exploit voxel-wise analysis techniques (VBM) used in the human literature to explore FA in mouse brain in-vivo. From the clinical literature we predicted widespread involvement of white matter tracks, especially in front-striatal-limbic circuits and the corpus callosum (Cheung et al., 2009; Cheung et al., 2008; Ellison-Wright and Bullmore, 2009). We expected that FA anomalies would be more extensive in the early exposed group. In addition we conducted a preliminary immunohistochemical exploration of the oligodendrocyte marker 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) to determine whether myelination processes might contribute to any changes in FA observed.

Material and Methods

Animals

Timed pregnant female C57BL6/N mice were supplied by The University of Hong Kong, Laboratory Animal Unit, (LAU) for a study approved by the Committee on the Use of Live Animals in Teaching and Research at The University of Hong Kong. Prenatal treatment comprised either saline control or a 5ml/kg injection volume of 5 mg/kg PolyI:C (potassium salt, Sigma Aldrich) administered via the tail vein on GD9 or GD17. Offspring were weaned and sexed at postnatal day 21. Three to 4 male littermates were caged together for use in the experiments. Adult behavioural phenotype and ventricular volumes in these animals have already been reported (Li et al., 2009). Total number of mice used for these present experiments were: control, n =12 (GD9=5, GD17=7), Poly I:C, n = 18 (GD9=10, GD17=8). Mice used for DTI scanning were, Control, n = 8 (GD9 n= 3, GD17 n= 5); PolyI:C, n = 14 (GD9 = 8; GD17 = 6). Four control mice (GD9=2, GD17=2), and 4 PolyI:C (GD9=2, GD17=2) mice that had not been scanned nor tested in accompanying behavioural paradigms (Li et al., 2009) were used for immunohistochemistry.

MRI

DTI scanning of 12-week old offspring was carried out in a dedicated small animal 7 T scanner with quadrature RF coil with 23 mm inner diameter and maximum gradient of 360 mT/m (70/16 PharmaScan, Bruker Biospin GmbH, Germany). Mice were weighed then anaesthetized via a nose cone with isoflurane/air mixture at 3% for induction and 1.5% for maintenance via a nose cone. Diffusion weighted images were acquired with a

respiration-gated 4-shot SE-EPI sequence with 2 different b-values (0.0 and 1.0 ms/ μm^2) along 30 gradient encoding directions (Hui et al., 2009; Wang et al., 2009a; Wang et al., 2009b; Wang et al., 2008; Wu et al., 2007; Wu and Wu, 2009) extending from bregma-2.92 to 1.98. The imaging parameters were TR/TE = 3000/30.3 ms, $\delta/\Delta = 5/17$ ms, slice thickness = 0.35 mm, FOV = 32.3 mm, data matrix = 128×128 (zero-filled to 256×256) and NEX = 4. The DTI component of the scan took 28 mins.

Voxel based Morphometry (VBM) method

MRI images were processed with the Statistical Parametric Mapping 2 (SPM2) software and the DTI toolbox, running under MATLAB 6.5. DTI toolbox was used to realign the DTI images to correct for motion and eddy current. FA map was calculated from the realigned DTI images (Cheung et al., 2008). There were 3 main preprocessing steps. First, the skull was removed from the DTI and the T2 images. Second, distortions in the DTI images were spatially corrected by non-linear registration to the T2 structural image of the same mouse. Third, the DTI images were normalized to standard space using the structural T2 image as reference.

In the first step, non-brain tissue were removed from the T2 image and the b0 image using the semi-automatic method previously described (Li et al., 2009). Distortion in the skull-free b0 and the T2 images was corrected as follows. The b0 and the T2 image were grossly co-registered by a rigid transformation. They were further registered by a secondary, two-dimensional affine transformation, where scaling and shearing were restricted to the X and to the Z direction, as the DTI coverage in the Y direction was short

of the whole brain T2. After two linear registrations, a third, non-linear registration was applied to further improve the mapping between DTI and the structural image. During this registration, a binary mask was applied to the T2 brain to mask regions that were out of the field of view of the b0 image. DTI distortions were mainly in the Z-X plane, therefore the number of bias functions in the Y direction was restricted to a single function. The parameters of the three transformations were merged together into a single transformation, and applied to correct the distortion of the skull-free FA map. The transformed FA map was modulated with the Jacobian determinant of the transformation to reflect the original FA value before the correction. Finally, the T2 brain was normalized to standard space (Li et al., 2009). The parameter of this transformation was applied to the distortion corrected FA map. The normalized FA map was then smoothed with a 0.3mm Gaussian kernel before the analysis.

In order to confine the statistical analysis to white matter only, a white matter mask was constructed by only including voxels that were above an FA value of 0.15 in all of the mice in the study. One mouse in each of the groups, control, GD9 and GD17 was rejected because of excessive motions in the DTI scans, thus the final numbers for DTI analysis were 7 controls, 7 GD9 PolyI:C and 5 GD17 PolyI:C. The data were entered into a single design matrix in SPM2 and ANOVA used to compare FA in the PolyIC groups to the control group. The VBM statistics were threshold with $p < .005$ and a minimum cluster size of 50 voxels.

Immunohistochemistry Protocol

Additional mice exposed to MIA or saline on GD9 or GD17, but not used for MRI or any other testing (Li et al., 2009) were deeply anesthetized by Hypnorm (12.5 mg/kg)/Dormicum (6.25 mg/kg) mixture and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in 0.1M sodium phosphate buffer pH 7.4. Brains were taken out and post-fixed with 4% paraformaldehyde for less than 24 h and then embedded in paraffin wax. Five μm thick sections were cut coronally from paraffin blocks of each brain. Sections were dewaxed and rehydrated before antigen retrieval with sodium citrate buffer (pH 6.0). They were then treated with 0.3% H_2O_2 in methanol for 15min to inactivate endogenous peroxidase. Sections were blocked with 1% BSA and 10% normal horse serum at room temperature for 1h, incubated with mouse monoclonal of CNPase (Abcam) diluted with 1:200 in 1% BSA at 4°C overnight. Next day, sections were incubated with horse radish peroxidase (HRP)-conjugated donkey anti-mouse IgG (Abcam) diluted 1: 2000 in 1% BSA for 1h at room temperature. All the slides were washed with tris buffered saline (TBS, pH 8.4) or TBS-Tween 20 (TBST) 5min each for three times after incubated with H_2O_2 , primary and secondary antibodies. Finally, sections were developed using DAB kit (3,3'-diaminobenzidine, Invitrogen) under the light microscope at room temperature for 2-3 min, and stopped in running tap water. After staining, the sections were dehydrated through an alcohol series, cleared with xylene, and mounted. Tissue sections for negative control were processed similarly but without addition of primary antibody.

Results

VBM

Voxel-wise analysis indicated widespread regions of significantly lower and higher FA in GD9 and GD17 PolyI:C exposed animals relative to controls. As can be seen in Table 1 and Figure 1, FA was lower in both GD9 and GD17 polyI:C exposed mice compared to controls in the left amygdala and cerebral peduncles and right fimbria. There were additional regions of lower FA in the GD 9 group including anterior cingulate, ventral striatum and external capsule. In contrast GD17 polyI:C animals had lower FA in right ventral subicular regions only. FA was significantly increased in a region corresponding to the left stria medullaris in both GD9 and GD17 polyI:C groups. The GD17 group also had increased FA in right stria medullaris and amygdala/piriform area while GD9 polyI:C exposed mice had multiple areas of increased FA including the left fimbria, lateral septal area and prefrontal lobe. (see Figure 1 and Table 1)

Immunohistochemistry

Inspection of CNPase stained sections confirmed a qualitative reduction in staining in the external capsule of GD9 polyI:C exposed mice, but not GD17 polyI:C exposed mice, in line with the VBM results. See Figure 2. In addition, CNPase staining appeared increased in the left fimbria of GD9 polyI:C mice but not GD17 polyI:C mice compared to controls, also consistent with the direction of VBM findings. See Figure 3.

Discussion

The present study yields direct experimental evidence that prenatal exposure to maternal inflammation disrupts white matter microstructure across a number of critical brain circuits. In either early or late gestation, MIA elicited widespread bidirectional changes in FA throughout fronto-striatal-limbic circuits. Regions with lower FA were more extensive in the early exposed group. In both groups there were regions with increased FA but again, these were more extensive in the early exposed group. These FA anomalies observed were predominantly left-sided. Preliminary immunohistochemical evidence revealed that mice exposed to MIA early in gestation had a qualitative reduction in the oligodendrocyte marker 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in the external capsule and increase in the fimbria of mice corresponding with the tracts showing decrease and increase in FA respectively. This is consistent with a white matter structural insult which at least partly affects myelination.

Our results extend previous observations that prenatal infection exposure in late gestation modifies white matter markers in offspring (Fatemi et al., 2008a). The present result adds to previous work by our team and others indicating that the impact of MIA on postnatal brain and behavioural phenotype depends on the timing of exposure during gestation (Li et al., 2009; Meyer et al., 2006; Meyer et al., 2008b; Meyer et al., 2007). Specifically, we reported that MIA on GD9 in the mouse causes lateral ventricular enlargement and sensorimotor gating abnormalities in offspring (Li et al., 2009). This closely mirrors schizophrenia in which lateral ventricular enlargement is the most consistent neuroimaging finding (Chua and McKenna, 1995) and sensorimotor gating impairment

constitutes an important endophenotype (Swerdlow et al., 2006, Quednow, 2009). MIA at a later time point in pregnancy (GD17) does not cause ventricular enlargement in the offspring, nor marked sensorimotor gating abnormalities (Li et al., 2009). The observation fits with more recent serological evidence indicating that early inflammation, during the first trimester of human pregnancy, may have a critical role in elevating the risk of schizophrenia in offspring (Brown et al., 2004; Brown et al., 2005) and precipitating brain morphological changes in schizophrenia (Brown et al., 2009).

A subsidiary aim of the present study was to apply voxel-based methods to analyse mouse DTI data. Although we have successfully used VBM to investigate CSF volume changes in the same animals (Li et al., 2009), the technique has not yet been applied to the more challenging DTI analysis of the rodent brain. Perhaps the most demanding component of the analysis was normalization of images to the T2 scan. The preprocessing routine applied a non-linear 2D normalization procedure to localize FA images to a common space within the mouse template (Chan et al., 2007). Thereafter a voxel-by-voxel comparison of FA could be performed and we suggest this method could be usefully applied in other MRI studies which collect similar composite datasets.

Our FA findings in the MIA mouse model are largely consistent with the pattern observed by ourselves and others in previous clinical imaging studies of schizophrenia and autism (Cheung et al., 2009; Cheung et al., 2008; Ellison-Wright and Bullmore, 2009). In a comprehensive meta-analysis of DTI studies of schizophrenia, Ellison-Wright and colleagues found significantly decreased FA was most consistently identified in left

frontal and temporal deep white matter (Ellison-Wright and Bullmore, 2009). They concluded that lower frontal FA supported a disruption to white matter circuits interconnecting frontal lobe, thalamus and cingulate gyrus, while the temporal FA reduction involved tracts interconnecting the frontal lobe, hippocampus–amygdala, and temporal and other targets. Our observation of predominantly left-sided anomalies in fronto-temporal regions, including those around the amygdala and hippocampus, is compelling both in the similarity of direction of change and distribution of anomalies to the human literature. Recently Rotarska-Jagiela conducted a DTI study of schizophrenia and also found lower FA in frontal pathways circuits, in addition to external capsule FA decreases as observed here (Rotarska-Jagiela et al., 2009).

The latter study and others additionally reported increased FA was associated with more severe positive symptoms in the arcuate fasciculus (Hubl et al., 2004; Shergill et al., 2007). The arcuate fasciculus links the parieto-temporal lobe with dorsal frontal lobe (BA 8, 46, and 6) (Catani et al., 2007; Catani et al., 2002; Catani et al., 2005; Catani and Mesulam, 2008; Catani and Thiebaut de Schotten, 2008) and, in connecting Wernicke’s area to Broca’s area, it is a key component of language pathways in man. Rotarska-Jagiela et al. interpreted their findings by linking a ‘strengthening’ of aberrant connections to the severity and duration of hallucinations experienced. In the mouse we also identified some restricted increases in FA. These were most evident in the offspring of early exposed animals. Sites of increased FA included regions around the stria medullaris, septum, fasciculus retroflexus and fimbria fornix. The stria medullaris connects septal and anterior thalamic nuclei to the habenula. The habenula is thought to have an

integrative function, acting in co-ordination with basal ganglia and dopaminergic/serotonergic systems in cognitive processing (Lecourtier and Kelly, 2007) and regulation of dopaminergic reward-driven behaviour (Matsumoto and Hikosaka, 2007).

White matter FA in a region corresponding to the fasciculus retroflexus was observed to be increased in the GD17 animals. This tract is of interest because it controls REM sleep through cholinergic projection to the hippocampus (Valjakka et al., 1998) and interacts with midbrain dopamine and serotonin activating systems (Carlson et al., 2000). Given the clinical observation of sleep pattern dysregulation in conditions such as autism (Taira et al., 1998) and schizophrenia (Chouinard et al., 2004), our results encourage closer investigation of the role of this projection in such manifestations of developmental change. Thus the present picture of abnormally increased FA in this key neurotransmitter control centre, fits well with the long-standing hypothesis of dopamine excess and dysregulation in schizophrenia (Murray et al., 2008). It also fits with the consistent reports of enhanced sensitivity to amphetamine in animals exposed to prenatal MIA particularly in the early exposed animals (Meyer et al., 2005; Meyer et al., 2008a).

The fornix is a fundamental limbic pathway in the rodent, connecting the hippocampal complex with the septum and mammillary bodies. The hippocampal-fornix system has long been implicated in the pathophysiology of schizophrenia, with neonatal lesions of the hippocampus driving phenotype changes in rodents analogous to features of schizophrenia ((Ellenbroek et al., 1998; Lipska and Weinberger, 2000). Similarly

hippocampal systems are implicated in autism, leading to a theory that autism involves developmental change in this brain region (DeLong, 1992). Post-mortem evidence for abnormality in this limbic pathway in schizophrenia has been recorded in a study by showing the fibres of the left fornix in males were denser than comparison control males (Chance et al., 1999). This is a striking parallel with the observation here of increased FA in the left fornix FA of GD9 exposed mice. Indeed the authors in the latter study suggested ‘a delay in late developmental processes, including myelination’ might explain their findings which were consistent with a ‘primary pathophysiological change in the development of global connectivity’.

The white matter microstructural differences we observed were clearly asymmetric. In recent years increasing attention has been focused on brain asymmetry in lower vertebrates. It has become apparent that cognitive asymmetry of the vertebrate brain is conserved through evolution (Vallortigara et al., 1999). In rodents, as in humans, there is appreciable anatomical asymmetry generally, but not always, involving left-hemispheric enlargement (Spring et al., 2010). Asymmetrical development is determined in early life through the Nodal signaling pathway which is regulated by the Wnt/Axin/b-catenin protein cascade (Carl et al., 2007). Disruption to the Wnt signaling pathway (Beasley et al., 2002; Beasley, 2001; Everall, 1999) and a reduction in brain asymmetry is recognised in schizophrenia (Sommer et al., 2001). In lower vertebrates the role of this signaling pathway in controlling patterning of the dorsal diencephalic conduction system (DDC), comprising the habenula nuclei, stria medullaris and fasciculus retroflexus has been closely studied. As discussed above, left-sided white matter FA was particularly

increased in components of the DDC in the immune-stimulated mouse model. The DDC has a highly conserved complex asymmetric organisation with right-ward lateralisation of the lateral habenula in the albino mouse, see review (Bianco and Wilson, 2009) and a larger right habenula and thicker fasciculus retroflexus in fish species (Adrio et al., 2000). Interestingly prenatal influenza A has been shown to specifically target the habenula complex (Mori et al., 1999). Taken together with our present findings, the evidence suggests that regions with asymmetric organization in mammalian brain are susceptible to inflammation occurring during prenatal life. Thus our findings are consistent with interruption of brain asymmetry patterning during early life leading to post-natal neurodevelopmental disorder (DeLisi et al., 1997). We propose that future study should incorporate a more detailed examination of the time-specific effects of prenatal immune activation on the development of brain asymmetry in the mouse.

We acknowledge that our work has a number of limitations. Our DTI study did not include the cerebellum. This is an important drawback as the cerebellum is implicated in both disorders, but particularly autism (Courchesne, 1997). However, we suggest that our VBM method to normalize DTI coverage to standard template brain has applications beyond the dataset acquired here. Moreover, given the practical difficulties of manual tracing approaches in the mouse FA map, VBM offers an operator independent option for other studies seeking to capitalize on in-vivo imaging of mouse models. The immunohistochemistry component of our study should be considered as a first step only in exploring the basis of these white matter anomalies precipitated by prenatal inflammatory insult. In indicating that myelination may contribute to the changes in FA

observed, our findings are consistent with accumulating evidence for dysregulation in the genetic control of myelination processes in schizophrenia (Dracheva et al., 2006).

In conclusion, our present results support the aetiological validity of the hypothesis that early inflammatory insult leads to widespread connectivity anomalies in the brain relevant to neurodevelopmental disorders such as schizophrenia and autism. The use of an in-vivo scanning method should pave the way for longitudinal study designs. For example, in our future studies we hope to investigate white matter developmental indices from an earlier time point with a view to identifying possible biomarkers for neurodevelopmental conditions and potentially test the usefulness of novel therapeutic interventions on white matter maturation.

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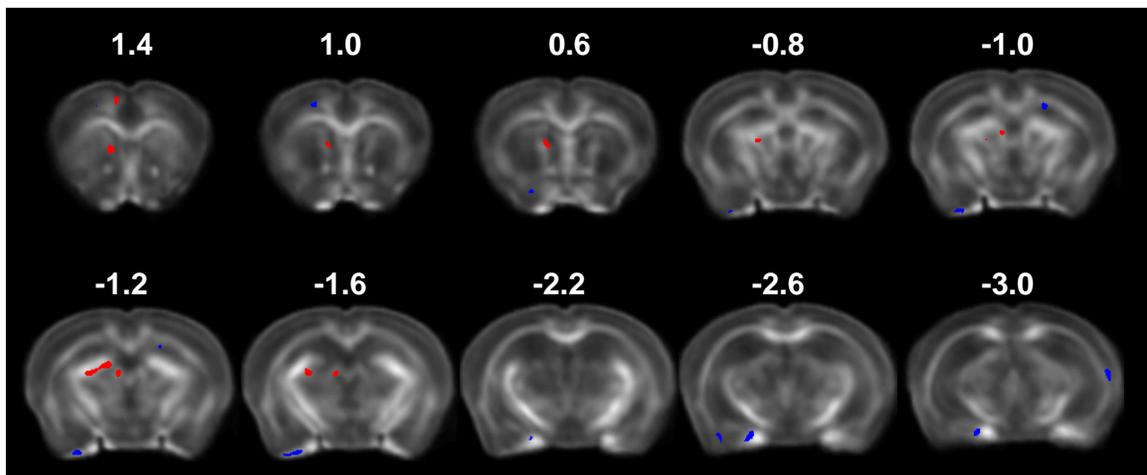
Table 1

Voxel-based analysis of white matter FA

FA lower than control	L/R	GD9			GD17		
		x	y	z	x	y	z
External Capsule	R	4.6	-3.1	-3.0			
Ventral subiculum/Entorhinal cortex	R				2.5	-5.5	-3.6
					2.1	-5.2	-3.5
Cerebral peduncle	L	-1.7	-6.0	-2.5	-1.6	-6.1	-2.1
Amygdala	L	-3.2	-6.0	-2.5	-3.8	-5.8	-1.1
	L	-2.3	-6.8	-1.4	-2.4	-6.8	-1.4
Corpus callosum/Cingulum Dorsal hippocampal commissure	L	-1.3	-1.4	2.2			
Fimbria/Ventral hippocampal commissure	R	1.5	-1.6	-1.1	0.7	-1.7	-0.7
Ventral striatum	L	-1.7	-5.8	0.7			
Anterior Cingulate/motor cortex	L	-1.6	-1.4	1.0			
FA higher than control	L/R	GD9			GD17		
Piriform area/Amygdala	R				3.1	-6.7	-1.7
Stria Medullaris/Fasciculus Retroflexus	R				0.2	-2.9	-1.6
Stria Medullaris	L	-0.5	-2.9	-1.1	-0.5	-2.9	-1.2
Fimbria	L	-2.0	-2.9	-1.4			
Fimbria/Stria terminalis	L	-1.4	-3.2	-0.8			
Orbital cortex	L	-1.6	-3.3	1.9			
Dorsal anterior cingulate	L	-0.5	-1.3	1.7			
Prelimbic cortex	L	-0.7	-4.4	2.5			
Lateral septum	L	-1.0	-3.4	0.7			

GD9, GD17 are mice exposed to PolyI:C on either gestation day 9 or day 17. x,y,z are co-ordinates based on the Allen C57/B6 Allen mouse brain atlas. z indicates the distance from Bregma in mm, x refers to horizontal plane, y refers to the vertical plane. L/R, left, right hemisphere.

A.



B.

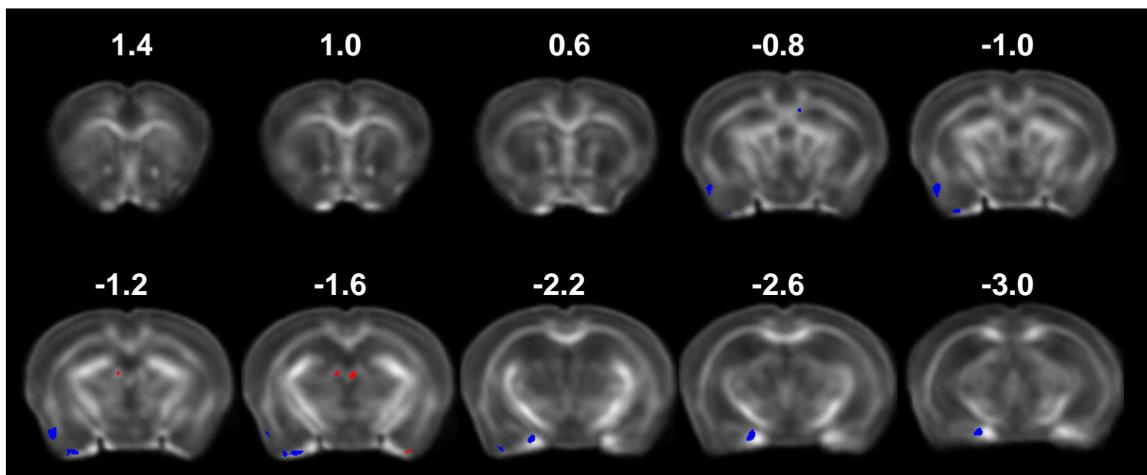


Fig. 1. Voxel-based analysis

A. FA differences in mice exposed to PolyI:C on gestation day 9 compared to control saline exposed animals. **B.** FA differences in mice exposed to PolyI:C on gestation day 17 compared to control saline exposed animals. Red indicates a relative increase in FA in PolyI:C exposed animals, blue indicates a relative decrease.

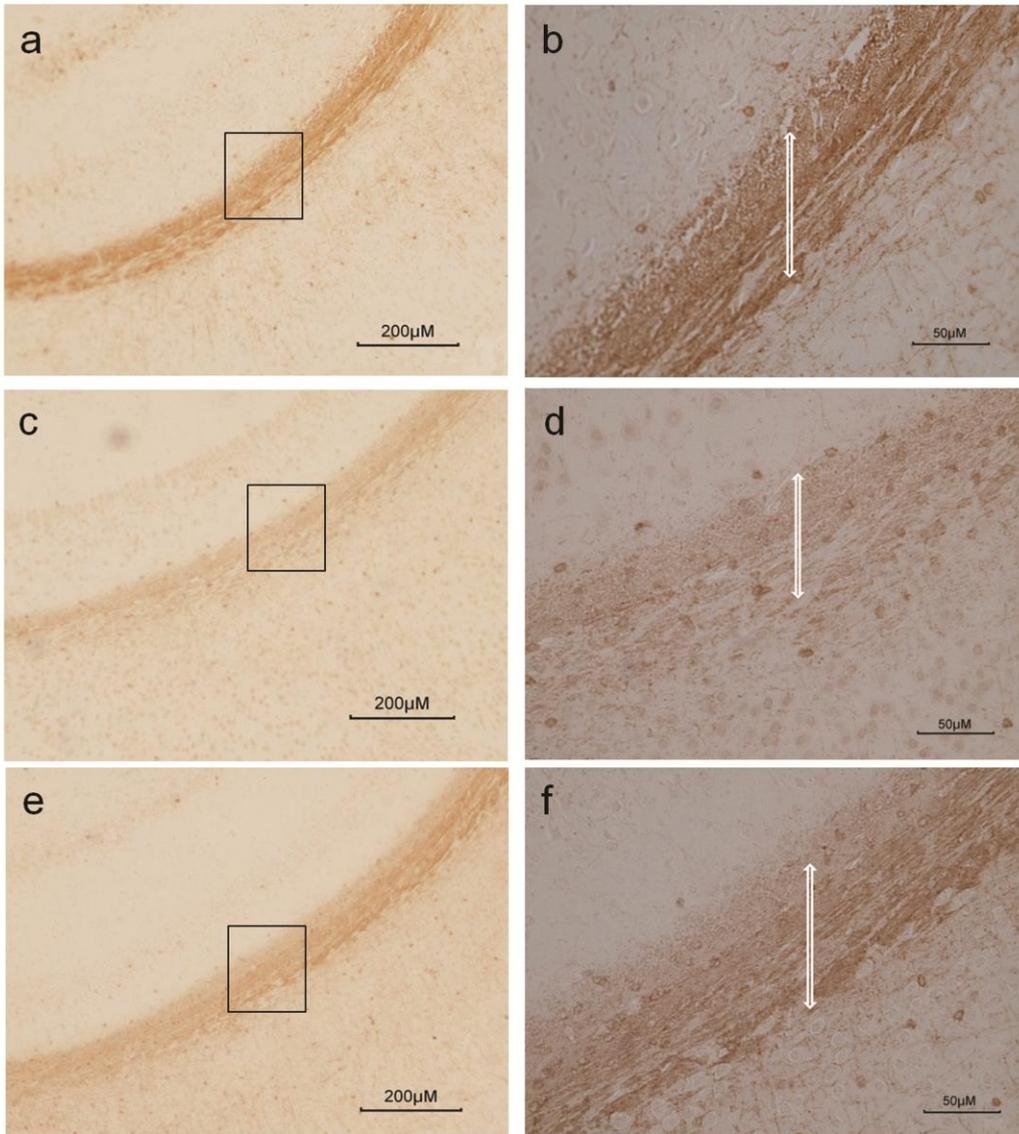
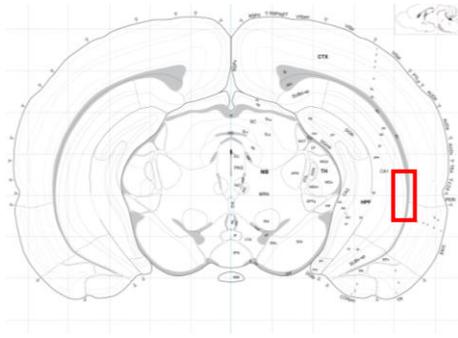


Fig.2. CNPase protein expression in right external capsule.

Images captured at approximate position of the red rectangle, about bregma -3.18mm, show greater CNPase protein expression in control compared to GD9 PolyI:C or GD17 PolyI:C. Mouse atlas is from Allen Institute (Lein et al., 2007). *a* .Control. *c*. GD9 PolyI:C. *e*. GD17 PolyI:C. *b,d,f*. images at higher magnification indicated by the squares in *a*, *c* and *e* respectively. The double-headed arrows indicate the thickness of EC. Scale bars: *a,c, e*, 200 μ M; *b,d, f*, 50 μ M.

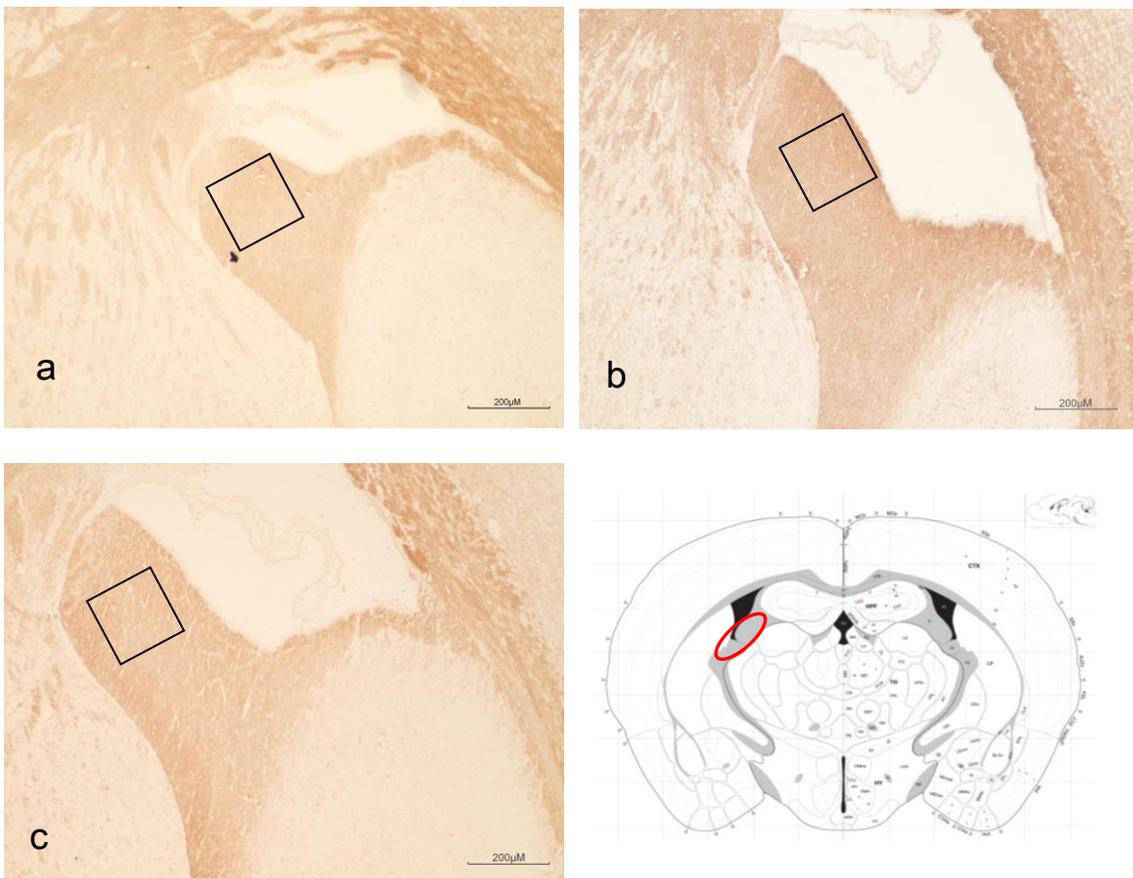


Fig.3. CNPase expression in left fimbria.

Fimbria images captured at the approximate position of the red oval, about bregma - 1.26mm, showed greater CNPase SIGNAL in GD9 PolyI:C group compared to control

group. Mouse atlas is from Allen Institute (Lein et al., 2007). *a.* control group. *b.* GD9 PolyI:C group. *c.* GD17 PolyI:C group. Scale bars: *a, b, c*, 200 μ M.