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# Estabelecimento de modelos de déficit de interação social em *zebrafish* (*Danio rerio*): avaliação de parâmetros neuroquímicos, moleculares e comportamentais

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Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul.

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Dedico este trabalho a minha família, pelo

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## RESUMO

Diversas doenças psiquiátricas e neurológicas são caracterizadas por apresentarem prejuízos no funcionamento social, como os transtornos do espectro do autismo (TEA) e esquizofrenia (SCZ). Neste estudo, realizamos uma triagem comportamental em diferentes períodos do desenvolvimento do peixe-zebra em 6, 30, 70 e 120 dpf (dias pós-fertilização) para estabelecer um modelo de déficit de interação social induzido por ácido valpróico (VPA) na concentração de 48 µM a fim de investigar o comportamento social, locomoção, agressividade e ansiedade. Para analisar o comportamento social cada peixe foi colocado num aquário experimental. De um lado do tanque experimental, foi colocado um aquário vazio e; do outro lado, um aquário de tamanho idêntico com 15 animais, designados como "peixe estímulo". Quantificamos o tempo de permanência do peixe experimental entre o lado do aquário "peixe estímulo" em relação ao aquário vazio. A locomoção dos animais foi analisada avaliando os seguintes parâmetros: distância percorrida, velocidade média, número de cruzamentos e o tempo gasto em cada zona do aquário (superior versus inferior) foi considerado índice de ansiedade. Para analisar a agressiviade os animais foram individualmente colocados em um aquário experimental. Um espelho foi colocado inclinado a 22,5 ° da parede do fundo do aquário, para que a borda esquerda vertical do espelho toque a borda direita do aquário. Assim, quando o peixe virar para o lado esquerdo do tanque sua imagem no espelho aparecerá mais perto, indicando preferência pelo adversário. Nossos resultados demonstram que a exposição ao VPA induz mudanças na atividade locomotora e ansiedade em diferentes períodos do desenvolvimento em peixe-zebra. Além disso, os animais tratados com VPA apresentaram um déficit de interação social aos 70 dpf e 120 dpf. A exposição ao VPA não alterou a agressividade no estágio adulto aos 70 dpf e 120 dpf. Neste estudo, também investigamos a sinalização purinérgica em um modelo de exposição embrionária ao VPA, que induz déficit de interação social em peixe-zebra adultos. Este estudo demonstra a primeira evidência de que a exposição embrionária ao VPA pode modular a hidrólise de nucleotídeos e nucleosídeo e a desaminação de adenosina em membranas de encéfalo de peixe-zebra em 120 dpf. Além disso, analisamos os efeitos da ocitocina, do agonista do receptor da ocitocina (carbetocina) e do antagonista do receptor da ocitocina (L-368,899) na reversão dos efeitos comportamentais induzidos pelo MK-801 em peixe-zebra em relação ao comportamento social, tais como, mudanças na interação social e

agressividade. Estes resultados sugerem o papel dos receptores NMDA e do sistema ocitocinérgico na regulação do comportamento social e agressividade. Este estudo pode contribuir para um melhor entendimento do comportamento social e da sinalização purinérgica sobre as principais desordens neuropsiquiátricas que afetam o comportamento social; como o autismo e a esquizofrenia. Além disso, este estudo apresenta modelos de déficit de interação social induzido pela exposição embrionária ao VPA e exposição ao MK-801 em peixe-zebra adulto. Estes achados também reforçam o envolvimento do sistema ocitocinérgico e o seu potencial farmacológico sobre doenças que afetam o comportamento social.

**Palavras-chaves:** adenosina, ácido valpróico, comportamento social, MK-801, ocitocina, peixe-zebra.

## ABSTRACT

Several psychiatric and neurological diseases are characterized by impairments in social function, such as autism spectrum disorders (ASD) and schizophrenia (SCZ). In this study, we conducted a behavioral screening at different times of zebrafish development at 6, 30, 70 and 120 dpf (postfertilization days) to establish a social interaction deficit model induced by valproic acid (VPA) at 48 µM concentration to investigate social behavior, locomotion, aggression and anxiety. To analyze the social behavior of each fish was placed in an experimental tank. On one side of the experimental tank, an empty fish tank was placed; on the other side, a tank of identical size held 15 zebrafish, which were designed the “stimulus fish”. We quantify the time of the experimental fish from the side of the tank "fish stimulus" in relation to empty tank. The locomotion of the animals was assessed by evaluating the following parameters: distance traveled, average speed, number of crossings and the time spent in each tank position (bottom vs. upper levels) was considered an index of anxiety. To analyze the aggression the animals were individually placed in an experimental tank. A mirror was placed at the side of the tank at an angle of 22.5° to the backwall of the tank so that the left vertical edge of the mirror touched the side of the tank and the right edge was further away.Thus, when the experimental fish swam to the left side of the tank, their mirror image appeared closer to them. indicating preference for the opponent.Our results showed that exposure to VPA induced changes on locomotor activity and anxiety in different periods of development in zebrafish. In addition, VPA-treated animals presented a deficit in social interaction to 70 dpf and 120 dpf. Exposure to VPA did not affect aggression in the adult stage after 70 dpf and 120 dpf. This study also investigated the purinergic signaling in a model of embryonic exposure to VPA, which induced deficit in social interaction in adult zebrafish. This study demonstrates the first evidence that exposure to VPA in embryonic zebrafish in 120 dpf can modulate the hydrolysis of nucleotide and nucleoside and adenosine deamination in zebrafish brain membranes. In addition, we analyzed the effects of oxytocin, the oxytocin receptor agonist (carbetocin) and oxytocin receptor antagonist (L-368,899) on the reversal of behavioral effects induced by MK-801 in zebrafish regarding behavior social, such as, changes in social interaction and aggression. These results support the role of NMDA receptors and

oxytocinergic system in the regulation of social behavior and aggression. This study can contribute to a better understanding of social behavior and purinergic signaling on the main neuropsychiatric disorders that affect social behavior; autism and schizophrenia, as well as to present social interaction deficit models induced by embryological exposure to VPA and exposure to MK-801 in adult zebrafish. These findings reinforce the involvement of oxytocinergic system and its pharmacological potential on diseases that affect social behavior.

**Keywords:** adenosine, MK-801, oxytocin, social behavior, valproic acid, zebrafish.

## LISTA DE ABREVIATURAS

**ADA -** adenosina desaminase

**ADAL -** adenosina desaminase “like”

**ADP -** adenosina 5´- difosfato

**AK -** adenosina quinase

**AMP -** adenosina 5´- monofosfato

**AMPc -** adenosina 5´- monofosfato cíclico

**ATP -** adenosina 5´- trifosfato

**$Ca^{+2}$ -** cálcio

**$Cl^{-}$ -** cloreto

**CNVs** - variações do número de cópia

**DPF** – dias pós-fertilização

**E- 5´NT** - Ecto-5’-nucleotidases

**E-NPPs** - Ecto-nucleotídeo pirofosfatase/fosfodiesterase

**E-NTPDases** - Ecto-nucleosídeo trifosfato difosfoidrolases

**GABA -** ácido gama-aminobutírico

**GPI –** glicosilfosfatidilinositol

**$K^{+}$ -** potássio

**$Mg^{+2}$ -** magnésio

**$Na^{+}$ -** sódio

**NMDA -** N-metil-D-aspartato

**NMDA-R -** receptores de glutamato N-metil-D-aspartato

**NTPDase -** nucleosídeo trifosfato difosfoidrolase

**SCZ -** esquizofrenia

**TEA** - transtornos do espectro do autismo

**VPA** - ácido valpróico

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# 1 INTRODUÇÃO

## 1.1 TRANSTORNOS DO COMPORTAMENTO SOCIAL

As espécies sociais são caracterizadas por formarem organizações que se sobrepõe ao comportamento individual. Estas estruturas organizacionais evoluíram em aspectos psicológicos, neurais, hormonais, celulares e genéticos, constituindo o comportamento social, já que este contribui para a herança genética e sobrevivência dos indivíduos (Cacioppo & Decety, 2011). A investigação dos mecanismos biológicos envolvidos na base das estruturas sociais, comportamento e processos é um elemento essencial para compreender o comportamento humano, vida social, educação e saúde (Cacioppo & Decety, 2011). Diversas doenças psiquiátricas e neurológicas são caracterizadas por apresentarem prejuízos no funcionamento social, como os transtornos do espectro do autismo (TEA) e esquizofrenia (SCZ), descritos a seguir.

### 1.1.1 AUTISMO

Os transtornos do espectro do autismo (TEA) compreendem um grupo heterogêneo e complexo de condições patológicas, incluindo as síndromes de Rett e Asperger, transtorno invasivo do desenvolvimento e autismo (Kumar et al., 2012; Grzadzinski et al., 2013). O termo TEA é usado para descrever um grupo de transtornos do desenvolvimento neurológico infantil, cujo aparecimento é geralmente antes de 3 anos de idade (Charman et al., 2008).

O autismo é diagnosticado com base na história clínica do paciente e normalmente é definido por sintomas relacionados com o desenvolvimento: déficits de comunicação como atrasos na linguagem, falta da língua falada ou incapacidade de sustentar uma conversa, irritabilidade, dificuldades na interação social como reciprocidade emocional, comportamentos repetitivos, padrões estereotipados e interesses restritos, incluindo a aderência inflexível a uma rotina específica e interesses do tipo obsessivo (American Psychiatric Association, 2013; Subramanian et al., 2015). Em termos de memória, os indivíduos com autismo apresentam uma disfunção na habilidade de processar informações complexas de caráter verbal e visual, porém apresentam habilidade de aprendizado associativo e memória de trabalho verbal (Williams et al, 2006). Embora deficiências motoras não sejam atualmente incluídas como sintomas de diagnóstico, vários estudos têm sugerido que anormalidades motoras podem servir como uma característica de identificação precoce do autismo (Landa & Garrett-Mayer, 2006).

Por alguns autores o autismo, é considerado uma desordem encefalopática estática sem qualquer cura específica e algumas intervenções biomédicas podem ser eficazes (American Psychiatric Association, 2013). No entanto, Dawson (2008) relata que o TEA claramente não é uma doença estática, mas sim é caracterizada por alterações dinâmicas no período pós-natal em relação ao cérebro e comportamento. O diagnóstico precoce oportuniza que crianças com TEA se beneficiem com uma intervenção terapêutica, pois o modelo teórico proposto por Dawson (2008) sugere a possibilidade de prevenir algumas manifestações de TEA, aproveitando a plasticidade inicial do cérebro e potencializando modificações em circuitos de recompensa no início do desenvolvimento (Dawson, 2008; Zwaigenbaum et al., 2013). A apresentação clínica da doença pode ser altamente heterogênea, pois alguns indivíduos

apresentam um elevado nível de deficiência intelectual, enquanto outros apresentam um nível médio ou superior de inteligência (Charman et al., 2011).

Ao longo das últimas décadas, a prevalência do autismo teve um aumento expressivo, podendo ser resultado das mudanças nos critérios de diagnóstico e maior conscientização global da doença (Nightingale, 2012). Embora a etiologia permaneça desconhecida, existem vários fatores envolvidos na etiopatogenia do autismo ou TEA, como prejuízo na resposta imune, neuroinflamação, neurotransmissão anormal, estresse oxidativo, disfunção mitocondrial, toxinas ambientais e eventos estressores. Esses achados levaram à hipótese de que um aumento na relação entre a excitação e a inibição sináptica durante um período crítico pode determinar um desenvolvimento disfuncional dos circuitos neurais, causando os sintomas típicos do TEA (Rubenstein & Merzenich, 2003; Gogolla et al. 2009).

O autismo possui uma alta herdabilidade (Dawson, 2008; Devlin et al., 2012). Fatores genéticos são conhecidos por terem um papel no desenvolvimento de muitos transtornos do desenvolvimento neurológico, incluindo TEA (Ginsberg et al., 2012). Um número considerável de casos de autismo é frequentemente associado com uma série de distúrbios genéticos identificáveis, como a síndrome do X frágil (Rutter et al., 1994), esclerose tuberosa, epilepsia e síndrome de Down (Kumar et al., 2012). No entanto, a maioria dos casos é idiopática (Ginsberg et al., 2012). Alterações hereditárias que influenciam a expressão gênica podem ser atribuídas à variação de sequência de DNA, bem como modificações epigenéticas (incluindo a metilação do DNA, modificações pós-tradução de proteínas histonas e silenciamento de RNA) (Coghlan et al., 2012), microdeleções ou microduplicações, conhecidas como variações do número de cópia (CNVs), que são anormalidades cromossômicas submicroscópicas, variando em tamanho de algumas bases até cerca de 500

kilobases (Feuk et al., 2006). Assim, o TEA parece resultar da interação de fatores genéticos e ambientais no período pré-natal e pós-natal (Tchaconas & Adesman, 2013).

É estimado que o autismo possa afetar 1 em 150 crianças, sendo que os homens são mais atingidos que as mulheres em uma proporção de 4:1 para o espectro como um todo (Kuehn, 2007; Brugha et al., 2011; Kim et al., 2011; Fombonne 2003; 2009).

Embora até o momento não haja nenhum marcador genético específico identificado como responsável pela condição, mutações genéticas têm sido observadas em todo o espectro do autismo (Sebat et al., 2007). Vários genes de susceptibilidade ao autismo são estudados, como o gene *En*-2, localizado no cromossomo 7, envolvido no desenvolvimento cerebelar (Cheh et al., 2006), e o gene do transportador de serotonina *SLC6A4* (Devlin et al., 2005). Indivíduos com autismo podem apresentar um alelo variante, o alelo short *5HTTLPR*, envolvido na transmissão serotoninérgica (Devlin et al., 2005). Levitt & Campbell (2009) analisaram o gene *MET* que codifica o receptor tirosina quinase e mostraram uma associação genética com o autismo. Dawson (2008) sugeriu uma associação direta entre a variação genética e o desenvolvimento atípico do cérebro no autismo.

Mutações raras no gene da neuroligina 3 e neuroligina 4, as quais são proteínas expressas nos neurônios, foram encontradas em indivíduos com autismo (Jamain et al., 2003). SHANK3 é outra proteína que está envolvida na via da neuroligina e mutações na *SHANK3* também foram encontradas (Durand et al., 2007). Mutações nos genes *JARID1C* (Adegbola et al., 2008), *HRAS* (Hérault et al., 1993), *SCN2A* (Weiss et al., 2003), *APC* (Zhou et al., 2007), *NLGN4X*, *NLGN3* (Yan et al., 2005) eneurexina 1-beta (Feng et al., 2006) também têm sido associadas com o autismo e disfunção cognitiva.

A literatura recente sugere que os insultos iniciais genéticos e/ou ambientais que causam o autismo convergem para vias e processos celulares (Tchaconas & Adesman, 2013; Subramanian et al., 2015). Estudos neuroquímicos em TEA apontam para o envolvimento de vários sistemas de neurotransmissores, sendo que os sistemas serotoninérgico, gabaérgico, colinérgico e glutamatérgico são intensamente investigados no autismo (Keller & Persico, 2003).

### 1.1.2 ESQUIZOFRENIA

A esquizofrenia é um transtorno psiquiátrico debilitante que prejudica o funcionamento mental e social, afetando cerca de 1% da população mundial (Nagai & Yamada, 2011). Os sintomas apresentam-se de forma heterogênea e podem ser analisados em três domínios: sintomas positivos (alucinações, delírios e transtornos do pensamento), sintomas negativos (déficits na interação social, expressão emocional e motivação) e disfunção cognitiva (prejuízos na atenção, velocidade de processamento, aprendizagem verbal e visual, e memória) (Carpenter et al, 2013; Young & Geyer, 2015). A esquizofrenia geralmente se manifesta na adolescência ou início da idade adulta através de um episódio psicótico. A base do neurodesenvolvimento deste transtorno ganhou maior evidência nas últimas três décadas (Lewis & Levitt, 2002).

De acordo com Thompson & Levitt (2010), o processo fisiopatológico da esquizofrenia ocorre em dois momentos: o distúrbio começa na vida pré-natal ou perinatal, com interrupção do desenvolvimento normal do cérebro (por exemplo, proliferação neuronal,

migração) por fatores genéticos e/ou ambientais, tais como infecções virais, deficiência de vitamina D e hipóxia (Byrne et al., 2007; McGrath et al., 2010). Posteriormente, adversidades sociais na infância e adolescência, tais como situações de *bullying* ou algum evento traumático, também podem promover o aparecimento de sintomas psicóticos (Varese et al., 2012). Estes fatores podem agir sinergicamente com o consumo de *cannabis*, podendo prejudicar o desenvolvimento neuronal, a deposição de mielina, e o equilíbrio normal entre vias inibitórias e excitatórios. Desta forma, estas alterações, podem gerar déficits sociais e cognitivos (Morgan et al., 2014) e, finalmente, o surgimento dos sintomas psicóticos (Insel, 2010).

Alterações em características neuroanatômicas são relatadas em pacientes esquizofrênicos, sendo que o achado mais comum é a diminuição do volume da substância cinzenta no giro temporal superior esquerdo e no lobo temporal medial esquerdo (Honea et al., 2005). Atualmente, os principais sistemas neurotransmissores que têm sido implicados na esquizofrenia são os sistemas dopaminérgico e glutamatérgico.

A desregulação do sistema dopaminérgico desempenha um papel crítico na psicose, transtornos cognitivos, função da recompensa anormal, e alterações no movimento, os quais se manifestam na esquizofrenia (Bissonette & Roesch, 2016; Mier & Kirsch, 2016). As evidências que relacionam a disfunção dopaminérgica com a esquizofrenia demonstram que fármacos, como a anfetamina, que aumentam os níveis de dopamina, pioram os sintomas psicóticos, enquanto que fármacos, como a reserpina, que diminuem os níveis de dopamina, reduzem os sintomas psicóticos (Howes et al., 2012). Os agentes antipsicóticos atualmente mais utilizados também validam esta hipótese: de fato, os primeiros fármacos com eficácia terapêutica para controlar os sintomas positivos foram caracterizados como sendo antagonistas

de receptores de dopamina D2. No entanto, a teoria dopaminérgica não é suficiente para explicar todos os sintomas da esquizofrenia, já que muitos antipsicóticos não tem efeito sobre os sintomas negativos e cognitivos. Além disso, alguns pacientes são refratários às estratégias terapêuticas com base na utilização de antagonistas do receptor de dopamina (Rial et al., 2014).

Outra hipótese para a patofisiologia da esquizofrenia é uma alteração na sinalização glutamatérgica, associada a uma diminuição da função dos receptores de glutamato N-metil-d--aspartato (NMDA-R). Observou-se que antagonistas do receptor NMDA (por exemplo, quetamina, fenilciclidina e MK-801) podem induzir diversos sintomas da esquizofrenia em humanos (Itil et al., 1967; Lefevre et al., 2015). Além disso, estudos mostram que os níveis de glutamato estão alterados em pacientes com esquizofrenia (Poels et al., 2014). A teoria da hipofunção glutamatérgica explica os sintomas positivos e também fornece uma explicação para os sintomas negativos e cognitivos da esquizofrenia (Neill et al., 2010). No entanto, existem questões a serem esclarecidas sobre o impacto dos NMDA-R sobre o funcionamento do circuito global e os efeitos comportamentais dos NMDA-R.

## 1.2 MODELOS FAMACOLÓGICOS DE DÉFICIT DE INTERAÇÃO SOCIAL

### 1.2.1 ÁCIDO VALPRÓICO

O ácido valpróico (VPA) é utilizado como um fármaco anti-epiléptico e estabilizador do humor. No entanto, a exposição pré-natal ao VPA em seres humanos tem sido associada com um aumento da incidência de autismo, além de poder afetar o desenvolvimento do cérebro fetal (Kim et al., 2011). O modelo de autismo utilizando VPA foi desenvolvido com base na observação de que o tratamento da epilepsia ou transtorno bipolar em gestantes que utilizavam VPA leva a um aumento da incidência de autismo em seus filhos (Williams & Hersh, 1997; Werler et al., 2011). Este modelo também é de importância clínica porque a exposição ao VPA durante o primeiro trimestre de gestação em seres humanos pode causar atrasos no desenvolvimento (Genton et al., 2006; Bromley et al., 2008). Assim, diferentes períodos gestacionais correspondem a janelas com distintas vulnerabilidades neuropatológicas e disfunções comportamentais da prole (Meyer et al., 2006).

A exposição ao VPA no dia 12,5 de gestação em roedores (Rodier et al., 1997) provoca alterações no funcionamento de sistemas cerebrais e respostas emocionais (Schneider et al., 2007). Diversos estudos relatam que ratos expostos ao VPA durante o período pré-natal apresentam movimentos repetitivos estereotipados (Schneider et al., 2008), diminuição na interação social (Schneider & Przewlocki, 2005), diminuição na preferência social por novidade (Bambini-Júnior et al., 2011), aumento da ansiedade, reforço nas respostas relacionadas a aprendizagem de medo e prejuízo na extinção da memória de medo (Kim et al.,

2013), sintomas semelhantes aos apresentados pelos pacientes humanos autistas. Um dos principais focos de modelos animais de autismo é mimetizar os déficits sociais da doença (Raza et al., 2015). Desta forma, o modelo animal de exposição ao VPA é útil para estudar os mecanismos envolvidos nos transtornos que afetam o comportamento social (Ranger et al., 2015).

Uma hipótese para a patologia cerebral do autismo consiste na hiper-funcionalidade e excessivo processamento neuronal em microcircuitos neuronais locais no córtex pré-frontal, somato-sensorial e amígdala, levando a alterações sociais e ambientais (Markram et al., 2007). Além disso, este modelo animal de déficit de interação social com VPA sugere que o número de sinapses excitatórias nos circuitos corticais locais é reforçado em TEA (Rinaldi et al. 2008a, b).

Novos modelos animais para estudar os efeitos do déficit de interação social provaram ser de valor por muitas razões. O peixe-zebra já está estabelecido como um excelente modelo para a biologia do desenvolvimento e neurociências (Norton et al., 2013). Portanto, é relevante estabelecer um modelo experimental de déficit de interação social induzido por VPA em peixe-zebra, tendo em vista que os mecanismos neuroquímicos e neurobiológicos pelos quais a interação social se desenvolve ainda não são completamente conhecidos.

### 1.2.2 MK-801

MK-801 é um antagonista não competitivo dos receptores NMDA de glutamato. Estudos indicam que uma hipofunção nos receptores NMDA está envolvida em transtornos que afetam o comportamento social, como a esquizofrenia (Brenner et al., 2005; Rujescu et al., 2006). Modelos animais que utilizam MK-801 apresentam prejuízos no comportamento social, e este antagonista pode ser administrado para simular aspectos do autismo e esquizofrenia (Neill et al., 2010; Seibt et al., 2011).

A administração de MK-801 em camundongos e peixe-zebra é descrita como um bom modelo farmacológico para esquizofrenia (O'Neill & Shaw, 1999; Seibt et al., 2010; Lefevre et al., 2015), já que este antagonista induz tanto os sintomas positivos quanto os negativos da doença (Bickel & Javitt, 2009; Yu et al., 2011). Seibt et al. (2010; 2011) demonstraram que peixe-zebra tratados com MK-801 apresentaram alterações comportamentais características da esquizofrenia, tais como prejuízos locomotores, déficits cognitivos e na interação social, que foram revertidos por antipsicóticos. Desta forma, a sinalização do receptor NMDA pode ser usada como um alvo para aprofundarmos o conhecimento dos aspectos envolvidos na interação social, bem como na modulação farmacológica.

## 1.3 PEIXE-ZEBRA (*Danio rerio*)

O peixe-zebra está firmemente estabelecido como um modelo de pesquisa para muitas áreas da biologia, genética, biologia do desenvolvimento, neurociências, medicina, investigação de doenças humanas e para a descoberta e desenvolvimento de medicamentos

(Liu & Leach, 2011; Matsui et al., 2014; Stednitz et al., 2015), oncologia, imunologia, farmacologia e toxicologia (Jia & Meng, 2012; Lu et al., 2015). É um dos mais importantes modelos de vertebrados e tem um papel cada vez mais expressivo na pesquisa (Xin et al., 2012). O peixe-zebra, também conhecido como paulistinha ou *zebrafish*, é um pequeno teleósteo (3-4 cm) que pertence à família Cyprinidae, originário de plantações de arroz adjacentes ao rio Ganges na Índia (Liu & Leach, 2011). O foco da pesquisa com peixe-zebra inicialmente estava na área de biologia do desenvolvimento, devido às diversas vantagens, tais como: rápido desenvolvimento, embriões transparentes, fecundação e reprodução externas, requer pouco espaço para manutenção, baixo custo e fácil observação comportamental (Beliaeva et al., 2010; Fleming & Rubinsztein, 2011).

O genoma do peixe-zebra foi totalmente sequenciado sendo comparativamente mais fácil o rastreio genético em grande escala para investigar doenças humanas relevantes, como a epilepsia, doença de Alzheimer e doença de Parkinson, doença renal policística e câncer quando comparado com outros modelos animais, como ratos (Xia et al., 2012). Além disso, mostrou-se que o genoma do peixe-zebra possui semelhanças com o genoma humano (Barbazuk et al., 2000; Howe et al., 2013). Novas tecnologias e o conhecimento sobre o genoma do peixe-zebra permitem o desenvolvimento e melhoria de estratégias sofisticadas como a geração de mutantes, morfolinos e tecnologia de microarranjos (Xia et al., 2012; Radev et al., 2015).

Devido a sua capacidade de absorver os componentes diretamente da água pelas suas brânquias e acumular em diferentes tecidos, este modelo têm sido muito utilizado em estudos toxicológicos (Hill et al., 2005; Yang et al., 2009).

Durante a última década, muitos sistemas de neurotransmissão excitatórios e inibitórios foram mapeados no sistema nervoso do peixe-zebra, tais como, sistemas dopaminérgico, serotoninérgico, colinérgico, purinérgico, histaminérgico, nitrérgico, glutamatérgico, glicinérgico e gabaérgico, enfatizando suas características como alvos farmacológicos e toxicológicos (Panula et al., 2010; Rico et al., 2011).

Há um interesse crescente em utilizá-lo em estudos comportamentais, visando à compreensão da base genética do comportamento (Miklósi & Andrew, 2006). O repertório comportamental do peixe-zebra é diversificado e permite a análise de uma série de parâmetros (Miklósi & Andrew, 2006) que podem ser facilmente observados e quantificados. Os aspectos comportamentais incluem alterações na locomoção, como movimentos erráticos, distância percorrida, tempo de permanência, velocidade média e número de cruzamentos (Giacomini et al., 2016)**.** Além disso, estudos avaliando características comportamentais do peixe-zebra foram desenvolvidos, envolvendo a análise da atividade locomotora, agressividade, interação social e aprendizado (Cognato et al., 2012; Karnik & Gerlai, 2012; Bailey et al., 2013). O rápido aprendizado e o envolvimento de mecanismos evolutivamente conservados permitem a caracterização de eventos envolvidos na formação da memória enquanto a clareza da resposta aprendida garante que déficits cognitivos sejam facilmente identificáveis.

O peixe-zebra apresenta comportamentos sociais altamente complexos, assim está emergindo como um importante modelo vertebrado para estudos de neurodesenvolvimento e investigação do comportamento social (Zimmermann et al., 2015). Uma característica marcante do seu comportamento é a interdependência dos membros do grupo, relações sociais ordenadas e interação social (Kalueff et al, 2014).

O peixe-zebra apresenta genes homólogos aos de mamíferos, os quais estão relacionados a transtornos psiquiátricos. Alguns estudos analisaram genes envolvidos no autismo e esquizofrenia, sendo que alguns desses também estão presentes no peixe-zebra. Morrow et al. (2008) analisaram a deleção do gene *c3orf58* implicado na etiologia do TEA. O gene foi, portanto, nomeado *DIA1* (gene de deleção do autismo-1). Aziz et al. (2011) caracterizaram o *DIA1* implicado no TEA e esquizofrenia. O genoma do peixe-zebra codifica dois genes estreitamente relacionados ao *DIA1, DIA1a e DIA1b* (Aziz et al., 2011). Outros genes relacionados são: AUTS2 (Oksenberg et al., 2013), 16p11.2 (Blaker-Lee et al., 2012; Maillard et al., 2015), gene do X frágil e retardo mental (*FMR1*), gene da subunidade GABAA β3 (*GABRB3), SHANK3, TSC1, NEUROLIGINA3 e NEUROLIGINA4* (Wright & Washbourne, 201; Zhang et al., 2015). Portanto, esses dados reforçam a utilização do peixe-zebra como modelo para investigar doenças que afetam o comportamento social.

## 1.4 OCITOCINA

A ocitocina é um hormônio neuroendócrino clássico, mas nos últimos 20 anos, emergiu como um influente hormônio liberado no cérebro que pode dar início a um amplo espectro de efeitos centrais (Baskerville & Douglas, 2010). A ocitocina é sintetizada principalmente no hipotálamo pelas células neurosecretoras do núcleo supraóptico e núcleo paraventricular, onde é transportada para a neuro-hipófise e liberada para o sangue. O receptor de ocitocina é um polipeptídeo de 389 aminoácidos, possui sete domínios transmembrana e faz

parte da família de receptores acoplados à proteína G. Quando a ocitocina se liga ao seu receptor, inicia uma cascata de eventos intracelulares (Gimpl & Fahrenholz, 2001). O receptor de ocitocina pode ligar-se a várias proteínas G, levando a diferentes efeitos funcionais, podem ser acoplados a Gq/11 que leva a ativação da fosfolipase Cβ, acúmulo de fosfatidilinositol-1,4,5-trifosfato e mobilização de $Ca^{+2}$ intracelular. A ocitocina também pode acoplar-se a Gi/o, exercendo efeitos antiproliferativos. Evidências indicam que a ocitocina pode também ativar a adenilato ciclase e aumentar o AMPc (Chaves et al., 2013).

Alterações no sistema da ocitocina podem ser associadas a diversos transtornos neuropsiquiátricos, como, por exemplo, o autismo e esquizofrenia (Feifel et al., 2016; Leckman et al., 1994). Modahl et al. (1998) relataram que a concentração plasmática de ocitocina é reduzida em crianças com autismo. Estudos também descobriram uma associação do gene do receptor da ocitocina e autismo (Wu et al., 2005; Jacob et al., 2007). Hollander et al. (2007) demonstraram que a administração intravenosa de ocitocina reduz os comportamentos repetitivos e aumenta a compreensão do afeto em indivíduos com TEA. Além disso, Rubin et al. (2010) demonstrou que níveis séricos elevados de ocitocina têm sido associados com sintomas positivos menos graves de psicose e com maior comportamento pró-social em pacientes com esquizofrenia.

Em peixes, a ocitocina tem efeitos sobre a resposta social, mudanças de sexo socialmente induzidas, vocalizações relacionadas à reprodução, comportamento de corte e comportamento social (Goodson & Bass, 2000; Black et al., 2004). A isotocina, também encontrada em teleósteos, é homóloga a ocitocina, mas está associada com o equilíbrio osmótico interrenal e iônico (Chou et al., 2011). No peixe, as células produtoras de ocitocina estão localizadas na área preóptica, enquanto que nos mamíferos, as células produtoras de

ocitocina localizam-se no núcleo paraventricular (PVN) e hipotálamo anterior (Eaton & Glasgow, 2006).

Braida et al. (2012) demonstraram que a ocitocina e vasopressina em peixe-zebra aumentaram o comportamento social e reduziram a resposta de medo ao predator, indicando um papel neuromodulatório nestes comportamentos complexos. Além disso, Filby et al. (2010) mostraram uma redução da agressividade após tratamento com vasopressina.

## 1.5 SISTEMA PURINÉRGICO

No sistema purinérgico, o ATP e seus metabólitos, ADP, AMP e adenosina, produzem uma grande variedade de efeitos fisiológicos que não estão necessariamente relacionadas com o seu papel no metabolismo energético.

Drury & Szent-Györgyi, em 1929, demonstraram pela primeira vez as potentes ações extracelulares do ATP e adenosina sobre o coração e vasos sanguíneos coronários. A hipótese da sinalização purinérgica, com o ATP como molécula responsável pela transmissão não adrenérgica e não colinérgica, no músculo liso do intestino e da bexiga foi proposta por Burnstock em 1972.

Os nucleosídeos e nucleotídeos derivados de purinas atuam como moléculas sinalizadoras extracelulares em vários tecidos e estão envolvidos em muitos mecanismos neuronais e não neuronais e em eventos de curta e longa duração, incluindo respostas imunes, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (Burnstock, 2014). A sinalização purinérgica está envolvida no trauma, epilepsia,

doenças neurodegenerativas, desordens neuropsiquiátricas e do humor e câncer (Burnstock, 2012; 2015). O ATP pode ser liberado nos terminais pré- e pós-sinápticos. Esta liberação pode ocorrer tanto como um mecanismo fisiológico ou em resposta a danos celulares, como hipóxia e injúrias (Burnstock, 2008).

A ação dos derivados das purinas se dá através dos receptores purinérgicos. A base para distinguir dois tipos de purinoceptores identificados como P1 (para adenosina) e P2 (para ATP e ADP) foi descrita por Burnstock em 1978 (Burnstock, 1978). Diversas evidências têm demonstrado o importante papel desempenhado por essas moléculas no SNC (Dunwiddie & Masino, 2001).

O ATP é reconhecido como um neurotransmissor, pois é sintetizado e armazenado em terminais sinápticos e liberado após estímulo destes terminais. Além disso, esse nucleotídeo é considerado uma molécula chave na sinalização presente no SNC, e recebe maior atenção devido ao seu papel como mensageiro durante diferentes eventos fisiológicos e patológicos (Tu & Wang, 2009). O ATP também atua como um co-transmissor sendo liberado com outros neurotransmissores clássicos no sistema nervoso periférico e central, apesar das proporções apresentarem variação entre as espécies e os tecidos e em diferentes condições de desenvolvimento e processos fisiológicos e patológicos. Esse nucleotídeo é liberado juntamente com a noradrenalina, neuropeptídeo Y, acetilcolina, óxido nítrico, glutamato, dopamina e serotonina (Burnstock, 2009).

O ATP exerce suas funções através da ativação de receptores purinérgicos do tipo P2. Este grupo de purinoreceptores é subdivido em duas famílias distintas de acordo com a base do mecanismo de ação, farmacologia e clonagem molecular (Burnstock & Kennedy, 1985; 2011). A primeira família é constituída pelos receptores P2X, os quais são ionotrópicos e estão

distribuídos em neurônios, células gliais e no músculo liso (Burnstock, 2006). A segunda família é constituída pelos receptores metabotrópicos P2Y, acoplados a proteína G e apresentam uma ampla distribuição nos tecidos (Burnstock & Knight, 2004; Burnstock, 2010). Os receptores do tipo P2X estão divididos em sete membros ($P2X_{1-7}$) que são ligados a canais iônicos, e quando ativados levam à abertura de um poro na membrana celular que permite a passagem dos íons $Na^{+}$, $K^{+}$ e $Ca^{+2}$. Estes receptores interagem com vários receptores ionotrópicos, incluindo receptores colinérgicos nicotínicos, receptores $GABA_{As}$ e receptores NMDA. Os mecanismos para estas interações parecem ser mediados pela fosforilação destes receptores, através da ação de cinases ativadas por $Ca^{+2}$ intracelular (Burnstock, 2010). Até o presente momento, foram descritos oito membros na família de receptores P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 e P2Y14) (Burnstock & Kennedy, 2011).

A clonagem e caracterização molecular dos receptores P2X em peixe-zebra já foram realizadas (Diaz-Hernandez et al., 2002). Kucenas & colaboradores (2003) mostraram que a subunidade P2X possui nove membros, sendo seis ortólogos a genes dos receptores P2X de mamíferos, dois parálogos em peixe-zebra e um gene que ainda precisa ser devidamente classificado (Kucenas et al., 2003). Os subtipos dos receptores P2X do peixe-zebra contêm resíduos altamente conservados, os quais são encontrados em subunidades de mamíferos.

Após ser liberado no espaço extracelular e exercer suas ações via receptores específicos, o ATP pode ser metabolizado pela ação de ecto-enzimas que fazem a conversão deste nucleotídeo até adenosina (Fredholm, 2011; Bonan, 2012). A ação da adenosina se dá através da ativação de receptores purinérgicos, do tipo P1 acoplados a proteínas G. Estes receptores são divididos em 4 subtipos de acordo com suas características, tais como estrutura molecular, distribuição tecidual e afinidade pelo seu ligante. São eles: o receptor $A_1$, $A_{2A}$, $A_{2B}$

e $A_3$. Os receptores $A_1$ e $A_3$ se ligam à família das proteínas $G_{i/o}$, responsáveis pela inibição da produção do segundo mensageiro AMP cíclico (AMPc). Os receptores $A_{2A}$ e $A_{2B}$ estimulam a produção de AMPc via ativação de proteínas $G_s$. A porção N-terminal do receptor está voltada para o meio extracelular e a porção C-terminal está voltada para o lado citosólico da membrana plasmática (Fredholm, 2011). A caracterização dos receptores $A_1$, $A_{2A1}$, $A_{2A2}$ e $A_{2B}$ foi realizada em peixe-zebra (Capiotti et al., 2011).

A adenosina extracelular é um importante neuromodulador do sistema nervoso e desempenha um papel crucial na excitabilidade neuronal e na transmissão sináptica e não-sináptica, modulando a liberação de neurotransmissores, as respostas pós-sinápticas e a ação de outros sistemas além de proteger o organismo em caso de isquemia e injúrias (Lane et al., 2011; Sperlágh & Vizi, 2011). Devido a este papel neuromodulador, a adenosina está envolvida na regulação de importantes mecanismos no SNC, podendo participar na regulação do sono, cognição, memória e nocicepção (para revisão Burnstock et al., 2011). Além disso, já foi sugerido que esse nucleosídeo pode estar relacionado com a patofisiopatologia de algumas doenças neurodegenerativas e neuropsiquiátricas, visto que os níveis de adenosina no cérebro se encontraram alterados em doença de Alzheimer, doença de Parkinson, epilepsia e esquizofrenia (Kovács et al., 2013).

São poucos os estudos que investigam o envolvimento do sistema purinérgico no autismo. Wyatt et al. (2013) mostraram que o receptor P2X4 está envolvido nas anormalidades fenotípicas, como funções sociocomunicativas, semelhantes às observadas em outros modelos murinos de TEA. Assim, os receptores P2X4 podem ser alvos para distúrbios associados com transtornos do desenvolvimento neurológico. Além disso, Masino e colaboradores (2011; 2013) relataram uma relação entre a adenosina no sistema nervoso central e autismo em

termos de sintomas e comportamentos, demonstrando o envolvimento da adenosina no comportamento social (Masino et al., 2011).

### 1.5.1 ECTONUCLEOTIDASES

Os nucleotídeos extracelulares podem ser hidrolisados por uma variedade de enzimas solúveis ou localizadas na superfície da célula, possuindo seu sítio ativo voltado para o meio extracelular denominadas ectonucleotidases (para revisão Bonan, 2012). Diversas enzimas constituem o grupo das ectonucleotidases, formado por quatro famílias de enzimas: Ecto-nucleosídeo trifosfato difosfoidrolases (E-NTPDases), Ecto-nucleotídeo pirofosfatase/fosfodiesterase (E-NPPs), fosfatases alcalinas e Ecto-5'-nucleotidases (E- 5´NT) (Robson et al., 2006; Abbracchio et al. 2009). Desta forma, as ectonucleotidases contribuem para o controle da disponibilidade de ligantes (ATP, ADP, AMP e adenosina) para os purinoreceptores e, conseqüentemente, a extensão e a duração da ativação destes receptores (Chen & Guidotti, 2001).

As E-NTPDases podem hidrolisar nucleosídeos 5´-trifosfatados e nucleosídeos 5´-difosfatados com ampla preferência, distribuição em diferentes tecidos e localização celular (Robson et al., 2006; Bonan, 2012). Os membros da família das NTPDases são codificados por oito genes diferentes, chamados de genes *ENTP*. Dos oito membros descritos até o momento, quatro estão localizados na membrana celular com o sítio ativo voltado para o meio extracelular (NTPDases 1, 2, 3 e 8). As NTPDases 5 e 6 se localizam intracelularmente, porém são secretadas após expressão heteróloga. As NTPDases 4 e 7 apresentam localização

intracelular com o sítio ativo voltado para o lúmen de organelas citoplasmáticas (Zimmermann, 2000; Robson et al., 2006). Os membros desta família diferem em relação à preferência pelo substrato; neste caso, nucleosídeos 5´-tri e difosfatados.

A Ecto-5´-NT desfosforila nucleosídeos monofosfatados não cíclicos, através da hidrólise da ligação fosfodiéster de 5´-ribonucleotídeos, levando à formação do correspondente ribonucleosídeo e fosfato. A principal função em animais é a hidrólise de AMP até adenosina (para revisão Bonan, 2012). As Ecto-5´-NT apresentam uma ampla distribuição tecidual sendo a principal enzima responsável pela formação extracelular de adenosina (Zimmermann, 2000).

No peixe-zebra, estudos demonstraram a presença de uma NTPDase e uma Ecto-5´-NT em membranas encefálicas. Estas duas enzimas foram caracterizadas como cátion-dependentes, apresentando atividade máxima à temperatura de 37 °C, pH ótimo entre 7,2 e 8,0, $K_M$ na faixa do micromolar e uma ampla especificidade por outros nucleotídeos (Rico et al., 2003; Senger et al., 2004). Estudos do nosso laboratório identificaram a presença de três isoformas diferentes de NTPDase 2 (NTPDase2mv, NTPDase2mg, NTPDase2mq) em encéfalo de peixe-zebra (Rico et al., 2006). A co-localização da NTPDases 1 e 2 já foi descrita em células de retina de peixe-zebra (Ricatti et al., 2009) e a NTPDase 3 foi caracterizada em neurônios sensoriais desta espécie (Appelbaum et al., 2007). A NTPDase4, duas isoformas da NTPDase5, sendo NTPDase5_ms e NTPDase5_me, NTPDase6 e NTPDase8 também já foram identificadas nesta espécie (Rosemberg et al., 2010). A hidrólise dos nucleotídeos em peixe-zebra adultos aumenta em função da concentração de proteína e a formação de produto mostra-se linear na taxa de 3-10 μg de proteína para cérebro e fígado e de 3-5 μg de proteína

para coração. O perfil da expressão gênica foi realizado, demonstrando a presença de diferentes níveis de transcritos das NTPDases em cérebro, coração e fígado desta espécie (Rosemberg et al., 2010).

Al-Mosalem et al. (2009) avaliaram as atividades ADPásica e ATPásica da NTPDase em plasma sanguíneo de pacientes autistas. Um aumento na hidrólise de ADP foi observado em plasma sanguíneo em crianças autistas, enquanto que a hidrólise de ATP pela NTPDase foi semelhante em comparação aos pacientes do grupo controle. Conforme Dorneles et al. (2008), este poderia ser um importante mecanismo de proteção para aumentar a concentração de adenosina. Naviaux e colaboradores (2014) verificaram que a terapia antipurinérgica foi capaz de reestabelecer o comportamento social num modelo de TEA. Assim, o metabolismo das purinas é uma importante via a ser investigada.

### 1.5.2 ADENOSINA DESAMINASE

O controle da sinalização adenosinérgica pode ser exercido pela captação de adenosina via transportadores bidirecionais, seguida pela fosforilação do AMP intracelular pela adenosina quinase (AK) ou desaminação pela adenosina desaminase (ADA) (Fredholm et al., 2007).

O nível intracelular é regulado pelo equilíbrio de diversas enzimas. A adenosina é formada pela ação de uma 5’-nucleotidase seletiva para AMP e, esta via é controlada principalmente pela quantidade de ATP e ADP. Portanto, um fator importante para determinar a taxa de formação de adenosina através deste caminho é a taxa relativa de hidrólise de ATP e

síntese de adenosina. Estas são determinadas, por sua vez, pela taxa de utilização da energia e da disponibilidade de substrato, principalmente o oxigênio, reduzido na síntese de ATP (Fredholm & Lerner, 1982).

Dessa forma, a ADA é uma enzima envolvida no metabolismo de purinas, responsável pela desaminação hidrolítica de adenosina à inosina e amônia. Como resultado de sua atividade enzimática, a ecto-ADA contribui para a concentração de adenosina na fenda sináptica (Martinez-Navio et al., 2010). Existem diferentes membros de ADA que incluem ADA1, ADA2, e uma seqüência de aminoácidos similar chamada de adenosina desaminase-like (ADAL) (Maier et al., 2005).

Em relação aos diferentes membros de proteínas relacionados à ADA, tem sido demonstrado que quase todas as atividades da ADA em humanos são atribuídas a ADA1 (Zavialov & Engstrom, 2005). Apesar da sua localização intracelular, ADA1 também atua como uma ecto-ADA, clivando adenosina extracelular (Franco et al., 1997).

Rosemberg e colaboradores (2007) demonstraram a existência de diferentes genes relacionados à ADA, com um padrão de expressão em diversos tecidos em peixe-zebra. Além disso, Rosemberg e colaboradores (2008) descreveram a cinética enzimática e propriedades da desaminação de adenosina a partir do encéfalo de peixe-zebra e a presença de atividade da ADA nas membranas encefálicas, sugerindo a existência de uma ecto-ADA em peixe-zebra. Assim, sugeriu-se que a desaminação da adenosina no SNC de peixe-zebra promovida por diferentes membros da família da ADA pode ser um elemento-chave para o controle da adenosina/inosina no meio intracelular e extracelular (Rosemberg et al., 2008).

Uma hipótese para o mecanismo patológico do desenvolvimento de sintomas autistas seria uma deficiência de adenosina desaminase (Okada et al., 1999; Benvenuto et al., 2009). Estudos demonstraram que uma redução dos níveis de adenosina produz sintomas comportamentais e fisiológicos típicos do autismo. Em roedores e humanos, foi demonstrado que o aumento dos níveis de adenosina pode melhorar comorbidades encontradas no autismo (Masino et al., 2013). Freitag et al. (2010) sugeriram um possível papel das variantes de ADORA2A na expressão fenotípica em TEA. Os receptores de adenosina podem ser novos alvos para o tratamento dos comportamentos repetitivos em autismo (Tanimura et al., 2010).

# 2 OBJETIVOS

## 2.1 OBJETIVO GERAL

O objetivo deste estudo é estabelecer um modelo animal de déficit de interação social induzido por VPA em peixe-zebra e avaliar a participação do sistema de neurotransmissão purinérgica. Além disso, investigar no modelo de déficit de interação social induzido por MK-801 em peixe-zebra a influência da ocitocina por meio de análise comportamental.

## 2.2 OBJETIVOS ESPECÍFICOS

- Padronizar e analisar as diferentes respostas comportamentais características do autismo induzidas por VPA, como: locomoção, interação social e agressividade no 6º, 30º, 70º e 120º dpf;

- Determinar a atividade enzimática e a expressão gênica das E-NTPDases, ecto-5´-nucleotidase e ADA em membranas e homogenato de encéfalo de peixe-zebra submetidos ao modelo de déficit de interação social induzido por VPA na idade de 120 dpf;

- Analisar o metabolismo do ATP em encéfalo de peixe-zebra submetidos ao modelo animal de déficit de interação social induzido por VPA, através da cromatografia líquida de alta eficiência (HPLC) em encéfalo de peixe-zebra na idade de 120 dpf;

- Analisar os níveis de glutamato, serotonina, dopamina e seus metabólitos em peixe-zebra submetidos ao modelo animal de déficit de interação social induzido por VPA, através da cromatografia líquida de alta eficiência (HPLC) em encéfalo de peixe-zebra na idade de 120 dpf;

- Analisar os efeitos da ocitocina na interação social em peixe-zebra adultos submetidos ao modelo de déficit de interação social induzido por MK-801;

- Analisar os efeitos do agonista (Carbetocina) e do antagonista (L-368,899) do receptor de ocitocina na interação social em peixe-zebra adultos submetidos ao modelo de déficit de interação social induzido por MK-801;

- Analisar os efeitos da ocitocina na agressividade em peixe-zebra adultos expostos ao MK-801;

- Analisar os efeitos do agonista (Carbetocina) e do antagonista (L-368,899) do receptor de ocitocina na agressividade em peixe-zebra adultos expostos ao MK-801.

## 3 RESULTADOS

## CAPÍTULO I

## ARTIGO CIENTÍFICO

*Embryological exposure to valproic acid induces social interaction deficits in zebrafish (Danio rerio): A developmental behavior analysis.*

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# Embryological exposure to valproic acid induces social interaction deficits in zebrafish (*Danio rerio*): A developmental behavior analysis

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ABSTRACT

Changes in social behavior are associated with brain disorders, including mood disorders, stress, schizophrenia, Alzheimer's disease, and autism spectrum disorders (ASD). Autism is a complex neurodevelopmental disorder characterized by deficits in social interaction, impaired communication, anxiety, hyperactivity, and the presence of restricted interests. Zebrafish is one of the most social vertebrates used as a model in biomedical research, contributing to an understanding of the mechanisms that underlie social behavior. Valproic acid (VPA) is used as an anti-epileptic drug and mood stabilizer; however, prenatal VPA exposure in humans has been associated with an increased incidence of autism and it can also affect fetal brain development. Therefore, we conducted a behavioral screening at different periods of zebrafish development at 6, 30, 70, and 120 dpf (days postfertilization) after VPA exposure in the early development stage to investigate social behavior, locomotion, aggression, and anxiety. VPA (48 μM) exposure during the first 48 hpf (hours postfertilization) did not promote changes on survival, morphology, and hatching rate at 24 hpf, 48 hpf, and 72 hpf. The behavioral patterns suggest that VPA exposure induces changes in locomotor activity and anxiety at different developmental periods in zebrafish. Furthermore, a social interaction deficit is present at 70 dpf and 120 dpf. VPA exposure did not affect aggression in the adult stage at 70 dpf and 120 dpf. This is the first study that demonstrated zebrafish exposed to VPA during the first 48 h of development exhibit deficits in social interaction, anxiety, and hyperactivity at different developmental periods.


## 1. Introduction

Several psychiatric and neurological disorders are characterized by alterations in the social domain (Kennedy and Adolphs, 2012). Impairments in social behavior abilities are considered features associated to mood disorders, stress, anxiety, schizophrenia, Alzheimer's disease, and autism spectrum disorders (American Psychiatric Association, 2013; Burns, 2006; Djukic and McDermott, 2012; Mahoney et al., 2014; Pelphrey et al., 2011; Rapin and Tuchman, 2008).

Autism spectrum disorders (ASD) comprise a heterogeneous group of neurodevelopmental disorders, which include Asperger's disorder, pervasive developmental disorder, childhood disintegrative disorder, Rett's disorder, and autism disorder (Grzadzinski et al., 2013). Autism is a complex neurodevelopmental disorder characterized by core symptoms related to stereotyped movements of behavior, deficits in social interaction, impaired communication, anxiety, hyperactivity, and the presence of restricted interests (Canitano, 2014; Schneider and Przewłocki, 2005). ASD has several forms and levels of severity; the clinical presentation of core symptoms can be heterogeneous because autistic patients exhibit moderate to severe symptoms, as well as different intellectual (Charman et al., 2011) and language profiles (Tager-Flusberg and Caronna, 2007). ASD are highly heritable (~90%) and are considered the most heritable brain disorder in humans (Constantino et al., 2013; Zafeiriou et al., 2013). Multiple genes, several cellular pathways and disordered molecular pathways (Pinto et al., 2014) have been implicated in the development of autism. Furthermore, environmental factors may also contribute to the risk of ASD (Banerjee et al., 2014; Persico and Bourgeron, 2006; Raff, 2014).

Currently, the neurological, behavioral, and genetic bases of autism represent a major problem for researchers because of the uncertainty of the origin of this neuropathology. Animal models of brain disorders are essential for the investigation of neuropathology development and

*Abbreviations:* ASD, autism spectrum disorders; dpf, days postfertilization; 5-HTT, serotonin transporter; VPA, valproic acid.

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provide a basis for new therapeutic approaches. The most commonly studied animal models use rodents; however, data indicate that several aspects of almost all brain disorders that can be studied in rodents can be modeled in zebrafish because this teleost is an excellent tool not only for basic research but also for the study of translational neurosciences (Kalueff et al., 2014; Miller and Gerlai, 2011). Zebrafish have been proposed as a model of Alzheimer's disease (Nery et al., 2014; Newman et al., 2014), schizophrenia (Seibt et al., 2011), drug abuse (Collier et al., 2014), and other brain disorders (Kalueff et al., 2014; Siebel et al., 2013). Zebrafish represent an useful model because they are one of the most social vertebrates used in biomedical research (Saverino and Gerlai, 2008). A remarkable feature of their behavior is the interdependence of the group members, which exhibits ordered social relationships and social interaction (Kalueff et al., 2014; Gerlai, 2014). Gerlai et al. (2000) identified preferences of animals for a group of animals of the same species. It is important to consider that these behaviors can change throughout zebrafish development (Buske and Gerlai, 2011). Furthermore, some behavioral assays for complex social interactions are easier to perform in zebrafish (Maaswinkel et al., 2013; Mahabir et al., 2013; Miller and Gerlai, 2011). Therefore, zebrafish represent an animal model that can significantly contribute toward understanding the mechanisms that underlie social behavior (Grossman et al., 1997; Morrison and Bellack, 1987).

Valproic acid (VPA) is extensively used as an antiepileptic and mood stabilizing drug; however, prenatal VPA exposure in humans has been associated with an increased incidence of autism, and it can also affect fetal brain development and decrease social interaction in rodents (Kim et al., 2011; Markram et al., 2007; Schneider and Przewłocki, 2005). VPA exposure produces similar patterns of abnormal development across species (Menegola et al., 1998; Petrere et al., 1986). This is a well-established animal model used to induce autism because it causes morphological and behavioral changes associated with the pathophysiology of autism (Arndt et al., 2005; Markram et al., 2007; Rodier et al., 1997; Schneider and Przewłocki, 2005); these symptoms predominately include alterations in social behavior, such as deficits in social interaction and anxiety symptoms.

Therefore, we conducted a behavioral screening at different periods of zebrafish development at 6, 30, 70, and 120 dpf (days postfertilization) after VPA exposure in the early development stage to investigate social behavior, locomotion, aggressiveness, and anxiety.

## 2. Materials and methods

### 2.1. Animals

Adult wild type zebrafish were maintained and bred according to standard procedures in an automated re-circulating system (Tecniplast, Buguggiate, VA, Italy) at a density of 1.5 fish per liter with a constant light–dark cycle (14–10 h) (Westerfield, 2000). For breeding, females and male (1:2) were placed in breeding tanks overnight and were separated by a transparent barrier that was removed after the lights were turned on the following morning. Embryos were collected after 15 min and transferred to sterile 6-well cell culture plates (20 embryos per well); the embryos were maintained in incubators at 28.5 °C with a controlled 14:10 h light–dark cycle. The embryos were maintained on Biochemical Oxygen Demand (BOD) incubators until 7 dpf at a density of 7 ml per larva. They were then immediately transferred to a tank with a density of one larva per 60 mL. When the animals reached the age of 30 dpf, they were maintained in a density of one animal per 200 mL until adulthood. The light and temperature control was performed in accordance with the previously described parameters (Westerfield, 2000). Survival assessments and general morphology were analyzed daily by visual inspection of the embryo under a dissection scope monitored under an inverted stereomicroscope.

### 2.2. Pharmacological treatment

Valproic acid (Sigma Aldrich, St. Louis, MO, USA) at a concentration of 48 μM diluted in water was administered in selected embryos from 0 to 48 h postfertilization (48 hpf). For the treatment, we used six wells that contained 15 embryos per well in 12 mL of VPA (treated group) or water (control) plates.

### 2.3. Analysis of VPA levels in treatment medium by ICP-MS

The levels of VPA in treatment medium for 48 h were assessed by inductively coupled plasma mass spectrometry (ICP-MS). The water of treatments were diluted (20×) with saline solution and filtrated for analysis (0.22 μm filter). The chromatographic parameters used were based on the method described by Gao et al. (2011). Briefly, 5 μl of diluted samples was injected into the UHPLC 1290/MS 6460 TQQQ — Agilent (all UHPLC components and software MassHunter were from Agilent Technologies®, Santa Clara, CA, USA). Chromatographic separation was performed using a Zorbax SB-C18 (4.6 × 50 mm 2.1 μm column). The flow rate of methanol:10 mM ammonium formate containing 0.1% formic acid (80:20, v/v) was 0.4 ml/min with a column temperature at 35 °C. VPA was detected using electrospray positive ionization and selected ion monitoring (SIM) for m/z = 143.1.

### 2.4. Behavioral assessment

#### 2.4.1. Locomotion and anxiety

The locomotor activity and anxiety of the animals were evaluated at 6 dpf, 30 dpf, 70 dpf, and 120 dpf. The 6-dpf larvae were individually placed in a 24-well plate filled with 3 ml of system water for locomotor performance analysis during a 5-min session following 1-min of acclimation. The performance was video recorded using a digital HD webcam (Logitech, Newark, CA, USA) for automated analysis (ANY-Maze, Stoelting Co., Wood Dale, IL, USA). The total distance traveled, mean speed, number of crossings, and absolute turn angle were considered the main parameters of locomotion, whereas the entries in the outer area represented a parameter of anxiety. At 30 dpf, 70 dpf, and 120 dpf, the animals were individually placed in the test tank (30 cm × 15 cm × 10 cm, length × height × width) and maintained for 30 s prior to the video recording as previously described (Gerlai et al., 2000). The locomotor activity was video recorded for 5 min after

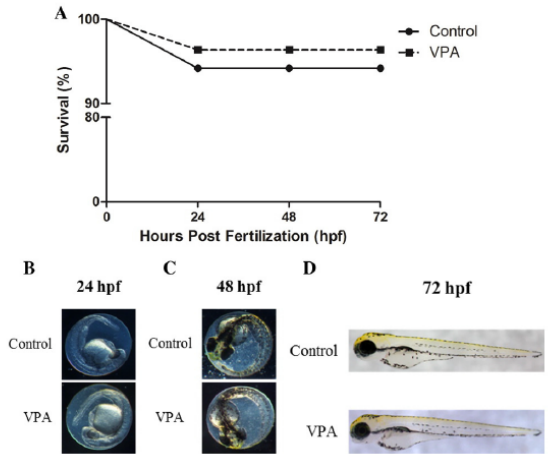


**Fig. 1.** Effect of VPA exposure on zebrafish embryos. Kaplan–Meier survival comparison for groups throughout the experiment showed no effects at 24 hpf, 48 hpf, and 72 hpf (log-rank (Mantel–Cox) test) that were not statistically significant when individual comparisons were performed (A) and no morphological changes at 24 hpf (B), 48 hpf (C) and 72 hpf (D).

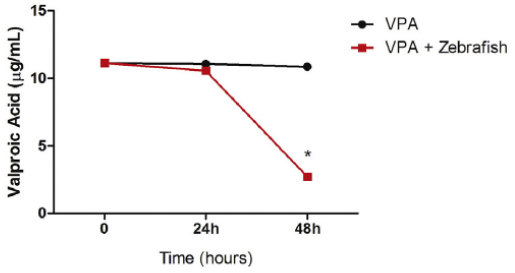


**Fig. 2.** VPA levels in treatment medium over 48 h of exposure. The asterisk (*) indicates a significant difference compared with the control group ($p < 0.05$). Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

the habituation period and simultaneously analyzed using ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA). The tank was divided into equal sections with four vertical lines and one horizontal line, and the following behavior patterns were measured: the number of crossings (vertical and horizontal lines), distance traveled and mean speed. The time spent in each tank position (bottom vs. upper levels) was considered an index of anxiety. This task exploits the natural tendency of zebrafish to spend most of the time at the bottom when introduced to a novel environment and then gradually extend the swimming range, over a period of minutes, to include the upper portions of the test tank (Levin et al., 2007). A longer time spent in the bottom and less time spent in the top part of the tank indicates increased anxiety (Levin et al., 2007).

#### 2.4.2. Social interaction

The social interaction of the animals was evaluated at 70 and 120 dpf because the zebrafish exhibit preference for its conspecifics. Each fish was placed in an experimental tank (30 cm × 15 cm × 10 cm, length × height × width). On one side of the experimental tank, an empty fish tank was placed; on the other side, a tank of identical size held 15 zebrafish, which were designed the "stimulus fish". The experimental fish was allowed to acclimate to the experimental tank for a 30 s period, after which behavior was video recorded over a period of 5 min. To quantify fish preference between the "stimulus fish" side of their tank in detriment of the empty tank, the experimental fish tank was divided in four equal sections. The zones 1 and 2 of the tank correspond to the segments closer to the conspecific school and the zones 3 and 4 are considered to the segments closer to the empty tank. The amount of time the experimental fish spent on each zone was measured using ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA) (Gerlai et al., 2000).

#### 2.4.3. Aggression

The aggression of the animals was evaluated on 70 dpf and 120 dpf. The mirror test was used to measure aggression (Gerlai et al., 2000; Moretz et al., 2007). Each fish was placed in an experimental tank (30 cm × 15 cm × 10 cm, length × height × width). A mirror (45 cm × 38 cm) was placed at the side of the tank at an angle of 22.5° to the back wall of the tank so that the left vertical edge of the mirror touched the side of the tank and the right edge was further away. Thus, when the experimental fish swam to the left side of the tank, their mirror image appeared closer to them. A test fish was added to the tank and was allowed acclimate for 60 s; the aggressive behaviors that a fish conducted toward its mirror image were subsequently recorded over a period of 5 min. The vertical lines divided the tank into four equal sections and allowed the number of entries to each section made by the fish to be counted. Entry to the left-most segment indicated preference for proximity to the "opponent", whereas entry to the right-most segments implied avoidance. The amount of time the experimental fish spent in each segment was measured using ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA).

### 2.5. Statistical analysis

Survival throughout the first 72 h was analyzed by Kaplan–Meier test. The ICP-MS data were analyzed by one-way ANOVA followed by Tukey's test. The behavioral assessment data were expressed as the mean ± standard error of the mean (S.E.M.) and analyzed by independent Student's t-tests, Multivariate analysis of variance (MANOVA) and analysis of variance (ANOVA). The α value determined is 5% to indicate different means between groups.

## 3. Results

We investigated the effect of VPA (48 μM) exposure during the first 48 h of development (48 hpf) on survival, morphology, and hatching rate at 24 hpf, 48 hpf, and 72 hpf. Survival rates were analyzed by Kaplan–Meier test and results indicated no significant difference on survival rate when groups were compared (log-rank (Mantel–Cox) test $p > 0.05$ $n = 85$) (Fig. 1A). VPA exposure did not promote significant changes in the morphology at 24 hpf, 48 hpf, and 72 hpf, respectively (Fig. 1B–D). We observed the normal appearance of the animals treated with VPA when compared to the control group, suggesting that perceived behavioral effects are not due to morphological changes. We observed that there was no significant alteration in the hatching rate of animals treated with VPA and the control group (data not shown).

In order to investigate if VPA levels alter during the exposure we analyzed VPA levels at treatment medium at 0 h, 24 h, and 48 h (hours) of exposure. As demonstrated in Fig. 2, the VPA levels decreased at 48 h of the treatment, suggesting that VPA was absorbed by the embryos.

**Table 1**
Locomotion parameters for VPA-treated and control groups at different ages.

| Age | Parameters of locomotor activity | Control group | | VPA group | | p value |
|---|---|---|---|---|---|---|
| | | Means ± S.E.M. | n | Means ± S.E.M. | n | |
| 6 dpf | Distance traveled | 0.7 ± 0.1 m | 38 | 1.6 ± 0.4 m | 38 | $p < 0.05$ |
| | Mean speed | 0.2 ± 0.03 cm/s | | 0.5 ± 0.1 cm/s | | |
| | Number of line crossings | 28.5 ± 3.5 | | 70 ± 14.3 | | |
| 30 dpf | Distance traveled | 1.6 ± 0.1 m | 17 | 2.2 ± 0.2 m | 17 | $p > 0.05$ |
| | Mean speed | 0.5 ± 0.04 cm/s | | 0.7 ± 0.1 cm/s | | |
| | Number of line crossings | 137.6 ± 10.7 | | 169.8 ± 11.1 | | |
| 70 dpf | Distance traveled | 3 ± 0.2 m | 32 | 2.7 ± 0.2 m | 32 | $p > 0.05$ |
| | Mean speed | 1 ± 0.08 cm/s | | 0.9 ± 0.07 cm/s | | |
| | Number of line crossings | 233.6 ± 14.52 | | 214.8 ± 14.58 | | |
| 120 dpf | Distance traveled | 2.6 ± 0.1 m | 26 | 2.5 ± 0.2 m | 26 | $p > 0.05$ |
| | Mean speed | 0.8 ± 0.04 cm/s | | 0.8 ± 0.07 cm/s | | |
| | Number of line crossings | 202 ± 14.1 | | 186 ± 16.2 | | |

Data are expressed as mean ± S.E.M.

### 3.1. VPA exposure results in differential changes in locomotor activity and anxiety expressed at different ages in zebrafish

VPA (48 μM) exposure during the first 48 h of development (48 hpf) induced significant changes in the parameters of locomotor activity and anxiety behavior at different developmental periods: 6 dpf, 70 dpf, and 120 dpf. Different parameters of zebrafish locomotor activity were evaluated and measured in the tank test. The same tank used to measure locomotor activity was also used to identify the index of anxiety, which was determined by the time spent in the bottom portion of the test tank.

MANOVA was performed in order to analyze the parameters related to locomotor activity (distance traveled, mean speed, and number of line crossings) simultaneously, and investigate the correlation between these parameters. At 6 dpf, the results of MANOVA presented a significant correlation between parameters of locomotor activity ($F = 2.62$, num df = 3, den df = 74, $p < 0.05$) (Table 1). The locomotion at 6 dpf of the animals treated with VPA exhibited an increase in the distance traveled (1.6 ± 0.4 m) compared with the control animals (0.7 ± 0.1 m) (Table 1). There was also an increase in the mean speed (0.5 ± 0.1 cm/s) compared with the control animals (0.2 ± 0.03 cm/s) (Table 1). The number of line crossings increased (70 ± 14.3) compared with the control animals (28.5 ± 3.5) (Table 1). Regarding to anxiety-related parameter, ANOVA showed that there were significant differences in the means between groups ($F = 5.56$, num df = 1, den df = 76, $p < 0.05$) (Table 2). The entries in the outer area also increased (35.8 ± 7.2) compared with the control group (15.1 ± 1.7) (Table 2). However, we did not identify changes in the absolute turn angle or rotations (data not shown).

At 30 dpf and 70 dpf, the results of MANOVA showed no significant correlations between the parameters of locomotor activity ($F = 1.35$, num df = 3, den df = 32, $p > 0.05$ and $F = 0.32$, num df = 3, den df = 58, $p > 0.05$, respectively) (Table 1). Furthermore, the anxiety-related parameter showed no significant differences at 30 dpf, in the mean between the groups ($F = 3.75$, num df = 1, den df = 34, $p > 0.05$ (Table 2). However, at 70 dpf, it has been observed differences in the means between groups ($F = 5.05$, num df = 1, den df = 60, $p < 0.05$) (Table 2). The time spent in the bottom portion of the test tank increased (268.5 ± 4.0 s) compared with the control group (248.9 ± 7.9 s) (Table 2).

At 120 dpf, the results of MANOVA did not present a significant correlation between parameters of locomotor activity ($F = 2.60$, num df = 3, den df = 48, $p > 0.05$) (Table 1). Regarding to anxiety-related parameter, ANOVA showed that there were significant differences in the means between groups ($F = 3.77$, num df = 1, den df = 50, $p < 0.05$) (Table 2). The time spent in the bottom portion of the test tank decreased (235.3 ± 10.9 s) compared with the control group (266.1 ± 12.6 s) (Table 2).

These results demonstrated that VPA exposure in embryos produced changes in parameters of locomotor activity at 6 dpf, which were demonstrated by the changes in the distance traveled, mean speed, and number of crossings compared with the control group. At 30 dpf, 70 dpf and 120 dpf, there were no significant differences in locomotion. The early exposure to VPA induced an increase in anxiety behavior at 6 dpf and 70 dpf, which may be interpreted as an indicator of anxiogenic behavior; however, this pattern changed at 120 dpf and indicated VPA exposure promoted anxiolytic behavior.

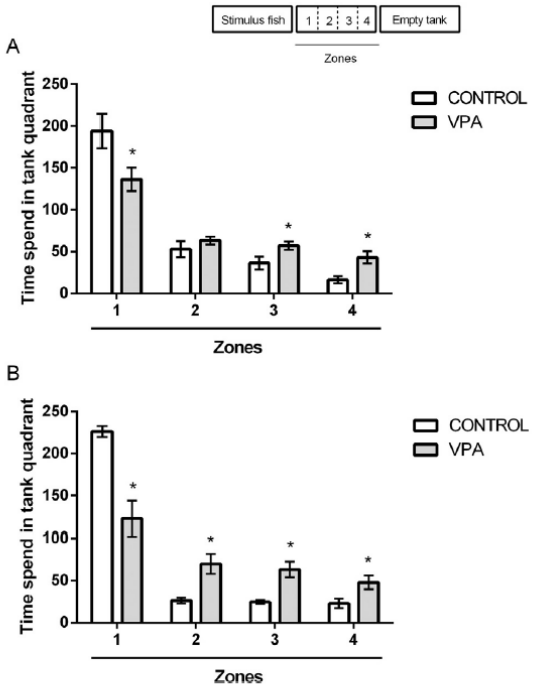


**Fig. 3.** Social interaction at 70 dpf (A) and 120 dpf (B) in the animals treated with VPA, which was determined during 5 min of video recording in the tank test. The inset of the figure demonstrates the segments of the test tank: the zone 1 of the tank corresponds to the segment closer to the conspecific school and the zone 4 is considered to the segment closer to the empty tank. The amount of time the experimental fish spent on each zone was measured using ANY-Maze recording software. The data were expressed as the mean ± S.E.M. The asterisk (*) indicates a significant difference compared with the control group ($p < 0.05$).

### 3.2. Social interaction deficits at different developmental periods in zebrafish exposed to VPA

Social interaction was assessed only at 70 and 120 dpf because at 6 and 30 dpf, the social behavior of zebrafish is not completely developed (Buske and Gerlai, 2012). Fig. 3 indicates that VPA induced impairment in social interaction in zebrafish at 70 and 120 dpf. We identified a significant ($p < 0.05$, n = 18) decrease in the time spent in the segment closest to the conspecific school (Zone 1; 136.2 ± 13.7 s) compared to the control group at 70 dpf (194 ± 20.6 s) (Fig. 3A). In addition, VPA treatment induced a significant increase ($p < 0.01$, n = 18) in the time spent in the zone 4 (43.1 ± 7.3 s), which is closest to the empty tank, when compared to control group (16.5 ± 4.4 s) (Fig. 3A). Similarly, at 120 dpf, we identified a significant ($p < 0.01$, n = 10) decrease in the

**Table 2**
Anxiety parameters for VPA-treated and control groups at different ages.

| Age | Anxiety parameter | Control group | | VPA group | | p value |
|---|---|---|---|---|---|---|
| | | Means ± S.E.M. | n | Means ± S.E.M. | n | |
| 6 dpf | Entries in the outer area | 15.1 ± 1.7 s | 38 | 35.8 ± 7.2 s | 38 | $p < 0.05$ |
| 30 dpf | Time spent in the bottom portion | 160.6 ± 12.6 s | 17 | 197.1 ± 10.9 s | 17 | $p > 0.05$ |
| 70 dpf | Time spent in the bottom portion | 248.9 ± 7.9 s | 32 | 268.5 ± 4.0 s | 32 | $p < 0.05$ |
| 120 dpf | Time spent in the bottom portion | 266.1 ± 12.6 s | 26 | 235.3 ± 10.9 s | 26 | $p < 0.05$ |

Data are expressed as means ± S.E.M.

time spent in the segment closest to the conspecific school (Zone 1; 123.0 ± 21 s) compared to the control group at 70 dpf (226.1 ± 6.6 s) (Fig. 3B). Furthermore, VPA treatment induced a significant increase ($p < 0.05$, $n = 10$) in the time spent in the zone 4 (47.9 ± 8.1 s), which is closest to the empty tank, when compared to control (22.9 ± 5.5 s). Thus, VPA induced a social interaction deficit in zebrafish at both ages (Fig. 3B).

### 3.3. VPA does not induce changes in aggressive behavior at different developmental periods in zebrafish

Aggressive behavior was evaluated at 70 dpf and 120 dpf. The embryological VPA exposure did not promote significant changes in the time spent in the segment nearest to the mirror image at 70 dpf and 120 dpf. Thus, in these conditions, VPA most likely did not affect the aggression of the animals.

## 4. Discussion

This study performed a behavioral screening at different development periods in zebrafish at 6 dpf, 30 dpf, 70 dpf, and 120 dpf to establish a model of social interaction deficits induced by VPA. Our results indicated the normal appearance of the animals treated with VPA, suggesting that perceived behavioral effects are not due to morphological changes. We observed that there was no significant alteration in the survival and hatching rate of animals treated with VPA. Likewise, Zellner et al. (2011) demonstrated the exposure to 48 μM VPA during 0–48 hpf without affecting the death or malformations. The behavioral patterns identified in our experiments suggest that VPA exposure results in a profile of hyperactivity in the early development stages in zebrafish, i.e., 6 dpf, because there was an increase in locomotion parameters, such as the total distance traveled, mean speed and number of line crossings. In addition, the animals exhibited a marked anxiety based on the increased entries in the outer area of the well. De Esch et al. (2012) demonstrated that zebrafish larvae can act as an alternative animal model to test toxicity; however, it is important to consider the age (and the brain stage of development) because it can generate changes in behavior. Studies have demonstrated that age influences the pattern of motor activity in zebrafish larvae (Colwill and Creton, 2011; Padilla et al., 2011). In addition, high doses of VPA caused histopathological alterations in zebrafish larvae at 72 hpf and 96 hpf (Beker van Woudenberg et al., 2014). These authors demonstrated that low doses of VPA (60 μM VPA) induced minimal disruption of normal brain structure, which was characterized by small regions of reduced cellularity. However, at 150 μM VPA, all larvae exhibited mild disruption of normal brain structure, which was characterized by large areas of reduced cellularity, in particular, in the developing preoptic region and tectum opticum. In addition, 60 μM VPA did not affect motor activity, whereas 150 μM VPA induced a reduction in total distance traveled (Beker van Woudenberg et al., 2014). However, Zellner et al. (2011) demonstrated that exposure to 48 μM VPA during 0–48 hpf promoted marked hyperactivity at 6 dpf without changes in neurotoxicological parameters. Therefore, differences in VPA concentrations and treatment times may influence the behavioral patterns observed in zebrafish.

Throughout development, the hyperactivity of 6 dpf zebrafish presented after embryological VPA treatment was attenuated. The locomotion parameters of 30 dpf, 70 dpf and 120 dpf were similar to the control animals. The anxiety index at 70 dpf remained increased; however, at 120 dpf, there was a decrease in anxiety. To support these results, it is important to consider that VPA exposure may affect brain development. Several studies have demonstrated that VPA can regulate signaling pathways and gene expression throughout brain development in rodents thereby interfering with critical windows of vulnerability (Almeida et al., 2014; Bartkowska et al., 2007; Kolozsi et al., 2009; Stodgell et al., 2006). Our results also demonstrated that animals treated with VPA during the first 48 h exhibited a significant deficit in social interaction at 70 dpf, and this effect was maintained throughout development at 120 dpf, suggesting that VPA can modulate differentially behavioral patterns throughout brain development. We also suggest that the profile of hyperactivity and anxiety may change throughout development and indicates neurochemical alterations in animals exposed to VPA at an early age. The serotoninergic system during early life can produce different behavioral responses, including changes in social behavior in zebrafish (Buske and Gerlai, 2011; Mahabir et al., 2013). It has been reported that anxiety and social interaction deficits are related to changes in the serotonergic system and these alterations are extremely relevant for several psychiatric disorders (Homberg et al., 2007; Homberg, 2013).

Several neuropsychiatric disorders are characterized by impaired social interactions and anxiety. Silverman et al. (2010) demonstrated that rodent models of autism exhibit deficits in both sociability and anxiety behaviors. These findings are consistent with our results at 70 dpf. However, at 120 dpf, a decrease in anxiety was identified. The literature shows the controversial results between social interaction and anxiety. For example, social interaction deficits can occur without affecting anxiety. Some autistic mouse models have deficits in social interaction but do not exhibit changes in anxiety behaviors (Liu and Smith, 2009; McFarlane et al., 2008). Schneider and Przewłocki (2005) demonstrated that the altered behaviors in rodents induced by VPA appeared prior to puberty. However, the social structure in the early developmental stage is primitive in zebrafish. Young larvae do not exhibit shoaling behavior, which develops later with age (Buske and Gerlai, 2012). Based on this finding, the results related to the anxiety index have different responses when evaluated at different periods of development; this is most likely because of changes in the behavioral response maturity. According to Buske and Gerlai (2012), changes in the serotonergic and dopaminergic systems may explain the age-dependent behavioral changes.

According to Roullet et al. (2013), behavioral studies have indicated that VPA in both rat and mouse models induced symptoms of social interaction deficits, stereotyped movements, disability in communication, and anxiety. However, aggression is poorly studied. In our study, we verified that VPA exposure in the embryonic phase did not affect aggression in the adult stage at 70 dpf and 120 dpf in zebrafish. Aggression in zebrafish may have several important functions (Gerlai, 2003), and this behavior is often used to maintain dominance (Larson et al., 2006; Spence and Smith, 2006). The quantification of this behavior could provide information regarding whether VPA and aggressive tendencies are related. However, our results indicated that animals treated with VPA at an early age may exhibit behavioral responses without expressing a domain of territory, and there is no direct association between a deficit in social interaction and aggression.

In summary, the present study evaluated a behavioral screening at different periods of development in zebrafish and established a model of social interaction deficit induced by VPA. This is the first study to demonstrate that zebrafish exposed to VPA during the first 48 h of life exhibit deficits in social interaction, anxiety, and locomotor changes at different periods of development. Together, these results highlight the importance of behavioral research for understanding the toxicological action of drugs, such as VPA; these drugs can have a significant impact on the behavioral neurodevelopment associated with neuropsychiatric disorders evaluated in a new model of social interaction deficits in zebrafish.

## Transparency document

The Transparency document associated with this article can be found, in the online version.

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## References


Almeida, L.E., Roby, C.D., Krueger, B.K., 2014. Increased BDNF expression in fetal brain in the valproic acid model of autism. Mol. Cell. Neurosci. 59, 57–62.

American Psychiatric Association, 2013. Diagnostic and Statistical Manual of Mental Disorders. 5th edn. American Psychiatric Publishing, Arlington, VA.

Arndt, T.L., Stodgell, C.J., Rodier, P.M., 2005. The teratology of autism. Int. J. Dev. Neurosci. 23, 189–199.

Banerjee, S., Riordan, M., Bhat, M.A., 2014. Genetic aspects of autism spectrum disorders: insights from animal models. Front. Cell. Neurosci. 8, 58.

Bartkowska, K., Paquin, A., Gauthier, A.S., Kaplan, D.R., Miller, F.D., 2007. Trk signaling regulates neural precursor cell proliferation and differentiation during cortical development. Development 134, 4369–4380.

Beker van Woudenberg, A., Snel, C., Rijkmans, E., de Groot, D., Bouma, M., Hermsen, S., Piersma, A., Menke, A., Wolterbeek, A., 2014. Zebrafish embryotoxicity test for developmental (neuro)toxicity: demo case of an integrated screening approach system using anti-epileptic drugs. Reprod. Toxicol. 49C, 101–116.

Burns, J., 2006. The social brain hypothesis of schizophrenia. World Psychiatry 5, 77–81.

Buske, C., Gerlai, R., 2011. Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotoninergic systems in adult zebrafish. Neurotoxicol. Teratol. 33, 698–707.

Buske, C., Gerlai, R., 2012. Maturation of shoaling behavior is accompanied by changes in the dopaminergic and serotoninergic systems in zebrafish. Dev. Psychobiol. 54, 28–35.

Canitano, R., 2014. New experimental treatments for core social domain in autism spectrum disorders. Front. Pediatr. 2, 61.

Charman, T., Pickles, A., Simonoff, E., Chandler, S., Loucas, T., Baird, G., 2011. IQ in children with autism spectrum disorders: data from the Special Needs and Autism Project (SNAP). Psychol. Med. 41, 619–627.

Collier, A.D., Khan, K.M., Caramillo, E.M., Mohn, R.S., Echevarria, D.J., 2014. Zebrafish and conditioned place preference: a translational model of drug reward. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 55, 16–25.

Colwill, R.M., Creton, R., 2011. Locomotor behaviors in zebrafish (*Danio rerio*) larvae. Behav. Process. 86, 222–229.

Constantino, J.N., Todorov, A., Hilton, C., Law, P., Zhang, Y., Molloy, E., Fitzgerald, R., Geschwind, D., 2013. Autism recurrence in half siblings: strong support for genetic mechanisms of transmission in ASD. Mol. Psychiatry 18, 137–138.

de Esch, C., van der Linde, H., Slieker, R., Willemsen, R., Wolterbeek, A., Woutersen, R., De Groot, D., 2012. Locomotor activity assay in zebrafish larvae: influence of age, strain and ethanol. Neurotoxicol. Teratol. 34, 425–433.

Djukic, A., McDermott, M.V., 2012. Social preferences in Rett syndrome. Pediatr. Neurol. 46, 240–242.

Gao, S., Miao, H., Tao, X., Jiang, B., Xiao, Y., Cai, F., Yun, Y., Li, J., Chen, W., 2011. LC–MS/MS method for simultaneous determination of valproic acid and major metabolites in human plasma. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879, 1939–1944.

Gerlai, R., 2003. Zebra fish: an uncharted behavior genetic model. Behav. Genet. 33, 461–468.

Gerlai, R., 2014. Social behavior of zebrafish: from synthetic images to biological mechanisms of shoaling. J. Neurosci. Methods 234, 59–65.

Gerlai, R., Lahav, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacol. Biochem. Behav. 67, 773–782.

Grossman, J.B., Carter, A., Volkmar, F.R., 1997. Social behavior in autism. Ann. N. Y. Acad. Sci. 807, 440–454.

Grzadzinski, R., Huerta, M., Lord, C., 2013. DSM-5 and autism spectrum disorders (ASDs): an opportunity for identifying ASD subtypes. Mol. Autism 4, 12.

Homberg, J.R., 2013. Measuring behaviour in rodents: towards translational neuropsychiatric research. Behav. Brain Res. 236, 295–306.

Homberg, J.R., Olivier, J.D., Smits, B.M., Mul, J.D., Mudde, J., Verheul, M., Nieuwenhuizen, O.F., Cools, A.R., Ronken, E., Cremers, T., Schoffelmeer, A.N., Ellenbroek, B.A., Cuppen, E., 2007. Characterization of the serotonin transporter knockout rat: a selective change in the functioning of the serotonergic system. Neuroscience 146, 1662–1676.

Kalueff, A.V., Stewart, A.M., Gerlai, R., 2014. Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol. Sci. 35, 63–75.

Kennedy, D.P., Adolphs, R., 2012. The social brain in psychiatric and neurological disorders. Trends Cogn. Sci. 16, 559–572.

Kim, K.C., Kim, P., Go, H.S., Choi, C.S., Yang, S.I., Cheong, J.H., Shin, C.Y., Ko, K.H., 2011. The critical period of valproate exposure to induce autistic symptoms in Sprague–Dawley rats. Toxicol. Lett. 201, 137–142.

Kolozsi, E., Mackenzie, R.N., Roullet, F.I., deCatanzaro, D., Foster, J.A., 2009. Prenatal exposure to valproic acid leads to reduced expression of synaptic adhesion molecule neuroligin 3 in mice. Neuroscience 163, 1201–1210.

Larson, E.T., O'Malley, D.M., Melloni Jr., R.H., 2006. Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. Behav. Brain Res. 167, 94–102.

Levin, E.D., Bencan, Z., Cerutti, D.T., 2007. Anxiolytic effects of nicotine in zebrafish. Physiol. Behav. 90, 54–58.

Liu, Z.H., Smith, C.B., 2009. Dissociation of social and nonsocial anxiety in a mouse model of fragile X syndrome. Neurosci. Lett. 454, 62–66.

Maaswinkel, H., Zhu, L., Weng, W., 2013. Assessing social engagement in heterogeneous groups of zebrafish: a new paradigm for autism-like behavioral responses. PLoS One 8, e75955.

Mahabir, S., Chatterjee, D., Buske, C., Gerlai, R., 2013. Maturation of shoaling in two zebrafish strains: a behavioral and neurochemical analysis. Behav. Brain Res. 247, 1–8.

Mahoney, E.B., Breitborde, N.J., Leone, S.L., Ghuman, J.K., 2014. An examination of social interaction profiles based on the factors measured by the screen for social interaction. Res. Dev. Disabil. 35, 2487–2494.

Markram, H., Rinaldi, T., Markram, K., 2007. The intense world syndrome—an alternative hypothesis for autism. Front. Neurosci. 1, 77–96.

McFarlane, H.G., Kusek, G.K., Yang, M., Phoenix, J.L., Bolivar, V.J., Crawley, J.N., 2008. Autism-like behavioral phenotypes in BTBR T + tf/J mice. Genes Brain Behav. 7, 152–163.

Menegola, E., Broccia, M.L., Prati, M., Giavini, E., 1998. Stage-dependent skeletal malformations induced by valproic acid in rat. Int. J. Dev. Biol. 42, 99–102.

Miller, N.Y., Gerlai, R., 2011. Shoaling in zebrafish: what we don't know. Rev. Neurosci. 22, 17–25.

Moretz, J.A., Martins, E.P., Robison, B.D., 2007. The effects of early and adult social environment on zebrafish (*Danio rerio*) behavior. Environ. Biol. Fish 80, 91–101.

Morrison, R.L., Bellack, A.S., 1987. Social functioning of schizophrenic patients: clinical and research issues. Schizophr. Bull. 13, 715–725.

Nery, L.R., Eltz, N.S., Hackman, C., Fonseca, R., Altenhofen, S., Guerra, H.N., Freitas, V.M., Bonan, C.D., Vianna, M.R., 2014. Brain intraventricular injection of amyloid-β in zebrafish embryo impairs cognition and increases tau phosphorylation, effects reversed by lithium. PLoS One 9, e105862.

Newman, M., Ebrahimie, E., Lardelli, M., 2014. Using the zebrafish model for Alzheimer's disease research. Front. Genet. 5, 189.

Padilla, S., Hunter, D.L., Padnos, B., Frady, S., MacPhail, R.C., 2011. Assessing locomotor activity in larval zebrafish: influence of extrinsic and intrinsic variables. Neurotoxicol. Teratol. 33, 624–630.

Pelphrey, K.A., Shultz, S., Hudac, C.M., Vander Wyk, B.C., 2011. Research review: constraining heterogeneity: the social brain and its development in autism spectrum disorder. J. Child Psychol. Psychiatry 52, 631–644.

Persico, A.M., Bourgeron, T., 2006. Searching for ways out of the autism maze: genetic, epigenetic and environmental clues. Trends Neurosci. 29, 349–358.

Petrere, J.A., Anderson, J.A., Sakowski, R., Fitzgerald, J.E., de la Iglesia, F.A., 1986. Teratogenesis of calcium valproate in rabbits. Teratology 34, 263–269.

Pinto, D., Delaby, E., Merico, D., Barbosa, M., Merikangas, A., Klei, L., Thiruvahindrapuram, B., Xu, X., Ziman, R., et al., 2014. Convergence of genes and cellular pathways dysregulated in autism spectrum disorders. Am. J. Hum. Genet. 94, 677–694.

Raff, M., 2014. Open questions: what has genetics told us about autism spectrum disorders? BMC Biol. 12, 45.

Rapin, I., Tuchman, R.F., 2008. Autism: definition, neurobiology, screening, diagnosis. Pediatr. Clin. N. Am. 55, 1129–1146.

Rodier, P.M., Ingram, J.L., Tisdale, B., Croog, V.J., 1997. Linking etiologies in humans and animal models: studies of autism. Reprod. Toxicol. 11, 417–422.

Roullet, F.I., Lai, J.K., Foster, J.A., 2013. In utero exposure to valproic acid and autism—a current review of clinical and animal studies. Neurotoxicol. Teratol. 36, 47–56.

Saverino, C., Gerlai, R., 2008. The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish. Behav. Brain Res. 191, 77–87.

Schneider, T., Przewłocki, R., 2005. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology 30, 80–89.

Seibt, K.J., Piato, A.L., da Luz, O.R., Capiotti, K.M., Vianna, M.R., Bonan, C.D., 2011. Antipsychotic drugs reverse MK-801-induced cognitive and social interaction deficits in zebrafish (*Danio rerio*). Behav. Brain Res. 224 (1), 135–139.

Siebel, A.M., Piato, A.L., Schaefer, I.C., Nery, L.R., Bogo, M.R., Bonan, C.D., 2013. Antiepileptic drugs prevent changes in adenosine deamination during acute seizure episodes in adult zebrafish. Pharmacol. Biochem. Behav. 104, 20–26.

Silverman, J.L., Yang, M., Lord, C., Crawley, J.N., 2010. Behavioural phenotyping assays for mouse models of autism. Nat. Rev. Neurosci. 11, 490–502.

Spence, R., Smith, C., 2006. Mating preferences of female zebrafish, *Danio rerio*, in relation to male dominance. Behav. Ecol. 17, 779–783.

Stodgell, C.J., Ingram, J.L., O'Bara, M., Tisdale, B.K., Nau, H., Rodier, P.M., 2006. Induction of the homeotic gene Hoxa1 through valproic acid's teratogenic mechanism of action. Neurotoxicol. Teratol. 28, 617–624.

Tager-Flusberg, H., Caronna, E., 2007. Language disorders: autism and other pervasive developmental disorders. Pediatr. Clin. N. Am. 54, 469–481.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). 4th ed. Univ of Oregon Press, Eugene.

Zafeiriou, D.I., Ververi, A., Dafoulis, V., Kalyva, E., Vargiami, E., 2013. Autism spectrum disorders: the quest for genetic syndromes. Am. J. Med. Genet. B Neuropsychiatr. Genet. 162B, 327–366.

Zellner, D., Padnos, B., Hunter, D.L., MacPhail, R.C., Padilla, S., 2011. Rearing conditions differentially affect the locomotor behavior of larval zebrafish, but not their response to valproate-induced developmental neurotoxicity. Neurotoxicol. Teratol. 33, 674–679.

# CAPÍTULO II

## ARTIGO CIENTÍFICO

*Analysis of extracellular nucleotide metabolism in adult zebrafish after embryological exposure to valproic acid*

Fernanda Francine Zimmermann, Karina Vidarte Gaspary, Anna Maria Siebel, Carlos Eduardo Leite, Luiza Wilges Kist, Mauricio Reis Bogo, Carla Denise Bonan

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**Responder A:** Zevie Davis <zdavis@lsuhsc.edu>

Dear Prof. Bonan:

I am pleased to inform you that your manuscript, "ANALYSIS OF EXTRACELLULAR NUCLEOTIDE METABOLISM IN ADULT ZEBRAFISH AFTER EMBRYOLOGICAL EXPOSURE TO VALPROIC ACID" has been accepted for publication in Molecular Neurobiology.. Whenever inquiring about your manuscript, remember to quote the number MOLN-D-16-00006R1 in your correspondence.

We would like to invite you to submit a figure or suggest a figure from your submission to be considered as a possible Cover for an upcoming issue of Molecular Neurobiology. If you choose to create a new figure, please submit it as a jpeg and a pdf file. Also, please submit a new legend describing the correlation of the figure to the manuscript as a Word document.

If you have any questions, please feel free to contact us.

Congratulations and best regards,

Nicolas G. Bazan, M.D., Ph.D.
Editor in Chief
Molecular Neurobiology

# ANALYSIS OF EXTRACELLULAR NUCLEOTIDE METABOLISM IN ADULT ZEBRAFISH AFTER EMBRYOLOGICAL EXPOSURE TO VALPROIC ACID

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ABSTRACT

Autism is a neurodevelopmental disorder characterized by symptoms related to stereotyped movements, deficits in social interaction, impaired communication, anxiety, hyperactivity, and the presence of restricted interests. Evidence indicates an important role of extracellular ATP and adenosine as signaling molecules in autism. ATP hydrolysis by ectonucleotidases is an important source of adenosine, and adenosine deaminase (ADA) contributes to the control of the nucleoside concentrations. Considering zebrafish is an animal model that may contribute toward understanding the mechanisms that underlie social behavior, we investigated the purinergic signaling in a model of embryological exposure to valproic acid (VPA) that induces social interaction deficit in adult zebrafish. We demonstrated embryological exposure to VPA did not change ATP and ADP hydrolysis in zebrafish at 120 dpf, as well as the cytosolic (soluble) ADA activity was not altered. However, we observed an increase of AMP hydrolysis (12.5%) whereas the ecto-ADA activity was decreased (19.2%) in adult zebrafish submitted to embryological exposure to VPA. Quantitative reverse transcription PCR (RT-PCR) analysis showed changes on *ntpd8,* ADA 2.1 and *A2a1* mRNA transcript levels. Brain ATP metabolism showed a rapid catabolism of ATP and ADP, whereas the extracellular metabolism of AMP and adenosine (ADO) occurred slowly. We demonstrated that embryological exposure to VPA altered biochemical and molecular parameters related to purinergic system in adult zebrafish. These findings indicate that the enzyme activities involved in the control of ATP and adenosine levels may be involved in the pathophysiological mechanisms of diseases related to the impairment of social interaction, such as autism.

KEYWORDS: adenosine; adenosine deaminase; autism; ectonucleotidases; purinergic system; zebrafish.

## 1. INTRODUCTION

Autism spectrum disorders (ASD) are neurodevelopmental conditions, characterized by deficits in social interaction, anxiety, impaired communication, behavioral abnormalities and the presence of restricted interests [1, 2]. ASD are genetically determined disorders with a heritability of ~90% and are considered the most heritable brain disorder in humans [3, 4]. Furthermore, emerging evidence suggests that environmental factors play a role in ASD [5]; however, the precise mechanism of inheritance remains unknown. As observed in autism, similar features are also showed in a variety of neurodevelopmental disorders, including epilepsy, Rett syndrome and Fragile X syndrome, which are characterized by dysfunctions in the balance between excitatory and inhibitory transmissions [6, 7].

Modifications in the pathways of the development of neurotransmitter systems, including GABAergic [8], dopaminergic [9, 10], serotoninergic [11] and purinergic system [12] may underlie the pathophysiological process leading to autism. The purinergic system is important for vital functions of the CNS and can modulate the actions of other neurotransmitter systems [13, 14]. Evidence indicates an important role of extracellular ATP and the purinergic signaling in autism [12, 15, 16]. Naviaux and collaborators (2014) [17] verified that the antipurinergic therapy was able to restored social behavior in an ASD model, showing that the behavioral alterations were not permanent, but treatable using this therapy [17].

In the purinergic system, the ectonucleotidase family is constituted by a set of ecto-enzymes, including ectonucleoside triphosphate diphosphohydrolases (E-NTPDases) and ecto-5′-nucleotidase (ecto-5'-NT) which are capable of hydrolyzing adenosine triphosphate (ATP)

to adenosine [18]. In addition, the sequential deamination of adenosine (ADO) to inosine is carried out by the enzyme adenosine deaminase (ADA) [18-21]. ADA is widely distributed across the tissues and catalyzes the hydrolytic deamination of adenosine to inosine in the cytosol and cell membranes [22, 23]. The activity of these enzymes is crucial for the efficient regulation of extracellular nucleotide under physiological and pathological conditions [18, 24]. Extracellular nucleotides and adenosine act on two classes of purinoceptors: P2 (P2X and P2Y receptors) and P1 ($A_1$, $A_{2A}$, $A_{2B}$, and $A_3$), respectively. Ecto- and cytosolic-ADA activities and different ADA-related genes have been described in zebrafish [25, 19]. Furthermore, biochemical and molecular studies have also characterized E-NTPDase and ecto-5′-nucleotidase in zebrafish brain [26, 27]. Persico and collaborators [15] showed that ADA plays a relevant role in purine metabolism, which may be altered in some autistic patients. In relation to the modulation of adenosine and autism, various reports suggested the therapeutic potential of the adenosine signaling due to several benefits [28-31]. Adenosine is known to be an important neuroprotective molecule [32], showing an essential relationship between metabolism and neuronal activity [33]. Masino and collaborators [29] reported a robust relationship between adenosine in the CNS and autism in terms of symptoms and behavior.

The zebrafish is an animal model that has been used to study Alzheimer's disease [34, 35], schizophrenia [36], drug abuse [37], and other brain disorders [38, 39]. Zebrafish is one of the most social vertebrates used in biomedical research [40]. It has a robust social behavior as a remarkable feature [38], becoming an interesting model to study disorders that affect social interaction, such as autism. Recently, it was performed a behavioral screening at different periods of zebrafish development at 6 dpf, 30 dpf, 70 dpf, and 120 dpf (days postfertilization) after valproic acid (VPA) exposure in the early development stage to investigate social

behavior, locomotion, aggressiveness, and anxiety [41]. This previous study demonstrated that animals treated with VPA during the first 48 hours exhibited deficit in social interaction at 120 dpf, and this effect mimics one of the main symptoms observed in autism [41].

Considering that: (I) autism is a complex neurodevelopmental disorder and represents a major problem for researchers due to the multifactorial origin of this neuropathology; (II) purinergic system may play a key role in the development of autism through modulation of ATP and ADO levels; (III) zebrafish is suitable to investigate mechanisms that underlie social behavior, the aim of this study was to investigate modifications in the control of purinergic signaling in a model of embryological exposure to VPA that induces social interaction deficit in adult zebrafish.

## 2.EXPERIMENTAL PROCEDURES

### *2.1. Chemicals*

Valproic acid, ATP, ADP, AMP, Adenosine, Trizma Base, ammonium molybdate, polyvinyl alcohol, Malachite Green, EDTA, EGTA, sodium citrate, calcium chloride were purchased from Sigma (St. Louis, MO, USA). Phenol, sodium nitroprusside, and magnesium chloride were purchased from Merck (Darmstadt, Germany). Trizol® Reagent, dNTPs, oligonucleotides, Taq polymerase, SYBR® Green I Low DNA Mass Ladder were purchased from Invitrogen (Carlsbad, California, USA) and ImProm-II™ Reverse Transcription System was obtained from Promega (São Paulo, SP, Brazil). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). All other reagents used were of analytical grade.

*2.2. Animals and housing*

Adult wild type zebrafish were maintained and bred according to standard procedures in an automated re-circulating system (Tecniplast, Buguggiate, VA, Italy) at a density of 1.5 fish per liter with a constant light-dark cycle (14–10 h) [42]. For breeding, females and male (1:2) were placed in breeding tanks overnight and were separated by a transparent barrier that was removed after the lights were turned on the following morning. Embryos were collected after 15 min and transferred to sterile 6-well cell culture plates (20 embryos per well); the embryos were maintained in incubators at 28.5 °C with a controlled 14:10 hour light-dark cycle. The embryos were maintained on Biochemical Oxygen Demand (BOD) incubators until 7 dpf at a density of 7 ml per larva. They were then immediately transferred to a tank with a density of one larva per 60 mL. When the animals reached the age of 30 dpf, they were maintained in a density of one animal per 200 mL until adulthood. The light and temperature control was performed in accordance with the previously described parameters [42]. All experimental procedures were conducted at 120 dpf (days postfertilization) after VPA exposure in the early developmental stage.

*2.3. Pharmacological treatment*

Valproic acid (Sigma Aldrich, St. Louis, MO, USA) at a concentration of 48 μM diluted in water was administered in selected embryos during the first 48 hours postfertilization (48 hpf). The dose and exposure time was chosen based on the study performed by Zellner et al. [43]. For the treatment, we used six wells that contained 15 embryos per well in 12 mL of VPA (treated group) or water (control).

*2.4. Preparation of soluble and membrane fractions*

At 120 days postfertilization (dpf) control and VPA fish were cryo-anaesthetized and euthanized [44]. The brains were removed by dissection and added to 60 volumes (v/w) of chilled Tris-citrate buffer (50 mM Tris citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4, adjusted with citric acid) (Sigma, St. Louis, MO, USA) for NTPDase and ecto-5'-nucleotidase assays [26, 27]. For ADA activity assays, brains were homogenized in 20 volumes (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA and 2 mM EGTA, pH 7.4 (Sigma, St. Louis, MO, USA) [19]. Each independent experiment was performed using biological preparations constituted by a ''pool'' of five brains. The preparation of brain membranes was according to a previously described method [45]. Briefly, samples were homogenized on ice in a motor-driven Teflon- glass homogenizer. The preparations were centrifuged at 800 *g* for 10 min at 4ºC to remove the nuclei and cell debris and the supernatant fractions were subsequently centrifuged at 40.000 *g* for 25 min. The resultant supernatant and the pellet obtained corresponded to the cytosolic and membrane fractions, respectively. For soluble ADA activity experiments, the supernatant was collected and kept on ice for enzyme assays. The pellets of both membrane preparations were frozen in liquid nitrogen, thawed, resuspended with the respective buffers and centrifuged at 40.000 *g* for 20 min. This freeze–thaw-wash procedure was used to ensure the lysis of the brain vesicles membranes. The final pellets were resuspended and used for the measurements of ectonucleotidase and ecto-ADA activities. All cellular fractions were maintained at 2–4ºC throughout preparation and they were immediately used for enzyme assays.

*2.5 Nucleotide Hydrolysis Assays*

Ectonucleotidase activities were determined as previously described [26, 27]. Brain membranes (3–5 μg protein) were added to the reaction mixture containing 50 mM Tris-HCl (pH 8.0) and 5 mM $CaCl_2$ (for E-NTPDase activities) and 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (Merck, Darmstadt, Germany) (for ecto-5'- nucleotidase activity) in a final volume of 200 μl. The samples were preincubated for 10 min at 37ºC before starting the reaction with the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reactions were stopped after 30 min with the addition of trichloroacetic acid (Sigma, St. Louis, MO, USA) at a final concentration of 5% and immediately placed on ice for 10 min. The inorganic phosphate (Pi) released was determined by colorimetric assay using Malachite Green reagent [46] and $KH_2PO_4$ (Sigma, St. Louis, MO, USA) as standard. To ensure that the concentration of Pi was within the linear range, dilutions of 1:8 and 1:2 to a volume of 400 μl were performed for the assessment of ATP and ADP hydrolysis, respectively. Samples were mixed to 1 ml of Malachite Green solution and nucleotide hydrolysis was determined spectrophotometrically at 630 nm after 20 min. Controls with membrane fractions after incubation period were used to correct nonenzymatic hydrolysis of substrates. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. NTPDase and ecto-5'-nucleotidase activities were expressed as nmol Pi $min^{-1}$ mg $protein^{-1}$.

*2.6 Adenosine deaminase Assay*

Ecto- and cytosolic ADA activities were determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported [47]. After the preparation of soluble and membrane fractions, the optimum conditions for adenosine hydrolysis were determined. The membrane and cytosolic fractions (5–10 μg protein) were added to the reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and 50 mM sodium phosphate buffer (pH 7.0) (Sigma, St. Louis, MO, USA), respectively, in a final volume of 200 μl. The samples were preincubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate adenosine (Sigma, St. Louis, MO, USA) to a final concentration of 1.5 mM. After incubated for 75 min (soluble fraction) and 120 min (membranes), the reaction was stopped by adding the samples on a 500 μL of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL) (Merck, Darmstadt, Germany). Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. The reaction mixtures were immediately mixed to 500 μL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6MNaOH) (Merck, Darmstadt, Germany) and vortexed. Samples were incubated at 37 °C for 15 min and the colorimetric assay was carried out at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Both ecto- and cytosolic ADA activities were expressed as nmol $NH_3$ $min^{-1}$ mg $protein^{-1}$

*2.7 Gene expression analysis by quantitative real time RT-PCR (RT-qPCR)*

The gene expression of ADA subfamilies (*ADA1, ADA2.1, ADA2.2*) including an alternative splicing isoform (*ADAasi*) and, an adenosine deaminase like related gene (*ADAL*), adenosine receptor subtypes (*A1, A2a1, A2a2,A2b*), ectonucleotidases (*entpd1, entpd2a.1, entpd2a.2, entpd2-like, entpd3, entpd8*) end *ecto-5'-nucleotidase* were determined. Total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, California, USA) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA, containing a final concentration of 0.2 x SYBR® Green I (Invitrogen), 100 μM dNTP, 1 x PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers [48, 49, 50] (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95°C, 40 cycles of 15 s at 95ºC for denaturation, 35 s at 60 °C for annealing and 15 s at 72°C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C and showed in all cases one single peak. *EF1α* and *Rpl13α* were used as reference genes for normalization. Relative expression levels were determined with 7500 and 7500 Fast Real-Time PCR Systems Software v.2.0.6 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR version 2012.3 Software (http://LinRegPCR.nl). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

*2.8 Analysis of ATP metabolism by high performance liquid chromatography (HPLC) in zebrafish brain*

Membrane samples were obtained as described in the subsection 2.4. The reaction medium contained 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for NTPDase activities) in a final volume of 200 µl. The membrane preparation (3-5 µg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. To start the reaction, ATP was added to the medium in a final concentration of 0.1 mM at 37 °C. Aliquots of the sample were collected at different incubation times (0–180 min), with the reaction being stopped with 5% TCA and immediately placed on ice. All samples were centrifuged 14.000 *g* for 15 min and stored on -80ºC until HPLC analysis. An HPLC system equipped with an isocratic pump, a diode array detector (DAD), a degasser, and a manual injection system was used (Agilent Technologies, Santa Clara, CA, USA). Aliquots of 20 µl were applied into HPLC system and chromatographic separations were performed using a reverse-phase column (150 mm x 4 mm, 5 µm Agilent® 100 RP-18 ec). The flow rate of the 60 mM $KH_2PO_4$, 5 mM tetrabutylammonium chloride, pH 6.0, in 13% methanol mobile phase was 1.2 mL/min. The absorbance was monitored at 260 nm, according to a method previously described, with few modifications [51]. The peaks of purines (ATP, ADP, AMP, and adenosine) were identified by their retention times and quantified by comparison with standards. The results are expressed as µM of the different compounds for each different incubation time. All incubations were carried out in triplicate and the controls to correct nonenzymatic hydrolysis of nucleotides were performed by measuring the peaks present into the same reaction medium without membrane. The control for intrinsic membrane purines was performed by incubation of the preparation without the substrate under the same conditions.

*2.9 Statistical analysis*

For enzyme assays, the data are shown as mean ± S.D of at least five (ATP, ADP and AMP hydrolysis and ADA activities) different experiments. For molecular and HPLC analysis, the results are expressed as mean ± S.E.M of four experiments. A pool of five whole zebrafish brains was used for each independent experiment. Statistical analysis was performed by Student's t-test. The statistical comparison of data regarding extracellular ATP metabolism was carried out at each timepoint of incubation and over-time of incubation. For assessing the global overtime changes, the area under the curve was obtained for each homogenate. Statistically significant differences between groups were considered for a $P<0.05$. All data were evaluated by GraphPad Prism 6 for Windows.

## 3.RESULTS

*3.1 Ectonucleotidase and ADA activities*

In this study, we verified the effects of embryological exposure to VPA in zebrafish at 120 dpf on ectonucleotidase (E-NTPDase and ecto-5'-NT) and ADA activities, which are responsible for regulating the extracellular concentrations of purine and pyrimidine nucleotides. Our results have demonstrated that there were no significant changes on ATP and ADP hydrolysis ($P=0.743$ and $P=0.258$, respectively) when compared to the control group (Fig. 1A and B). In relation to ecto-5′-nucleotidase activity, the results showed that embryological exposure to VPA in zebrafish at 120 dpf promoted an increase of AMP hydrolysis in brain membranes (12.5%; 14.4±0.3 nmol Pi $min^{-1}$ $mg^{-1}$ of protein; $P<0.05$; $n=5$) when compared to control group (12.6±0.6 nmol Pi $min^{-1}$ $mg^{-1}$ of protein; $n=5$; Fig. 1C). In

contrast, ecto-ADA activity was significantly decreased (19.2%; 9.9±0.4 $NH_3$ $min^{-1}$ $mg^{-1}$ of protein; *P<0.01*; *n*=5) in brain membranes of adult zebrafish submitted to embryological VPA exposure when compared to the control group (12.3±0.5 $NH_3$.$min^{-1}$ $mg^{-1}$ of protein *n*=5; Fig. 2A). However, the cytosolic (soluble) ADA activity was not altered in adult zebrafish submitted to embryological exposure to VPA (Fig.2B).

*3.2 Gene expression analysis*

Since we have observed significant changes on ectonucleotidase and ADA activities, we investigated whether embryological exposure to VPA has any effect in the expression of ectonucleotidases and ADA genes in zebrafish at 120 dpf. Quantitative RT-qPCR analysis did not show significant changes on *entpd1, entpd2a.1, entpd2a.2, entpd2-like* and *entpd3* (Fig. 3A-E). However, we observed a significant increased on *ntpd8 (P<0.05; n*=4*)* gene expression in zebrafish at 120 dpf submitted to embryological exposure to VPA (Fig. 3F). *Ecto-5'-nucleotidase* gene expression was not changed by treatment (Fig. 3G). In relation to ADA gene expression we observed that expressions of *ADA 1, ADA 2.2, ADAasi* and *ADA L* were not affected (Fig. 4A, C-D, respectively); nonetheless, ADA 2.1 (*P*<0.05*; n*=4) showed decreased expression in the treated group when compared to control (Fig. 4B).

The effects of embryological exposure to VPA in zebrafish at 120 dpf on gene expression pattern of adenosine receptors were also analyzed. We evaluated the *A1, A2a1, A2a2, A2b* mRNA transcript levels. The results have demonstrated that the relative amount of *A2a1* mRNA levels was increased after exposure to VPA in zebrafish at 120 dpf (*P*<0.05; *n*=4; Fig. 5B). However, the results did not show significant effects on A1 (Fig. 5A), A2a2 (Fig. 5C), A2b (Fig. 5D).

*3.3 Analysis of ATP metabolism*

The results revealed a rapid catabolism of ATP and ADP, which were completely consumed after 1 h of incubation (Figs. 6A, B). We observed a significant increase in ATP level from 0 min to 10 min of incubation in the VPA- treated group at 120 dpf (Fig. 6A). In contrast, ADP level showed a decrease at 0 min and from 10 min to 60 min of incubation in the treated group (Fig. 6B). The extracellular metabolism of AMP and adenosine (ADO) (Figs. 6C and D) occurred slowly during the incubation period of 3 h. The AMP level showed an increase at 60 min of incubation and decreased at 120 min of incubation (Fig. 6C). In relation to the adenosine level, it has been observed a decrease at 30 min of incubation (Fig. 6D). The areas under the curve were calculated for all groups and the statistical analysis confirmed the data described above (Fig. 6 - inset).

## 4.DISCUSSION

In the present study, we demonstrated that embryological exposure to VPA altered biochemical and molecular parameters related to purinergic system in adult zebrafish. These findings indicate that the ATP and adenosine-metabolizing enzymes may be involved in the pathophysiological mechanisms of diseases related to the impairment of social interaction, such as autism.

Our experiments showed that embryological exposure to VPA did not influence ATP and ADP hydrolysis in zebrafish brain membranes. These findings may be due to an adaptive plasticity in E-NTPDase induced by embryological exposure to VPA. However, this condition

was able to promote an increase in ecto-5´-nucleotidase activity. Furthermore, embryological exposure to VPA decreased the ecto-ADA activity but it was unable to alter cytosolic ADA activity in adult zebrafish. These data suggest that embryological exposure to VPA could alter adenosine levels in zebrafish brain. Beyond the adenosine release by nucleoside transporters, extracellular ATP hydrolysis promoted by ectonucleotidases is another important source of extracellular adenosine. Triphosphonucleosides and diphosphonucleosides may be hydrolyzed by nucleoside triphosphate diphosphohydrolases (NTPDases), whereas ecto-5′-nucleotidase hydrolyzes nucleoside monophosphates producing adenosine [18]. Thus, this enzyme cascade serves a double purpose, because it removes the excitatory signaling ATP molecule and, simultaneously, generates nucleoside adenosine. The control of the adenosinergic signaling can be performed by adenosine uptake via bi-directional transporters, followed by intracellular phosphorylation to AMP by adenosine kinase (AK) or deamination to inosine by adenosine deaminase. Therefore, the increased ecto-5´-nucleotidase activity and the decreased ecto-ADA activity observed in our study could lead to an increase on extracellular adenosine levels.

To assess the ATP hydrolysis in zebrafish brain, the nucleotide levels was evaluated at different times and analyzed by HPLC. We observed an increase in ATP levels and a decrease in ADP levels. The AMP levels showed an increase at 60 min of incubation and decreased at 120 min of incubation. The results presented in Fig. 6D showed that there was a decreased in ADO levels. It is our knowledge that there are other pathways involved in controlling the levels of adenosine. Extracellular adenosine concentrations can be regulated by neural cell uptake through bidirectional nucleoside transporters followed by phosphorylation to AMP by adenosine kinase [22, 52]. A hypothesis for the observed decrease in adenosine levels in the

brain of the zebrafish may be due to the nucleoside reuptake, through the bidirectional nucleoside transporters and the action of enzyme adenosine kinase.

Abnormalities in purine metabolism have been reported in ASD [53, 54]. Adenosine is an important neuromodulator and, together with ATP, these purine molecules form a unique connection between cellular energy and neuronal excitability [55]. Based on behavioral and physiological characteristics of ASD, insufficient adenosine levels may be related to some symptoms (e.g., poor eye contact, repetitive movements) [28, 56]. Several studies suggest the therapeutic potential of adenosine in relation to autism [28, 30, 31, 55, 57]. Masino and collaborator [28] report that interventions that generate an increase in adenosine levels are an important strategy to alleviate symptoms related to autism. Thus, it is critical to explore the therapeutic potential of adenosine, a neuroprotective molecule, with strong effects on neural activity in ASD. During excessive neuronal activity, increased adenosine provides a local feedback inhibition reducing the excitability, and as a result, protects neurons from excitotoxicity [33]. Multiple adenosine receptor agonists are in clinical trials for various conditions, including cardiac arrhythmias, neuropathic pain, myocardial perfusion imaging, cardiac ischemia, inflammatory diseases and cancer [58]. Ghanizadeh (2010) [31] proposed that caffeine, an adenosine receptor antagonist with differential effects depending on acute or chronic administration, could have beneficial effects in ASD. Furthermore, Tanimura and collaborators [57] showed that adenosine $A_{2A}$ receptor ($A_{2A}R$) activation has been associated with reduced perseverative behaviors. Thus, adenosine receptors may be new target for the treatment of repetitive behaviors in autism [57].

Stubbs and collaborators [59] observed a reduction of ADA activity in serum of autistic children. Based on this study, some authors reported an association between the genetic

polymorphism of adenosine deaminase and a risk factor for the development of autism [15, 60, 61]. Franco and collaborators [22] reported that the ecto-ADA in the CNS can act as a cell adhesion molecule exerting functions in growth processes and neuronal plasticity. In addition, ADA is abundant in some areas of the CNS, whose alterations in the development may be involved in the pathogenesis of autism [14, 62]. These findings corroborate with our results that showed a decrease in the ecto-ADA activity. Besides their enzymatic functions, there is evidence that is ecto-ADA linked to adenosine receptor, modulating their affinity and has co-stimulatory functional roles [63-65]. The $A_{2A}R$ is a promising candidate for genetic association studies in ASD [28, 30]. Studies have demonstrated the involvement of the $A_{2A}R$ on locomotion, anxiety, inhibition of excitatory neuronal activity and sleep regulation [57, 65].These studies are consistent with our results, since we observed that the relative amount of *A2a1* mRNA levels was increased after exposure to VPA in zebrafish at 120 dpf.

Changes in enzyme activity promoted by embryological exposure to VPA may be a consequence of transcriptional control. To determine if the transcriptional regulation has occurred, a RT-qPCR analysis was performed. Interestingly, the results demonstrated that the relative gene expression level of E-NTPDase member (*entpd8*) was significantly higher after embryological exposure to VPA, whereas there was no change in enzyme activity. Thus, the change in gene expression was not sufficient to affect the enzymatic activity of E-NTPDase. Al-Mosalem and collaborators (2009) [12] assessed E-NTPDases (ATPase and ADPase) in plasma of 30 autistic patients and observed that ATPase was non-significantly elevated compared to control whereas ADPase was significantly higher in autistic patients. While we did not observe E-NTPDase changes in brain membranes of embryological exposure to VPA in zebrafish at 120 dpf, our results demonstrated a similar effect to other studies increasing

ADPase activity, since we observed decreased ADP levels in HPLC analysis. Moreover, the results showed that there was no change in ecto-5´-nucleotidase mRNA levels after exposure to VPA in zebrafish at 120 dpf, indicating that the enhancement observed in the enzyme activity did not occur at the transcriptional level. We observed a decrease on the ecto-ADA activity, as well as a reduction on *ada2.1* gene expression after the embryological exposure to VPA in zebrafish at 120 dpf that could be a consequence of transcriptional control. Regarding the relative gene expression of adenosine receptors, we verified an increase in mRNA transcripts of $A2_{a.1}$ receptor in zebrafish brain after VPA treatment. The increase in gene expression in adenosine receptor may be a response to compensate higher levels of adenosine that occur due to increased ecto-5'-nucleotidase and decreased ecto-ADA activity. Thus, there is adenosine available for binding to adenosine receptors and this could, subsequently, generate a decrease in adenosine levels, as observed by HPLC analysis.

In summary, our data demonstrated the first evidence that embryological exposure to VPA in zebrafish at 120 dpf could modulate nucleotide and nucleoside hydrolysis and adenosine deamination in zebrafish brain membranes. In addition, our study contributes to elucidate the mechanisms underlying the modulatory effects of purinergic signaling in social interaction deficit in zebrafish.

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**References**

[1] Schneider T, Przewłocki R (2005) Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology 30:80-9

[2] Banerjee A, García-Oscos F, Roychowdhury S, Galindo LC, Hall S, Kilgard MP, Atzori M (2013) Impairment of cortical GABAergic synaptic transmission in an environmental rat model of autism. Int J Neuropsychopharmacol 16(6):1309-18

[3] Zafeiriou DI, Ververi A, Dafoulis V, Kalyva E, Vargiami E (2013) Autism spectrum disorders: the quest for genetic syndromes. Am J Med Genet B Neuropsychiatr Genet 162B:327-66

[4] Constantino JN, Todorov A, Hilton C, Law P, Zhang Y, Molloy E, et al (2013) Autism recurrence in half siblings: strong support for genetic mechanisms of transmission in ASD. Mol Psychiatry 18:137-8

[5] Volk HE, Lurmann F, Penfold B, Hertz-Picciotto I, McConnell R (2013) Traffic-related air pollution, particulate matter, and autism. JAMA Psychiatry 70(1):71-7

[6] Chao HT, Chen H, Samaco RC, Xue M, Chahrour M, Yoo J et al (2010) Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. Nature 468(7321):263-9

[7] Giovedí S, Corradi A, Fassio A, Benfenati F (2014) Involvement of synaptic genes in the pathogenesis of autism spectrum disorders: the case of synapsins. Front Pediatr 2:94

[8] Banerjee S, Riordan M, Bhat MA (2014) Genetic aspects of autism spectrum disorders: insights from animal models. Front Cell Neurosci 8:58

[9] Nguyen M, Roth A, Kyzar EJ, Poudel MK, Wong K, Stewart AM et al (2014) Decoding the contribution of dopaminergic genes and pathways to autism spectrum disorder (ASD). Neurochem Int 66:15-26

[10] Nikolac Perkovic M, Nedic Erjavec G, Stefulj J, Muck-Seler D, Pivac N et al (2014) Association between the polymorphisms of the selected genes encoding dopaminergic system with ADHD and autism. Psychiatry Res 215(1):260-1

[11] Nakamura K, Sekine Y, Ouchi Y, Tsujii M, Yoshikawa E, Futatsubashi M et al (2010) Brain serotonin and dopamine transporter bindings in adults with high-functioning autism. Arch Gen Psychiatry 67(1):59-68

[12] Al-Mosalem OA, El-Ansary A, Attas O, Al-Ayadhi L (2009) Metabolic biomarkers related to energy metabolism in Saudi autistic children. Clin Biochem 42(10-11):949-57

[13] Zhang YX, Yamashita H, Ohshita T, Sawamoto N, Nakamura S (1995) ATP increases extracellular dopamine level through stimulation of P2Y purinoceptors in the rat striatum. Brain Res 691(1-2):205-12

[14] Okada M, Kawata Y, Murakami T, Wada K, Mizuno K, Kondo T, Kaneko S (1999) Differential effects of adenosine receptor subtypes on release and reuptake of hippocampal serotonin. Eur J Neurosci 11(1):1-9

[15] Persico AM, Militerni R, Bravaccio C, Schneider C, Melmed R, Trillo S, et al (2000) Adenosine deaminase alleles and autistic disorder: case-control and family-based association studies. Am J Med Genet 96(6):784-90

[16] Theoharides TC (2013) Extracellular mitochondrial ATP, suramin, and autism? Clin Ther 35(9):1454-6

[17] Naviaux JC, Schuchbauer MA, Li K, Wang L, Risbrough VB, Powell SB, et al (2014) Reversal of autism-like behaviors and metabolism in adult mice with single-dose antipurinergic therapy. Transl Psychiatry 4:e400

[18] Bonan CD (2012) Ectonucleotidases and nucleotide/nucleoside transporters as pharmacological targets for neurological disorders. CNS Neurol Disord Drug Targets 11(6):739-50

[19] Rosemberg DB, Rico EP, Senger MR, Dias RD, Bogo MR, Bonan CD et al (2008) Kinetic characterization of adenosine deaminase activity in zebrafish (Danio rerio) brain. Comp Biochem Physiol B Biochem Mol Biol 151(1):96-101

[20] Rosemberg DB, Rico EP, Langoni AS, Spinelli JT, Pereira TC, Dias RD et al (2010) NTPDase family in zebrafish: Nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart. Comp Biochem Physiol B Biochem Mol Biol 155(3):230-40

[21] Yegutkin GG (2014) Enzymes involved in metabolism of extracellular nucleotides and nucleosides: functional implications and measurement of activities. Crit Rev Biochem Mol Biol 49(6):473-97

[22] Franco R, Casadó V, Ciruela F, Saura C, Mallol J, Canela EI et al (1997) Cell surface adenosine deaminase: much more than an ectoenzyme. Prog Neurobiol 52(4):283-94

[23] Maier SA, Galellis JR, McDermid HE (2005) Phylogenetic analysis reveals a novel protein family closely related to adenosine deaminase. J Mol Evol 61(6):776-94

[24] Zimmermann H, Zebisch M, Sträter N (2012) Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal 8(3):437-502

[25] Rosemberg DB, Rico EP, Guidoti MR, Dias RD, Souza DO, Bonan CD et al (2007) Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (Danio rerio). Life Sci 81(21-22):1526-34

[26] Rico EP, Senger MR, Fauth Mda G, Dias RD, Bogo MR, Bonan CD (2003) ATP and ADP hydrolysis in brain membranes of zebrafish (Danio rerio). Life Sci 73(16):2071-82

[27] Senger MR, Rico EP, Dias RD, Bogo MR, Bonan CD (2004) Ecto-5'-nucleotidase activity in brain membranes of zebrafish (Danio rerio). Comp Biochem Physiol B Biochem Mol Biol 139(2):203-7

[28] Masino SA, Kawamura M Jr, Plotkin LM, Svedova J, DiMario FJ Jr, Eigsti IM (2011) The relationship between the neuromodulator adenosine and behavioral symptoms of autism. Neurosci Lett 500(1):1-5

[29] Masino SA, Kawamura M Jr, Cote JL, Williams RB, Ruskin DN (2013) Adenosine and autism: a spectrum of opportunities. Neuropharmacology 68:116-21

[30] Freitag CM, Agelopoulos K, Huy E, Rothermundt M, Krakowitzky P, Meyer J (2010) Adenosine A(2A) receptor gene (ADORA2A) variants may increase autistic symptoms and anxiety in autism spectrum disorder. Eur Child Adolesc Psychiatry 19(1):67-74

[31] Ghanizadeh A (2010) Possible role of caffeine in autism spectrum disorders, a new testable hypothesis. J Food Sci 75(6):ix

[32] Gomes CV, Kaster MP, Tomé AR, Agostinho PM, Cunha RA (2011) Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. Biochim Biophys Acta 1808(5):1380-99

[33] Fredholm BB (2007) Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ 14(7):1315-23

[34] Nery LR, Eltz NS, Hackman C, Fonseca R, Altenhofen S, Guerra HN et al (2014) Brain intraventricular injection of amyloid-β in zebrafish embryo impairs cognition and increases tau phosphorylation, effects reversed by lithium. PLoS One 9:e105862

[35] Newman M, Ebrahimie E, Lardelli M (2014) Using the zebrafish model for Alzheimer's disease research. Front Genet 5:189

[36] Seibt KJ, Piato AL, da Luz Oliveira R, Capiotti KM, Vianna MR, Bonan CD (2011) Antipsychotic drugs reverse MK-801-induced cognitive and social interaction deficits in zebrafish (Danio rerio). Behav Brain Res 224(1):135-9

[37] Collier AD, Khan KM, Caramillo EM, Mohn RS, Echevarria DJ (2014) Zebrafish and conditioned place preference: a translational model of drug reward. Prog Neuropsychopharmacol Biol Psychiatry 55:16-25

[38] Kalueff AV, Stewart AM, Gerlai R (2014) Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol Sci 35:63-75

[39] Siebel AM, Piato AL, Schaefer IC, Nery LR, Bogo MR, Bonan CD (2013) Antiepileptic drugs prevent changes in adenosine deamination during acute seizure episodes in adult zebrafish. Pharmacol Biochem Behav 104:20-6

[40] Saverino C, Gerlai R (2008) The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish. Behav Brain Res 191(1):77-87

[41] Zimmermann FF, Gaspary KV, Leite CE, De Paula Cognato G, Bonan CD (2015) Embryological exposure to valproic acid induces social interaction deficits in zebrafish (Danio rerio): A developmental behavior analysis. Neurotoxicol Teratol 52(Pt A):36-41

[42] Westerfield M (2000) The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). 4th ed. Eugene: Univ of Oregon Press.

[43] Zellner D, Padnos B, Hunter DL, MacPhail RC, Padilla S. (2011) Rearing conditions differentially affect the locomotor behavior of larval zebrafish, but not their response to valproate-induced developmental neurotoxicity. NeurotoxicolTeratol. 33:674-679.

[44] Wilson JM, Bunte RM, Carty AJ (2009) Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (Danio rerio). J Am Assoc Lab Anim Sci 48(6):785-9

[45] Barnes JM, Murphy PA, Kirkham D, Henley JM (1993) Interaction of guanine nucleotides with [3H]kainate and 6-[3H]cyano-7-nitroquinoxaline-2,3-dione binding in goldfish brain. J Neurochem 61(5):1685-91

[46] Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca2+ -stimulated ATPase activity. Anal Biochem 157(2):375-80

[47] Weisman MI, Caiolfa VR, Parola AH (1988) Adenosine deaminase-complexing protein from bovine kidney. Isolation of two distinct subunits. J Biol Chem 263(11):5266-70

[48] Tang R, Dodd A, Lai D, Mcnabb WC, Love DR (2007) Validation of Zebrafish (*Danio rerio*) Reference Genes for Quantitative Real-time RT-PCR Normalization. Acta Biochim Biophys Sin (39)384-390

[49] Leite CE, Maboni Lde O, Cruz FF, Rosemberg DB, Zimmermann FF, Pereira TC et al (2013) Involvement of purinergic system in inflammation and toxicity induced by copper in zebrafish larvae. Toxicology and Applied Pharmacology 272(3):681-9

[50] Capiotti KM, Fazenda L, Nazario LR, Menezes FP, Kist LW, Bogo MR et al (2013) Arginine exposure alters ectonucleotidase activities and morphology of zebrafish larvae (*Danio rerio*). Int J Dev Neurosci 31(1):75-81

[51] Voelter W, Zech K, Arnold P, Ludwig G (1980) Determination of selected pyrimidines, purines and their metabolites in serum and urine by reversed-phase ion-pair chromatography. J Chromatogr 199:345-54

[52] Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM (2005) Adenosine and brain function. Int Rev Neurobiol 63:191-270

[53] Nyhan WL, James JA, Teberg AJ, Sweetman L, Nelson LG (1969) A new disorder of purine metabolism with behavioral manifestations. J Pediatr 74(1):20-7

[54] Page T, Coleman M (1998) De novo purine synthesis is increased in the fibroblasts of purine autism patients. Adv Exp Med Biol 431:793-6

[55] Masino SA, Kawamura M, Wasser CD, Pomeroy LT, Ruskin DN (2009) Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity. Curr Neuropharmacol 7(3):257-68

[56] Malow BA (2004) Sleep disorders, epilepsy, and autism. Ment Retard Dev Disabil Res Rev 10(2):122-5

[57] Tanimura Y, Vaziri S, Lewis MH (2010) Indirect basal ganglia pathway mediation of repetitive behavior: attenuation by adenosine receptor agonists. Behav Brain Res 210(1):116-22

[58] Gao ZG, Jacobson KA (2011) Emerging adenosine receptor agonists: an update. Expert Opin Emerg Drugs 16(4):597-602

[59] Stubbs G, Litt M, Lis E, Jackson R, Voth W, Lindberg A et al (1982) Adenosine deaminase activity decreased in autism. J Am Acad Child Psychiatry 21(1):71-4

[60] De Luca D, Lucarelli P, Bottini N, Porfirio C, Curatolo P (1999) Adenosine deaminase polymorphism. J Child Neurol 14:323-324

[61] Bottini N, De Luca D, Saccucci P, Fiumara A, Elia M, Porfirio MC et al (2001) Autism: evidence of association with adenosine deaminase genetic polymorphism. Neurogenetics 3(2):111-3

[62] Yamamoto T, Geiger JD, Daddona PE, Nagy JI (1987) Subcellular, regional and immunohistochemical localization of adenosine deaminase in various species. Brain Res Bull 19(4):473-84

[63] Ciruela F, Saura C, Canela EI, Mallol J, Lluis C, Franco R (1996) Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. FEBS Lett 380(3):219-23

[63] Herrera C, Casadó V, Ciruela F, Schofield P, Mallol J, Lluis C et al (2001) Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. Mol Pharmacol 59(1):127-34

[64] Saura CA, Mallol J, Canela EI, Lluis C, Franco R (1998) Adenosine deaminase and A1 adenosine receptors internalize together following agonist-induced receptor desensitization. J Biol Chem 273(28):17610-7

[65] Moreau JL, Huber G (1999) Central adenosine A(2A) receptors: an overview. Brain Res  Brain Res Rev 31(1):65-82

**Table 1.** Primer sequences for RT-qPCR experiments included in the study

| Gene | Primer sequences (5'-3') | Accession number (mRNA) | Amplicon size (bp) |
|---|---|---|---|
| *Rpl13α*[a] | F-TCTGGAGGACTGTAAGAGGTATGC<br>R-AGACGCACAATCTTGAGAGCAG | NM_212784 | 147 |
| *EF1α*[a] | F- CTGGAGGCCAGCTCAAACAT<br>R-<br>ATCAAGAAGAGTAGTACCGCTAGCATTAC | NSDART00000023156 | 86 |
| *ADA1*[b] | F-GCACAGTGAATGAGCCGGCCAC<br>R-AATGAGGACTGTATCTGGCTTCAACG | BC076532.1 | 168 |
| *ADA2.1*[b] | F-TTCAACACCACACGTATCGGGCAC<br>R-ATCAGCACTGCAGCCGGATGATC | AF384217.1 | 161 |
| *ADA2.2*[b] | F-TTGCAATTGTTCATCATCCCGTAGC<br>R-TCCCGAATAAACTGGGATCATCG | XM_682627.1 | 186 |
| *ADAasi*[b] | F-CTTTGTGGTACTTCAAGGACGCTTTG<br>R-TTGTAGCAGATAAAAGAAGCGAGACG | AF384217.1 | 121 |
| *ADAL*[b] | F-CTCTAATGTGAAAGGTCAAACCGTGC<br>R-AAGACGCCCTTATCATCCGTGC | NM_001033744.1 | 108 |
| *entpd1*[c] | F-TTATGGCCTACATTTATTTCCGTCG<br>R-GATTCTTTGAAATGTAAAACCGCTTG | BC078240.1 | 176 |
| *entpd2a.1*[c] | F-TTAAATCCAATGCTATATGCCGGTG<br>R-TCTGTGATGGATGTGTCGGACAAAGG | BC078419.1 | 103 |
| *entpd2a.2*[c] | F-AAAGTTGAAGACACCTCTGTCGGCTG<br>R-CCATTCTTTTGGTAGCTTCGCAAC | XM_682630.2 | 188 |
| *entpd2-like*[c] | F-AGGCGTCTGTTGGCTGGGCTC<br>R-GAAACATCAAACCAGTCCATGCTGC | XM_692508.3 | 117 |
| *entpd3*[c] | F-GCTACAATACCTCCATACCTGCAGAGG<br>R-GATACTCCTGACCAAGGCTTTGCAC | EF446129.1 | 146 |
| *entpd8*[c] | F-GTTGCAGATACAGATATTGGTTGGACG<br>R-GTAGAGTGAGGAAGAGGGCAAATGC | NM_001002379.2 | 154 |
| *ecto-5'-nucleotidase*[c] | F-TGGACGGAGGAGACGGATTCACC<br>R-GGAGCTGCTGAACTGGAAGCGTC | BC055243.1 | 149 |
| *A1*[b] | F-GTTCCTCATTTACATTGCCATTCTGC<br>R-TGGTTGTTATCCAGTCTCTCGCTCG | NM_001128584.1 | 180 |
| *A2a1*[b] | F-GCGAACTGTACGCCGAGCAGAG<br>R-TTATTCCCAGTGAGCGGCGACTC | AY945800 | 178 |
| *A2a2*[b] | F-GGATTGGGTCATGTACCTGGCCATC<br>R-GCTGTTTCCAATGGCCAGCCTG | AY945801.1 | 160 |
| *A2b*[b] | F-GTTTGTTCGCTCTCTGTTGGCTGC<br>R-CTAAAAGTGACTCTGAACTCCCGAATG | AY945802.1 | 178 |

According to [a]Tang et al. (2007), [b]Leite et al. (2013), [c]Capiotti et al. (2013).

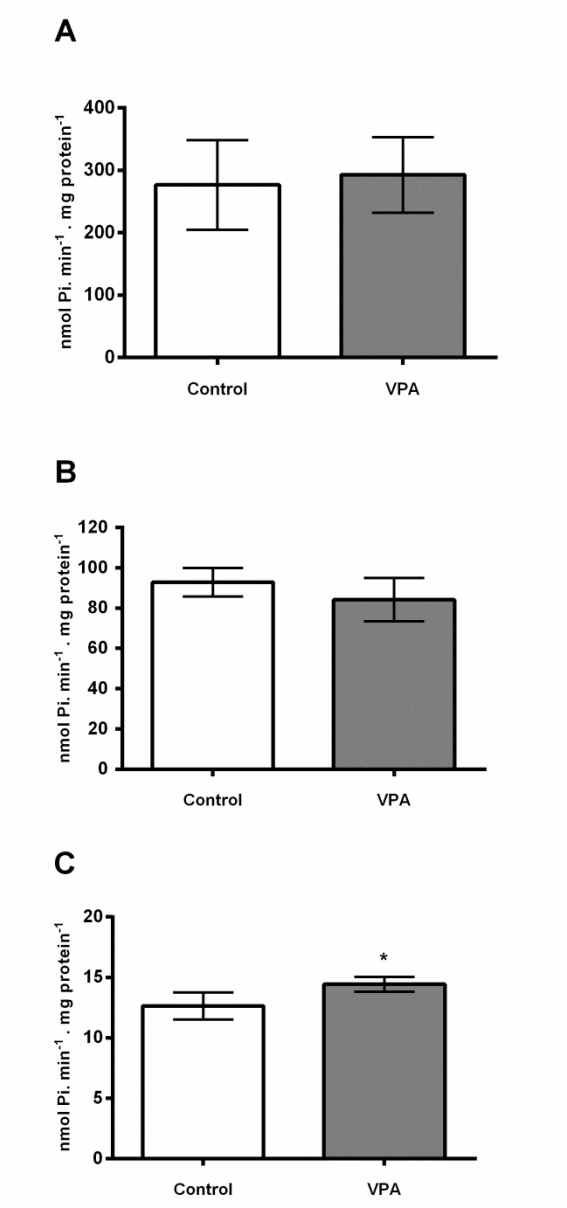


**Fig. 1** Effect of VPA treatment on ATP (A), ADP (B) and AMP (C) hydrolysis from zebrafish brain. Bars represent the mean ± SD (n=8). The symbol (asterisk) represents a significant difference from control group (Students t test, $P<0.05$). The specific enzyme activity is expressed as nmol Pi $min^{-1}$ mg $protein^{-1}$.

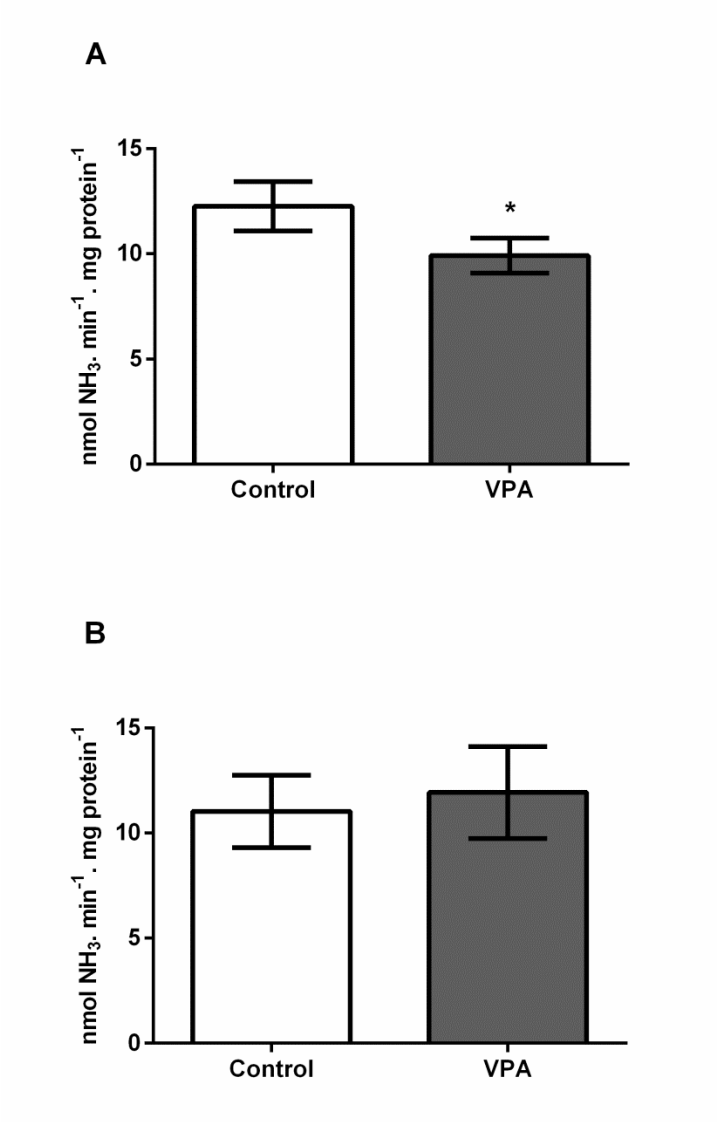


**Fig. 2** Effect of VPA treatment on membrane-bound (A) and soluble (B) ADA activity from zebrafish brain. Bars represent the mean±SD (n=8). The symbol (asterisk) represents a significant difference from control group (Students t test, $P<0.05$). The specific enzyme activity is expressed as nmol NH3$min^{-1}$ $mg^{-1}$ of protein.

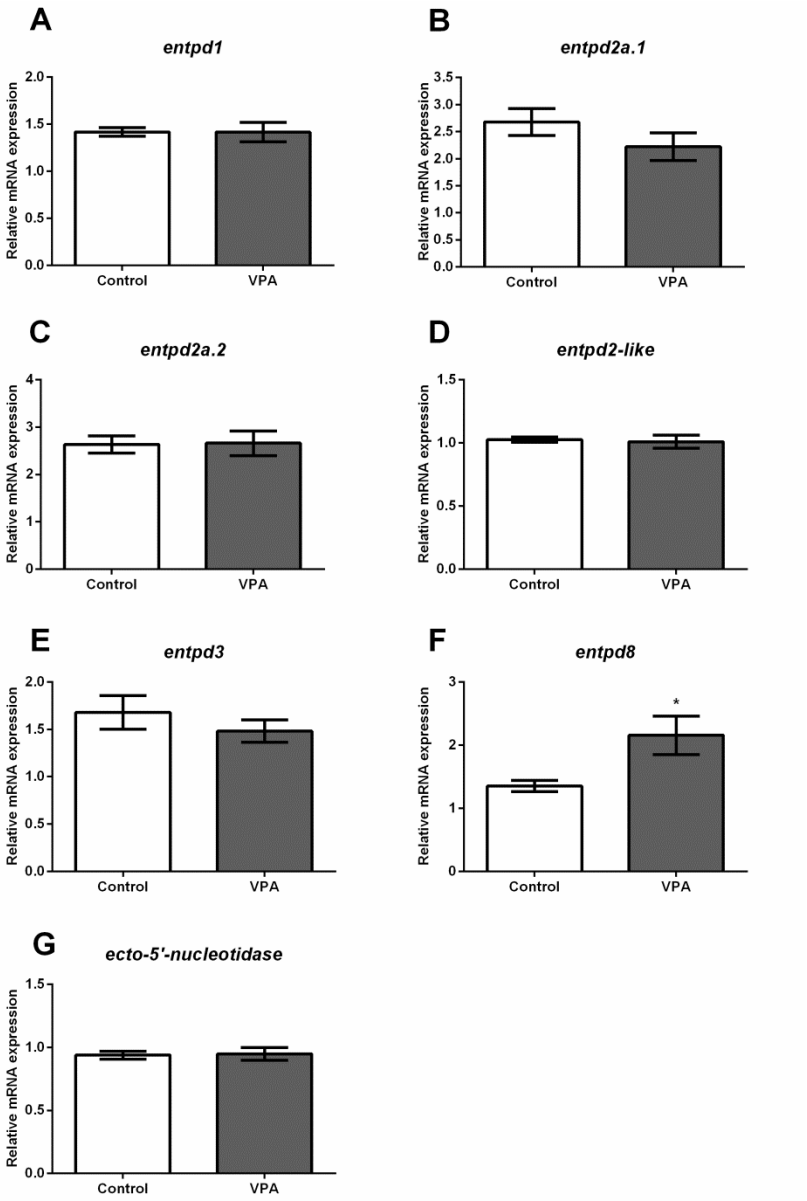


**Fig. 3** Effect of VPA treatment on ectonucleotidases gene expression in zebrafish brain. The figures show the expression patterns of *entpd1* (A), *entpd2a.1* (B), *entpd*2a.2 (C), *entpd2-like* (D), *entpd3* (E), *entpd8* (F) and *ecto-5´- nucleotidase* (G) in adult zebrafish brain. Data are expressed as mean±SEM of four independent experiments (n=4) performed in quadruplicate. The symbol (asterisk) represents a significant difference from control group (Students t test, $P<0.05$).

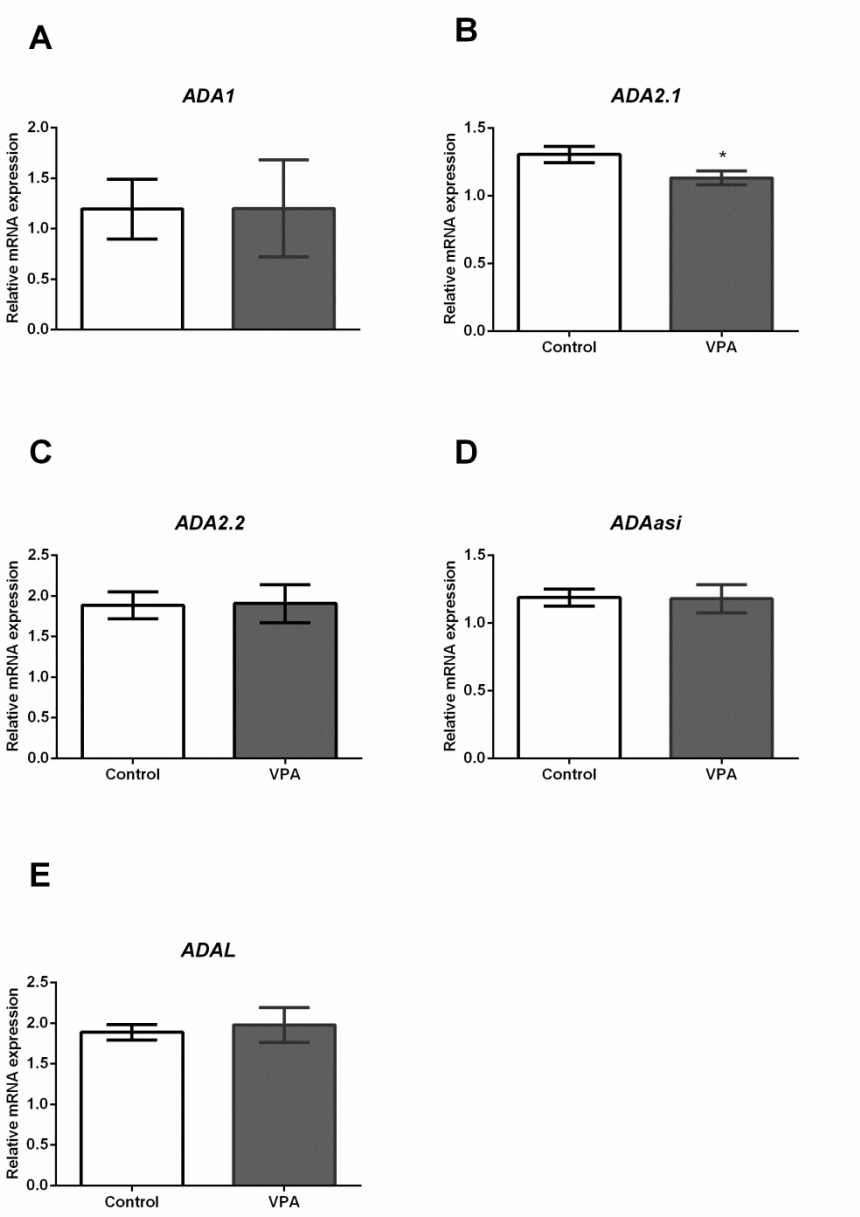


**Fig. 4** Effect of VPA treatment on ADA gene expression in zebrafish brain. The figures show the expression patterns of *ada1* (A), *ada2.1* (B) *ada2.2* (C), *adaasi* (D) and *adal* (E) in adult zebrafish brain. Data are expressed as mean±SEM of four independent experiments (n=4) performed in quadruplicate. The symbol (asterisk) represents a significant difference from control group (Students t test, $P<0.05$).

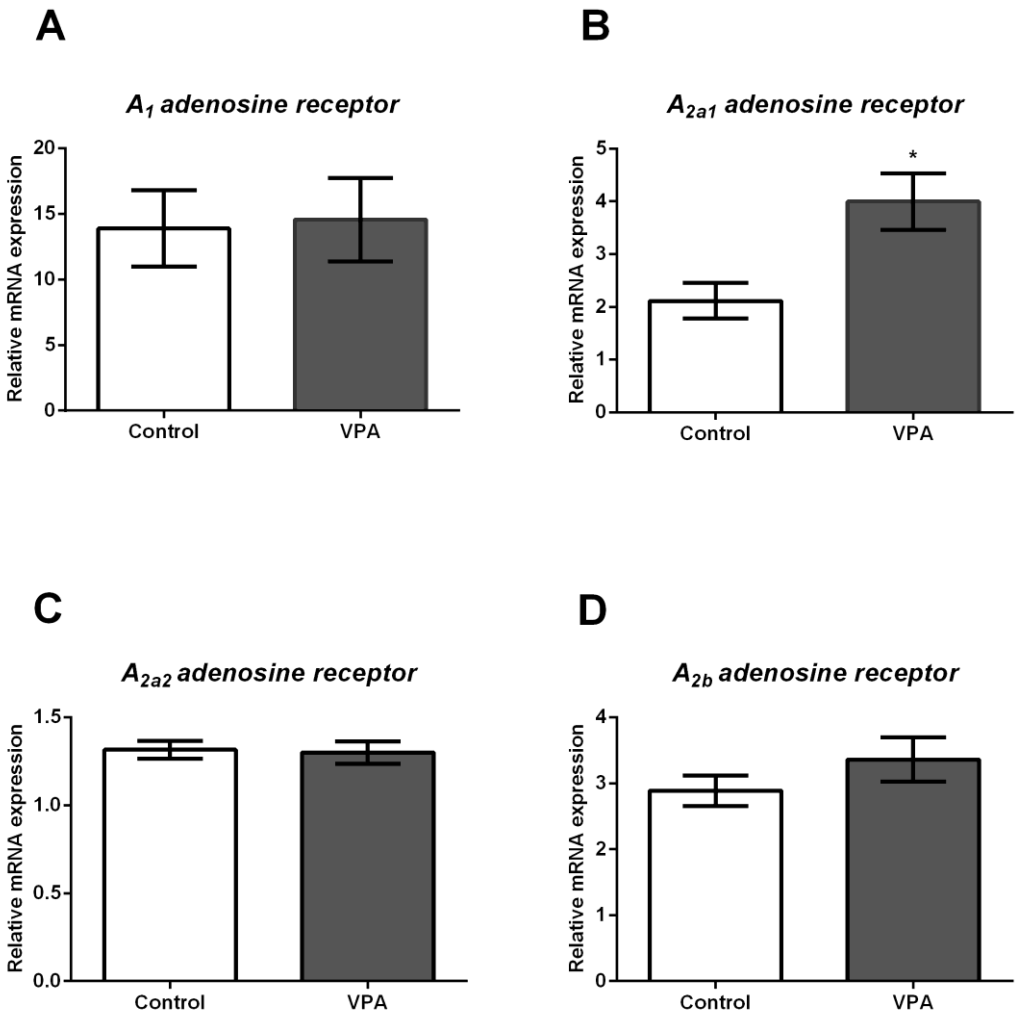


**Fig. 5** Effect of VPA treatment on adenosine receptor gene expression in zebrafish brain. The figures show the expression patterns of $A_1R$ (A), $A_{2a1}R$ (B), $A_{2a2}R$ (C) and $A_{2b}R$ (D) in adult zebrafish brain. Data are expressed as mean±SEM of four independent experiments (n=4) performed in quadruplicate. The symbol (asterisk) represents a significant difference from control group (Students t test, $P<0.05$).

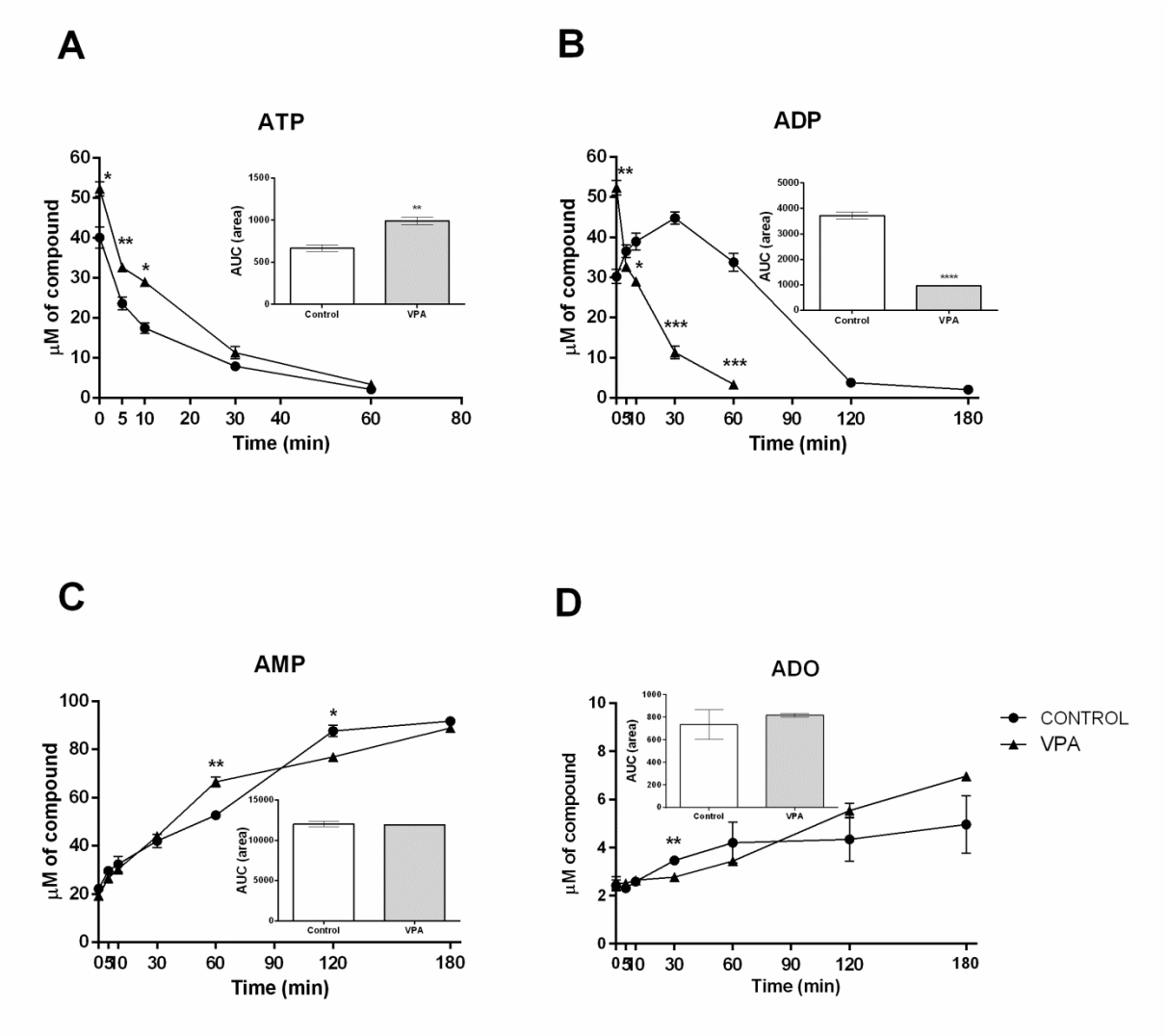


**Fig. 6** VPA effects on extracellular ATP hydrolysis and, its degradation products. ATP (A), ADP (B), AMP (C), and ADO (D) were assayed by HPLC-DAD. The data are mean ± S.D. of four homogenates The symbols represent statistical difference from control group (Students t test, * $P<0.05$, ** $P < 0.005$, *** $P < 0.0005$). The groups were compared at each time of incubation (lines) and over time of incubation (inset). For assessment over time, the area under the curve was obtained for each homogenate.

# CAPÍTULO III

## ARTIGO CIENTÍFICO

*Oxytocin reversed MK-801-induced social interaction and aggression deficits in zebrafish*

Fernanda Francine Zimmermann, Karina Vidarte Gaspary, Anna Maria Siebel,

Carla Denise Bonan

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Authors: Fernanda F Zimmermann; Karina V Gaspary; Anna M Siebel; Carla Denise Bonan
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# OXYTOCIN REVERSED MK-801-INDUCED SOCIAL INTERACTION AND AGGRESSION DEFICITS IN ZEBRAFISH

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**Abstract**

Changes in social behavior occur in several neuropsychiatric disorders such as schizophrenia and autism. The interaction between individuals is an essential aspect and an adaptive response of several species, among them the zebrafish. Oxytocin is a neuroendocrine hormone associated with social behavior. The aim of the present study was to investigate the social interaction and aggression produced by MK-801 exposure in the zebrafish. We also examined the effects of oxytocin, oxytocin receptor agonist (carbetocin) and the oxytocin receptor antagonist (L-368,899) on the reversal of behavioral effects induced by MK-801 in zebrafish. Our results have shown that MK-801 induced a decrease in the time spent in the segment closest to the conspecific school and in the time spent in the segment nearest to the mirror image, suggesting an effect on social behavior. The treatment with oxytocin after the exposure to MK-801 was able to reestablish the time spent in the segment closest to the conspecific school, as well as the time spent in the segment nearest to the mirror image. In addition, to support the modulation of oxytocin pathway, we verified that the oxytocin receptor agonist (carbetocin) reestablished the social and aggressive behavioral deficits induced by MK-801. However, the oxytocin receptor antagonist (L-368,899) was not able to reverse the behavioral changes induced by MK-801. This study supports the critical role for NMDA receptors and the oxytocinergic system in the regulation of social behavior and aggression which may be relevant for the mechanisms associated to autism and schizophrenia.

**Keywords:** aggression; MK-801; oxytocin; social behavioral; zebrafish

## Introduction

Neurodevelopmental disorders comprise a group of neuropsychiatric manifestations caused by disturbances in brain development, including autism spectrum disorders [1]. Similarly, schizophrenia has also been proposed as a result of alterations in neurodevelopment, usually expressing only in the adult stage [2]. The symptoms of schizophrenia can be classified into cognitive (decreased attention, memory problems), negative (anhedonia, social withdrawal) or positive (hallucinations, disorganized behavior, high levels of aggression) types [1]. The autistic patients often also show symptoms, such as deficits in social communication and interaction, repetitive patterns of behavior, restricted interests, anxiety and hyperactivity [1,3]. Historically, autism and schizophrenia were considered to be intimately related [4,5]. Several studies indicate that these diseases share overlapping characteristics [6]. A major feature of the clinical symptoms presented by both autism and schizophrenia is the impairment in social functions [7]. Furthermore, abnormal aggression levels are observed in human patients with psychiatric disorders such as ASD [8] and SZ [9].

Aggression is an adaptive behavior that is essential for the establishment of social hierarchies, mating, competition for food and territory [10]. Evidence shows that zebrafish exhibit a rich repertoire of aggressive behavior and the neural mechanisms can be studied for understanding the core pathogenesis of aggression [11,12]. In this way, a certain level of aggression can be beneficial for survival of an individual or species, as well as in the social function.

Zebrafish is a highly social species which plays an excellent role as a model organism to study neuropathological disorders that affect the social behavior, such as autism and

schizophrenia [13,14]. Social interaction is a significant and complex aspect of zebrafish behavior [15,16]; however, the mechanisms that regulate the social behavior in zebrafish are not well understood. Studies suggest that fish are suitable model to evaluate social behavior and its evolution, since the structure of the brain and physiology are conserved among vertebrates [17,18]. An extensive network of nuclei that is critical for social behavior is highly conserved within vertebrates [17,19]. Furthermore, the fish are able to perform complex decisions on social context [18].

A hypo-function of ionotropic N-methyl-D-aspartate (NMDA) receptors is implicated in schizophrenia [20,21]. Dizocilpine (MK-801), a non-competitive antagonist of the glutamate NMDA-receptor, has been most strongly implicated in social behavior in animal models and is applied to mimic some aspects of autism and schizophrenia [22-24]. Behavior and pharmacological actions of NMDA appear to be conserved in zebrafish [25]. Several studies showed that MK-801 induced deficit in the social interaction parameters in rodents and zebrafish [23,26].

The role of the neuropeptide oxytocin (OT) in the different types of social behavior (including aggression) is one of the earliest discoveries in social neuroscience [27,28]. Oxytocin is a promising molecule for the treatment of psychotic symptoms in patients with several brain disorders, including schizophrenia and autism [29,30]. Modahl et al. (1998) reported that the plasma concentration of oxytocin is reduced in children with autism [31]. Hollander et al. (2007) showed that intravenous administration of oxytocin reduces repetitive behaviors and increases the understanding of the emotional meaning in individuals with ASD [32]. Studies reported the improvement of social deficits by administering oxytocin for schizophrenic patients [33,34]. In fish, oxytocin has effects on courtship and social behavior

[35,36]. Braida et al. (2012) demonstrated that oxytocin and vasopressin increased social behavior and reduced the fear response to the predator, indicating a neuromodulatory role in these complex behaviors in zebrafish [37].

The aim of the present study was to investigate the social behavior, i.e., changes in the social interaction and aggression produced by MK-801 exposure in the zebrafish. Since that oxytocin can modulate social behavior, we also examined the effects of oxytocin, oxytocin receptor agonist (carbetocin) and the oxytocin receptor antagonist (L-368,899) on the reversal of behavioral effects induced by MK-801 in zebrafish.

## 1. Materials and methods

### 2.1. Animals

Adult (6–8 months old) wild-type zebrafish (D. rerio) used in this study were obtained from our breeding stock held at the Pontifícia Universidade Católica do Rio Grande do Sul. The animals were housed in a 50 L-thermostated aquarium filled with unchlorinated water constantly aerated at a targeted temperature of 26 ± 2 °C. Fish were kept under a 14–10 h light/dark cycle photoperiod and fed twice a day with commercial flake fish food supplemented with live brine shrimp. The protocol was approved by the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) under the number 13/00346-CEUA.

### 2.2 Materials

The drugs hydrogen maleate (MK-801), oxytocin, carbetocin, L-368,899 and tricaine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.3 Pharmacological treatments

A group of animals was individually exposed in a 300-ml beaker to 5 μM MK-801 hydrogen maleate, dissolved in tank water, for 15 min. To assess the effects on the reversal of the behavioral changes induced by MK-801, we conducted the anesthesia of the animals prior to the injection, which was obtained by immersion in a 100 mg/L tricaine solution until the animal showed lack of motor coordination and reduced respiration rate. The animals received i.p. injection with 10 ng/kg oxytocin, 10 ng/kg carbetocin, 10 ng/kg L-368,899 or saline. After the injection, the animals were placed in a separate tank with highly aerated unchlorinated tap water (26±2 °C) to facilitate their recovery from anesthesia. Intraperitoneal injections were conducted using a 3/10-mL U-100 BD Ultra- Fine™ Short Insulin Syringe 8 mm (5/16 in.) × 31 G Short Needle (Becton Dickinson and Company, New Jersey, USA). The same animals were transferred to another beaker containing water, and remained there for additional 15 min. After this period, the animals were individually placed in a test tank to analyze the social behavior.

### 2.4 Behavioral assessment

#### 2.4.1. Social interaction

The zebrafish is a schooling fish that may exhibit preference for its conspecifics under certain circumstances. Each fish was placed in an experimental tank (30 cm × 15 cm × 10 cm, length × height × width). On one side of the experimental tank, an empty fish tank was placed; on the other side, a tank of identical size held 15 zebrafish, which were designed the “stimulus fish”. The experimental fish was allowed to acclimate to the experimental tank for a 30 s period, after which behavior was video recorded over a period of 5 min. To quantify fish

preference between the “stimulus fish” side of their tank in detriment of the empty tank, the experimental fish tank was divided in four equal sections. The zones 1 and 2 of the tank correspond to the segments closer to the conspecific school and the zones 3 and 4 are considered to the segments closer to the empty tank. The amount of time the experimental fish spent on each zone was measured using ANYMaze recording software (Stoelting Co., Wood Dale, IL, USA) [38] (Gerlai et al., 2000).

#### 2.4.2. Aggression

The mirror test was used to measure aggression [38]. Each fish was placed in an experimental tank (30 cm × 15 cm × 10 cm, length × height × width). A mirror (45 cm × 38 cm) was placed at the side of the tank at an angle of 22.5° to the backwall of the tank so that the left vertical edge of the mirror touched the side of the tank and the right edge was further away. Thus, when the experimental fish swam to the left side of the tank, their mirror image appeared closer to them. A test fish was added to the tank and was allowed acclimate for 60 s; the aggressive behaviors that a fish conducted toward its mirror image were subsequently recorded over a period of 5 min. The vertical lines divided the tank into four equal sections and allowed the number of entries to each section made by the fish to be counted. Entry to the left-most segment indicated preference for proximity to the “opponent”, whereas entry to the right most segments implied avoidance. The amount of time the experimental fish spent in each segment was measured using ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA).

### 2.5. Statistical analysis

The data are expressed as the mean ± S.E.M., and were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. For all comparisons, the significance level was set at $P < 0.05$.

## 3. Results

### *3.1. Modulation with oxytocin on social interaction deficit induced by MK-801*

Our results have shown that MK-801 treatment induces social interaction deficit in zebrafish. Fig. 1 shows the effect of oxytocin on MK-801-induced social interaction impairment in zebrafish. As expected, animals treated with MK-801 significantly decreased (193.1±17.8 s) the time spent in the segment closest to the conspecific school compared to the control group (treated with saline and exposed to tank water) (248.8 ± 17.8 s). Animals treated with oxytocin *per se* were devoid of effects. However, when zebrafish were treated with oxytocin after MK-801 pretreatment spent a longer time (254 ± 20.9 s) in the segment closest to the conspecific school when compared to animals that were exposed to MK-801 after saline treatment (193.1 ± 17.8 s) (Fig 1; $F(1,45) = 2.5$, $P < 0.05$). In this way the treatment with oxytocin reversed the effects of MK-801 in the social interaction test.

### *3.2. Effects of oxytocin receptor antagonist and agonist on social interaction response to MK-801*

In order to investigate the effects of oxytocin receptor antagonist and agonist on social interaction response to 5 μM MK-801, we treated zebrafish with L-368,899 (10 ng/kg) or carbetocin (10 ng/kg), respectively, during 15 min after MK-801 exposure. Our results showed carbetocin (oxytocin receptor agonist) was able to reverse the MK-801-induced changes on the social interaction whereas the L-368,899 (oxytocin receptor antagonist) did not alter

behavioral responses induced by MK-801. Animals treated with carbetocin after MK-801 treatment spent 273.4 ± 28.6 s in the segment closest to the conspecific school, while animals that were exposed to saline after MK-801 pre-treatment spent 188.6 ± 28.6 s (Fig. 2). Control animals (treated with saline and exposed to tank water) and animals treated with carbetocin and exposed to tank water remained in the segment closest to the conspecific school during 285 ± 25.4 and 262.7 ± 25.4 s, respectively (Fig. 2; $F(1,39) = 7.9$, $P > 0.05$). L-368,899 was not able to change behavioral responses induced by MK-801 pretreatment (Fig. 3; $F(1,44) = 1.2$, $P > 0.05$).

*3.3. MK-801 induces changes in aggressive behavior*

The MK-801 promoted significant changes in the aggressive behavior and was evaluated by the time spent in the segment nearest to the mirror image. Our results demonstrated that animals treated with MK-801 remained less time in the segment nearest to the mirror (36.6 ± 5.4 s) when compared with the control group (89.1 ± 16.8 s) ($P < 0.05$), indicating that treatment with MK-801 induced a decrease in aggressive behavior (Fig. 4).

*3.4. Modulation with oxytocin on aggressive behavior deficit induced by MK-801*

The results showed that MK-801 treatment induced decrease in aggressive behavior in zebrafish. Fig. 5 demonstrates the effect of oxytocin on MK-801-induced aggressive behavior impairment in zebrafish. Animals treated with MK-801 significantly decreased (44.4 ± 22.3 s) the time spent in the segment nearest to the mirror image compared to the control group (treated with saline and exposed to tank water) (107 ± 22.3 s). Animals treated with oxytocin *per se* were devoid of effects. However, zebrafish treated with oxytocin after MK-801 pretreatment spent 117.3 ± 22.3 s in the segment nearest to the mirror image school, while animals that were exposed to MK-801 after saline treatment spent 44.4 ± 22.3 s, (Fig 5; F (1,

52) = 15.7, P < 0.05). In this way, the treatment with oxytocin reversed the effects of MK-801 in the aggressive behavior test.

*3.5. Effects of oxytocin receptor antagonist and agonist on aggressive behavior response to MK-801*

In order to investigate the effects of oxytocin receptor agonist and antagonist on aggressive behavior to 5 μM MK-801, we treated zebrafish with carbetocin (10 ng/kg) and L-368,899 (10 ng/kg), respectively, during 15 min after MK-801 exposure. Our results have shown that the oxytocin receptor agonist carbetocin is able to reverse the aggressive behavior changes induced by MK-801 pretreatment and the oxytocin receptor antagonist L-368,899, is not able to change behavioral responses induced by MK-801 pretreatment. Animals treated with carbetocin after MK-801 treatment spent 91.6 ± 20 s in the segment nearest to the mirror image, while animals that were exposed to saline after MK-801 pretreatment spent 35.3 ± 15 s. Control animals (treated with saline and exposed to tank water) and animals treated with carbetocin and exposed to tank water remained in the segment nearest to the mirror image during 127.5 ± 15 and 62 ± 15 s, respectively (Fig. 6; F (1,50) = 22.8, P < 0.05). The oxytocin receptor antagonist L-368,899 was not able to change behavioral responses induced by MK-801 pretreatment (Fig. 7; F (1,30) = 1.9, P > 0.05).

## 4. Discussion

In this study, our results have shown that MK-801 induced a decrease in the time spent in the segment closest to the conspecific school and in the time spent in the segment nearest to the image, suggesting an effect on social behavior. The treatment with oxytocin after the

exposure to MK-801 showed that oxytocin was able to reestablish the time spent in the segment closest to the conspecific school, as well as the time spent in the segment nearest to the mirror image. To support the modulation of oxytocin pathway, we verified that the oxytocin receptor agonist (carbetocin) recovered the social and aggressive behavioral patterns. However, the oxytocin receptor antagonist (L-368,899) was not able to reverse the time spent in the segment closest to the conspecific school as well as the time spent in the segment nearest to the mirror image. Neuropsychiatric disorders, such as autism and schizophrenia, share an important feature of clinical symptoms, such as impairment in social functions [7,39]. Schizophrenia and autism are considered complex diseases with multiple factors contributing to pathogenesis [40,41]. Traditional medicines currently available are not absolutely effective, since it deals with only some of the symptoms.

MK-801 is one of the NMDA receptor antagonists, and this agent has been used for inducing schizophrenia and autism symptoms [24,42,43]. Of the three subtypes of receptors for glutamate, the NMDA receptor has been most strongly implicated in social behavior [44-46]. The effect of MK-801 on social behavior was induced in different animal models [23,47]. Several studies have shown social behavior changes due to NMDA receptor antagonists in rodents and zebrafish [23,43,47]. Moy et al. (2013) showed that MK-801 led to a loss of significant social preference measured by the time spent in each side of the chamber [43]. The neurobehavioral actions of NMDA receptor antagonism are conserved in zebrafish [25]. In zebrafish, it has been already described the impact of MK-801 on social behavior. According to Maaswinkel et al. (2013), MK-801-treated zebrafish reduced social cohesion of the entire shoal [24]. Seibt et al. (2011) demonstrated that MK-801 reduces the preference of zebrafish for a stimulus group of zebrafish and Echevarria et al. (2008) reported a disrupted shoaling

[23,48]. Although there are studies evaluating the effect of MK-801 on social interaction, it is unclear its effects on aggression behavior. The neurobiology of aggressive behavior involves a complex network [49]. McAllister (1990) found that MK-801, among other compounds, tends to increase the aggression and social behavior in mice [50]. In agreement to our study, Kalinine et al. (2014) demonstrated that a single intraperitoneal dose of the MK-801 shortly before the intruder test decreased aggressive behavior in mice [51]. Our results showed that MK-801 induced deficit in social interaction and decreased aggressive behavior since animals exposed to MK-801 spent time in the segment closest to the conspecific school as well as in the segment nearest to the mirror image.

Zebrafish models of neuropsychiatric diseases provide a base for mechanistic understanding and development of new therapies. Evidence suggests that the deregulation of oxytocinergic system may be involved in the pathophysiology of certain neuropsychiatric disorders, such as autism and schizophrenia [52,53]. The oxytocinergic system plays a crucial role in several and complex social behaviors and social interaction [54,55]. The neuropeptide oxytocin is involved in the modulation of different aspects of social behavior.

In addition to its vital function as a hormone, oxytocin also acts on an important central network as neurotransmitter peptide [56]. Most oxytocin neurons project to the posterior pituitary but some also project within the central nervous system and the endogenous central oxytocin plays a physiologically significant role in social behavior [57].

Considering that there is no standard treatment for social dysfunction and clinical studies have identified oxytocin as a potential therapeutics, we investigated the effects of oxytocin, oxytocin receptor agonist (carbetocin) and oxytocin receptor antagonist (L-368,899) in MK-801-induced changes on social interaction and aggression. Our data demonstrated that

the treatment with oxytocin after the exposure to MK-801 was able to reestablish the time spent in the segment closest to the conspecific school as well as the time spent in the segment nearest to the mirror image. In addition, we verified the oxytocin receptor agonist carbetocin reestablished the social and aggressive behavioral patterns whereas and oxytocin receptor antagonist L-368,899 was not able to reverse the decrease in the social interaction and aggression induced by MK-801. Behavioral studies employing similar approach indicated that oxytocin treatment can increase sociability in rodents and zebrafish [37, 58]. Braida et al. (2012) demonstrated that the oxytocin increased social preference in zebrafish and the antagonists dose-dependently inhibited the effect induced by the neuropeptides, which corroborates with our findings [37]. At the present moment, there are no studies showing the effect of oxytocin and aggression in zebrafish; however, Filby et al. (2010) demonstrated that treatment with arginine vasotocin (AVT) (is found in fishes and a key modulators of social and nonsocial behavior, similar to oxytocin) significantly reduced aggression in dominant male zebrafish [59]. Furthermore, studies demonstrated that oxytocin has also been linked to social dominance and aggression in rodents [60,61]. The potent effects of intranasal oxytocin suggest that may be beneficial for a variety of psychiatric disorders, including schizophrenia and autism [32,62,63]. Modahl et al. (1998) observed a significant correlation between oxytocin levels and social impairment in children [31]. Hollander et al. (2007) verified that oxytocin administration facilitated the processing and retention of social information in adults diagnosed with autism or Asperger's disorder [32]. Other study suggests that oxytocin may relieve the positive symptoms, debilitating and reducing social and cognitive deficits in schizophrenic patients [29].

The oxytocin receptors are also highly conserved in evolution. In this way, to test whether the effects on social behavior and aggression were mediated by oxytocin receptor in zebrafish, we tested the oxytocin receptor antagonist (L-368,899) and agonist (carbetocin). Our findings indicated that the oxytocin receptor antagonist (L-368,899) was unable to reverse the effect induced by MK-801 on social interaction and aggression. On the other hand, oxytocin receptor agonist (carbetocin) reversed the effects caused by MK-801 in social behavior and aggression. Thus, we suggest that these modulatory effects may be mediated by oxytocin. There are few studies assessing the effect of oxytocin receptor pharmacologically in relation to social behavior and aggression. Mooney et al. (2014) showed that oxytocin increases the social behavior and these effects were blocked by co-administration of oxytocin antagonist [64]. Similarly, Goodson et al. (2009) found that peripheral oxytocin antagonist administration decreases the time spent in close proximity both to a familiar cagemate and to a larger group of conspecifics in zebra finches [65]. Other study demonstrated that blocking central oxytocin receptors in primates disrupts parental-like behavior and female sexual behavior [66]. Furthermore, Suraev et al. (2014) demonstrated that oxytocin, but not agonist [Thr4, Gly7]-oxytocin, significantly increased the amount of time spent close to a social stimulus in social preference test performed in late adolescence in rodents [67]. In relation to aggression, Calcagnoli et al. (2013) reported an increased offensive aggression in low aggressive residents after icv administration of a selective oxytocin receptor antagonist [68]. However, Calcagnoli et al. (2015) showed that anti-aggressive and pro-social changes were entirely blocked when the binding of exogenous oxytocin to the oxytocin receptors was impeded by pretreatment with a selective oxytocin receptors antagonist [69]. These data

demonstrated the involvement of oxytocin and oxytocin receptor in social and aggression behavior.

In summary, our results support the critical role for NMDA receptors and the oxytocinergic system in the regulation of social behavior and aggression which may be relevant for the mechanisms associated to autism and schizophrenia.

**ACKNOWLEDGMENTS**

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**References**


[1] American Psychiatric Association. Diagnostic and statistical manual of mental disorders: DSM-5. 5th edn. Arlington, VA: American Psychiatric Publishing, 2013.

[2] Lewis DA, Levitt P. Schizophrenia as a disorder of neurodevelopment. Annu Rev Neurosci. 2002;25:409-32.

[3] Canitano R. New experimental treatments for core social domain in autism spectrum disorders. Front Pediatr. 2014 Jun 20;2:61.

[4] Parnas J, Bovet P. Autism in schizophrenia revisited. Compr Psychiatry. 1991 Jan-Feb;32(1):7-21.

[5] Crespi BJ. Revisiting Bleuler: relationship between autism and schizophrenia. Br J Psychiatry. 2010 Jun;196(6):495; author reply 495-6.

[6] Mealey A, Abbott G, Byrne LK, McGillivray J. Overlap between autistic and schizotypal personality traits is not accounted for by anxiety and depression. Psychiatry Res. 2014 Oct 30;219(2):380-5.

[7] Couture SM, Penn DL, Losh M, Adolphs R, Hurley R, Piven J. Comparison of social cognitive functioning in schizophrenia and high functioning autism: more convergence than divergence. Psychol Med. 2010 Apr;40(4):569-79.

[8] Parikh MS, Kolevzon A, Hollander E. Psychopharmacology of aggression in children and adolescents with autism: a critical review of efficacy and tolerability. J Child Adolesc Psychopharmacol. 2008 Apr;18(2):157-78.

[9] Arango C, Calcedo Barba A, González-Salvador, Calcedo Ordóñez A. Violence in patients with schizophrenia: a prospective study. Schizophr Bull. 1999;25(3):493-503.

[10] Brain PF. Animal conflict Felicity Huntingford and Angela Turner. Behav Processes. 1988 Jul;17(1):75.

[11] Oliveira RF, Silva JF, Simões JM. Fighting zebrafish: characterization of aggressive behavior and winner-loser effects. Zebrafish. 2011 Jun;8(2):73-81.

[12] Teles MC, Dahlbom SJ, Winberg S, Oliveira RF. Social modulation of brain monoamine levels in zebrafish. Behav Brain Res. 2013 Sep 15;253:17-24.

[13] Mathur P, Guo S. Use of zebrafish as a model to understand mechanisms of addiction and complex neurobehavioral phenotypes. Neurobiol Dis. 2010 Oct;40(1):66-72.

[14] Tropepe V, Sive HL. Can zebrafish be used as a model to study the neurodevelopmental causes of autism? Genes Brain Behav. 2003 Oct;2(5):268-81.

[15] Pagnussat N, Piato AL, Schaefer IC, Blank M, Tamborski AR, Guerim LD, Bonan CD, Vianna MR, Lara DR. One for all and all for one: the importance of shoaling on behavioral and stress responses in zebrafish. Zebrafish. 2013 Sep;10(3):338-42.

[16] Saif M, Chatterjee D, Buske C, Gerlai R. Sight of conspecific images induces changes in neurochemistry in zebrafish. Behav Brain Res. 2013 Apr 15;243:294-9.

[17] Oliveira RF. Mind the fish: zebrafish as a model in cognitive social neuroscience. Front Neural Circuits. 2013 Aug 8;7:131.

[18] Bshary R, Gingins S, Vail AL. Social cognition in fishes. Trends Cogn Sci.2014 Sep;18(9):465-71.

[19] Goodson JL. The vertebrate social behavior network: evolutionary themes and variations. Horm Behav. 2005 Jun;48(1):11-22.

[20] Amann LC, Gandal MJ, Halene TB, Ehrlichman RS, White SL, McCarren HS, Siegel SJ. Mouse behavioral endophenotypes for schizophrenia. Brain Res Bull. 2010 Sep 30;83(3-4):147-61.

[21] Kraeuter AK, Loxton H, Lima BC, Rudd D, Sarnyai Z. Ketogenic diet reverses behavioral abnormalities in an acute NMDA receptor hypofunction model of schizophrenia. Schizophr Res. 2015 Nov 4. pii: S0920-9964(15)30041-4.

[22] Neill JC, Barnes S, Cook S, Grayson B, Idris NF, McLean SL, Snigdha S, Rajagopal L, Harte MK. Animal models of cognitive dysfunction and negative symptoms of schizophrenia: focus on NMDA receptor antagonism. Pharmacol Ther. 2010 Dec;128(3):419-32.

[23] Seibt KJ, Piato AL, da Luz Oliveira R, Capiotti KM, Vianna MR, Bonan CD. Antipsychotic drugs reverse MK-801-induced cognitive and social interaction deficits in zebrafish (Danio rerio). Behav Brain Res. 2011 Oct 10;224(1):135-9.

[24] Maaswinkel H, Zhu L, Weng W. Assessing social engagement in heterogeneous groups of zebrafish: a new paradigm for autism-like behavioral responses. PLoS One. 2013 Oct 8;8(10):e75955.

[25] Chen J, Patel R, Friedman TC, Jones KS. The Behavioral and Pharmacological Actions of NMDA Receptor Antagonism are Conserved in Zebrafish Larvae. Int J Comp Psychol. 2010;23(1):82-90.

[26] Kamińska K, Rogóż Z. The effect of combined treatment with risperidone and antidepressants on the MK-801-induced deficits in the social interaction test in rats. Parmacol Rep. 2015 Dec;67(6):1183-7.

[27] Anacker AM, Beery AK. Life in groups: the roles of oxytocin in mammalian sociality. Front Behav Neurosci. 2013 Dec 11;7:185.

[28] Andari E. Editorial: Oxytocin's routes in social behavior: into the 21st century. Front Behav Neurosci. 2015 Aug 25;9:224.

[29] Feifel D, Shilling PD, MacDonald K. A Review of Oxytocin's Effects on the Positive, Negative, and Cognitive Domains of Schizophrenia. Biol Psychiatry. 2015 Aug 13. pii: S0006-3223(15)00650-2.

[30] Yatawara CJ, Einfeld SL, Hickie IB, Davenport TA, Guastella AJ. The effect of oxytocin nasal spray on social interaction deficits observed in young children with autism: a randomized clinical crossover trial. Mol Psychiatry. 2015 Oct 27.

[31] Modahl C, Green L, Fein D, Morris M, Waterhouse L, Feinstein C, Levin H. Plasma oxytocin levels in autistic children. Biol Psychiatry. 1998 Feb 15;43(4):270-7.

[32] Hollander E, Bartz J, Chaplin W, Phillips A, Sumner J, Soorya L, Anagnostou E, Wasserman S. Oxytocin increases retention of social cognition in autism. Biol Psychiatry. 2007 Feb 15;61(4):498-503.

[33] Averbeck BB, Bobin T, Evans S, Shergill SS. Emotion recognition and oxytocin in patients with schizophrenia. Psychol Med. 2012 Feb;42(2):259-66.

[34] Fischer-Shofty M, Brüne M, Ebert A, Shefet D, Levkovitz Y, Shamay-Tsoory SG. Improving social perception in schizophrenia: the role of oxytocin. Schizophr Res. 2013 May;146(1-3):357-62.

[35] Goodson JL, Bass AH. Forebrain peptides modulate sexually polymorphic vocal circuitry. Nature. 2000; 403(6771):769-72.

[36] Black MP, Reavis RH, Grober MS. Socially induced sex change regulates forebrain isotocin in *Lythrypnus dalli*. Neuroreport. 2004;15(1):185–189.

[37] Braida D, Donzelli A, Martucci R, Ponzoni L, Pauletti A, Sala M. Neurohypophyseal hormones protect against pentylenetetrazole-induced seizures in zebrafish: role of oxytocin-like and V1a-like receptor. Peptides. 2012 Oct;37(2):327-33.

[38] Gerlai R, Lahav M, Guo S, Rosenthal A. Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. Pharmacol Biochem Behav. 2000 Dec;67(4):773-82.

[39] Zhang L, Tang J, Dong Y, Ji Y, Tao R, Liang Z, Chen J, Wu Y, Wang K. Similarities and Differences in Decision-Making Impairments between Autism Spectrum Disorder and Schizophrenia. Front Behav Neurosci. 2015 Sep 23;9:259.

[40] Nagai T, Ibi D, Yamada K. Animal model for schizophrenia that reflects gene-environment interactions. Biol Pharm Bull. 2011;34(9):1364-8.

[41] van Loo KM, Martens GJ. Genetic and environmental factors in complex neurodevelopmental disorders. Curr Genomics. 2007 Nov;8(7):429-44.

[42] Yu J, Qi D, Xing M, Li R, Jiang K, Peng Y, Cui D. MK-801 induces schizophrenic behaviors through downregulating Wnt signaling pathways in male mice. Brain Res. 2011 Apr 18;1385:281-92.

[43] Moy SS, Nonneman RJ, Shafer GO, Nikolova VD, Riddick NV, Agster KL, Baker LK, Knapp DJ. Disruption of social approach by MK-801, amphetamine, and fluoxetine in adolescent C57BL/6J mice. Neurotoxicol Teratol. 2013 Mar-Apr;36:36-46.

[44] de Moura Linck V, Herrmann AP, Goerck GC, Iwu MM, Okunji CO, Leal MB, Elisabetsky E. The putative antipsychotic alstonine reverses social interaction

withdrawal in mice. Prog Neuropsychopharmacol Biol Psychiatry. 2008 Aug 1;32(6):1449-52.

[45] Silvestre JS, Nadal R, Pallarés M, Ferré N. Acute effects of ketamine in the holeboard, the elevated-plus maze, and the social interaction test in Wistar rats. Depress Anxiety. 1997;5(1):29-33.

[46] Snigdha S, Neill JC. Improvement of phencyclidine-induced social behavior deficits in rats: involvement of 5-HT1A receptors. Behav Brain Res. 2008 Aug 5;191(1):26-31.

[47] Morales M, Spear LP. The effects of an acute challenge with the NMDA receptor antagonists, MK-801, PEAQX, and ifenprodil, on social inhibition in adolescent and adult male rats. Psychopharmacology (Berl). 2014 Apr;231(8):1797-807.

[48] Echevarria DJ, Hammack CM, Pratt DW. Hosemann JD (2008) A novel behavioral test battery to assess global drug effects using zebrafish. Int. J. Comp. Psychol 21: 19 -34.

[49] Carrillo M, Ricci LA, Melloni RH. Glutamate and the aggression neural circuit in adolescent anabolic steroid-treated Syrian hamsters (Mesocricetus auratus). Behav Neurosci. 2011 Oct;125(5):753-63.

[50] McAllister K H. Ethological analysis of the effects of MK-801 upon aggressive male mice: similarity to chlordiazepoxide. Pharmacol Biochem Behav. 1990 Sep;37(1):101-6.

[51] Kalinine E, Zimmer ER, Zenki KC, Kalinine I, Kazlauckas V, Haas CB, Hansel G, Zimmer AR, Souza DO, Müller AP, Portela LV. Nandrolone-induced aggressive behavior is associated with alterations in extracellular glutamate homeostasis in mice. Horm Behav. 2014 Jul;66(2):383-92.

[52] Macdonald K, Feifel D. Oxytocin in schizophrenia: a review of evidence for its therapeutic effects. Acta Neuropsychiatr. 2012 Jun 1;24(3):130-146.

[53] Tachibana M, Kagitani-Shimono K, Mohri I, Yamamoto T, Sanefuji W, Nakamura A, Oishi M, Kimura T, Onaka T, Ozono K, Taniike M. Long-term administration of intranasal oxytocin is a safe and promising therapy for early adolescent boys with autism spectrum disorders. J Child Adolesc Psychopharmacol. 2013 Mar;23(2):123-7.

[54] Bosch OJ, Neumann ID. Both oxytocin and vasopressin are mediators of maternal care and aggression in rodents: from central release to sites of action. Horm Behav. 2012 Mar;61(3):293-303.

[55] Romano A, Tempesta B, Micioni Di Bonaventura MV, Gaetani S. From Autism to Eating Disorders and More: The Role of Oxytocin in Neuropsychiatric Disorders. Front Neurosci. 2016 Jan 12;9:497.

[56] Buijs RM, Van Heerikhuize JJ. Vasopressin and oxytocin release in the brain—a synaptic event. Brain Res. 1982 Dec 2;252(1):71-6.

[57] Caldwell JD, Johns JM, Faggin BM, Senger MA, Pedersen CA. Infusion of an oxytocin antagonist into the medial preoptic area prior to progesterone inhibits sexual receptivity and increases rejection in female rats. Horm Behav. 1994 Sep;28(3):288-302.

[58] Teng BL, Nikolova VD, Riddick NV, Agster KL, Crowley JJ, Baker LK, Koller BH, Pedersen CA, Jarstfer MB, Moy SS. Reversal of social deficits by subchronic oxytocin in two autism mouse models. Neuropharmacology. 2015 Dec 31;105:61-71.

[59] Filby AL, Paull GC, Hickmore TF, Tyler CR. Unravelling the neurophysiological basis of aggression in a fish model. BMC Genomics. 2010 Sep 16;11:498.

[60] Ebner, K., Wotjak, C.T., Landgraf, R., Engelmann, M., 2000. A single social defeat experience selectively stimulates the release of oxytocin, but not vasopressin, within the septal brain area of male rats. Brain Res. 872, 87–92.

[61] Hathaway GA, Faykoo-Martinez M, Peragine DE, Mooney SJ, Holmes MM. Subcaste differences in neural activation suggest a prosocial role for oxytocin in eusocial naked mole-rats. Horm Behav. 2015 Dec 21;79:1-7.

[62] Meyer-Lindenberg A, Domes G, Kirsch P, Heinrichs M. Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. Nat Rev Neurosci. 2011 Aug 19;12(9):524-38.

[63] Feifel D, Macdonald K, Nguyen A, Cobb P, Warlan H, Galangue B, Minassian A, Becker O, Cooper J, Perry W, Lefebvre M, Gonzales J, Hadley A. Adjunctive intranasal oxytocin reduces symptoms in schizophrenia patients. Biol Psychiatry. 2010 Oct 1;68(7):678-80.

[64] Mooney SJ, Douglas NR, Holmes MM. Peripheral administration of oxytocin increases social affiliation in the naked mole-rat (Heterocephalus glaber). Horm Behav. 2014 Apr;65(4):380-5.

[65] Goodson JL, Schrock SE, Klatt JD, Kabelik D, Kingsbury MA. Mesotocin and nonapeptide receptors promote estrildid flocking behavior. Science. 2009 Aug 14;325(5942):862-6.

[66] Boccia ML, Goursaud AP, Bachevalier J, Anderson KD, Pedersen CA. Peripherally administered non-peptide oxytocin antagonist, L368,899, accumulates in limbic brain areas: a new pharmacological tool for the study of social motivation in non-human primates. Horm Behav. 2007 Sep;52(3):344-51.

[67] Suraev AS, Bowen MT, Ali SO, Hicks C, Ramos L, McGregor IS. Adolescent exposure to oxytocin, but not the selective oxytocin receptor agonist TGOT, increases social behavior and plasma oxytocin in adulthood. Horm Behav. 2014 May;65(5):488-96.

[68] Calcagnoli F, de Boer SF, Althaus M, den Boer JA, Koolhaas JM. Antiaggressive activity of central oxytocin in male rats. Psychopharmacology (Berl). 2013 Oct;229(4):639-51.

[69] Calcagnoli F, Stubbendorff C, Meyer N, de Boer SF, Althaus M, Koolhaas JM. Oxytocin microinjected into the central amygdaloid nuclei exerts anti-aggressive effects in male rats. Neuropharmacology. 2015 Mar;90:74-81.

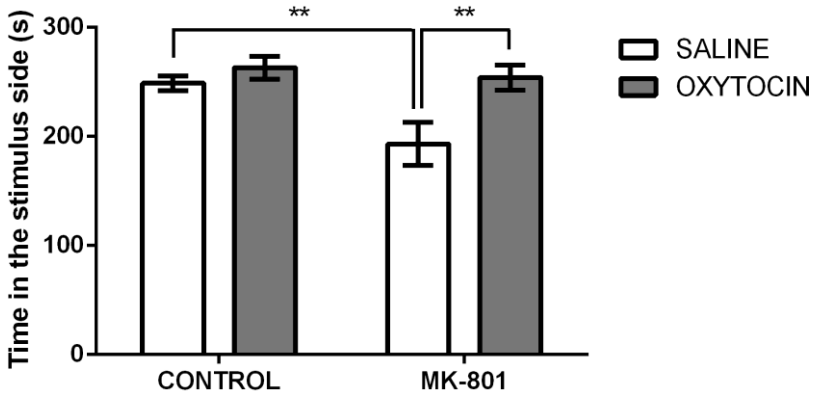



**Figure 1.** Effects of oxytocin on MK-801-induced social interaction deficits in zebrafish. The data are expressed as the mean ± S.E.M. (n = 16 per group), and were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. The symbol ** represents statistical difference when compared to the respective control group, $P < 0.05$.

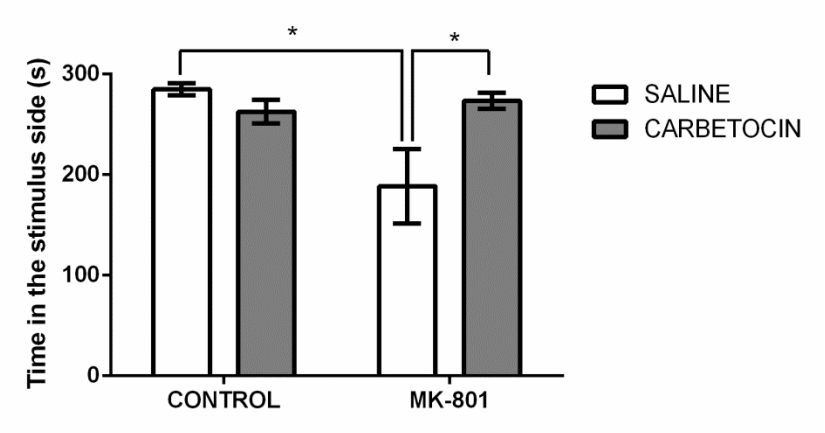



**Figure 2.** Effects of carbetocin on MK-801-induced social interaction deficits in zebrafish. The data are expressed as the mean ± S.E.M. (n = 12 per group), and were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. The symbol * represents statistical difference when compared to the respective control group, $P > 0.05$.

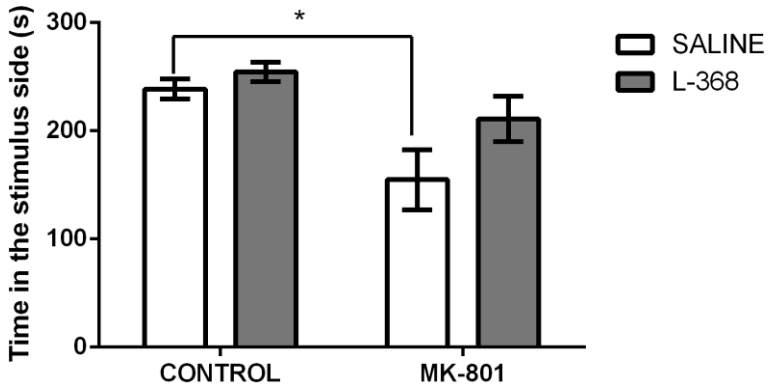



**Figure 3.** Effects of L-368,899 on MK-801-induced social interaction deficits in zebrafish. The data are expressed as the mean ± S.E.M. (n = 14 per group), and were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. The symbol * represents statistical difference when compared to the respective control group, $P > 0.05$.

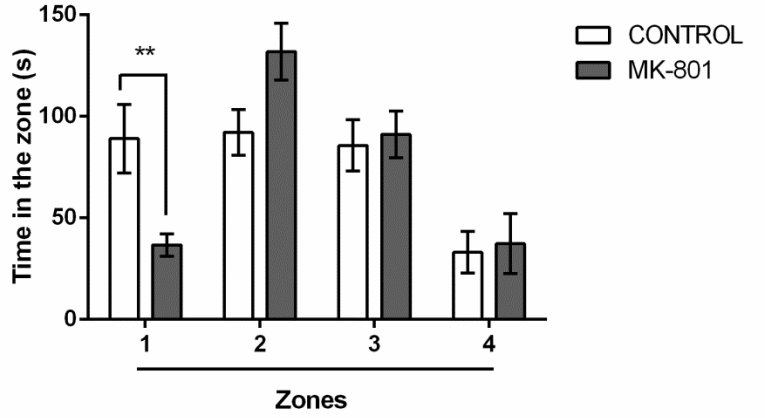



**Figure 4.** Effects of MK-801-induced aggression deficits in zebrafish. The data are expressed as the mean ± S.E.M. (n = 24 per group), and were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. The symbol ** represents statistical difference when compared to the respective control group, $P < 0.05$.

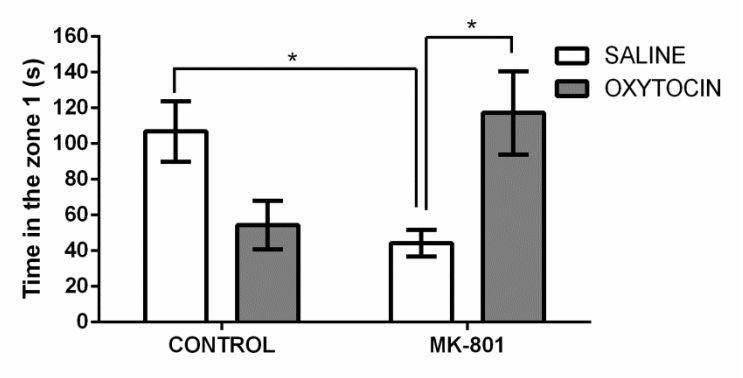



**Figure 5.** Effects of oxytocin on MK-801-induced aggression deficits in zebrafish. The data are expressed as the mean ± S.E.M. (n = 15 per group), and were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. The symbol * represents statistical difference when compared to the respective control group, $P < 0.05$).

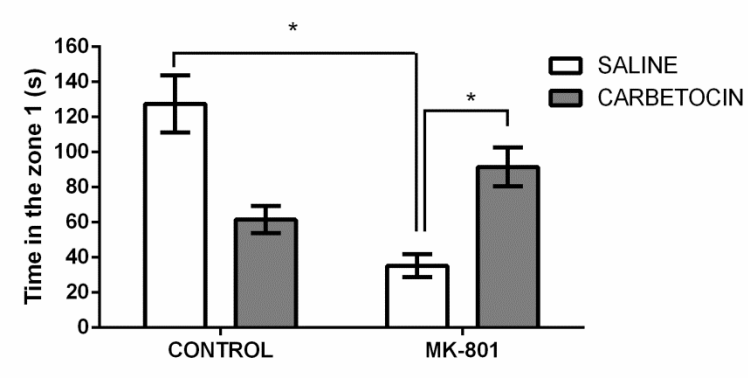



**Figure 6.** Effects of carbetocin on MK-801-induced aggression deficits in zebrafish. The data are expressed as the mean ± S.E.M. (n = 14 per group), and were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. The symbol * represents statistical difference when compared to the respective control group, $P < 0.05$.

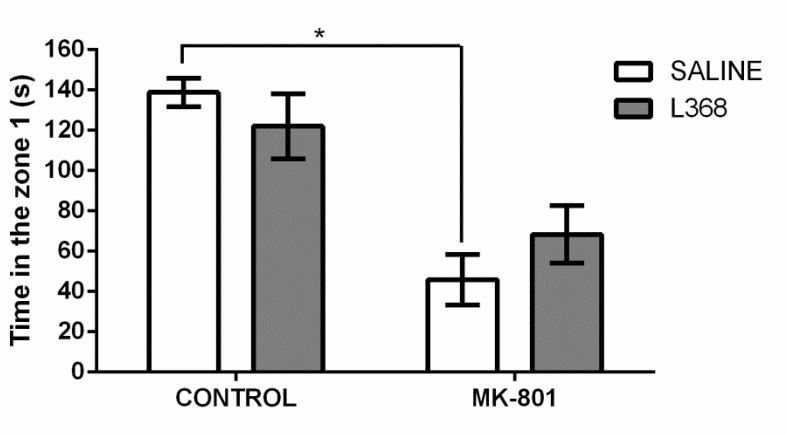



**Figure 7.** Effects of L-368,899 on MK-801-induced aggression deficits in zebrafish. The data are expressed as the mean ± S.E.M. (n = 10 per group), and were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. The symbol * represents statistical difference when compared to the respective control group, $P > 0.05$.

# 4 RESULTADOS ADICIONAIS

## 4.1 ANÁLISES DOS NÍVEIS DE GLUTAMATO, SEROTONINA, DOPAMINA E DOPAC EM ENCÉFALO DE PEIXE-ZEBRA SUBMETIDOS AO MODELO ANIMAL DE DÉFICIT DE INTERAÇÃO SOCIAL INDUZIDO POR VPA

Evidências sugerem que anormalidades na neurotransmissão serotonérgica, glutamatérgica e dopaminérgica podem estar implicadas no autismo e esquizofrenia (McDougle et al., 2005; Huang et al., 2008; Howes et al., 2012; Bissonette & Roesch, 2016). Devido a isso, analisamos os níveis de glutamato, serotonina, dopamina e DOPAC em peixe-zebra submetidos ao modelo animal de déficit de interação social induzido por VPA, através da cromatografia líquida de alta eficiência (HPLC) em encéfalo de peixe-zebra na idade de 120 dpf.

Conforme demonstra a figura 1, não observamos alterações significativas nos níveis de serotonina, glutamato e dopamina. No entanto, verificamos um aumento de DOPAC, metabólito da dopamina, em encéfalos de peixe-zebra na idade de 120 dpf submetidos ao modelo de déficit de interação social induzido por VPA.

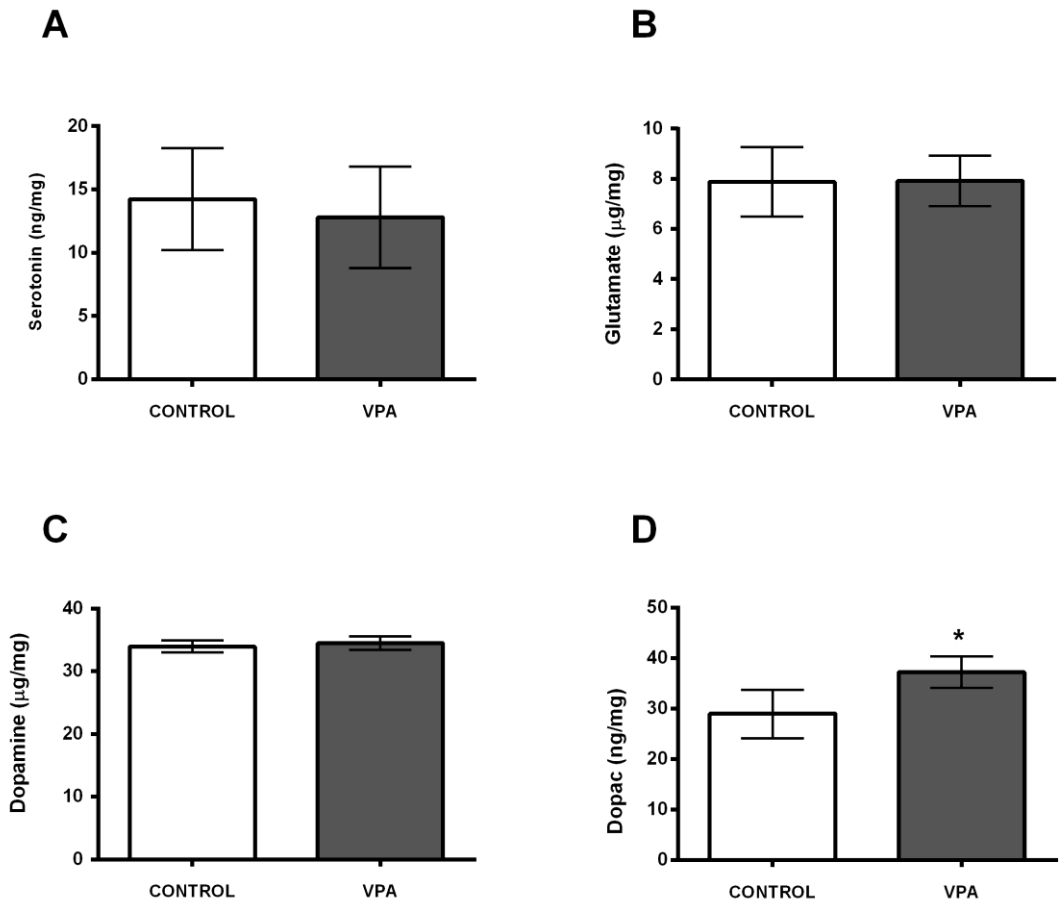



**Figura 1**. Níveis de serotonina (A), glutamato (B), dopamina (C) e DOPAC (D) foram analisados por LC-MSMS. Os dados estão representados em média ± S.D. (n=4). Cada experimento independente foi realizado utilizando preparações biológicas que consistiram de um "pool" de cinco encéfalos. O símbolo (asterisco) representa uma diferença significativa em relação ao grupo controle (Teste t de Student, $P<0.05$).

## 4.2 RESULTADOS ADICIONAIS REFERENTES AO MODELO DE DÉFICIT DE INTERAÇÃO SOCIAL INDUZIDO POR MK-801 E PREVENÇÃO COM OCITOCINA

Os animais foram tratados com MK-801 (5 µM) durante 15 minutos e o comportamento foi analisado durante 5 minutos em diferentes segmentos de tempo: 0 min, 15 min, 2 h, 4 h e 24 h para verificarmos o tempo de permanência do efeito do MK-801 sobre a resposta na interação social do peixe-zebra. Para os testes de interação social, após esta exposição, o peixe foi colocado individualmente em um aquário central, contendo em uma extremidade um aquário estímulo e na outra um aquário vazio. A permanência do animal perto do aquário estímulo foi avaliada.

Os dados demonstram que os animais expostos ao MK-801 (5 µM) aos 15 min permaneceram menos tempo ao lado do aquário estímulo ($p < 0.05$) quando comparados com o grupo controle (Figura 2).

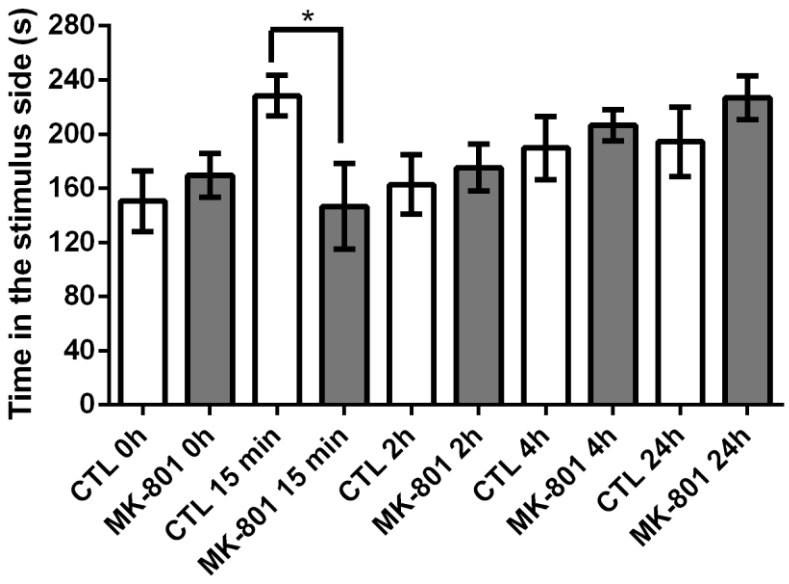



**Figura 2.** Tempo de permanência do efeito induzido por MK-801 sobre a resposta na interação social do peixe-zebra. Animais foram expostos ao MK-801 na concentração de 5 µM durante 0, 15 minutos, 2h, 4h e 24h. A resposta do comportamento induzida por MK-801 foi mensurada como o tempo de permanência no lado do estímulo. Os dados foram expressos em média ± SEM (n=16) e avaliados por análise de variância de duas vias (ANOVA) seguida de testes *post-hoc* de Bonferroni's. Os símbolos representam estatisticamente a diferença quando comparados com o grupo controle. * $P<0.05$.

Para verificar a capacidade da ocitocina em prevenir o déficit de interação social causado pelo MK-801, os animais foram injetados com salina ou ocitocina (10 ng/kg) 15 minutos antes da exposição ao MK-801. A administração de ocitocina antes da exposição ao MK-801 aumenta o tempo de permanência ao lado do aquário estímulo, quando comparados com animais somente expostos ao MK-801 (Figura 3, $p <0.05$). Estes dados demonstram que

MK-801 provoca um efeito de déficit de interação social, e que a ocitocina é capaz de prevenir o déficit de interação social induzido pelo MK-801.

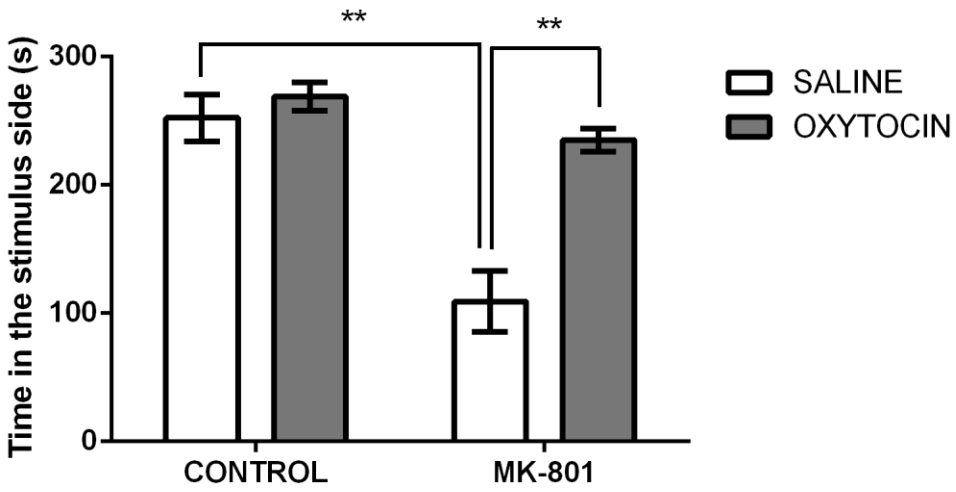



**Figura 3.** Prevenção pela ocitocina sobre o déficit de interação social induzido por MK-801. A resposta do comportamento induzida por MK-801 e ocitocina foi mensurada como o tempo de permanência no lado do estímulo. Animais reberam uma injeção intraperitoneal de ocitocina ou salina (grupo controle) e após 15 minutos foram expostos ao MK-801 na concentração de 5 µM por 15 min adicionais. Os dados foram expressos em média ± SEM (n=16) e avaliados por análise de variância de duas vias (ANOVA) seguida de testes *post-hoc* de Bonferroni's. Os símbolos representam estatisticamente a diferença quando comparados com o grupo controle. * $P<0.05$, ** $P<0.005$.

Para investigar os efeitos do antagonista do receptor de ocitocina (L-368,899) sobre a interação social após tratamento com MK-801 em peixe-zebra, animais foram injetados com L-368,899 (10 ng/kg) 15 minutos antes da exposição ao MK-801. A administração de L-368,899 antes da exposição ao MK-801 não alterou o tempo de permanência ao lado do aquário estímulo, quando comparados com animais somente expostos ao MK-801 (Figura 4, p

> 0.05). Estes dados demonstram que MK-801 provoca um efeito de déficit de interação social, mas o antagonista do receptor da ocitocina (L-368, 899) não foi capaz de prevenir o déficit de interação social induzido pelo MK-801, sugerindo o envolvimento dos receptores de ocitocina neste efeito.

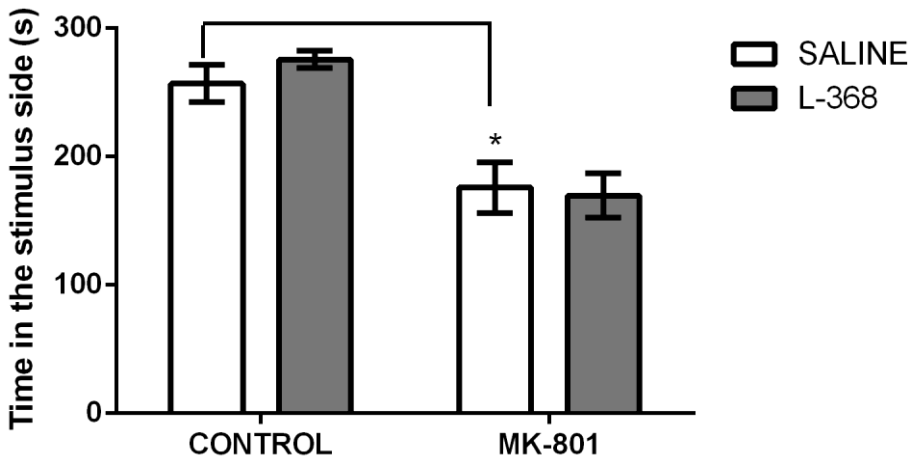



**Figura 4.** Efeito do L-368, 899 sobre o déficit de interação social, induzido por MK-801 em peixe-zebra. Os dados foram expressos em média ± SEM (n=16) e avaliados por análise de variância de duas vias (ANOVA) seguida de testes *post-hoc* de Bonferroni's. O símbolo * representa diferença estatística quando comparado com o respectivo grupo controle. * P<0.05.

# 5 DISCUSSÃO

Desordens neurológicas, como o transtorno do espectro do autismo (TEA) e esquizofrenia, compartilham sintomas (Mealey et al., 2014), sendo que um dos principais sintomas clínicos apresentados por ambos, TEA e SCZ, é o prejuízo nas funções sociais (Couture et al., 2010). Os transtornos do espectro do autismo compreendem um grupo heterogêneo e complexo de condições patológicas, incluindo as síndromes de Rett e Asperger, transtorno invasivo do desenvolvimento e autismo (Kumar et al., 2012; Grzadzinski et al., 2013). Os pacientes autistas muitas vezes apresentam déficits na comunicação e interação social, padrões repetitivos de comportamento, interesses restritos, ansiedade e hiperatividade (American Psychiatric Association, 2013; Canitano, 2014). Os sintomas da esquizofrenia podem ser classificados em cognitivos (diminuição da atenção, problemas de memória), negativos (anedonia, isolamento social) ou positivos (alucinações, comportamento desorganizado, altos níveis de agressão) (American Psychiatric Association, 2013).

O ácido valpróico (VPA) é utilizado como um fármaco anti-epiléptico e estabilizador do humor. No entanto, a exposição pré-natal de VPA em seres humanos tem sido associada com um aumento da incidência de autismo, além de poder afetar o desenvolvimento do cérebro fetal (Kim et al., 2011). A exposição ao VPA durante o período pré-natal em roedores provoca alterações no funcionamento de sistemas cerebrais e respostas emocionais (Rodier et al., 1997; Schneider et al., 2007). Além disso, foram observados movimentos repetitivos (Schneider et al., 2008), diminuição na interação social (Schneider & Przewlocki, 2005) e aumento da ansiedade, sintomas semelhantes aos apresentados pelos pacientes humanos autistas. No estudo, apresentado no Capítulo 1 desta tese, realizamos um *screening*

comportamental em diferentes períodos do desenvolvimento do peixe-zebra em 6, 30, 70 e 120 dpf (dias pós-fertilização) para estabelecer um modelo de déficit de interação social induzido por VPA, a fim de investigar o comportamento social, locomoção, agressividade e ansiedade.

Nossos resultados demonstram que a exposição de VPA (48 µM) durante as primeiras 48 hpf (horas pós-fertilização) não promoveu mudanças na sobrevivência, morfologia e taxa de eclosão em 24 hpf, 48 hpf e 72 hpf, sugerindo que os efeitos comportamentais observados não são devido a alterações morfológicas macroscópicas. Os padrões comportamentais sugerem que a exposição ao VPA induz mudanças na atividade locomotora e ansiedade em diferentes períodos do desenvolvimento em peixe-zebra. Além disso, apresentou um déficit de interação social aos 70 dpf e 120 dpf. A exposição ao VPA não alterou a agressividade no estágio adulto aos 70 dpf e 120 dpf.

Similarmente aos nossos resultados, Zellner et al. (2011) verificaram que a exposição a 48 µM VPA durante 0-48 hpf não afetou a sobrevivência nem causou malformações. Os padrões comportamentais identificados em nossos experimentos sugerem que a exposição de VPA gerou um perfil de hiperatividade nas fases iniciais de desenvolvimento em peixe-zebra, isto é, 6 dpf, porque houve um aumento nos parâmetros de locomoção. Os animais também exibiram um aumento da ansiedade. As larvas de peixe-zebra são uma alternativa de modelo animal para testes de toxicidade (de Esch et al., 2012). No entanto, é importante considerar a idade (e a fase de desenvolvimento do cérebro), pois pode gerar mudanças no comportamento. Estudos analisam que a idade pode influenciar no padrão de atividade motora em larvas de peixe-zebra (Colwill & Creton, 2011; Padilla et al., 2011). Além disso, Beker van Woudenberg e colaboradores (2014) mostraram que a administração de 60 µM de VPA em

peixe-zebra não afetou a atividade motora. No entanto, quando administrado 150 µM VPA houve uma redução da distância total percorrida. Zellner et al. (2011) demonstraram que a exposição a 48 µM de VPA durante 0-48 hpf promoveu uma hiperatividade acentuada em 6 dpf. Resaltamos que as diferenças nas concentrações de VPA e tempo de tratamento podem influenciar os padrões comportamentais observados no peixe-zebra.

Nossos achados demonstram que a hiperatividade apresentada pelo peixe-zebra aos 6 dpf após o tratamento embriológico com VPA foi atenuada ao longo do desenvolvimento. Verificamos que os parâmetros de locomoção de 30 dpf, 70 dpf e 120 dpf são semelhantes aos observados nos animais controle. O índice de ansiedade em 70 dpf permaneceu aumentado, mas em 120 dpf, houve uma diminuição da ansiedade. Desta forma, é importante considerarmos que a exposição ao VPA pode afetar o desenvolvimento cerebral. Vários estudos têm demonstrado que o VPA pode regular vias de sinalização e a expressão de genes durante todo o desenvolvimento do cérebro em roedores, interferindo assim com janelas críticas de vulnerabilidade (Almeida et al., 2014; Bartkowska et al., 2007; Kolozsi et al., 2009; Stodgell et al., 2006). Em relação ao comportamento social, nossos resultados também demonstraram que os animais tratados com VPA durante as primeiras 48 h apresentaram um déficit significativo na interação social em 70 dpf, e este efeito foi mantido durante o desenvolvimento em 120 dpf, sugerindo que o VPA pode modular padrões comportamentais ao longo do desenvolvimento cerebral. Além disso, sugerimos que alterações neuroquímicas em animais expostos ao VPA em uma idade precoce podem estar envolvidas na mudança do perfil de hiperatividade e ansiedade ao longo do desenvolvimento. Estudos indicam que a modulação de sistemas de neurotransmisssão, como o sistema serotonérgico pode produzir diferentes respostas comportamentais, incluindo mudanças no comportamento social no peixe-

zebra (Buske & Gerlai, 2011; Mahabir et al, 2013). Silverman e colaboradores (2010) verificaram que modelos de autismo em roedores apresentam déficit na sociabilidade e comportamento de ansiedade. Estes resultados são consistentes com os nossos achados em 70 dpf. Contudo, em 120 dpf, identificamos uma diminuição na ansiedade. A literatura mostra resultados controversos entre interação social e ansiedade. Diferentes estudos mostram que os déficits da interação social podem ocorrer sem afetar a ansiedade em modelos de autismo em roedores (Liu & Smith, 2009; McFarlane et al., 2008). Mudanças na maturidade podem gerar alterações na resposta comportamental e isso pode explicar as respostas em relação ao índice de ansiedade quando avaliados em diferentes períodos do desenvolvimento.

Em nosso estudo, ao verificarmos a agressividade observamos que a exposição ao VPA na fase embrionária não foi alterada significativamente na fase adulta de 70 dpf e 120 dpf no peixe-zebra. Os nossos resultados indicaram que os animais tratados com VPA no início do desenvolvimento podem apresentar respostas comportamentais sem expressar um domínio do território, e não há associação direta entre um déficit de interação social e agressividade.

Modificações em sistemas de neurotransmissores, incluindo sistema GABAérgico (Banerjee et al., 2014), dopaminérgico (Nguyen et al., 2014), serotonérgico (Nakamura et al., 2010) e purinérgico (Al-Mosalem et al., 2009) podem estar envolvidos no processo fisiopatológico que leva ao autismo. O sistema purinérgico é essencial para as funções vitais do SNC e pode modular as ações de outros sistemas de neurotransmissores (Zhang et a., 1995, Okada et al., 1999). Diversos estudos indicam um papel importante do ATP extracelular e da sinalização purinérgica no autismo (Al-Mosalem et al., 2009; Persico et al., 2000; Theoharides, 2013). Considerando que o peixe-zebra é um modelo animal que pode contribuir para a compreensão dos mecanismos que fundamentam o comportamento social, no Capítulo

2 desta tese investigamos a sinalização purinérgica em um modelo de exposição embrionária ao VPA, que induz déficit de interação social em peixe-zebra adultos (Capítulo 1). Nos resultados apresentados no Capítulo 2, demonstramos que a exposição embrionária ao VPA não alterou a hidrólise de ATP e ADP no peixe-zebra em 120 dpf, bem como a atividade da ADA citosólica (solúvel) não foi alterada. No entanto, observou-se um aumento da hidrólise de AMP (12,5%) enquanto que a atividade da ecto-ADA diminuiu (19,2%) no encéfalo de peixe-zebra adultos submetidos à exposição embrionária ao VPA. A análise quantitativa da transcrição reversa de PCR (RT-PCR) mostrou alterações nos níveis de RNAm da *ntpd 8*, ADA 2.1 e *A2a1*. O metabolismo de ATP cerebral mostrou um catabolismo rápido de ATP e ADP, enquanto o metabolismo extracelular de AMP e adenosina (ADO) ocorreu lentamente.

Nossos experimentos mostraram que a exposição embrionária ao VPA não alterou significativamente a hidrólise de ATP e ADP em membranas de encéfalo de peixe-zebra. Estes resultados podem ser devidos a uma plasticidade adaptativa das E-NTPDase induzida pela exposição embrionária ao VPA. No entanto, houve um aumento da atividade da ecto-5'-nucleotidase. A exposição embrionária ao VPA também reduziu a atividade da ecto-ADA, mas não alterou a atividade da ADA citosólica em peixe-zebra adulto. Estes dados sugerem que a exposição embrionária ao VPA pode alterar os níveis de adenosina em encéfalo de peixe zebra. É de conhecimento que os transportadores de nucleosídeos e a hidrólise de ATP extracelular promovida por ectonucleotidases são fontes de adenosina extracelular. O controle da sinalização adenosinérgica pode ser realizado pela recaptação de adenosina através dos transportadores bidirecionais, seguida por fosforilação intracelular de AMP através da adenosina quinase (AK) ou por desaminação da inosina pela adenosina desaminase. Portanto, o aumento da atividade da ecto-5'-nucleotidase e a diminuição da atividade da ecto-ADA

observada em nosso estudo poderia conduzir a um aumento nos níveis de adenosina extracelular. As análises em HPLC foram conduzidas para avaliarmos a hidrólise de ATP em encéfalo de peixe-zebra, em diferentes períodos de incubação. Observou-se um aumento nos níveis ATP e uma diminuição nos níveis de ADP. Os níveis de AMP mostraram uma diminuição em 120 min de incubação. Como podemos observar na Fig. 6D (Capítulo 2), houve uma diminuição nos níveis de ADO. Esta diminuição observada nos níveis de adenosina em encéfalo de peixe-zebra pode ser devido à recaptação do nucleosideo, através dos transportadores de nucleosídeos bidirecionais e a ação da enzima AK.

Estudos sugerem que níveis insuficientes de adenosina podem estar relacionados com alguns sintomas comportamentais e fisiológicos do TEA (por exemplo, pouco contato visual, movimentos repetitivos) (Masino et al., 2011; Malow, 2004). Além disso, é descrito na literatura o potencial terapêutico da adenosina em relação ao autismo (Masino et al., 2011; Freitag et al., 2010; Ghanizadeh, 2010; Masino et al., 2009; Tanimura et al., 2010). Masino e colaboradores (2011) demonstram que intervenções que geram um aumento nos níveis de adenosina aliviam os sintomas relacionados com o autismo. Os receptores de adenosina podem ser um novo alvo para o tratamento dos comportamentos repetitivos do autismo (Tanimura et al., 2010). Ghanizadeh (2010) propôs que a cafeína, um antagonista do receptor de adenosina, pode ter efeitos benéficos no TEA. Além disso, Tanimura e colaboradores (2010) mostraram que a ativação do receptor de adenosina $A_{2A}$ ($A_{2A}R$) tem sido associada com uma redução dos comportamentos perseverativos.

É descrito que alterações da ADA no desenvolvimento podem estar envolvidas na patogênese do autismo (Okada et al., 1999, Yamamoto et al., 1987). Diversos estudos corroboram com os nossos resultados (Persico et al., 2000; Stubbs et al., 1982; De Luca et

al., 1999; Bottini et al., 2001). Por exemplo, Stubbs e colaboradores (1982) observaram uma redução da atividade da ADA em soro de crianças autistas. Bottini e colaboradores (2001) relataram uma associação entre um polimorfismo genético da adenosina desaminase e um fator de risco para o desenvolvimento de autismo. Além das suas funções enzimáticas, há evidências de que a ecto-ADA está relacionada com o receptor de adenosina, modulando a sua afinidade e exercendo papel co-estimulatório funcional (Ciruela et al., 1996; Herrera et al., 2001; Saura et al., 1998). Freitag e colaboradores (2010) relatam que $A_{2A}R$ possui uma associação genética em TEA. Os receptores $A_{2A}$ podem estar envolvidos na locomoção, ansiedade, inibição da atividade neuronal excitatória e regulação do sono (Tanimura et al., 2010; Moreau &Huber, 1999). Estes dados corroboram com nossos resultados, uma vez que observamos que a quantidade relativa dos níveis de RNAm de *A2a1* aumentou após a exposição ao VPA em encéfalo de peixe-zebra aos 120 dpf.

As mudanças observadas na atividade das enzimas promovidas pela exposição embrionária ao VPA poderiam ocorrer devido a mudanças transcricionais na expressão das enzimas, assim realizamos uma análise de RT-qPCR. Curiosamente, nossos achados demonstraram que o nível de expressão do gene relativo ao membro da E-NTPDase (*entpd8*) foi significativamente maior após a exposição embrionária ao VPA, enquanto que não houve alteração na atividade da enzima. Desta forma, sugere-se que a alteração na expressão do gene não foi suficiente para afetar a atividade enzimática da E-NTPDases. Al-Mosalem e colaboradores (2009) avaliaram as E-NTPDases (ATPase e ADPase) em plasma de 30 pacientes autistas e observaram que a ADPase foi significativamente maior em pacientes autistas. No entanto, em nosso estudo não observamos alterações na atividade da E-NTPDase em membranas de encéfalo expostas ao VPA no período embrionário em peixe-zebra aos 120

dpf. Nossos resultados demonstraram um efeito semelhante a outros estudos, já que a análise de HPLC demonstrou uma diminuição dos níveis de ADP (Al-Mosalem et al., 2009). Mostramos também que os níveis de RNAm da ecto-5'-nucleotidase foram significativamente aumentados após a exposição embrionária de VPA em peixe-zebra aos 120 dpf, sugerindo que o aumento da atividade da ecto-5'-nucleotidase pode estar diretamente relacionado com a expressão elevada de CD73. Nossos achados estão de acordo com a diminuição dos níveis de AMP em 120 min de incubação verificada na análise por HPLC. Da mesma forma, observamos uma diminuição na atividade da ecto-ADA, bem como uma redução na expressão do gene *ada2.1* após a exposição embrionária de VPA no peixe-zebra aos 120 dpf que poderia ser uma consequência do controle transcricional. O aumento na expressão do gene do receptor de adenosina pode ser uma resposta para compensar os níveis mais elevados de adenosina que ocorrem devido ao aumento da ecto-5'-nucleotidase e diminuição da atividade da ecto-ADA. Desta forma, este incremento de adenosina observado, pode produzir um aumento na ligação dos receptores de adenosina, capaz de gerar, posteriormente, uma resposta compensatória, diminuindo os níveis de adenosina disponível, como observado no HPLC por análise em HPLC.

Portanto, este estudo apresenta a primeira evidência de que a exposição embrionária ao VPA no peixe-zebra em 120 dpf pode modular a hidrólise de nucleotídeo e nucleosídeo e desaminação de adenosina em membranas de encéfalo de peixe-zebra. Por conseguinte, os nossos dados contribuem na elucidação dos mecanismos envolvidos nos efeitos modulatórios da sinalização purinérgica no déficit de interação social no peixe-zebra. Outro fármaco que induz aspectos do autismo e esquizofrenia, gerando déficits de interação social é o MK-801 (Maaswinkel et al., 2013).

O MK-801 é um antagonista não-competitivo do receptor de glutamato NMDA. Estudos demonstram que está fortemente implicado no comportamento social em modelos animais e é utilizado para mimetizar alguns aspectos do autismo e esquizofrenia (Neill et al, 2010; Seibt et al., 2011; Maaswinkel et al, 2013). A esquizofrenia e autismo são consideradas doenças complexas com vários fatores que contribuem para a patogênese (Nagai et al, 2011;. Van Loo e Martens, 2007), além de compartilharem uma característica importante dos sintomas clínicos, tais como prejuízos nas funções sociais (Couture et al., 2010; Zhang et al., 2015). As ações farmacológicas do receptor NMDA parecem ser conservadas em peixes-zebra (Chen et al., 2010). Seibt e colaboradores (2011) demonstraram que o MK-801 induz déficits nos parâmetros de interação social em peixe-zebra (Seibt et al, 2011). Dados na literatura demonstram que a ocitocina é uma molécula promissora para o tratamento de sintomas psicóticos em pacientes com vários transtornos psiquiátricos, incluindo a esquizofrenia e o autismo (Feifel et al., 2016; Yatawara et al., 2015). Estudos relataram a melhora dos déficits sociais através da administração de ocitocina em pacientes esquizofrênicos (Averbeck et al., 2012; Fischer-shofty et al., 2013).

Sendo assim, no Capítulo 3 desta tese investigamos o comportamento social, isto é, mudanças na interação social e agressividade através da exposição ao MK-801 em peixe-zebra. Devido ao fato da ocitocina poder modular o comportamento social, analisamos os efeitos da ocitocina, do agonista do receptor da ocitocina (carbetocina) e do antagonista do receptor da ocitocina (L-368, 899) na reversão dos efeitos comportamentais induzidos pelo MK-801 em peixe-zebra. Nossos dados revelaram que o MK-801 promoveu uma diminuição no tempo de permanência no segmento mais próximo ao grupo de animais coespecíficos e no tempo gasto no segmento mais próximo da imagem do espelho, sugerindo um efeito sobre o

comportamento social. O tratamento com ocitocina após a exposição ao MK-801 reestabeleceu o tempo de permanência no segmento mais próximo ao estímulo, assim como o tempo gasto no segmento mais próximo da imagem do espelho. Além disso, para suportar a modulação via ocitocina, observamos que o agonista do receptor de ocitocina (carbetocina) reestabeleceu os padrões do comportamento social e da agressividade. No entanto, o antagonista do receptor da ocitocina (L-368,899) não foi capaz de reverter o tempo de permanência no segmento mais próximo ao estímulo, assim como o tempo de permanência no segmento mais próximo da imagem no espelho.

Moy e colaboradores (2013) demonstraram em roedores que o MK-801 leva a alterações no comportamento social. Em relação ao peixe-zebra, diversos estudos vão ao encontro dos nossos resultados, demonstrando o impacto do MK-801 sobre o comportamento social. De acordo com Maaswinkel e colaboradores (2013), um único peixe-zebra tratado com MK-801 reduziu a coesão social de todo o cardume. Seibt e colaboradores (2011) demonstraram que o MK-801 reduz a preferência do peixe-zebra por um grupo estímulo da mesma espécie. Além disso, Echevarria e colaboradores (2008) relataram uma interrupção do comportamento de *shoaling* pelo MK-801. Em relação à agressividade, são poucos os estudos que investigam o efeito do MK-801 sobre este padrão comportamental. É descrito o efeito deste fármaco em roedores, como demonstra o estudo desenvolvido por McAllister (1990) em que o MK-801, entre outros compostos, tende a aumentar a agressividade e o comportamento social. Kalinine e colaboradores (2014) demonstraram que uma única dose intraperitoneal de MK-801, antes do teste de agressividade, diminuiu o comportamento agressivo.

O sistema ocitocinérgico desempenha um papel crucial em vários e complexos comportamentos sociais (Bosch & Neumann, 2012; Romano et al., 2016). Evidências sugerem

que a desregulação do sistema ocitocinérgico pode estar envolvida na fisiopatologia de certas desordens neuropsiquiátricas, como o autismo e esquizofrenia (Macdonald et al, 2012; Tachibana et al, 2013). Até o presente momento, não há nenhum tratamento padrão para a disfunção social e estudos clínicos identificaram a ocitocina como um agente terapêutico em potencial. Estudos comportamentais em roedores e em peixe-zebra indicaram que o tratamento com ocitocina pode aumentar a sociabilidade (Braida et al, 2012; Teng et al, 2015). Nossos achados estão de acordo com o estudo de Braida e colaboradores (2012) que identificaram que a ocitocina aumentou a preferência social no peixe-zebra e os antagonistas inibiram o efeito induzido pelo neuropeptídeo. Em relação ao comportamento de agressividade não identificamos nenhum estudo que investigue a influência da ocitocina sobre este padrão comportamental em peixe-zebra. No entanto, um estudo desenvolvido por Filby e colaboradores (2010) demonstrou que o tratamento com arginina vasotocina (AVT) (neuropeptídeo, encontrado em peixes e um modulador chave do comportamento social e não social, semelhante à ocitocina) reduziu significativamente a agressividade em peixe-zebra. Em roedores, estudos demonstraram que a ocitocina também tem sido associada à dominância social e agressividade (Ebner et al, 2000; Hathaway et al, 2015). Baseado nos dados da literatura é evidente o potencial terapêutico da ocitocina para auxiliar alguns sintomas comportamentais de desordens, como o autismo e a esquizofrenia (Meyer-Lindenberg et al., 2011; Feifel et al., 2016; Hollander et al., 2007; Modahl et al.,1998). Os receptores da ocitocina são altamente conservados na evolução. Os resultados obtidos no Capítulo 3 indicam que o antagonista do receptor da ocitocina (L-368,899) não foi capaz de reverter o efeito induzido pelo MK-801 na interacção social e agressividade. Por outro lado, o agonista do receptor da ocitocina (carbetocina) reverteu os efeitos causados pelo MK-801 no

comportamento social e agressividade. Assim, sugerimos que estes efeitos modulatórios podem ser mediados pela ocitocina. São poucos os estudos que avaliam farmacologicamente o efeito do receptor da ocitocina em relação ao comportamento social e agressividade. Mooney e colaboradores (2014) observaram que a ocitocina aumenta o comportamento social e esses efeitos foram bloqueados pela co-administração do antagonista da ocitocina. Boccia e colaboradores (2007) demonstraram que o bloqueio dos receptores de ocitocina em primatas perturbou o comportamento parental, bem como o comportamento sexual feminino. Suraev e colaboradores (2014) também demonstraram que o agonista da ocitocina não afetou significativamente o tempo gasto perto de um estímulo social em roedores. No que tange à agressividade, Calcagnoli e colaboradores (2013) relataram um aumento da agressividade ofensiva em residentes com baixa agressividade após a administração icv de um antagonista do receptor de ocitocina seletivo. No entanto, Calcagnoli e colaboradores (2015) mostraram que mudanças anti-agressividade e pró-sociais foram totalmente bloqueadas quando a ligação da ocitocina com seu receptor foi impedida pelo pré-tratamento com o antagonista do receptor da ocitocina. Concluindo, os nossos resultados apoiam o papel dos receptores NMDA e do sistema ocitocinérgico na regulação do comportamento social e agressividade, podendo ser relevantes para os mecanismos associados ao autismo e esquizofrenia.

Além dos resultados apresentados no decorrer dos três capítulos desta tese, produzimos resultados adicionais, os quais serão perspectivas futuras a serem desenvolvidas. Evidências na literatura sugerem que anormalidades na neurotransmissão serotonérgica, glutamatérgica e dopaminérgica podem estar implicadas no autismo e esquizofrenia (McDougle et al., 2005; Huang et al., 2008; Howes et al., 2012; Bissonette & Roesch, 2016). Devido a isso, analisamos os níveis de glutamato, serotonina, dopamina e DOPAC em peixe-zebra

submetidos ao modelo animal de déficit de interação social induzido por VPA, através da cromatografia líquida de alta eficiência (HPLC) em encéfalo de peixe-zebra na idade de 120 dpf. Observamos que não houve alterações significativas nos níveis de serotonina, glutamato e dopamina. Diferentemente dos nossos resultados, Shinohe et al. (2006) demonstraram através de uma análise bioquímica que o nível de glutamato no soro é elevado em pacientes autistas. Gabriele e colaboradores (2014) observaram níveis elevados de serotonina no sangue em pacientes autistas. Uma possível justificativa é que nossos resultados são referentes a amostras de encéfalo e não sangue periférico. No entanto, verificamos um aumento de DOPAC, metabólito da dopamina, em encéfalos de peixe-zebra na idade de 120 dpf submetidos ao modelo de déficit de interação social induzido por VPA. Martineau e colaboradores (1992) investigaram os níveis de dopamina (DA), ácido 3-4 dihidroxifenilacético (DOPAC), serotonina (5HT), entre outros, na urina de crianças autistas e observaram alterações nos níveis destas monoaminas quando comparado com o grupo controle. Desta forma, podemos sugerir que o tratamento com VPA no período embrionário não causou alteração significativa nos níveis de serotonina, glutamato e dopamina em encéfalo de peixe-zebre em 120 dpf. O aumento observado do metabólito da dopamina, DOPAC, pode ser devido a alterações do transportador de dopamina. Assim, uma perspectiva futura é avaliar a expressão gênica dos transportadores de dopamina em encéfalos de peixe-zebra na idade de 120 dpf submetidos ao modelo de déficit de interação social induzido por VPA.

Em relação aos resultados adicionais referentes ao modelo de déficit de interação social induzido por MK-801 e prevenção com ocitocina desenvolvemos primeiramente uma curva de tempo para verificarmos o tempo de permanência do efeito induzido pelo MK-801 sobre a resposta na interação social do peixe-zebra. Desta forma, determinamos que o período mais

adequado para analisar o efeito do déficit de interação social é após 15 min de exposição ao MK-801. Nossos dados vão ao encontro dos resultados obtidos por Seibt e colaboradores (2011), onde utilizaram um protocolo similar ao desenvolvido em nosso estudo. Posteriormente, nosso objetivo foi verificar a capacidade da ocitocina e do antagonista do receptor de ocitocina em prevenir o déficit de interação social causado pelo MK-801. Assim, observamos que a ocitocina é capaz de prevenir o déficit de interação social induzido pelo MK-801. No entanto, a administração de L-368,899 antes da exposição ao MK-801 não alterou o tempo de permanência ao lado do aquário estímulo. Estes dados corroboram os resultados apresentados no capítulo 3 reforçando o envolvimento do sistema ocitocinérgico no déficit de interação social induzido pelo MK-801.

De forma conjunta, os resultados apresentados nos três capítulos desta tese, somados aos resultados adicionais, podem contribuir para um melhor entendimento do comportamento social e da influência da sinalização purinérgica, além de apresentar modelos de déficit de interação social induzido pela exposição embrionária ao VPA e exposição ao MK-801 em peixe-zebra adulto. Além disso, nossos dados mostraram uma modulação do sistema ocitocinérgico após tratamento com MK-801, reforçando o potencial farmacológico deste sistema sobre as desordens neuropsquiátricas estudadas.

## REFERÊNCIAS BIBLIOGRÁFICAS


Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signaling in the nervous system: an overview. Trends Neurosci. 2009; 32(1):19-29.

Adegbola A, Gao H, Sommer S, Browning M. A novel mutation in JARID1C/SMCX in a patient with autism spectrum disorder (ASD). Am J Med Genet A. 2008; 146A(4):505-11.

Almeida LE, Roby CD, Krueger BK. Increased BDNF expression in fetal brain in the valproic acid model of autism. Mol Cell Neurosci. 2014; 59:57–62.

Al-Mosalem OA, El-Ansary A, Attas O, Al-Ayadhi L. Metabolic biomarkers related to energy metabolism in Saudi autistic children. Clin Biochem. 2009; 42(10-11):949-57.

American Psychiatric Association. Diagnostic and statistical manual of mental disorders, 5th edn. American Psychiatric Association. 2013. Disponível em <http:\\dsm5.org>

Appelbaum L, Skariah G, Mourrain P, Mignot E. Comparative expression of P2x receptors and ecto-nucleoside triphosphate diphosphohydrolase 3 in hypocretin and sensory neurons in *zebrafish*. Brain Res. 2007; 1174: 66-75.

Averbeck BB, Bobin T, Evans S, Shergill SS. Emotion recognition and oxytocin in patients with schizophrenia. Psychol Med. 2012; 42(2):259-66.

Aziz A, Harrop SP, Bishop NE. Characterization of the deleted in autism 1 protein family: implications for studying cognitive disorders. PLoS One. 2011; 6(1):e14547.

Bailey J, Oliveri A, Levin ED. Zebrafish model systems for developmental neurobehavioral toxicology. Birth Defects Res C Embryo Today. 2013; 99(1):14-23.

Bambini-Junior V, Rodrigues L, Behr G A, Moreira J C., Riesgo R. and Gottfried C. Animal model of autism induced by prenatal exposure to valproate: behavioral changes and liver parameters. Brain Res. 2011; 1408: 8–16.

Banerjee A, García-Oscos F, Roychowdhury S, Galindo LC, Hall S, Kilgard MP, Atzori M. Impairment of cortical GABAergic synaptic transmission in an environmental rat model of autism. Int J Neuropsychopharmacol. 2013; 16(6):1309-18.

Barbazuk WB, Korf I, Kadavi C, Heyen J, Tate S, Wun, E., Bedell JA., Mcpherson JA, Johnson SL. The syntenic relationship of the *zebrafish* and human genomes. Genome Res. 2000; 10(9):1351-1358.

Bartkowska K, Paquin A, Gauthier AS, Kaplan DR, Miller FD. Trk signaling regulates neural precursor cell proliferation and differentiation during cortical development. Development. 2007; 134,4369–4380.

Baskerville TA, Douglas AJ. Dopamine and oxytocin interactions underlying behaviors: potential contributions to behavioral disorders. CNS Neurosci Ther. 2010; 16(3):e92-123.

Beker van Woudenberg A, Snel C, Rijkmans E, de Groot D, Bouma M, et al. Zebrafish embryotoxicity test for developmental (neuro)toxicity: demo case of an integrated

screening approach system using anti-epileptic drugs. Reprod. Toxicol. 2014; 49C,101–116.

Beliaeva NF, Kashirtseva VN, Medvedeva NV, Khudoklinova IuIu, Ipatova OM, Archakov AI. *Zebrafish* as a model organism for biomedical studies. Biomed Khim. 2010; 56(1):120-31.

Benvenuto A, Moavero R, Alessandrelli R, Manzi B, Curatolo P. Syndromic autism: causes and pathogenetic pathways. World J Pediatr. 2009; 5(3):169-76.

Bickel S, Javitt DC. Neurophysiological and neurochemical animal models of schizophrenia: focus on glutamate. Behav Brain Res. 2009; 204(2):352-62.

Bissonette GB, Roesch MR. Development and function of the midbrain dopamine system: what we know and what we need to. Genes Brain Behav. 2016; 15(1):62-73.

Black MP, Reavis RH, Grober MS. Socially induced sex change regulates forebrain isotocin in *Lythrypnus dalli*. Neuroreport. 2004; 15(1):185–189.

Blaker-Lee A, Gupta S, McCammon JM, De Rienzo G, Sive H. *Zebrafish* homologs of genes within 16p11.2, a genomic region associated with brain disorders, are active during brain development, and include two deletion dosage sensor genes. Dis Model Mech. 2012; 5(6):834-51.

Boccia ML, Goursaud AP, Bachevalier J, Anderson KD, Pedersen CA. Peripherally administered non-peptide oxytocin antagonist, L368,899, accumulates in limbic brain areas: a new pharmacological tool for the study of social motivation in non-human primates. Horm Behav. 2007; 52(3):344-51.

Bonan CD. Ectonucleotidases and Nucleotide/Nucleoside Transporters as Pharmacological Targets for Neurological Disorders. CNS Neurol Disord Drug Targets. 2012; 11(6): 739-50.

Bosch OJ, Neumann ID. Both oxytocin and vasopressin are mediators of maternal care and aggression in rodents: from central release to sites of action. Horm Behav. 2012; 61(3):293-303.

Bottini N, De Luca D, Saccucci P, Fiumara A, Elia M, Porfirio MC et al. Autism: evidence of association with adenosine deaminase genetic polymorphism. Neurogenetics. 2001; 3(2):111-3

Braida D, Donzelli A, Martucci R, Capurro V, Busnelli M, Chini B, Sala M. Neurohypophyseal hormones manipulation modulate social and anxiety-related behavior in *zebrafish*. Psychopharmacology (Berl). 2012; 220(2):319-30.

Brenner E, Kondziella D, Håberg A, Sonnewald U. Impaired glutamine metabolism in NMDA receptor hypofunction induced by MK801. J Neurochem. 2005; 94(6):1594-603.

Bromley RL, Mawer G, Clayton-Smith J, Baker GA; Liverpool and ManchesterNeurodevelopment Group. Autism spectrum disorders following in utero exposure to antiepileptic drugs. Neurology. 2008; 71(23):1923-4.

Brugha, TS, McManus, S, Bankart, J, Scott, F, Purdon, S, Smith, J, et al. Epidemiology of autism spectrum disorders in adults in the community in England. Archives of General Psychiatry. 2011; 68(5): 459–466.

Burnstock G. Purinergic Nerves. Pharmacol Rev. 1972; 24(3): 509-581.

Burnstock G. A basis for distinguishing two types of purinergic receptor. In Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach New York: Raven Press. 1978; 45: 107-118.

Burnstock G, Knight, GE. Cellular Distribution and Functions of P2 Receptor Subtypes In Different Systems. Rev Cytol Clin. 2004; 240: 231–304.

Burnstock G. Historical review: ATP as a neurotransmitter. TRENDS in Pharmacolog Sci. 2006; 27: 166-176.

Burnstock G. Purinergic Signalling And Disorders Of The Central Nervous System. Nat Rev Drug Discov. 2008; 7(7): 575-590.

Burnstock G. Purinergic cotransmission. F1000 Biol Rep. 2009; 9: 1-46.

Burnstock G. Control of vascular tone by purines and pyrimidines. Br J Pharmacol. 2010; 161(3): 527-9.

Burnstock G. Discovery of purinergic signalling, the initial resistance and current explosion of interest. Br J Pharmacol. 2012; 167(2): 238-55.

Burnstock G, Kennedy C. P2X receptors in health and disease. Adv Pharmacol. 2011; 61:333-72.

Burnstock G, Krügel U, Abbracchio MP, Illes P. Purinergic signalling: from normal behaviour to pathological brain function. Prog Neurobiol. 2011; 95(2): 229-74.

Burnstock G. Purinergic signalling: from discovery to current developments. Exp Physiol. 2014; 99(1):16-34.

Burnstock G. Physiopathological roles of P2X receptors in the central nervous system. Curr Med Chem. 2015; 22(7):819-44.

Buske C, Gerlai R. Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotoninergic systems in adult zebrafish. Neurotoxicol Teratol. 2011; 33, 698–707.

Byrne M, Agerbo E, Bennedsen B, Eaton WW, Mortensen PB. Obstetric conditions and risk of first admission with schizophrenia: a Danish national register based study. Schizophr Res. 2007; 97(1-3):51-9.

Cacioppo JT, Decety J. Social neuroscience: challenges and opportunities in the study of complex behavior. Ann N Y Acad Sci. 2011; 1224:162-73.

Calcagnoli F, de Boer SF, Althaus M, den Boer JA, Koolhaas JM. Antiaggressive activity of central oxytocin in male rats. Psychopharmacology (Berl). 2013; 229(4):639-51.

Calcagnoli F, Stubbendorff C, Meyer N, de Boer SF, Althaus M, Koolhaas JM. Oxytocin microinjected into the central amygdaloid nuclei exerts anti-aggressive effects in male rats. Neuropharmacol. 2015; 90:74-81.

Canitano R. New experimental treatments for core social domain in autism spectrum disorders. Front Pediatr. 2014; 2:61.

Capiotti KM, Menezes FP, Nazario LR, Pohlmann JB, de Oliveira GM, Fazenda L, Bogo MR, Bonan CD, Da Silva RS. Early exposure to caffeine affects gene expression of adenosine receptors, DARPP-32 and BDNF without affecting sensibility and morphology of developing zebrafish (Danio rerio). Neurotoxicol Teratol. 2011; 33(6): 680-5.

Carpenter Jr WT, Silverstein SM, Moghaddam B, Wykes T. How the diagnosis of schizophrenia impeded the advance of knowledge (and what to do about it). Schizophrenia: Evolution and Synthesis: Strüngmann Forum Reports 2013. vol. 13. MIT Press, Cambridge, MA.

Charman T, Loucas T, Pickles A, Simonoff E, Chandle S, Meldrum D, Baird G: Autistic symptomatology and language ability in autism spectrum disorder and specific language impairment. J Child Psychol Psychiatry. 2008; 49: 1184–1192.

Charman T, Pickles A, Simonoff E, Chandler S, Loucas T, Baird G. IQ in children with autism spectrum disorders: data from the Special Needs and Autism Project (SNAP). Psychol Med. 2011; 41(3):619-27.

Chaves VE, Tilelli CQ, Brito NA, Brito MN. Role of oxytocin in energy metabolism. Peptides. 2013; 45:9– 14.

Cheh MA, Millonig JH, Roselli LM, Ming X, Jacobsen E, Kamdar S, Wagner GC. En2 knockout mice display neurobehavioral and neurochemical alterations relevant to autism spectrum disorder. Brain Res. 2006; 1116(1):166-76.

Chen W, Guidotti G. Soluble apyrases release adp during ATP hydrolysis. Biochem Biophys Res Commun. 2001; 23: 90-5.

Chou MY, Hung JC, Wu LC, Hwang SP, Hwang PP. Isotocin controls ion regulation through regulating ionocyte progenitor differentiation and proliferation. Cell Mol Life Sci. 2011; 68(16):2797-809.

Ciruela F, Saura C, Canela EI, Mallol J, Lluis C, Franco R. Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. FEBS Lett. 1996; 380(3):219-23.

Coghlan S, Horder J, Inkster B, Mendez MA, Murphy DG, Nutt DJ. GABA system dysfunction in autism and related disorders: from synapse to symptoms. Neurosci Biobehav Rev. 2012; 36(9):2044-55.

Cognato G de P, Bortolotto JW, Blazina AR, Christoff RR, Lara DR, Vianna MR, Bonan CD. Y-Maze memory task in *zebrafish* (Danio rerio): the role of glutamatergic and cholinergic systems on the acquisition and consolidation periods. Neurobiol Learn Mem. 2012; 98(4):321-8.

Colwill RM, Creton R. Locomotor behaviors in zebrafish (Danio rerio) larvae. Behav Process. 2011; 86, 222–229

Couture SM, Penn DL, Losh M, Adolphs R, Hurley R, Piven J. Comparison of social cognitive functioning in schizophrenia and high functioning autism: more convergence than divergence. Psychol Med. 2010; 40(4):569-79.

Dawson G. Early behavioral intervention, brain plasticity, and the pre-vention of autism spectrum disorder. Dev Psychopathol. 2008; 20:775–803.

de Esch C, van der Linde H, Slieker R, Willemsen R, Wolterbeek A, Wouterse R, De Groot D. Locomotor activity assay in zebrafish larvae: influence of age, strain and ethanol. Neurotoxicol Teratol. 2012; 34, 425–433.

De Luca D, Lucarelli P, Bottini N, Porfirio C, Curatolo P. Adenosine deaminase polymorphism. J Child Neurol. 1999; 14:323-324.

Devlin B, Cook EH Jr, Coon H, Dawson G, Grigorenko EL, McMahon W, Minshew N, Pauls D, Smith M, Spence MA, Rodier PM, Stodgell C, Schellenberg GD; CPEA Genetics

Network. Autism and the serotonin transporter: the long and short of it. Mol Psychiatry. 2005; 10(12):1110-6.

Devlin B, Scherer SW. Genetic architecture in autism spectrum disorder. Curr Opin Genet Dev. 2012; 22(3):229-37.

Diaz-Hernandez M, Cox JA, Migita K, Haines W, Egan TM, Voigt MM. Biochem Biophys Res Commun. 2002 ;4: 849-53.

Dorneles AG, Menezes C, Sperotto RL, Duarte MM, Morsch VM, Schetinger MR, Loro VL. Adenine nucleotide hydrolysis in patients with aseptic and bacterial meningitis. Neurochem Res. 2009; 34(3):463-9.

Drury AN, Szent-Györgyi A. The physiological activity of adenine compounds with special reference to their action upon the mammalian heart. J Physiol. 1929; 68: 213–237.

Dunwiddie TE, Masino S. The Role and Regulation of Adenosine in the Central Nervous System. Annu Rev Neurosci. 2001; 24:31-55.

Durand CM, Betancur C, Boeckers TM, Bockmann J, Chaste P, Fauchereau F, Nygren G, Rastam M, et al. Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. Nat Genet. 2007; 39(1):25-7.

Eaton JL, Glasgow E. The *zebrafish* bHLH PAS transcriptional regulator, single-minded 1 (sim1), is required for isotocin cell development. Dev Dyn. 2006; 235(8):2071-82.

Ebner, K., Wotjak, C.T., Landgraf, R., Engelmann, M. A single social defeat experience selectively stimulates the release of oxytocin, but not vasopressin, within the septal brain area of male rats. Brain Res. 2000; 872, 87–92.

Echevarria DJ, Hammack CM, Pratt DW. Hosemann JD. A novel behavioral test battery to assess global drug effects using zebrafish. Int J Comp Psychol. 2008; 21: 19 -34.

Feifel D, Shilling PD, MacDonald K. A Review of Oxytocin's Effects on the Positive, Negative, and Cognitive Domains of Schizophrenia. Biol Psychiatry. 2016; 79(3):222-33.

Feng J, Schroer R, Yan J, Song W, Yang C, Bockholt A, Cook EH Jr, Skinner C, Schwartz CE, Sommer SS. High frequency of neurexin 1beta signal peptide structural variants in patients with autism. Neurosci Lett. 2006; 409(1):10-3.

Feuk L, Carson AR, Scherer SW. Structural variation in the human genome. Nat Rev Genet. 2006; 7(2):85-97.

Filby AL, Paull GC, Hickmore TF, Tyler CR. Unravelling the neurophysiological basis of aggression in a fish model. BMC Genomics. 2010; 11:498.

Fischer-Shofty M, Brüne M, Ebert A, Shefet D, Levkovitz Y, Shamay-Tsoory SG. Improving social perception in schizophrenia: the role of oxytocin. Schizophr Res. 2013; 146(1-3):357-62.

Fleming A, Rubinsztein DC. *Zebrafish* as a model to understand autophagy and its role in neurological disease. Biochim Biophys Acta. 2011; 1812(4):520-6.

Fombonne E. Epidemiological surveys of autism and other pervasive developmental disorders: an update. J Autism Dev Disord. 2003; 33(4):365-82.

Fombonne E. Epidemiology of pervasive developmental disorders. Pediatr Res. 2009; 65(6):591-8.

Franco R, Casadó V, Ciruela F, Saura C, Mallol J, Canela EI, Lluis C. Cell surface adenosine deaminase: much more than an ectoenzyme. Prog Neurobiol. 1997; 4: 283-94.

Fredholm BB, Lerner U. Metabolism of adenosine and 20-deoxyadenosine by fetal mouse calvaria in culture. Med Biol. 1982; 60: 267–271.

Fredholm BB, Chern Y, Franco R, Sitkovsky M. Aspects of the general biology of adenosine A2A signaling. Prog Neurobiol. 2007; 83(5):263-76.

Fredholm BB, IJzerman AP, Jacobson KA, Linden J, and Müller CE. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and Classification of Adenosine Receptors-An Update. Pharmacol Rev. 2011; 63:1-34.

Freitag CM, Agelopoulos K, Huy E, Rothermundt M, Krakowitzky P, Meyer J, Deckert J, von Gontard A, Hohoff C. Adenosine A(2A) receptor gene (ADORA2A) variants may increase autistic symptoms and anxiety in autism spectrum disorder. Eur Child Adolesc Psychiatry. 2010; 19(1):67-74.

Gabriele S, Sacco R, Persico AM. Blood serotonin levels in autism spectrum disorder: a systematic review and meta-analysis. Eur Neuropsychopharmacol. 2014; 24(6):919-29.

Genton P, Semah F, Trinka E. Valproic acid in epilepsy: pregnancy-related issues. Drug Saf. 2006; 29(1):1-21.

Ghanizadeh A. Possible role of caffeine in autism spectrum disorders, a new testable hypothesis. J Food Sci. 2010; 75:6.

Giacomini AC, Abreu MS, Giacomini LV, Siebel AM, Zimerman FF, Rambo CL, Mocelin R, Bonan CD, Piato AL, Barcellos LJ. Fluoxetine and diazepam acutely modulate stress induced-behavior. Behav Brain Res. 2016; 296:301-10.

Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. Physiol Rev. 2001; 81(2):629-83.

Ginsberg MR, Rubin RA, Falcone T, Ting AH, Natowicz MR. Brain transcriptional and epigenetic associations with autism. PLoS One. 2012; 7(9):e44736.

Gogolla N, Leblanc JJ, Quast KB, Südhof TC, Fagiolini M, Hensch TK. Common circuit defect of excitatory-inhibitory balance in mouse models of autism. J Neurodev Disord. 2009; 1(2):172-81.

Goodson JL, Bass AH. Forebrain peptides modulate sexually polymorphic vocal circuitry. Nature. 2000; 403(6771):769-72.

Grzadzinski R, Huerta M, Lord C. DSM-5 and autism spectrum disorders (ASDs): an opportunity for identifying ASD subtypes. Mol Autism. 2013; 4(1):12.

Hathaway GA, Faykoo-Martinez M, Peragine DE, Mooney SJ, Holmes MM. Subcaste differences in neural activation suggest a prosocial role for oxytocin in eusocial naked mole-rats. Horm Behav. 2015; 79:1-7.

Hérault J, Perrot A, Barthe´le´my C, Buchler M, Cherpi C, Leboyer M, Sauvage D. Possible association of c-Harvey-ras-1 (HRAS) marker with autism. Psychiatr Res. 1993; 46:261–267.

Herrera C, Casadó V, Ciruela F, Schofield P, Mallol J, Lluis C et al. Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. Mol Pharmacol. 2001; 59(1):127-34

Hill AJ, Teraoka H, Heideman W, Peterson RE. *Zebrafish* as a model vertebrate for. investigating chemical toxicity. Toxicol Sci. 2005; 86: 6-19.

Hollander E, Bartz J, Chaplin W, Phillips A, Sumner J, Soorya L, Anagnostou E, Wasserman S. Oxytocin increases retention of social cognition in autism. Biol Psychiatry. 2007; 61(4):498-503.

Honea R, Crow TJ, Passingham D, Mackay CE. Regional deficits in brain volumein schizophrenia: a meta-analysis of voxel-based morphometry studies. Am J Psychiatry. 2005; 162(12):2233-45.

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C., et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013; 496(7446): 498-503.

Howes OD, Kambeitz J, Kim E, Stahl D, Slifstein M, Abi-Dargham A, Kapur S. The nature of dopamine dysfunction in schizophrenia and what this means for treatment. Arch Gen Psychiatry. 2012; 69(8):776-86.

Huang CH, Santangelo SL. Autism and serotonin transporter gene polymorphisms: a systematic review and meta-analysis. Am J Med Genet B Neuropsychiatr Genet. 2008; 147B(6):903-13.

Insel TR. Rethinking schizophrenia. Nature. 2010; 468(7321):187-93.

Itil T, Keskiner A, Kiremitci N, Holden JM. Effect of phencyclidine in chronic schizophrenics. Can Psychiatr Assoc J. 1967; 12(2):209-12.

Jacob S, Brune CW, Carter CS, Leventhal BL, Lord C, Cook EH Jr. Association of the oxytocin receptor gene (OXTR) in Caucasian children and adolescents with autism. Neurosci Lett. 2007; 417(1):6-9.

Jamain S, Quach H, Betancur C, Råstam M, Colineaux C, Gillberg IC, Soderstrom H, Giros B, Leboyer M, Gillberg C, Bourgeron T; Paris Autism Research International Sibpair Study. Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. Nat Genet. 2003; 34(1):27-9.

Jia SJ, Meng AM. The development of *zebrafish* research in China]. Yi Chuan. 2012; 34: 1082-8.

Kalinine E, Zimmer ER, Zenki KC, Kalinine I, Kazlauckas V, Haas CB, Hansel G, Zimmer AR, Souza DO, Müller AP, Portela LV. Nandrolone-induced aggressive behavior is associated with alterations in extracellular glutamate homeostasis in mice. Horm Behav. 2014; 66(2):383-92.

Kalueff AV, Stewart AM, Gerlai R . Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol Sci. 2014; 35:63-75.

Karnik I, Gerlai R. Can zebrafish learn spatial tasks? An empirical analysis of place and single CS-US associative learning. Behav Brain Res. 2012; 233(2): 415-21.

Keller F, Persico AM. The neurobiological context of autism. Mol Neurobiol. 2003; 28(1):1-22.

Kim KC, Kim P, Go HS, Choi CS, Yang SI, Cheong JH, Shin CY, Ko KH. The critical period of valproate exposure to induce autistic symptoms in Sprague-Dawley rats. Toxicol Lett. 2011; 201(2):137-42.

Kim KC, Kim P, Go HS, Choi CS, Park JH, Kim HJ, Jeon SJ, Dela Pena IC, Han SH, Cheong JH, Ryu JH, Shin CY. Male-specific alteration in excitatory post-synaptic development and social interaction in pre-natal valproic acid exposure model of autism spectrum disorder. J Neurochem. 2013; 124(6):832-43.

Kolozsi E, Mackenzie RN, Roullet FI, de Catanzaro D, Foster JA. Prenatal exposure to valproic acid leads to reduced expression of synaptic adhesion molecule neuroligin 3 in mice. Neuroscience 2009; 163,1201-1210.

Kovács Z, Dobolyi A, Kékesi KA, Juhász G. 5'-nucleotidases, nucleosides and their distribution in the brain: pathological and therapeutic implications. Curr Med Chem. 2013; 20(34):4217-40.

Kucenas S, Li Z, Cox Ja, Egan TM, Voigt MM. Molecular characterization of the *zebrafish* P2X receptor subunit gene family. Neuroscience. 2003; 121: 935-945.

Kuehn BM. CDC: autism spectrum disorders common. JAMA. 2007; 297(9):940.

Kumar B, Prakash A, Sewal RK, Medhi B, Modi M. Drug therapy in autism: a present and future perspective. Pharmacol Rep. 2012; 64(6):1291-304.

Landa R, Garrett-Mayer E. Development in infants with autism spectrum disorders: a prospective study. J Child Psychol Psychiatry. 2006; 47(6):629-38.

Lane JR, Jaakola VP, Ijzerman AP. The structure of the adenosine receptors implications for drug discovery. Adv Pharmacol. 2011; 61:1-40.

Leckman JF, Goodman WK, North WG, Chappell PB, Price LH, Pauls DL, Anderson GM, Riddle MA, McSwiggan- Hardin M, McDougle CJ. Elevated cerebrospinal fluid levels of oxytocin in obsessive-compulsive disorder. Comparison with Tourette's syndrome and healthy controls. Arch Gen Psychiatry. 1994; 51:782–792.

Lefevre EM, Eyles DW, Burne TH. Behavioural sensitisation to MK-801 is dose-dependent and independent of environmental context. Behav Brain Res. 2015; 298(Pt B):241-245.

Levitt P, Campbell DB. The genetic and neurobiologic compass points toward common signaling dysfunctions in autism spectrum disorders. J Clin Invest. 2009; 119(4):747-54.

Lewis DA, Levitt P. Schizophrenia as a disorder of neurodevelopment. Annu Rev Neurosci. 2002; 25:409-32.

Liu S, Leach SD. *Zebrafish* models for cancer. Annu Rev Pathol. 2011; 28:71-93.

Liu ZH, Smith CB. Dissociation of social and nonsocial anxiety in a mouse model of fragile X syndrome. Neurosci Lett. 2009; 454, 62–66.

Lu JW, Ho YJ, Yang YJ, Liao HA, Ciou SC, Lin LI, Ou DL. Zebrafish as a disease model for studying human hepatocellular carcinoma. World J Gastroenterol. 2015; 21(42):12042-58.

Maaswinkel H, Zhu L, Weng W. Assessing social engagement in heterogeneous groups of zebrafish: a new paradigm for autism-like behavioral responses. PLoS One. 2013; 8(10):e75955.

Macdonald K, Feifel D. Oxytocin in schizophrenia: a review of evidence for its therapeutic effects. Acta Neuropsychiatr. 2012; 24(3):130-146.

Mahabir S, Chatterjee D, Buske C, Gerlai R. Maturation of shoaling in two zebrafish strains: a behavioral and neurochemical analysis. Behav Brain Res. 2013; 247, 1–8.

Maier SA, Galellis JR, McDermid HE. Phylogenetic analysis reveals a novel protein family closely related to adenosine deaminase. J Mol Evol. 2005; 61: 776-794.

Maillard AM, Hippolyte L, Rodriguez-Herreros B, Chawner SJ, Dremmel D, Agüera Z, et al. 16p11.2 locus modulates response to satiety before the onset of obesity. Int J Obes (Lond). 2015.

Malow BA. Sleep disorders, epilepsy, and autism. Ment Retard Dev Disabil Res Rev. 2004; 10(2):122-5

Markram H, Rinaldi T, Markram K. The intense world syndrome an alternative hypothesis for autism. Front Neurosci. 2007; 1(1):77-96.

Martineau J, Hérault J, Petit E, Guérin P, Hameury L, Perrot A, Mallet J, Sauvage D, Lelord G, Müh JP. Catecholaminergic metabolism and autism. Dev Med Child Neurol. 1994; 36(8):688-97.

Martinez-Navio JM, Casanova V, Pacheco R, Naval-Macabuhay I, Climent N, Garcia F, Gatell JM, Mallol J, Gallart T, Lluis C, Franco R. Adenosine deaminase potentiates the generation of effector, memory, and regulatory CD4+ T cells. J Leukoc Biol. 2011; 89:127-36

Masino SA, Kawamura M, Wasser CD, Pomeroy LT, Ruskin DN. Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity. Curr Neuropharmacol 2009; 7(3):257-68.

Masino SA, Kawamura M Jr, Plotkin LM, Svedova J, DiMario FJ Jr, Eigsti IM. The relationship between the neuromodulator adenosine and behavioral symptoms of autism. Neurosci Lett. 2011; 500(1):1-5.

Masino SA, Kawamura M Jr, Cote JL, Williams RB, Ruskin DN. Adenosine and autism: a spectrum of opportunities. Neuropharmacol. 2013; 68:116-21.

Matsui H, Namikawa K, Köster RW. Identification of the zebrafish red nucleus using Wheat Germ Agglutinin transneuronal tracing. Commun Integr Biol. 2014; 7(6):e994383.

McAllister KH. Ethological analysis of the effects of MK-801 upon aggressive male mice: similarity to chlordiazepoxide. Pharmacol Biochem Behav. 1990; 37(1):101-6

McDougle CJ, Erickson CA, Stigler KA, Posey DJ. Neurochemistry in the pathophysiology of autism. J Clin Psychiatry. 2005; 66(suppl 10):9–18.

McFarlane HG, Kusek GK, Yang M, Phoenix JL, Boliva V, Crawley JN. Autism-like behavioral phenotypes in BTBR T + tf/J mice. Genes Brain Behav. 2008;7,152–163.

McGrath JJ, Burne TH, Feron F, Mackay-Sim A, Eyles DW. Developmental vitamin D deficiency and risk of schizophrenia: A 10-year update. Schizophrenia Bulletin. 2010; 36, 1073–1078.

Mealey A, Abbott G, Byrne LK, McGillivray J. Overlap between autistic and schizotypal personality traits is not accounted for by anxiety and depression. Psychiatry Res. 2014; 219(2):380-5.

Meyer U, Feldon J, Schedlowski M, Yee BK. Immunological stress at the maternal-foetal interface: a link between neurodevelopment and adult psychopathology. Brain Behav Immun. 2006; 20(4):378-88.

Meyer-Lindenberg A, Domes G, Kirsch P, Heinrichs M. Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. Nat Rev Neurosci. 2011; 12(9):524-38.

Mier D, Kirsch P. Social-Cognitive Deficits in Schizophrenia. Curr Top Behav Neurosci. 2016 Jan 5.

Miklósi A, Andrew RJ. The *Zebrafish* As A Model For Behavioral Studies. *Zebrafish*. 2006; 3:227-234.

Modahl C, Green L, Fein D, Morris M, Waterhouse L, Feinstein C, Levin H. Plasma oxytocin levels in autistic children. Biol Psychiatry. 1998; 43(4):270-7.

Mooney SJ, Douglas NR, Holmes MM. Peripheral administration of oxytocin increases social affiliation in the naked mole-rat (Heterocephalus glaber). Horm Behav. 2014; 65(4):380-5.

Moreau JL, Huber G. Central adenosine A(2A) receptors: na overview. Brain Res Brain Res Rev. 1999; 1:65-82 .

Morgan C, Reininghaus U, Reichenberg A, Frissa S; SELCoH study team, Hotopf M, Hatch SL. Adversity, cannabis use and psychotic experiences: evidence of cumulative and synergistic effects. Br J Psychiatry. 2014; 204:346-53.

Morrow EM, Yoo SY, Flavell SW, Kim TK, Lin Y. Identifying autism loci and genes by tracing recent shared ancestry. Science. 2008; 321: 218–223.

Moy SS, Nonneman RJ, Shafer GO, Nikolova VD, Riddick NV, Agster KL, Baker LK, Knapp DJ. Disruption of social approach by MK-801, amphetamine, and fluoxetine in adolescent C57BL/6J mice. Neurotoxicol Teratol. 2013; 36:36-46.

Nagai T, Ibi D, Yamada K. Animal model for schizophrenia that reflects gene-environment interactions. Biol Pharm Bull. 2011; 34(9):1364-8.

Nakamura K, Sekine Y, Ouchi Y, Tsujii M, Yoshikawa E, Futatsubashi M et al. Brain serotonin and dopamine transporter bindings in adults with highfunctioning autism. Arch Gen Psychiatry. 2010; 67(1):59-68

Naviaux JC, Schuchbauer MA, Li K, Wang L, Risbrough VB, Powell SB, Naviaux RK. Reversal of autism-like behaviors and metabolism in adult mice with single-dose antipurinergic therapy. Transl Psychiatry. 2014; 4:e400.

Neill JC, Barnes S, Cook S, Grayson B, Idris NF, McLean SL, Snigdha S, Rajagopal L, Harte MK. Animal models of cognitive dysfunction and negative symptoms of schizophrenia: focus on NMDA receptor antagonism. Pharmacol Ther. 2010; 128(3):419-32.

Nguyen M, Roth A, Kyzar EJ, Poudel MK, Wong K, Stewart AM et al. Decoding the contribution of dopaminergic genes and pathways to autism spectrum disorder (ASD). Neurochem Int. 2014; 66:15-26.

Nightingale S. Autism spectrum disorders. Nat Rev Drug Discov. 2012; 11(10):745-6.

Norton WH. Toward developmental models of psychiatric disorders in *zebrafish*. Front Neural Circuits. 2013; 26;7:79.

Okada M, Kawata Y, Murakami T, Wada K, Mizuno K, Kaneko S. Interaction between purinoceptor subtypes on hippocampal serotonergic transmission using *in vivo* microdialysis. Neuropharmacology. 1999; 38:707-715.

O'Neill MF, Shaw G. Comparison of dopamine receptor antagonists on hyperlocomotion induced by cocaine, amphetamine, MK-801 and the dopamine D1 agonist C-APB in mice. Psycopharmacology (Berl) 1999; 145: 237-50.

Padilla S, Hunter DL, Padnos B, Frady S, MacPhail RC. Assessing locomotor activity in larval zebrafish: influence of extrinsic and intrinsic variables. Neurotoxicol Teratol. 2011; 33, 624–630.

Panula P, Chen YC, Priyadarshini M, Kudo H, Semenova S, Sundvik M, Sallinen V. The comparative neuroanatomy and neurochemistry of *zebrafish* CNS systems of relevance to human neuropsychiatric diseases. Neurobiol Dis. 2010; 40(1):46-57.

Persico AM, Militerni R, Bravaccio C, Schneider C, Melmed R, Trillo S, et al Adenosine deaminase alleles and autistic disorder: case-control and familybased association studies. Am J Med Genet. 2000; 96(6):784-90

Poels EM, Kegeles LS, Kantrowitz JT, Javitt DC, Lieberman JA, Abi-Dargham A, Girgis RR. Glutamatergic abnormalities in schizophrenia: a review of proton MRS findings. Schizophr Res. 2014; 152(2-3):325-32.

Radev Z, Hermel JM, Elipot Y, Bretaud S, Arnould S, Duchateau P, Ruggiero F, Joly JS, Sohm F. A TALEN-Exon Skipping Design for a Bethlem Myopathy Model in Zebrafish. PLoS One. 2015; 10(7):e0133986.

Ranger P, Ellenbroek BA. Perinatal Influences of Valproate on Brain and Behaviour: An Animal Model for Autism. Curr Top Behav Neurosci. 2015

Raza S, Himmler BT, Himmler SM, Harker A, Kolb B, Pellis SM, Gibb R. Effects of prenatal exposure to valproic acid on the development of juvenile-typical social play in rats. Behav Pharmacol. 2015; 707-19.

Rial D, Lara DR, Cunha RA. The adenosine neuromodulation system in schizophrenia. Int Rev Neurobiol. 2014; 119:395-449.

Ricatti MJ, Alfie LD, Lavoie EG, Sévigny J, Schwarzbaum PJ, Faillace MP. Immunocytochemical localization of NTPDases1 and 2 in the neural retina of mouse and *zebrafish*. Synapse. 2009; 63: 291-307.

Rico EP, Senger MR, Fauth M da G, Dias RD, Bogo MR, Bonan CD. ATP and ADP hydrolysis in brain membranes of *zebrafish* (Danio rerio). Life Sci. 2003; 73:2071-82.

Rico EP, Rosemberg DB, Senger MR, Arizi M de B, Bernardi GF, Dias RD, Bogo MR, Bonan CD. Methanol alters ecto-nucleotidases and acetylcholinesterase in *zebrafish* brain. Neurotoxicol Teratol. 2006; 28:489-96.

Rico EP, Rosemberg DB, Seibt KJ, Capiotti KM, Da Silva RS, Bonan CD. *Zebrafish* neurotransmitter systems as potential pharmacological and toxicological targets. Neurotoxicol Teratol. 2011; 33:608-17.

Rinaldi T, Perrodin C, Markram H. Hyper-connectivity and hyper-plasticity in the medial prefrontal cortex in the valproic acid animal model of autism. Front Neural Circuits. 2008a; 2:4.

Rinaldi T, Silberberg G, Markram H. Hyperconnectivity of local neocortical microcircuitry induced by prenatal exposure to valproic acid. Cereb Cortex. 2008b; 18:763–770

Robson SC, Sévigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signal. 2006; 2:409-430.

Rodier PM, Ingram JL, Tisdale B, Croog VJ. Linking etiologies in humans and animal models: studies of autism. Reprod Toxicol. 1997; 11(2-3):417-22.

Romano A, Tempesta B, Micioni Di Bonaventura MV, Gaetani S. From Autism to Eating Disorders and More: The Role of Oxytocin in Neuropsychiatric Disorders. Front Neurosci. 2016; 9:497.

Rosemberg DB, Rico EP, Senger MR, Arizi MB, Dias RD, Bogo MR, Bonan CD. Acute and subchronic copper treatments alter extracellular nucleotide hydrolysis in *zebrafish* brain membranes. Toxicology. 2007; 236:132-139.

Rosemberg DB, Rico EP, Senger MR, Dias RD, Bogo MR, Bonan CD, Souza DO. Kinetic characterization of adenosine deaminase activity in *zebrafish* (Danio rerio) brain. Comp Biochem Physiol B Biochem Mol Biol. 2008; 151:96-101.

Rosemberg DB, Rico EP, Langoni AS, Spinelli JT, Pereira TC, Dias RD, Souza OD, Bonan CD, Bogo MR. NTPDase family in *zebrafish*: Nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart. Comp Biochem Physiol B Biochem Mol Biol. 2010; 155:230-240.

Rubenstein JL, Merzenich MM. Model of autism: increased ratio of excitation/inhibition in key neural systems. Genes Brain Behav. 2003; 2(5):255-67.

Rubin LH, Carter CS, Drogos L, Pournajafi-Nazarloo H, Sweeney JA, Maki PM. Peripheral oxytocin is associated with reduced symptom severity in schizophrenia. Schizophr Res. 2010; 124(1-3):13-21.

Rujescu D, Bender A, Keck M, Hartmann AM, Ohl F, Raeder H, et al. A pharmacological model for psychosis based on N-methyl-d-aspartate receptor hypofunction: molecular, cellular, functional and behavioral abnormalities. Biol Psychiatry 2006; 59:721–9.

Rutter M, Bailey A, Bolton P, Le Couteur A. Autism and known medical conditions: myth and substance. J Child Psychol Psychiatry. 1994; 35(2):311-22.

Saura CA, Mallol J, Canela EI, Lluis C, Franco R. Adenosine deaminase and A1 adenosine receptors internalize together following agonist-induced receptor desensitization. J Biol Chem. 1998; 273(28):17610-7.

Schneider T, Przewlocki R. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacol. 2005; 30:80–89.

Schneider T, Ziolkowska B, Gieryk A, Tyminska A, Przewloki R. Prenatal exposure to valproic acid disturbs the enkephalinergic system functioning, basal hedonic tone, and emotional responses in an animal model of autism. Psychopharmacol (Berl). 2007; 193: 547–555.

Schneider T, Roman A, Basta-Kaim A, Kubera M, Budziszewska B, Schneider K, Przewłocki R. Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. Psychoneuroendocrinol. 2008; 33(6):728-40.

Sebat J, Lakshmi B, Malhotra D, Troge J, Lese-Martin C, et al. Strong association of de novo copy number mutations with autism. Science. 2007; 316(5823):445-9.

Seibt KJ, Oliveira RL, Rico EP, Dias RD, Bogo MR, Bonan CD. Antipsychotic drugs inhibit nucleotide hydrolysis in *zebrafish* (Daniorerio) brain membranes. Toxicol In Vitro. 2010; 23:78-82.

Seibt KJ, Piato AL, da Luz Oliveira R, Capiotti KM, Vianna MR, Bonan CD. Antipsychotic drugs reverse MK-801-induced cognitive and social interaction deficits in *zebrafish* (Danio rerio). Behav Brain Res. 2011; 224(1):135-9.

Senger MR, Rico EP, Dias RD, Bogo MR, Bonan CD. Ecto-5′-nucleotidase activity in brain membranes of *zebrafish* (Danio rerio). Comp Biochem Physiol B Biochem Mol Biol. 2004; 139: 203-7.

Shinohe A, Hashimoto K, Nakamura K, Tsujii M, Iwata Y, et al. Increased serum levels of glutamate in adult patients with autism. Prog Neuropsychopharmacol Biol Psychiatry. 2006; 30(8):1472–1477.

Silverman JL, Yang M, Lord C, Crawley JN. Behavioural phenotyping assays for mouse models of autism. Nat Rev Neurosci. 2010; 11, 490–502.

Sperlágh B, Vizi ES. The role of extracellular adenosine in chemical neurotransmission in the hippocampus and Basal Ganglia: pharmacological and clinical aspects. Curr Top Med Chem. 2011; 11:1034-46.

Stednitz SJ, Freshner B, Shelton S, Shen T, Black D, Gahtan E. Selective toxicity of L-DOPA to dopamine transporter-expressing neurons and locomotor behavior in zebrafish larvae. Neurotoxicol Teratol. 2015; 52(Pt A):51-56.

Stodgell CJ, Ingram JL, O'Bara M, Tisdale BK, Nau H, Rodier PM. Induction of the homeotic gene Hoxa1 through valproic acid's teratogenic mechanism of action. Neurotoxicol Teratol. 2006; 28, 617–624.

Stubbs G, Litt M, Lis E, Jackson R, Voth W, Lindberg A et al. Adenosine deaminase activity decreased in autism. J Am Acad Child Psychiatry. 1982; 21(1):71-4.

Subramanian M, Timmerman CK, Schwartz JL, Pham DL, Meffert MK. Characterizing autism spectrum disorders by key biochemical pathways. Front Neurosci. 2015; 9:313.

Suraev AS, Bowen MT, Ali SO, Hicks C, Ramos L, McGregor IS. Adolescent exposure to oxytocin, but not the selective oxytocin receptor agonist TGOT, increases social behavior and plasma oxytocin in adulthood. Horm Behav. 2014; 65(5):488-96.

Tchaconas A, Adesman A. Autism spectrum disorders: a pediatric overview and update. Curr Opin Pediatr 2013; 25:130–44.

Tachibana M, Kagitani-Shimono K, Mohri I, Yamamoto T, Sanefuji W, Nakamura A, Oishi M, Kimura T, Onaka T, Ozono K, Taniike M. Long-term administration of intranasal oxytocin is a safe and promising therapy for early adolescent boys with autism spectrum disorders. J Child Adolesc Psychopharmacol. 2013; 23(2):123-7.

Tanimura Y, Vaziri S, Lewis MH. Indirect basal ganglia pathway mediation of repetitive behavior: Attenuation by adenosine receptor agonists. Behav Brain Res. 2010; 210(1):116–22.

Teng BL, Nikolova VD, Riddick NV, Agster KL, Crowley JJ, Baker LK, Koller BH, Pedersen CA, Jarstfer MB, Moy SS. Reversal of social deficits by subchronic oxytocin in two autism mouse models. Neuropharmacol. 2015; 105:61-71

Theoharides TC. Extracellular mitochondrial ATP, suramin, and autism? Clin Ther. 2013; 35(9):1454-6.

Thompson BL, Levitt P. Now you see it, now you don't--closing in on allostasis and developmental basis of psychiatric disorders. Neuron. 2010; 65(4):437-9.

Tu J, Wang LP. Therapeutic potential of extracellular ATP and P2 receptors in nervous system diseases. Neurosci Bull. 2009; 25:27-32.

van Loo KM, Martens GJ. Genetic and environmental factors in complex neurodevelopmental disorders. Curr Genomics. 2007; 8(7):429-44.

Varese F, Smeets F, Drukker M, Lieverse R, Lataster T, Viechtbauer W, Read J, van Os J, Bentall RP. Childhood adversities increase the risk of psychosis: a meta-analysis of patient-control, prospective- and cross-sectional cohort studies. Schizophr Bull. 2012; 38(4):661-71.

Weiss LA, Escayg A, Kearney JA, Trudeau M, MacDonald BT, Mori M, Reichert J, Buxbaum JD, Meisler MH. Sodium channels SCN1A, SCN2A and SCN3A in familial autism. Mol Psychiatry. 2003; 8:186–194.

Werler MM, Ahrens KA, Bosco JL, Mitchell AA, Anderka MT, Gilboa SM, Holmes LB; National Birth Defects Prevention Study. Use of antiepileptic medications in pregnancy in relation to risks of birth defects. Ann Epidemiol. 2011; 21(11):842-50.

Williams DL, Goldstein G, Minshew NJ. The profile of memory function in children with autism. Neuropsychol. 2006; 20(1):21-9.

Williams PG, Hersh JH. A male with fetal valproate syndrome and autism. Dev Med Child Neurol. 1997; 39(9):632-4.

Wright GJ, Washbourne P. Neurexins, neuroligins and LRRTMs: synaptic adhesion getting fishy. J Neurochem. 2011; 117(5):765-78.

Wu S, Jia M, Ruan Y, Liu J, Guo Y, Shuang M, Gong X, Zhang Y, Yang X, Zhang D. Positive association of the oxytocin receptor gene (OXTR) with autism in the Chinese Han population. Biol Psychiatry. 2005; 58(1):74-7.

Wyatt LR, Godar SC, Khoja S, Jakowec MW, Alkana RL, Bortolato M, Davies DL. Sociocommunicative and Sensorimotor Impairments in Male P2X4-Deficient Mice. Neuropsychopharmacol. 2013.

Xia S, Zhu Y, Xu X, Xia W. Computational techniques in *zebrafish* image processing and analysis. J Neurosci Methods. 2012; S0165-0270: 00465-7.

Xin SC, Zhao YQ, Li S, Lin S, Zhong HB. Application of *zebrafish* models in drug screening. Yi Chuan. 2012; 34: 1144-52.

Yamamoto T, Geiger JD, Daddona PE, Nagy JI. Subcellular, regional and immunohistochemical localization of adenosine deaminase in various species. Brain Res Bull. 1987;19(4):473-84.

Yan J, Oliveira G, Coutinho A, Yang C, Feng J, et al. Analysis of the neuroligin 3 and 4 genes in autism and other neuropsychiatric patients. Mol Psychiatry. 2005; 10:329–332.

Yang L, Ho NY, Alshut R, Legradi J, Weiss C, Reischl M, Mikut R, Liebel U, Müller F, Strähle U. *Zebrafish* embryos as models for embryotoxic and teratological effects of chemicals. Reprod Toxicol. 2009; 2: 245-53

Yatawara CJ, Einfeld SL, Hickie IB, Davenport TA, Guastella AJ. The effect of oxytocin nasal spray on social interaction deficits observed in young children with autism: a randomized clinical crossover trial. Mol Psychiatry. 2015.

Young JW, Geyer MA. Developing treatments for cognitive deficits in schizophrenia: the challenge of translation. J Psychopharmacol. 2015; 29(2):178-96.

Yu J, Qi D, Xing M, Li R, Jiang K, Peng Y, Cui D. MK-801 induces schizophrenic behaviors through downregulating Wnt signaling pathways in male mice. Brain Res. 2011; 1385:281-92.

Zavialov AV, Engstrom A. Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity. Biochem J. 2005; 391: 51-57.

Zellner, D., Padnos, B., Hunter, D.L., MacPhail, R.C., Padilla, S. Rearing conditions differentially affect the locomotor behavior of larval zebrafish, but not their response to valproate-induced developmental neurotoxicity. Neurotoxicol Teratol. 2011; 33, 674–679.

Zhang J, Sun XY, Zhang LY. MicroRNA-7/Shank3 axis involved in schizophrenia pathogenesis. J Clin Neurosci. 2015; 22(8):1254-7.

Zhang YX, Yamashita H, Ohshita T, Sawamoto N, Nakamura S. ATP increases extracellular dopamine level through stimulation of P2Y purinoceptors in the rat striatum. Brain Res. 1995; 691(1-2):205-12.

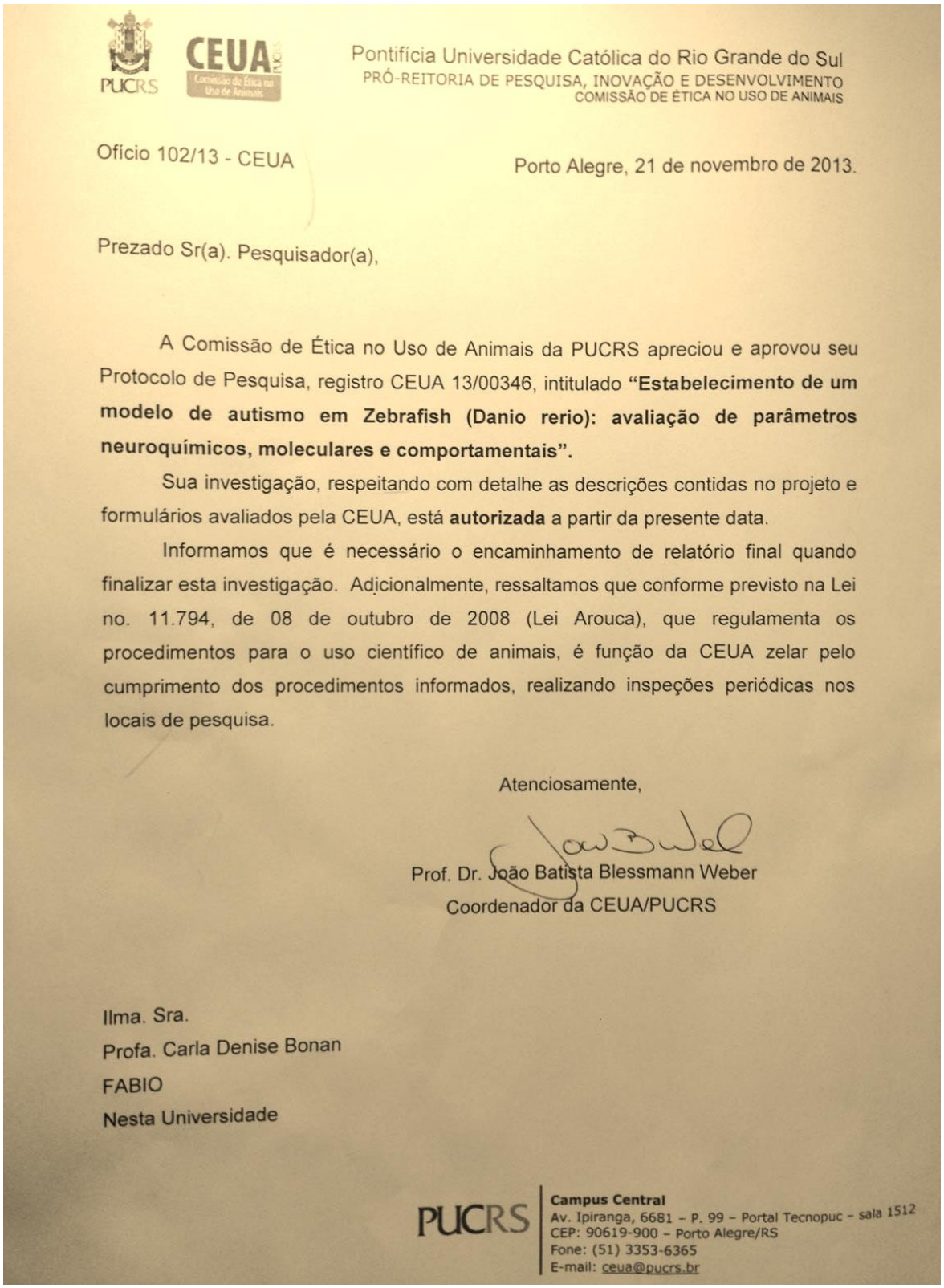
Zimmermann FF, Gaspary KV, Leite CE, De Paula Cognato G, Bonan CD. Embryological exposure to valproic acid induces social interaction deficits in zebrafish (Danio rerio): A developmental behavior analysis. Neurotoxicol Teratol. 2015; 52(Pt A):36-41

Zimmermann H. Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch Pharmacol. 2000; 362: 299-309.

Zhou X-L, Giacobini M, Anderlid B-M, Anckarsa¨ter H, Omrani D, Gillberg C, Nordenskjo¨ld M, Lindblom A. Association of adenomatous polyposis coli (APC) gene polymorphisms with autism spectrum disorder (ASD). Am J Med Genet Part B Neuropsychiatr Genet. 2007; 144B:351–354.

Zwaigenbaum L, Bryson S, Garon N. Early identification of autism spectrum disorders. Behav Brain Res. 2013.

**ANEXO**



PUCRS CEUA

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 102/13 - CEUA

Porto Alegre, 21 de novembro de 2013.

Prezado Sr(a). Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 13/00346, intitulado **"Estabelecimento de um modelo de autismo em Zebrafish (Danio rerio): avaliação de parâmetros neuroquímicos, moleculares e comportamentais".**

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está **autorizada** a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

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