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Decreased expression of mGluR5 within the dorsolateral prefrontal cortex in autism and increased microglial number in mGluR5 knockout mice: Pathophysiological and neurobehavioral implications

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ABSTRACT

Metabotropic glutamate receptor 5 (mGluR5) and microglial abnormalities have been implicated in autism spectrum disorder (ASD). However, controversy exists as to whether the receptor is down or upregulated in functioning in ASD. In addition, whilst activation of mGluR5 has been shown to attenuate microglial activation, its role in maintaining microglial homeostasis during development has not been investigated. We utilised published microarray data from the dorsolateral prefrontal cortex (DLPFC) of control ($n = 30$) and ASD ($n = 27$) individuals to carry out regression analysis to assess gene expression of mGluR5 downstream signalling elements. We then conducted a post-mortem brain stereological investigation of the DLPFC, to estimate the proportion of mGluR5-positive neurons and glia. Finally, we carried out stereological investigation into numbers of microglia in mGluR5 knockout mice, relative to wildtype littermates, together with assessment of changes in microglial somal size, as an indicator of activation status. We found that gene expression of mGluR5 was significantly decreased in ASD versus controls ($p = 0.018$) as well as downstream elements *SHANK3* ($p = 0.005$) and *PLCB1* ($p = 0.009$) but that the pro-inflammatory marker NOS2 was increased ($p = 0.047$). Intensity of staining of mGluR5-positive neurons was also significantly decreased in ASD versus controls ($p = 0.016$). Microglial density was significantly increased in mGluR5 knockout animals versus wildtype controls ($p = 0.011$). Our findings provide evidence for decreased expression of mGluR5 and its signalling components representing a key pathophysiological hallmark in ASD with implications for the regulation of microglial number and activation during development. This is important in the context of microglia being considered to play key roles in synaptic pruning during development, with preservation of appropriate connectivity relevant for normal brain functioning.

1. Introduction

To date, the aetiology of autism spectrum disorder (ASD) remains poorly understood with diagnosis relying solely on clinical interview. Evidence linking the metabotropic glutamate receptor (mGluR5) to ASD pathogenesis has come from studies demonstrating that mGluR5 is strongly linked to fragile X syndrome (FXS) and tuberous sclerosis (Tsc); two genetically defined disorders with significantly increased prevalence of ASD and similar core

Abbreviations: CAK β, cell adhesion kinase-β; CREB, cyclic AMP response binding protein; DAG, diacylglycerol; ER, endoplasmic reticulum; GKAP, guanylate kinase associated protein; Gq/Gq11, guanine nucleotide binding protein q/q11; IP3, inositol 1,4,5 trisphosphate; NMDA, N-methyl-D-aspartate; GRM5/mGluR5, metabotropic glutamate receptor-5; MAPK, mitogen activated protein kinase; PIP2, Phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLCB1, phospholipase C-beta 1; PSD95, post-synaptic density 95; SHANK3, SH3 and multiple ankyrin repeat domain.

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symptomatology (Krueger and Bear, 2011). Recently, we have found that single nucleotide polymorphisms (SNPs) in *GRM5* (the gene encoding mGluR5) had a strong weighting in our predictive genetic classifier for ASD (Skafidas et al., 2014). MGluR5 plays a role in many critical neuronal processes, including synapse formation (Piers et al., 2012), long term depression (LTD) (Luscher and Huber, 2010), as well as regulation of astrocyte-mediated increase in excitatory post-synaptic currents (EPSCs) following activation of microglia, ATP release, and subsequent activation of the P2Y1 receptor on astrocytes (Pascual et al., 2012). In addition, it has been demonstrated that activation of mGluR5 *in vitro* can attenuate microglial activation as well as associated neurotoxicity following exposure to lipopolysaccharide (LPS) (Loane et al., 2009). This is of relevance to the aetiological investigation of ASD as increased microglial numbers and/or activation has been demonstrated in post-mortem investigations of the DLPFC, white matter and cerebellum in individuals with ASD (Morgan et al., 2010; Vargas et al., 2005). In addition, microglial activation in ASD has been demonstrated *in vivo* via positron emission tomography (PET) in the cerebellum, midbrain, pons, fusiform gyri, and the anterior cingulate and orbitofrontal cortices (Suzuki et al., 2013). These findings are interesting in light of the fact that microglia have been shown to play an important role in synaptic development and pruning, including postnatal circuits (Paolicelli et al., 2011; Schafer et al., 2012), and with a recent RNA sequencing study of post-mortem ASD brains demonstrating that a gene expression module associated with microglial activation is negatively correlated with a neuronal functioning module (Gupta et al., 2014).

Whilst mGluR5 has been implicated in ASD pathogenesis, controversy exists as to whether mGluR5 signalling is increased or decreased in the brains of individuals with ASD, with evidence for the use of drugs to potentiate or inhibit this receptor having therapeutic potential (Carlson, 2012). With respect to potentiation of mGluR5 signalling being beneficial to symptoms associated with ASD, it has been demonstrated that a positive allosteric modulator (PAM) of mGluR5, 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl) benzamide (CDPPB) corrected synaptic and biochemical defects in the hippocampus of tuberous sclerosis 2 ($Tsc2^{+/-}$) mutant mice, as well as restoring cognitive deficits present in these mice (Auerbach et al., 2011). MGluR5 also has a strong interaction with N-methyl-D-aspartate (NMDA) receptors causing enhancement of NMDAR signalling (Benquet et al., 2002). This is of interest given that NMDAR hypofunction is thought to play a role in ASD, with autoantibodies to NMDA being shown to cause autistic like regression in a case study of a toddler meeting criteria for ASD diagnosis (Scott et al., 2013). ASD-like behaviours have also been exhibited in SH3 and multiple ankyrin repeat domains 3 (*SHANK3*) mouse models (Peca et al., 2011) with mutations and copy number variations in *SHANK3* being strongly associated with ASD (Durand et al., 2007; Sykes et al., 2009) and with SHANK3 interacting directly with mGluR5 through binding of Homer and phospholipase C (PLC) to regulate signalling (Hwang et al., 2005; Tu et al., 1999). In addition, a recent meta-analysis has demonstrated that mutations in *SHANK3* are present in 0.69% of patients with ASD, with *SHANK1* and *2* mutations to a lesser extent present in 0.04% and 0.17% respectively (Leblond et al., 2014) with SNPs and CNVs in *PLCB1* also recently been identified and linked to ASD (Girirajan et al., 2013; St Pourcain et al., 2014). Furthermore, dysregulation in the methylation status of *SHANK3* in post-mortem brains of individuals with ASD versus controls has also been demonstrated (Zhu et al., 2013) as well as inhibition of SHANK3 being shown to cause a reduction in synaptic expression of mGluR5 in hippocampal and cortical neuronal cultures, leading to reduced spine density and mini EPSCs (Verpelli et al., 2011).

Activation of PLCB1 by mGluR5 has also been shown to be critical for the co-ordinated development of pre- and post-synaptic elements in mice (Hannan et al., 2001; Hannan et al., 1998; Spires et al., 2005). In addition, mGluR5 knockout (KO) mice also demonstrate hyperlocomotion and deficits in spatial working memory (Burrows et al., 2015; Gray et al., 2009; Jew et al., 2013), together with a lack of novelty-seeking behaviour (Parkitna et al., 2013) and decreased pre-pulse inhibition (Brody et al., 2004; Chen et al., 2010). These findings are of interest given that sensorimotor deficits are seen in individuals with ASD, including excessive movement (De Jong et al., 2011) together with spatial working memory deficits (Steele et al., 2007) and low novelty-seeking behaviours and reward dependence also seen in individuals with ASD (Anckarsater et al., 2006).

As *GRM5* was the strongest candidate gene in our predictive genetic classifier as well as strong evidence implicating mGluR5 in ASD pathophysiology, we firstly decided to investigate whether gene expression of mGluR5 and molecules downstream of its activation are dysregulated within DLPFC of individuals with ASD versus controls together with gene expression changes in pro-inflammatory markers associated with microglial activation.. We chose to investigate the DLPFC as dysfunction or lack of normal maturation of the DLPFC is heavily implicated in ASD and is thought to underpin many ASD symptoms, including behavioural deficits (Bachevalier and Loveland, 2006), with neuroimaging studies also supporting this hypothesis (Schmitz et al., 2007; Sun et al., 2012). This part of our investigation was carried out utilising recently published microarray gene expression data patients with ASD and controls (Chow et al., 2012). We then investigated whether mGluR5 protein levels were altered in the DLPFC of individuals with ASD utilising post-mortem stereological quantitation and investigating the number of mGluR5-positive neurons and glia within the DLPFC of individuals with ASD versus normal controls. Finally, to assess the role of mGluR5 in microglial homeostasis we undertook further stereological quantitation of microglial numbers and somal size in mGluR5 KO mice.

2. Materials and methods

2.1. Microarray gene expression analysis

2.1.1. Gene expression data

Microarray gene expression data were obtained from the National Centre for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) as dataset GSE28475. The authors of the original data set submitted 74 microarray files, generated from RNA extracted from DLPFC brain tissue from 57 individuals (30 ASD and 27 Controls), followed by cDNA synthesis, DASL based labelling and hybridisation to Illumina HumanRef8 v3 microarray. Full details of RNA extraction and processing can be found in the Materials and Methods by Chow et al. (2012).

2.1.2. Interrogation of gene expression candidates

We compared gene expression differences in the DLPFC from individuals with ASD versus controls for mGluR5 and related downstream, signalling elements, including PLCB1, Phosphatidylinositol 4,5-bisphosphate (PIP2), protein kinase C (PKC), Inositol trisphosphate (IP3), mitogen activated protein kinase (MAPK) and SHANK3. In addition, we assessed pro-inflammatory markers interleukin-6 (IL-6), interleukin 1β (IL1β), Interleukin 1 receptor, type I (IL1R1), Allograft inflammatory factor 1 (AIF-1) (also known as ionised calcium-binding adapter molecule 1 (IBA1), tumour necrosis-α (TNF-α), cluster of differentiation molecule 11B (CD11B), Chemokine (C–C motif) ligand 2 (CCL2) and CCL3, nitric oxide synthase-2 (NOS2), Interferon regulatory factor 7 (IRF7) and IRF8 PU.1 that have been shown to be associated with changes in microglial activation.

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