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Altered Gene Expression in Early Postnatal Monoamine Oxidase A Knockout Mice

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Abstract

We reported previously that monoamine oxidase (MAO) A knockout (KO) mice show increased serotonin (5-hydroxytryptamine, 5-HT) levels and autistic-like behaviors characterized by repetitive behaviors, and anti-social behaviors. We showed that administration of the serotonin synthesis inhibitor para-chlorophenylalanine (pCPA) from post-natal day 1 (P1) through 7 (P7) in MAO A KO mice reduced the serotonin level to normal and reverses the repetitive behavior. These results suggested that the altered gene expression at P1 and P7 may be important for the autistic-like behaviors seen in MAO A KO mice and was studied here. In this study, Affymetrix mRNA array data for P1 and P7 MAO A KO mice were analyzed using Partek Genomics Suite and Ingenuity Pathways Analysis to identify genes differentially expressed versus wild-type and assess their functions and relationships. The number of significant differentially expressed genes (DEGs) varied with age: P1 (664) and P7 (3307) [false discovery rate (FDR) <0.05, fold-change (FC) >1.5 for autism-linked genes and >2.0 for functionally categorized genes]. Eight autism-linked genes were differentially expressed in P1 (upregulated: *NLGN3*, *SLC6A2*; down-regulated: *HTR2C*, *MET*, *ADSL*, *MECP2*, *ALDH5A1*, *GRIN3B*) while four autism-linked genes were differentially expressed at P7 (upregulated: *HTR2B*; downregulated: *GRIN2D*, *GRIN2B*, *CHRNA4*). Many other genes involved in neurodevelopment, apoptosis, neurotransmission, and cognitive function were differentially expressed at P7 in MAO A KO mice. This result suggests that modulation of

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these genes by the increased serotonin may lead to neurodevelopmental alteration in MAO A KO mice and results in autistic-like behaviors.

Keywords

serotonin; monoamine oxidase; autism; brain; development

1. Introduction

Monoamine oxidase (MAO) A and B are two mitochondrial isoenzymes that catalyze the deamination of neurotransmitters and dietary amines (Bach et al., 1988). MAO A has a higher affinity for serotonin [5-hydroxytryptamine (5-HT)] and norepinephrine (NE). Phenylethylamine (PEA) is the preferred substrate of MAO B. Both isoenzymes degrade dopamine (DA), tyramine and tryptamine (Shih et al, 1999a). MAO A knockout (KO) mice exhibit aggression (Cases et al., 1995; Scott et al., 2008). MAO A and MAO A/B KO mice showed autistic-like features including decreased ultrasonic vocalization (communication), increased marble burying (repetitive behavior), decreased sniffing (social interaction), and aggression (Bortolato et al, 2013a). MAO A deficiency in humans is characterized by aggression, sleep disorders, and mild mental retardation (Brunner et al, 1993). MAO A and MAO A/B deletion is typified by autistic-like behaviors, developmental delays, short stature, and problems with muscle tone (Bortolato et al, 2013a, Saito et al, 2014). MAO A polymorphisms, resulting in lower promoter activity, have been associated with increased severity of ASDs (Davis et al, 2008).

The etiology of ASDs, which represents a heterogeneous group of diseases including autistic disorder, Asperger's syndrome, and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) remains largely unknown with a higher rate of prevalence amongst males than females (Wijngaarden-Cremers et al, 2014). A variety of neuropathological anomalies in ASD patients have been documented, which includes decreased Purkinje cell number in the cerebellum (Fatemi et al, 2002), increased cell packing, fewer dendrites, and smaller neuronal cell size in the hippocampus (Bauman and Kemper, 2003), and cortical dysgenesis (Santangelo and Tsatsanis, 2005). MAO A KO mice exhibit decreased basilar dendritic length and increased apical dendritic length orbitofrontal cortex in orbitofrontal cortex (Bortolato et al, 2011), reduced corpus callosum thickness, and disrupted microarchitecture of the cerebellum (Bortolato et al., 2013a). MAO A/B KO mice showed significantly altered cerebellar cortex including the loss of Purkinje cells and the enlargement of the molecular layer (Bortolato et al., 2013a).

5-HT is elevated in the blood of approximately one-third of ASD patients (Veenstra-VanderWeele et al., 2012), and has been linked to early postnatal brain overgrowth (Flood et al., 2012). In MAO A KO mice, the brain level of 5-HT is elevated and is particularly high during the first few weeks postnatally (Cases et al., 1995, Bortolato et al., 2008). Attenuating the 5-HT level in MAO A KO mice during early development by administering the 5-HT synthesis inhibitor pCPA from post-natal day 1 (P1) through 7 (P7) reduces repetitive behaviors in adult mice, underscoring the significance of 5-HT in early neurodevelopment

(Bortolato et al., 2013b). The present study was undertaken to identify and characterize differentially expressed genes at P1 or P7 in MAO A KO mice to delineate why parachlorophenylalanine (pCPA) treatment from P1 through P7 reverses its autistic-like behaviors.

2. Results

2.1. Gene expression changes in MAO A knockout mice

The gene expression profiles of age-matched MAO A KO and Wt mice were determined by microarray analysis. RNA was isolated from the brain tissues at P1 and P7 postnatally and converted to cDNA to generate probes (targets) (Tabakoff et al., 2008). Targets corresponding to individual mutant or Wt mice were hybridized with Affymetrix GeneChip arrays. Partek Genomics Suite was then used to identify genes with altered expression in the MAO A mutant. To avoid excluding small yet significant changes in gene expression, DEGs were defined as those with a FDR < 0.05 and a fold-change (MAO A KO versus Wt mice) >1.5 or -1.5 for the analysis of autism-linked genes. For the analysis of functional categories (axonogenesis, axon guidance, growth cone morphology, synaptogenesis, actin reorganization, neuronal differentiation, neuronal migration, neuronal survival, and apoptosis), DEGs were defined as those with FDR < 0.05 and a fold-change (MAO A KO versus Wt mice) >2 or -2. Figure 1 shows the results from comparing differential gene expression between P1 and P7. A greater number of genes were differentially expressed at P7 (3307) than at P1 (664). 297 DEGs were commonly detected at P1 and P7 while 367 unique DEGs were detected at P1 and 3010 unique DEGs at P7.

2.2. Autism-linked genes expressed in MAO A KO mice

Functional annotation database of Ingenuity Pathways Analysis was used to identify genes linked to ASD. Figure 2 shows the fold-change at P1 and P7 for autism-linked genes that are statistically significant (FDR < 0.05). At P1, eight autism-linked genes were differentially expressed with a fold-change greater than 1.5, and, at P7, four autism-linked genes were differentially expressed. At P1, *NLGN3* and *SLC6A2* were upregulated and *HTR2C*, *MET*, *ADSL*, *MECP2*, *ALDH5A1*, and *GRIN3B* were downregulated. At P7, *HTR2B* was upregulated and *GRIN2D*, *GRIN2B*, *CHRNA4* were downregulated.

2.3. Differentially expressed genes in MAO A knockout mice involved in neurogenesis, synaptogenesis, axonogenesis and axon guidance

DEGs linked to neurogenesis and related cellular and developmental processes (axonogenesis, axon guidance, growth cone morphology, synaptogenesis, actin reorganization, neuronal differentiation, neuronal migration, neuronal survival, and apoptosis) were identified using the functional annotation database of Ingenuity Pathways Analysis. Figure 3 displays these DEGs based on fold-change at P7. At P7, among the upregulated genes were *CASP14* (caspase involved in apoptosis), *DDX5* (RNA helicase, a component of the ribonucleoprotein translational regulatory complex), *FCGR2B* (receptor for Fc fragment of IgG that inhibits the function of immune and inflammatory response), *SEMA3F* (secreted protein that regulates axon guidance and promotes migration), and *SIPR2* (G protein-coupled receptor that interacts with the membrane protein Nogo-A, which

limits neurite growth and synaptic plasticity). The downregulated genes include *CAMK4* (calcium/calmodulin-dependent protein kinase type IV that regulates FMR1 to affect protein synthesis for neuronal plasticity), *DNM1L* (dynamin-related GTPase that regulates mitochondrial fission essential for proper brain development), *EXTL3* (putative receptor for Reg-1a, whose overexpression stimulates neurite overgrowth), *SYT1* (integral membrane protein mediating synaptic vesicle fusion and exocytosis of neurotransmitters), *SLC1A1* (sodium-dependent glutamate uptake system), and *SEMA7A* (membrane anchored protein that promotes neurite outgrowth) (Figure 3).

In addition, the expression of *MYH9* (myosin heavy chain 9), *SULF2* (extracellular sulfatase Sulf-2), *ARHG* (a member of the *RAS* homolog gene family), *RPL24* (ribosomal protein L24), *CREB1* (cAMP responsive element binding protein, involved in proliferation of neural progenitor cells), *GPR12* (G-protein coupled receptor 12), *DFNA5* (deafness, autosomal dominant 5), AKAP5 [a kinase (PRKA) anchor protein 5]), *DNAJA* [DnaJ (Hsp40) homolog], and *SLC1A* was decreased at both P1 and P7. The transcription of *NR4A3* (nuclear receptor subfamily 4), *AGTR* (angiotensin receptor), *EGR3* (early growth response 3), *IL1RA* (interleukin 1 receptor antagonist), *NPY2R* (neuropeptide Y receptor Y2), *S100B* (S100 calcium binding protein B, involved in neurite formation), *SALL1* [a human homologue of *Drosophila* Spa/t (*sal*) gene, involved in neuronal differentiation and migration], *SPP1* (secreted phosphoprotein 1, involved in neurite growth), *CALB1* (calbindin 1) was suppressed at P1 only. In contrast, the expression of *IGFBP* (insulin-like growth factor binding protein 3, a neurotrophic factor), *NEO1* (neogenin 1a), *SMO* (smoothed homolog), *SHH* (sonic hedgehog), *SEMA3* and *SIPR2* was increased at both P1 and P7. The transcription of *DPYSL* (dihydropyrimidinase-like 2, function in axonal outgrowth and path finding during brain development) was elevated at P1 only.

Finally, the molecular functions of DEGs were analyzed using the PANTHER (Protein Analysis Through Evolutionary Relationships) database, which categorized them into 10 different molecular functions (Figure 4). Two of these categories, i.e. binding and catalytic activity, were highly represented at both P1 and P7. Transcription regulatory activity also constituted a significant fraction of total DEGs. Despite having different number of DEGs at P1 and P7 in MAO A KO mice, the proportion of genes in each category remained similar at P1 and P7.

Next, the biological processes that may be affected by the gene expression changes were addressed using the Ingenuity Pathways Analysis (IPA) regulation z-score algorithm (Figure 5). A z-score >2 or <-2 predicts that a function will be increased or decreased in MAO A KO vs Wt. In MAO A KO mice, the downstream effect analysis using the algorithm predicted alteration in multiple neuronal activities. For neurodevelopment, it forecasted a reduction in the development of brain (cerebral cortex) and central nervous system, the migration of brain cells, and the formation, extension and growth of neurites. For neurotransmission, it predicted diminished neurotransmission of ileocolonic junction, release of neurotransmitter, and neurotransmission of synapse. For cognitive function, a decline in cognition, learning, long-term potentiation, and memory was predicted. For movement, decreased coordination and spatial learning were prognosticated. In contrast, increased apoptosis (apoptosis of neurons), hyperactivity and seizure disorder were

predicted. The algorithm also predicted a decline in molecular processes pertaining to cytoskeleton (microtubule dynamics, formation of cellular protrusion or actin filaments), nucleotide metabolism (hydrolysis or metabolism of nucleotide), and protein metabolism (ubiquitination, catabolism or transport of protein). The predicted decrease in the size and length of the animal is consistent with the low body weight of postnatal MAO A KO pups observed (Scott et al., 2008).

3. Discussion

The first neurobiological finding for ASD was increased mean head circumference in autistic children compared to normal subjects based on height and weight reported in the first clinical description of autism by Kanner in 1943, which has been replicated by numerous other studies (Kanner, 1943). Accelerated brain growth has been observed through neuroimaging studies conducted during early years and is concurrent with the onset of core ASD symptoms; however, it is unclear whether the brain enlargement occurs later during development or represents increased neurons throughout or in specific regions of the brain. Large and consistent enlargement of the frontal lobes has been described in numerous reports (Carper et al. 2002). Within the frontal lobes, anatomical changes are less distinct, but there is evidence for decreased orbitofrontal cortex (Hardan et al., 2006) and increased medial frontal and dorsolateral prefrontal cortex (Carper and Courchesne, 2005). Yet, these magnetic resonance imaging (MRI) studies are limited by inconsistent delineation of cortical regions, smaller samples sizes, and the use of older and higher-functioning ASD cases. Although the evidentiary value of grey matter enlargement and regional differences remains nebulous, increasingly the reports suggest that the increase in white matter may account primarily for the brain enlargement in autism (Ben Bashat et al., 2007). Although only a few studies of postmortem cortical neuropathology have been reported, the identification of poorly laminated and coarse anterior cingulate cortex, increased thickness, high neuronal density and abnormal patterns of lamination in the cortex represent their key findings (Santangelo and Tsatsanis, 2005).

Functional MRI studies (Valk et al., 2015) and many recent diffusion tensor imaging (DTI) studies (Ameis and Catani, 2015) documented abnormal neural connectivity in ASD. Overall connectivity findings in ASD revealed decreased long-range connectivity (both interhemispheric connectivity and connections to and from the cortex, especially to sensory association and higher-order association areas) and increased local connectivity, including corticocortical connections from layers II and III. These findings of abnormal connectivity are consistent with the view that key ASD-associated deficits concern brain functions that rely on integration of information or coordination of multiple neural systems whereas functions that have lower information processing demands or rely mainly on local neural connections remain unaffected or are enhanced (Minshew and Williams, 2007). Although these studies strongly support the connectivity hypothesis of autism, functional MRI and DTI are relatively nonspecific and cannot directly measure the structure of neural connections due to the lack of resolution. Conventional histological techniques are too painstaking for tracing axons over large distances. The use of fluorescent neural tracers may allow detailed and quantifiable mapping of the connectivity in the MAO A KO mouse model of ASD with elevated serotonin as we have recently demonstrated (Hirtiryan et al., 2016).

Deficits in ASD are thought to be caused by abnormalities in neuronal organization and connectivity, especially in the cortex, which may be due to aberrant synaptogenesis. Recent reports documented distinct neurogligin-3 mutations in mice that augment repetitive behaviors implicated in ASD through selective synaptic impairment in the nucleus accumbens/ventral striatum, which occurs via impeding synaptic inhibition onto D1-dopamine receptor-expressing medium spiny neurons (Rothwell et al., 2014). Changes in the structure and function of synapses and dendrites result from abnormal neurochemistry, with most evidence supporting the role of altered serotonin and glutamate in ASD pathology thus far.

The function of synaptic neurotransmission in the adult brain as well as the maturation of neural networks in the developing brain depends on the proper regulation of neurotransmitter levels. In the adult, serotonin is responsible for the modulation of sleep, appetite, emotions, memory and learning while it is particularly significant in the development of serotonergic neurons and neurons in the cerebral cortex and hippocampus, the targets of the serotonergic system. Since serotonin receptors are found on many glutamate and GABA neurons, serotonergic overdrive may lead to altered GABA and glutamate signaling. Glutamate is a non-essential amino acid, which is involved in synaptic plasticity, learning, memory, sensory perception and aggression. During early development, glutamate levels play a critical role in glutamatergic synapse maturation. The autistic-like behaviors of the MAO A KO mice result from the effects of elevated serotonin on early neuronal development. Increased serotonergic neurotransmission alters glutaminergic and GABAergic pathways and leads to altered expression of genes regulating neurogenesis, synaptogenesis, axonogenesis, and axon guidance in 5-HT, GABA and glutamate target cells. This view is recapitulated by our analysis of DEGs in MAO A KO mice, reinforcing the concept that serotonin plays a critical role in the development of autistic-like behaviors.

Many of the autism-linked DEGs identified function in serotonergic, glutamergic, and/or GABAergic neurotransmission. Following the initial impact of 5-HT elevation, down-regulation of 5-HT receptors (HTRs) would be expected as a compensatory mechanism. HTR2C is a member of serotonin receptor family, which mediates the post-synaptic effect of serotonergic neurotransmission through its G protein coupled receptor (GPCR) activity. The effects of HTR2C are modulated through Gq/G11 activation of signaling through the PLC/DAG/IP3/PKC cascade. 5-HT receptor 2C (HTR2C) was the only serotonin receptor down-regulated in the microarray data and was down-regulated significantly at P1. 5-HT receptor 2B (HTR2B) is located on the pre-synaptic side of serotonergic synapses and mediates inhibition of serotonergic neurotransmission through its effects, which includes promoting the phosphorylation of serotonin transporter (Launay et al., 2006). HTR2B, a pre-synaptic inhibitory receptor, was up-regulated at P7. It suggests that some compensatory mechanisms may be occurring with HTRs in response to increased brain level of 5-HT in MAO A KO mice.

The *CHRNA4* gene encodes the alpha 4 subunit of neuronal nicotinic acetylcholine receptor ($\alpha 4\beta 2$). Activation of $\alpha 4\beta 2$ leads to increased sodium and potassium permeability that causes excitation, which is involved in modulating sensory processing potential transmission in the cortex as well as cognitive processes related to memory and learning. CHRN4A

expression was decreased at P7 in the MAO A KO mice consistent with the previous studies that its expression was decreased in cerebellar cortex of ASD (Martin-Luiz et al., 2004). Decreased nicotinic acetylcholine receptors causing impaired cholinergic neurotransmission in the brains of MAO A KO mice and ASD patients may contribute to the cognitive abnormalities in learning and attention. The effects of cholinergic neurotransmission are mediated through stimulation of 5-HT, NE, and DA release via increased glutamate release, which may point to a mechanistic link with increased serotonin. Since the microarray analysis was performed using whole-brain samples, it remains unclear whether CHRN4A was down-regulated in specific cell types or in certain populations of neurons such as those that stimulate serotonin release.

N-methyl-D-aspartate receptors (NMDARs) constitute a subtype of ionotropic glutamate receptors and represent the main excitatory neurotransmitters in the brain. The *GRIN* (Glutamate Receptor, Ionotropic, NMDA) gene family encodes NMDAR subunits. NMDA receptors are heterotetramers comprised of two NR1 subunits (GRIN1 variants) and two NR2 or NR3 subunits (GRIN2 and GRIN3 variants). For activation, NMDA receptors must bind glutamate as well as glycine and the neuron must be depolarized. The NR2 and NR3 subunits contain the glutamate binding sites. Activation of NMDA receptors on serotonergic neurons by glutamate has an excitatory effect, promoting the release of serotonin. The other group of neurotransmitter receptors differentially expressed in the MAO A KO mice was NMDARs. It was previously suggested that the role of MAO A on aggression may be mediated through NMDARs as NR2A and NR2B subunit levels were increased in adult MAO A KO mice (Bortolato et al., 2012). To the contrary, the microarray analysis showed that the expression of GRIN2B, GRIN2D and GRIN3B was down-regulated. During normal brain development, NR2B and NR2D represent the predominant NR2 subunits in the prenatal and early postnatal periods with the switch to NR2A and NR2C occurring by P15. The NR3 expression follows a similar pattern; it is highly expressed during early development but significantly attenuated in adults. NMDA receptors with NR2B subunits stay open longer upon activation. NR2B subunit is essential as its knockout leads to developmental lethality while NR2A subunit knockout mice are viable (Ohno et al., 2010). As MAO A KO mice were compared to Wt mice, the decrease in GRIN2B and GRIN2D mRNA cannot be attributed to normal developmental processes but may occur due to acceleration or intensification of this phenomenon (i.e. early decrease in NR2B and NR2D). Stimulation of serotonin receptors in adult rats causes increased expression of glutamate decarboxylase (GAD1). GAD1 catalyzes the decarboxylation of glutamate to glutamate and 4-aminobutyric acid (GABA), so elevated serotonin may lead to decreased glutamatergic neurotransmission and increased GABAergic neurotransmission.

NLGN3, neuroligin 3, is a synaptic adhesion-type protein that mediates glutamatergic and GABAergic synapse formation and alignment, affecting the learning and social behaviors (Rothwell et al., 2014). Neuroligin 3 is one of the downstream targets of serotonergic signaling that causes altered glutamatergic and GABAergic synaptogenesis and neurodevelopment. Neuroligins—on what will become the post-synaptic dendritic spine—interact with neurexins on an emerging pre-synaptic axon and induces proper differentiation into a synapse. NLGN3 is associated with autism. Acetylcholine receptors functionally associate with presynaptic cell adhesion molecules including neurexins and neuroligins that

assist in targeting the receptors to the synapse. A mutation in mice (equivalent to that found in a minor subset of ASD cases) causes increased inhibitory synaptic transmission, but knockout of NLGN3 did not have this effect (Tabuchi et al., 2007).

ALDH5A1, mitochondrial NAD(+)-dependent succinic semialdehyde dehydrogenase, is an enzyme that functions in the catabolism of GABA. ALDH5A1 deficiency causes learning disability and its low activity is linked to low IQ (Plomin et al., 2004). Severe adenylosuccinate lyases (ADSL) deficiency leads to a build-up of toxic metabolites that leads to autistic-like behavior, mental retardation, seizures and motor difficulties (Sivendran et al., 2004). ADSL is an enzyme that catalyzes the conversion of N6-(1, 2-dicarboxyethyl) AMP (adenylosuccinate) to fumarate and AMP as part of the purine metabolic cycle.

MECP2, methyl CpG binding protein 2, regulates gene expression through binding to methylated DNA and repressing transcription by condensing the chromatin structure and blocking transcription factors or activating transcription by recruiting CREB1. Its expression increases in neurons as they mature and is completely absent in glial cells. MECP2 interacts with CREB1. MECP2 mutations are implicated in the etiology of Rett Syndrome, a pervasive developmental disorder, through its ability to maintain parental imprinting. Individuals with ASD exhibit abnormal MECP2 expression with decreased long versus short form of MECP2 as well as overall decreased MECP2 in some patients (Samaco et al., 2004).

The human norepinephrine transporter (*NET*) gene (solute carrier family 6, member 2; SLC6A2) has been implicated in various psychiatric disorders including attention deficit hyperactive disorder (ADHD), depression, and autonomic dysfunction (Kim et al., 2008). NET mediates the re-uptake of norepinephrine into presynaptic nerve terminals. Norepinephrine plays a significant modulatory role in brain activities such as attention, learning, emotion and attention.

In the brain, hepatocyte growth factor (HGF) is a survival factor and chemoattractant that causes growth of axons, differentiation of neurons, and proliferation of microglia and Schwann cells. *MET*, Met proto-oncogene, is a hepatocyte growth factor receptor. The MET signaling in neurons may involve the PI3K-AKT pathway and MAP kinase pathways. The MET signaling is required for neural lineage commitment. The HGF/MET signaling is also necessary for the postnatal proliferation of cerebellar granule neurons. This occurs by modulating the migration of specialized neuron types, e.g. transtencephalic migration of interneurons during forebrain development and the migration of olfactory interneuron precursors in the rostral migratory stream (Garzotto et al., 2008). Additionally, MET signaling affects neuronal growth and morphology as its abrogation cause reduction in the size and complexity of the dendritic factors. The loss of MET function leads to abnormal development of GABAergic interneurons in the hippocampus. *MET* is considered a predisposing gene for ASD. Through genotyping, a MET promoter variant (rs1858830 C allele) was identified, which is linked to the onset of ASD (Campbell et al., 2008).

Apoptosis plays a significant role during the brain development as mice deficient in apoptotic pathway genes such as caspase-3 or caspase-9 show severely malformed brain. Though the exact underlying mechanism remains unknown, it is presumed that apoptosis

prevents overgrowth of the neuroepithelium through regulating neural cell numbers in the developing brain. Sequential activation of caspases plays a central role in the execution of apoptosis. Caspase 14 encoded by the *CASP14* gene (increased 3.055 fold at P7; Figure 3), is activated by caspases 9 and caspases 10 and functions in terminal keratinocyte differentiation.

Disruption in neuronal protein synthesis implicated in the pathophysiology of ASD. DDX5 is an RNA-dependent ATPase, an RNA helicase containing a 'DEAD (Asp-Glu-Ala-Asp) box'. It plays a role in the alteration of RNA secondary structure and may function in translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly. DDX5 (increased 4.843 fold at P7; Figure 3) is involved in embryogenesis, cellular growth and division, and spermatogenesis. It is a component of the RNP (ribonucleoprotein) translational regulatory complex that includes JAKMIP1 (Janus kinase and microtubule interacting protein 1), whose expression is altered in ASD patients (Berg et al., 2015). JAKMIP1 knockout mice display ASD-related behaviors with reduced new translation in neurons (Berg et al., 2015).

Humoral response plays an important role in the defense against pathogens. The Fc fragment of IgG possesses a suppressive property of the humoral immunity. This effect is mediated by the interaction of Fc fragments with the cognate receptor (Fc γ R) expressed in many immune cells. Whereas most of these receptors tend to activate, Fc γ RIIB encoded by the *FCGR2B* gene (increased 6.614 fold at P7; Figure 3) has an inhibitory function of immune and inflammatory response. If it is crosslinked to the B cell receptor, it suppresses antibody production.

Alteration in synaptic plasticity and glutamatergic signaling is implicated in the pathogenesis of ASD. Fragile X syndrome is a genetic disorder with a spectrum of intellectual disabilities with approximately 15–60% of the affected exhibiting autism. The syndrome is caused by a CGG trinucleotide expansion in the promoter region of the *FMR1* gene. FMR1, along with TSC1/2 (tuberous sclerosis complex gene 1 and 2), represent the downstream of effectors of metabotropic glutamate receptor GRM1/5 signaling cascade. Activation of the postsynaptic receptor GRM1/5 causes long-term plasticity of synaptic transmission through altering local protein synthesis regulated by FMR1 and TSC1/2. The transcriptional level of the *FMR1* gene is regulated by CAMK4 (decreased 2.964 fold at P7; Figure 3), a calcium/calmodulin-dependent protein kinase type IV. The altered expression of CAMK4 through single nucleotide polymorphism or gene copy number variation is associated with ASD (Waltes et al., 2014).

Multiple observations indicate that mitochondrial dysfunction may contribute to the development of ASD. These include the mitochondrial energy deficiency in ASD lymphoid cell lines, higher rate of mitochondrial hydrogen peroxide production or mitochondrial DNA overreplication in a subset of ASD children, and decreased expression of mitochondrial respiratory chain complexes in the postmortem cerebellum, temporal and frontal lobes of ASD children (Tang et al., 2013). Dynamin-related protein (Drp1) encoded by the *DNM1L* gene (decreased 2.311 fold at P7; Figure 3) is a GTPase that regulates mitochondrial fission. Mitochondria maintain a dynamic reticular network by routinely undergoing fission and

fusion and the process of fission is necessary for mitophagy, i.e. to breakdown and recycle damaged mitochondria. Drp1 is essential for proper brain development as Drp1 knockout mice show impaired synapse formation and neurite growth and die early. In humans, a dominant mutation in DRP1 was associated with early death and microencephaly, abnormal brain development, and optic atrophy (Westermann et al., 2010). This finding is intriguing as numerous mitochondrial genes ranked highly amongst differentially expressed genes in the brain of ASD patients (Ch'ng et al., 2015).

Regenerating islet-derived 1 α (Reg-1 α), a member of a family of secreted protein containing a C-type lectin domain, is involved in the proliferation and differentiation of cells in the digestive system. The expression of Reg-1 α is also detected in fetal and infant brain though very low in adult brain. Its transient overexpression stimulates neurite outgrowth. The deposits of Reg-1 α was found in the brain of very early stage Alzheimer disease patients and may reflect aberrant neuritic sprouting associated with synaptic disconnection and dementia (Acquatella-Tran Van Ba et al., 2012). Its putative receptor is 'exostosin tumor-like 3' protein encoded by the *EXTL3* gene (decreased 2.396 fold at P7; Figure 3). Its expression is also developmentally regulated and contributes to brain development. A girl with delayed mental and motor development, peripheral neuropathy, and ophthalmological abnormalities was shown to harbor chromosome 8 deletion encompassing the *EXTL3* gene (Klopocki et al., 2006).

Defects in synaptic function are increasingly implicated in various mental disorders including autism. The release of neurotransmitters by calcium-triggered synaptic vesicle exocytosis represents one of the key events for the interneuronal communication. The fusion of synaptic vesicles at the presynaptic plasma membrane for the release of neurotransmitters is mediated by SNARE complex formed of synaptobrevin-2/VAMP2, syntaxin-1A/STX1A, and SNAP25, a process regulated by STXBP1 and SYT1 (decreased 2.051 fold at P7; Figure 3). Synaptotagmin-1 encoded by the *SYT1* gene is an integral membrane protein of synaptic vesicles and represents the main calcium sensor in neurons that mediates synaptic vesicle fusion and exocytosis of neurotransmitters. The comprehensive association analysis with single nucleotide polymorphisms performed on a sample of Chinese Han descent identified several candidate susceptibility genes for ADHD (attention deficit hyperactivity disorder), which includes *MAOA* and *STY1* (Guan et al., 2009).

Homeodomain proteins are transcriptional factors that regulate pattern formation during the embryonic development of diverse organisms. They are encoded by *HOX* gene clusters in the genome. The homeobox domain allows sequence-recognition of their target genes. The expression of HOXA1, which encodes a homeodomain protein that functions in the placement of hindbrain segments in the proper location along the anterior-posterior axis during development, was altered in the brain of ASD patients (Ch'ng et al., 2015). In the case of MAOA KO mice, the expression of HOXA5, which is involved in positional specification in the embryonic neuraxis, was altered (increased 2.582 fold at P7; Figure 3).

SEMAPs, semaphorins, represent a family of secreted and membrane proteins important for axonogenesis and axon guidance through chemorepulsion of other neurons. SEMA7A (decreased 2.032 fold at P7; Figure 3) is a glycosylphosphatidylinositol (GPI) membrane-

anchored protein that promotes neurite outgrowth (Scott et al., 2008). SEMA6C is a transmembrane protein that regulates axon guidance. SEMA3F (increased 2.263 fold at P7; Figure 3) is a secreted protein that regulates axon guidance and promotes migration (Nasarre et al., 2003). Whole exome sequencing of genomic DNA from females with ASD implicated SEMA3F in autism (Butler et al., 2015). Genotyping of individuals with ASD in Croatia found chromosomal abnormalities altering the copy number of the genes encoding *SEMA3A*, *SEMA3C*, *SEMA3D*, *SEMA3E* or *SEMA7A* (Wang et al., 2010). The expression of SEMA3 was also increased at P1.

Sphingosine-1-phosphate receptor 2 (S1PR2) represents a G protein-coupled receptor (GPCR) and interacts with the membrane protein Nogo-A, which limits the neurite growth and synaptic plasticity in the central nervous system. S1PR2 (increased 2.367 fold at P7; Figure 3) regulates blood brain barrier (BBB) permeability by destabilizing adherens junctions (Cruz-Orengo et al., 2014). Altered function of BBB is characteristic of multiple sclerosis (MS), which is an inflammatory disorder of the central nervous system with a considerably higher incidence amongst females. A gene ontology study on the role of neurexin-neuroglin-SHANK complex in autism described S1PR2 as relevant to synaptogenesis in the context of ASD (Patel et al., 2015). The S1PR2 expression was also increased at P1.

Glutamate represents the major excitatory neurotransmitter of the vertebrate central nervous system and its extracellular level is tightly regulated to modulate its potency as a neurotoxin, which is achieved by sodium-dependent glutamate uptake systems of the SLC1A (decreased 2.136 fold at P7; Figure 3) family (Gegelashvili and Bjerrum, 2014). Thus far, 5 subtypes of glutamate plasma membrane transporters have been identified, each with varying cellular expression pattern and the corresponding regulatory mechanism. A single nucleotide polymorphism in SLC1A1 has been associated with severe anxiety observed in children with ASD (Gadow et al., 2010). The expression of *SLC1A* was also decreased at P1.

In conclusion, our study identified a number of genes including autism-linked genes whose expression levels are altered at P1 or P7, a critical window during development that is susceptible to pCPA treatment to reverse autistic-like behaviors. Among them are multiple genes whose molecular functions involved with serotonin. Taken together, the results corroborate that modulation of these genes by the increased serotonin may lead to neurodevelopmental alteration in MAO A KO mice, resulting in autistic-like behaviors.

4. Experimental Procedure

4.1 Microarray analysis

RNA was isolated from the whole brain of age-matched MAO A KO and wild-type (Wt) littermates at P1 and P7, and converted to cDNA to generate probes for hybridization (Saba et al., 2006; Tabakoff et al., 2008). Gene expression levels were determined using Affymetrix GeneChip® arrays (Santa Clara, CA). Partek Genomics Suite (PGS) (St. Louis, MO) was used to identify differentially expressed genes between MAO A KO and Wt mice. Differentially expressed genes (DEGs) are defined as MAO KO vs Wt [false discovery rate (FDR) < 0.05, fold-change (FC) > 1.5 for autism-linked genes and > 2.0 for functionally

categorized genes]. Ingenuity Pathway Analysis (IPA) and Biobase (Venlo, The Netherlands) were utilized to integrate the microarray data with functional annotations from the literature. All procedures were performed with the approval from the Institutional Animal Care and Use Committee of the University of Southern California.

4.2 Animals

For MAO A KO mice, we used MAOA^{A1863T} KO mice harboring a spontaneous point mutation (adenine to thymine), which causes a nonsense mutation (lysine to premature stop codon) in exon 8 of the MAO A gene (Scott et al., 2008). The mice was serendipitously isolated in a 129/ScEvTac colony. The mutation is similar to the mutation that causes Brunner syndrome, a recessive X-linked condition associated with mild retardation and violent aggression (Brunner et al., 1993). The mice were housed in cages with access to food and water on a 12 h light cycle following a protocol approved by the University of Southern California Institutional Animal Care and Use Committee.

4.3 Computational analysis of protein functions

To identify and classify the functions of DEGs, we used the PANTHER program containing a curated database of gene or protein families, molecular functions, and biological pathways.

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Abbreviations

MAO	monoamine oxidase
pCPA	para-chlorophenylalanine
DEG	differentially expressed gene
ASD	autism spectrum disorder
PGS	Partek Genomics Suite
IPA	Ingenuity Pathway Analysis
PANTHER	Protein Analysis Through Evolutionary Relationships

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Research Highlights

- First study to show differentially expressed genes in MAO A KO mice at P1 and P7
- Altered genes function in neurogenesis, synaptogenesis, axonogenesis, axon guidance
- Autism-linked genes are altered at P1 and P7 MAO A KO mice
- MAO A KO mice may be used as model for autistic-like behavior with high serotonin

Figure 1. Number of genes differentially expressed in monoamine oxidase A (MAO A) knockout (KO) mice at P1 and P7 are different

Venn diagram of differentially expressed genes (DEGs) at P1 versus P7 from whole brain cDNA microarray. DEGs are defined as MAO KO vs wild-type (Wt) [false discovery rate (FDR) < 0.05, fold-change (FC) >1.5 for autism-linked genes and >2.0 for functionally categorized genes].

Gene Symbol	Fold Change P1 (MAO A KO vs. WT)	Fold Change P7 (MAO A KO vs. WT)	Description	Autism
MET	-2.03	-1.49	Met proto-oncogene, a hepatocyte growth factor receptor	↓
HTR2C	-1.75	-1.29	5-hydroxytryptamine receptor 2C	antagonist for treatment
ALDH5A1	-1.64	-1.29	Aldehyde dehydrogenase 5 family member A1, GABA catabolism	↓
GRIN3B	-1.56	--	Glutamate receptor ionotropic N-methyl-D-aspartate (NMDA) receptor subunit	variants
MECP2	-1.56	-1.32	Methyl CpG binding protein-2, DNA methylation	↓
ADSL	-1.50	-1.38	Adenylosuccinate lyase, purine metabolic cycle	mutation
GRIN2B	1.33	-1.60	Glutamate receptor ionotropic N-methyl-D-aspartate (NMDA) receptor subunit	variants
SLC6A2	1.55	1.15	Solute carrier family 6 neurotransmitter transporter noradrenaline	antagonist for treatment
NLGN3	1.91	-1.38	Neurologin, synaptic adhesion	mutation
GRIN2D	--	-1.77	Glutamate receptor ionotropic N-methyl-D-aspartate (NMDA) receptor subunit	variants
CHRNA4	--	-1.51	Cholinergic nicotinic receptor subunit	↓
HTR2B	--	1.92	5-hydroxytryptamine receptor 2B	antagonist for treatment

Figure 2. Altered expression of autism-linked genes in monoamine oxidase A (MAO A) knockout (KO) mice at P1 and P7

Ingenuity Pathways Analysis (IPA) annotation database was used to identify autism-linked differentially expressed genes (DEGs) [false discovery rate (FDR) < 0.05; fold-change (FC) > 1.5]. Fold change of each gene is displayed at P1 and P7 as well as a gene description and autism association. Up-regulated genes are shown in red and down-regulated genes are shown in blue.

Neurodevelopment		Apoptosis		Neurotransmission		Cognitive Function	
Gene	Fold Change	Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
CAMK4	-2.964	ATRX	-2.298	AP3M2	-1.527	ADCYAP1R1	-2.245
CAPZB	-1.899	C6	2.834	ATP6V0C	-1.586	CALCA	1.989
CLASP2	-1.918	CAMK4	-2.964	DVL1	-1.632	CAMK2A	-1.793
DCLK1	-1.927	CASP14	3.055	GABPA	-1.584	CAMK4	-2.964
DNM1L	-2.311	CASR	2.206	GRIK2	-1.643	CD47	-1.799
DYRK1A	-2.014	CTTN	-2.378	NRXN1	-1.570	GABBR1	-2.029
EXTL3	-2.396	DDX5	4.843	NRXN2	-1.614	GABBR3	-1.887
GJA1	-2.081	EIF4G1	-2.228	PCP4	-1.516	GJA1	-2.081
KRAS	-1.975	FCGR2B	6.614	PRKCB	-1.528	GRIN2D	-1.766
NCK2	-1.962	GAS2	2.991	RAB3A	-1.678	IL2	1.760
NTRK3	-1.886	HOXA5	2.582	SGTA	-1.520	KCNH3	1.791
OPA1	-2.098	IGFBP5	3.051	SIAH1	-1.971	NCAM1	-1.720
PARD3	1.930	NEDD9	2.539	SLC32A1	-1.697	NPAS3	-1.982
POLR3F	-1.952	OPA1	-2.098	SLC8A3	-1.731	NTRK3	-1.886
PRKCE	-1.920	PEBP1	2.638	SST	-1.691	PRKAR1B	-1.869
S1PR2	2.367	PLA2G6	2.623	STX1A	-1.662	RHOB	-1.720
SEMA3D	1.862	PRKRA	2.423	SYT1	-2.051	SLC1A1	-2.136
SEMA3F	2.263	RPS3	3.253	THY1	-1.511	SOD3	1.752
SEMA7A	-2.032	S1PR2	2.367	UCN	-1.799	UBE3A	-1.730

Figure 3. Top differentially expressed genes in 4 functional categories in monoamine oxidase A (MAO A) knockout (KO) mice at P7

The 20 genes with the largest fold change between MAO A KO vs wild-type (Wt) at P7 in each category are displayed. The altered genes in these functional categories: neurodevelopment, apoptosis, neurotransmission, and cognitive function, may explain the autistic-like behavior in the MAO A KO mice. Differentially expressed genes (DEGs) are defined as MAO KO vs Wt [false discovery rate (FDR) < 0.05; fold-change (FC) > 2.0 for functionally categorized genes].

Figure 4. Molecular functions of differentially expressed genes in monoamine oxidase A (MAO A) knockout (KO) mice at P1 and P7 according to PANTHER (Protein Analysis Through Evolutionary Relationships) database

Although the MAO A KO mice had different numbers of differentially expressed genes (DEGs) at P1 and P7, the percentage of genes with each molecular function was largely the same at P1 and P7. The top molecular functions were binding (P1–33%, P7–31%) and catalytic activity (P1–26%, P7–30%). Transcription regulator activity was a significant portion of total genes at P1 (13%) and P7 (7%). DEGs are defined as MAO KO vs wild-type (Wt) [false discovery rate (FDR) < 0.05; fold-change (FC) > 1.5 for autism-linked genes and > 2.0 for functionally categorized genes].

Figure 5. IPA functional analysis of differentially expressed genes in monoamine oxidase A (MAO A) knockout (KO) mouse brain at P7 based on z-score and p-value

Z-score algorithm predicts the biological functions that are expected to be increased or decreased according to the gene expression changes in the data and literature annotations for each gene in the category. A z-score >2 or <-2 predicts that a function is increased or decreased in MAO A KO vs wild-type (Wt). The corresponding *p-values* for the biological functions listed are shown schematically in a graph (with a logarithmic scale) embedded in the figure. Differentially expressed genes (DEGs) are defined as MAO KO vs Wt [false discovery rate (FDR) <0.05 ; fold-change (FC) >1.5 for autism-linked genes and >2.0 for functionally categorized genes].