UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA
CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

ALTERAÇÕES NEUROQUÍMICAS E COMPORTAMENTAIS INDUZIDAS PELA EXPOSIÇÃO À PROLINA EM PEIXE-ZEBRA (*Danio rerio*)

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PORTO ALEGRE

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito parcial para obtenção do título de Mestre em Ciências Biológicas: Bioquímica.

PORTO ALEGRE

2012

À minha mãe e meus irmãos, pelo amor, carinho, apoio e constante incentivo durante meus estudos.

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"Os que se encantam com a prática sem a ciência são como os timoneiros que entram no navio sem timão nem bússola, nunca tendo certeza do seu destino."

Leonardo Da Vinci

SUMÁRIO

RESUMO

A hiperprolinemia é uma doença metabólica que pode ser causada por dois distintos erros inatos do metabolismo da prolina. A hiperprolinemia tipo I ocorre por uma deficiência na enzima prolina oxidase, enquanto que a hiperprolinemia tipo II é causada pela ausência da atividade da enzima Δ^1-pirrolino-5-carboxilato desidrogenase. Os pacientes afetados por essa doença geralmente apresentam manifestações neurológicas, tais como convulsões, déficit cognitivo e retardo mental. Além disso, tem sido descrita uma associação entre a hiperprolinemia moderada e doenças psiquiátricas. Entretanto, os mecanismos neuroquímicos relacionados a esses sintomas neurológicos ainda são pouco compreendidos. Portanto, no presente estudo, investigamos o efeito da exposição aguda e crônica à prolina sobre parâmetros comportamentais em peixe-zebra, tais como: atividade locomotora, ansiedade e interação social. Além disso, avaliamos o efeito *in vivo* e *in vitro* da prolina sobre a atividade da acetilcolinesterase (AChE), bem como sobre a atividade e expressão gênica das ectonucleotidases em cérebro de peixe-zebra. Para os estudos *in vivo*, os animais foram expostos a duas concentrações de prolina (1,5 e 3,0 mM) durante 1 hora ou 7 dias (tratamento agudo e crônico, respectivamente). Para os ensaios *in vitro*, diferentes concentrações de prolina foram testadas (variando de 3,0 a 1000 µM). A exposição aguda à prolina não promoveu alterações significativas nos parâmetros bioquímicos e comportamentais analisados. Entretanto, a exposição crônica à prolina na concentração de 1,5 mM provocou um aumento na atividade locomotora do peixe-zebra, caracterizada pelo aumento no número de linhas cruzadas (47%), na distância total percorrida (29%) e na velocidade média (33%). Um aumento significativo no tempo gasto na parte superior do aquário (91%) também foi observado após esse mesmo tratamento, o que pode ser interpretado como um comportamento ansiolítico. A prolina na concentração de 1,5 mM também induziu o déficit de interação social (78%), quando comparado ao grupo controle. Além disso, exposição crônica aumentou significativamente a atividade da AChE em ambos os grupos tratados (34% e 39%). Esse mesmo tratamento também aumentou a hidrólise de ATP em ambas as concentrações testadas (14% e 22%, respectivamente), enquanto que a hidrólise de ADP e AMP aumentou apenas na concentração de 3,0 mM (21% e 17%, respectivamente). A expressão gênica da E-NTPDase3 aumentou em ambos os grupos tratados após a exposição crônica à prolina, enquanto que a E-NTPDase1 teve seus níveis de transcritos aumentados apenas na concentração de 3,0 mM. A prolina, quando avaliada *in vitro,* não promoveu alterações significativas nas atividades das ectonucleotidase e da AChE. Por fim, demonstramos, ainda, que as alterações comportamentais e o aumento da atividade da AChE induzidos pela prolina foram completamente revertidos pela administração aguda de um antipsicótico atípico (sulpirida), mas não por um típico (haloperidol). Em conjunto, estes dados demonstram que a exposição crônica à prolina induz alterações comportamentais, bem como aumenta a atividade da AChE e catabolismo de nucleotídeos em cérebro de peixe-zebra. Esses achados podem contribuir, pelo menos em parte, para uma melhor compreensão dos mecanismos relacionados às manifestações neurológicas verificadas em pacientes hiperprolinêmicos, como os transtornos psicóticos e cognitivos. Além disso, este estudo pode facilitar o uso do peixe-zebra como modelo experimental para o estudo de erros inatos do metabolismo que afetam o sistema nervoso central.

ABSTRACT

Hyperprolinemia is a metabolic disease that may be caused by two distinct inborn errors of proline metabolism. Hyperprolinemia type I occurs by a deficiency in proline oxidase, while the hyperprolinemia type II is caused by an absence of Δ^1-pyrroline-5-carboxylic acid dehydrogenase. Patients affected by this disease usually present neurological manifestations, such as seizures, cognitive impairment, and mental retardation. Moreover, an association between psychiatry disorders and moderate hyperprolinemia has been reported. However, the mechanisms related to these neurological symptoms still remain poorly understood. Therefore, in the present study, we investigated the effect of short- and long-term proline exposure on behavioral parameters in zebrafish, such as locomotor activity, anxiety, and social interaction. In addition, we evaluated the *in vivo* and *in vitro* effects of proline on acetylcholinesterase (AChE) activity, as well as on ectonucleotidase activities and gene expression in zebrafish brain. For the *in vivo* studies, animals were exposed at two proline concentrations (1.5 and 3.0 mM) during 1 hour or 7 days (short- or long-term treatments, respectively). For the *in vitro* assays, different proline concentrations (ranging from 3.0 μM to 1000 μM) were tested. Short-term proline exposure did not promote significant changes on the behavioral and biochemical parameters analyzed. Long-term exposure at 1.5 mM proline caused an increase in locomotor activity in zebrafish, characterized by an increase in the number of line crossings (47%), in the total distance traveled (29%), and in the mean speed (33%). A significant increase in the time spent in the upper portion of the test tank was also observed after the same treatment (91%), which may be interpreted as an indicator of anxiolytic behavior. Proline at 1.5 mM also induced social interaction impairment (78%), when compared to the untreated group. Moreover, long-term proline exposure significantly increased AChE activity for both treated groups (34% and 39%). This treatment also increased ATP catabolism in both concentrations tested (14% and 22%, respectively), whereas ADP and AMP hydrolysis were increased only at 3.0 mM proline (21% and 17%, respectively). The gene expression of E-NTPDase3 increased in both treated groups after long-term proline, whereas E-NTPDase1 transcript levels increased only at concentration of 3.0 mM. Proline, when assessed *in vitro,* did not promote significant changes on AChE and ectonucleotidase activities. At last, we demonstrated the proline-induced behavioral changes and increase in AChE activity were completely reversed by acute administration of an atypical antipsychotic drug (sulpiride), but not by a typical (haloperidol). Altogether, these data demonstrate that long-term proline exposure induces behavioral changes as well as increases AChE activity and nucleotide catabolism in zebrafish brain. These findings may contribute, at least in part, to better understand the mechanisms related to the neurological manifestations observed in hyperprolinemic patients, such as the psychotic and cognitive dysfunctions. Moreover, this study might facilitate the use of the zebrafish as experimental model for studying inborn errors of amino acid metabolism that affect the central nervous system.

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LISTA DE ABREVIATURAS

ACh: acetilcolina

AChE: acetilcolinesterase

ADP: adenosina 5´-difosfato

AMPA: ácido α-amino-3-hidróxi-5-metil-4-isoxazol-epropionato

AMP: adenosina 5´-monofosfato

ATP: adenosina 5'-trifosfato

BuChE: butirilcolinesterase

CAT: catalase

ChAT: colina acetiltransferase

CPC: fenciclidina

DA: dopamina

EIM: erros inatos do metabolismo

E-NTPDases: ecto-nucleosídeo trifosfato difosfoidrolases

E-NPPs: ecto-nucleotídeo pirofosfatase/fosfodiesterase

$FADH_2$: flavina adenina dinucleotídeo (forma reduzida)

GSH-Px: glutationa peroxidase

HPI: hiperprolinemia tipo I

HPII: hiperprolinemia tipo II

MK-801: maleato de dizocilpina

NAD^+: nicotinamida adenina dinucleotídeo (forma oxidada)

NADH: nicotinamida adenina dinucleotídeo (forma reduzida)

$NADP^+$: nicotinamida adenina dinucleotídeo fosfato (forma oxidada)

NADPH: nicotinamida adenina dinucleotídeo fosfato (forma reduzida)

NMDA: N-metil-D-aspartato

OAT: ornitina aminotransferase

POX: prolina oxidase

P5C: pirrolino-5-carboxilato

P5CDh: Δ^1-pirrolino-5-carboxilato desidrogenase

TRAP: potencial antioxidante total

1. INTRODUÇÃO

1.1 Erros Inatos do Metabolismo

Os Erros Inatos do Metabolismo (EIM) são distúrbios de natureza genética que se manifestam pela síntese de uma proteína anômala, geralmente uma enzima, podendo resultar na diminuição parcial ou total de sua atividade. Esse comprometimento enzimático é capaz de acarretar o bloqueio de rotas metabólicas, ocasionando, assim, alguma falha de síntese, degradação, armazenamento ou transporte de moléculas no organismo. Como consequência, pode ocorrer tanto o acúmulo de metabólitos tóxicos como a falta de produtos essenciais, levando ao comprometimento dos processos celulares e à doença subsequente (Scriver et al., 2001).

Os EIM são classificados de acordo com a área do metabolismo afetada, dentre os quais podemos citar: EIM de aminoácidos, ácidos orgânicos, carboidratos, lipídios, glicosaminoglicanos, glicoproteínas, purinas e pirimidinas, lipoproteínas, entre outros (Scriver et al., 2001). Em geral, os EIM são doenças graves que afetam todo o organismo e manifestam-se, principalmente, na infância, mas podem surgir em qualquer faixa etária. O seu diagnóstico deve ser realizado o mais breve possível, a fim de possibilitar ao indivíduo um tratamento específico e imediato (Giugliani, 1988). Entretanto, para algumas dessas doenças a fisiopatologia é pouco conhecida e não há um tratamento específico, por isso muitos estudos na área de EIM ainda são necessários. Nesta dissertação, foram estudadas as hiperprolinemias, que podem ser causadas por dois distintos EIM do

metabolismo da prolina e caracterizam-se pelo acúmulo tecidual desse aminoácido.

1.2 L-Prolina

A L-prolina (prolina) é classificada como um aminoácido apolar de cadeia lateral alifática, na qual se insere um grupo o pirrolidina cíclico que lhe confere certa rigidez conformacional e promove estabilidade física às proteínas que possuem esse aminoácido em sua estrutura (Phang et al., 2001). Além disso, tem sido demonstrado um importante papel desse aminoácido no reconhecimento proteína-proteína e na sinalização molecular (Lu et al., 2003).

A prolina é um aminoácido não essencial, sendo condicionalmente essencial em prematuros (Phang et al., 2001). Em sua via de síntese, a prolina pode ser formada a partir de ornitina ou glutamato. A ornitina é convertida em glutamato-γ-semialdeído, um tautômero acíclico com o qual o Δ^1-pirrolino-5-carboxilato (P5C) está em equilíbrio espontâneo, por ação da enzima ornitina aminotransferase (OAT), numa reação na qual o α-cetoglutarato é utilizado como aceptor amino. Da mesma forma, o glutamato também é convertido em glutamato-γ-semialdeído numa reação catalisada pela enzima P5C sintase. O P5C, por sua vez, é transportado para o citosol e reduzido à prolina pela P5C redutase que tem como cofator NADH ou NADPH (Flynn et al., 2002; Phang et al., 2001).

A enzima prolina oxidase (POX; EC 1.5.1.2), localizada na matriz mitocondrial, é a primeira enzima envolvida na degradação da prolina, dando

origem ao P5C. Essa primeira reação envolve a transferência dos elétrons da prolina a uma flavoproteína (FAD), gerando $FADH_2$. A segunda reação é espontânea e envolve a conversão de P5C a glutamato-γ-semialdeído. Este, por sua vez, pode formar ornitina na reação reversível catalisada pela OAT ou glutamato pela Δ^1-pirrolino-5-carboxilato desidrogenase (P5CDh; EC 1.5.1.12). A Δ^1-pirrolino-5-carboxilato desidrogenase utiliza NAD^+ como aceptor de elétrons, formando NADH (Phang et al., 2001) (Figura 1).

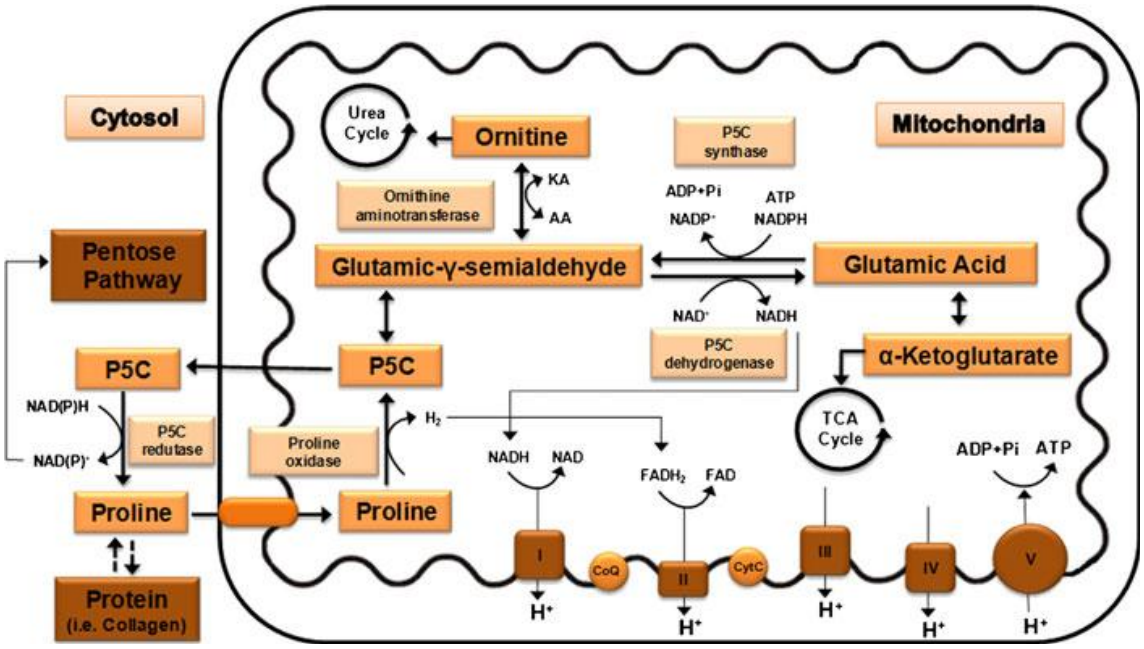

Figura 1. Rota metabólica da prolina. P5C: Δ^1-pirrolino-5-carboxilato; CoQ: coenzima Q; Cyt c: citocromo c; I–IV: complexos da cadeia transportadora de elétrons e FoFi-ATP sintase; AA: aminoácido; KA: α-cetoácido (adaptado de Wyse e Netto, 2011).

O papel fisiológico da rota metabólica da prolina é, basicamente, fornecer prolina para a síntese de proteínas, gliconeogênese hepática e síntese de ornitina e arginina (Phang et al., 2001). Entretanto, outras funções bioquímicas e fisiológicas têm sido descritas para o seu metabolismo. Sabe-

se, por exemplo, que as interconversões da prolina em sua rota metabólica auxiliam na regulação celular por mecanismos redox (Phang et al., 2001). Além disso, as coenzimas reduzidas NADH e $FADH_2$ geradas durante o catabolismo da prolina podem entregar seus elétrons na cadeia respiratória mitocondrial, contribuindo para a produção de ATP no processo de fosforilação oxidativa (Adams e Frank, 1980; Hagedorn e Phang, 1983; Phang et al., 2001).

Os estudos sugerem, ainda, que a prolina tem uma ação na neurotransmissão excitatória no sistema nervoso central (SNC), pois este aminoácido apresenta algumas características típicas de moléculas classificadas como neurotransmissores, tais como: biossíntese e acúmulo em sinaptossomas e distribuição regional no cérebro, principalmente em vias de neurotransmissão glutamatérgica (Cohen e Nadler, 1997; Phang et al., 2001). Além disso, algumas pesquisas demonstraram que populações de neurônios glutamatérgicos expressam transportadores de prolina, reforçando a ideia de que a prolina pode desempenhar um importante papel na modulação de rotas específicas na transmissão sináptica excitatória no SNC (Fremeau et al., 1992; Velaz-Faircloth et al., 1995). Entretanto, ainda não há um completo entendimento acerca dessas funções (Wyse e Netto, 2011).

1.3 As Hiperprolinemias

As hiperprolinemias podem ser causadas por dois distintos erros inatos do metabolismo da prolina. A hiperprolinemia tipo I (HPI) é provocada por uma deficiência na atividade da enzima prolina oxidase (POX; EC 1.5.1.2).

Essa enzima é codificada pelo gene da prolina desidrogenase (PRODH), localizado na região cromossomal 22q11. Na hiperprolinemia tipo II (HPII), o defeito enzimático envolve a enzima Δ^1-pirrolino-5-carboxilato desidrogenase (P5CDh; EC 1.5.1.12) devido a uma mutação no gene P5CDH (Figura 1) (Phang et al., 2001, Mitsubuchi et al., 2008). Ambos os defeitos enzimáticos citados acima resultam no acúmulo tecidual de prolina. No entanto, cada tipo de hiperprolinemia possui sua sintomatologia característica (Flynn et al., 1989; Mitsubuchi et al., 2008).

Na HPI, níveis plasmáticos de prolina podem variar entre 500 e 2600 µM (valores indivíduos normais: 50-270 µM). A HPI é diagnosticada com base nos níveis elevados de prolina sérica, sem a presença de P5C na urina. Entretanto, o diagnóstico é feito por exclusão, pois a determinação direta da atividade da prolina oxidase não é realizada. Alguns pacientes com HPI podem apresentar doenças renais, defeitos auditivos, alterações oculares ou neurológicas, enquanto outros podem ser assintomáticos (Phang et al., 2001, Wyse e Netto, 2011). Além disso, estudos têm demonstrado que níveis moderadamente elevados de prolina, como aqueles geralmente verificados na HPI, podem estar associados a doenças psiquiátricas, tais como a esquizofrenia (Jacquet et al., 2005; Oresic et al., 2011).

A HPII caracteriza-se por apresentar níveis séricos de prolina ainda mais elevados do que aqueles verificados nos pacientes diagnosticados com HPI, os quais oscilam entre 500-3700 µM (Phang et al., 2001). A presença de P5C na urina, associada aos elevados níveis plasmáticos de prolina, caracteriza a HPII. Ao contrário da HPI, a confirmação do diagnóstico da

HPII pode ser realizada pela determinação da atividade enzimática da P5C desidrogenase em leucócitos ou cultura de fibroblastos. Os portadores dessa doença podem apresentar manifestações neurológicas, como convulsões, epilepsia e retardo mental. Entretanto, os mecanismos pelos quais essas manifestações ocorrem ainda são pouco compreendidos (Flynn et al., 1989; Phang et al., 2001).

Com a finalidade de compreender melhor as alterações fisiológicas e comportamentais induzidas por níveis séricos elevados de prolina, alguns estudos foram desenvolvidos em ratos utilizando um modelo animal em que os níveis de prolina são artificialmente elevados até alcançarem valores semelhantes aos encontrados em humanos que apresentam hiperprolinemia (Moreira et al., 1989). Utilizando esse modelo, nosso grupo tem mostrado que a prolina reduziu a atividade da citocromo c oxidase (Delwing et al., 2007a), as atividades da Na^+/K^+-ATPase (Franzon et al., 2003) e acetilcolinesterase (Delwing et al., 2003a), bem como a captação de glutamato em córtex cerebral de ratos (Delwing et al., 2007b). Além disso, a prolina provocou déficit de memória (Delwing et al., 2006) e alterou vários parâmetros de estresse oxidativo, como a redução do potencial antioxidante total não-enzimático (TRAP) e alterações nas atividades das enzimas catalase (CAT) e glutationa peroxidase (GSH-Px) (Delwing et al., 2003b, 2005a) (Figura 2). Esses resultados sugerem que altos níveis de prolina sejam neurotóxicos ou, pelo menos, serem capazes de induzir um dano neuronal (Wyse e Netto, 2011).

Apesar dos estudos na área de EIM terem aumentado nos últimos anos, até o presente momento, parece não existir tratamento específico para

as hiperprolinemias. A restrição dietética desse aminoácido é difícil e não provoca impacto nas manifestações clínicas da doença, uma vez que a prolina é um aminoácido não essencial sintetizado a partir de outros precursores. Além disso, a maioria das proteínas contém resíduos de prolina. Assim, muitos estudos ainda são necessários para que seja possível conhecer os mecanismos que levam às manifestações clínicas observadas, facilitando a busca de alternativas para o tratamento desta doença (Phang et al., 2001; Mitsubuchi et al., 2008; Wyse e Netto, 2011).

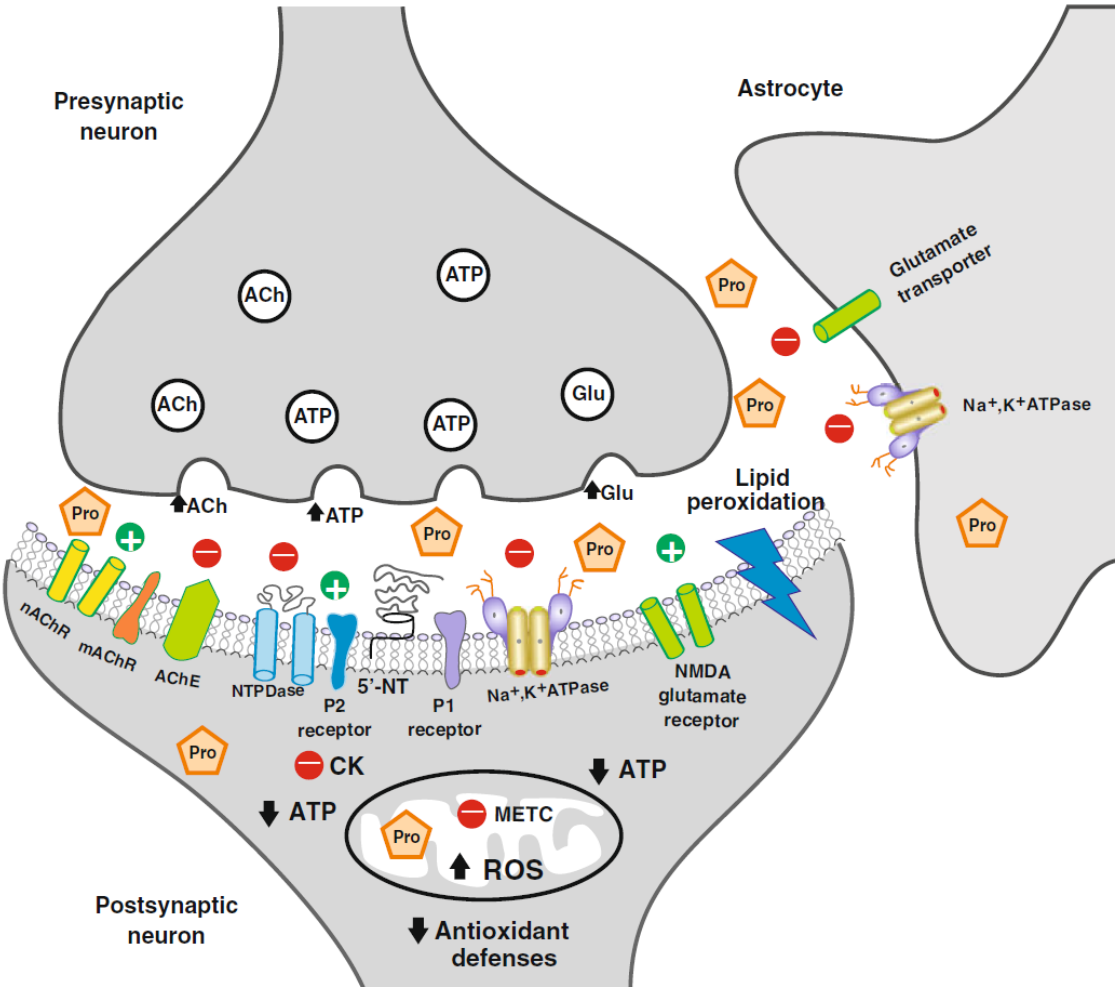

Figura 2. Alterações neuroquímicas induzidas pela hiperprolinemia. O acúmulo de prolina pode induzir estresse oxidativo, prejudicar o metabolismo energético e provocar excitotoxicidade (adaptado de Wyse e Netto, 2011).

1.4 Antipsicóticos

Os antipsicóticos são fármacos amplamente utilizados no tratamento de doenças neuropsiquiátricas como, por exemplo, a esquizofrenia. A ação terapêutica dos antipsicóticos deve-se, principalmente, ao bloqueio de receptores de dopamina. Entretanto, o antagonismo desses receptores também é responsável pelos efeitos adversos extrapiramidais induzidos por esses fármacos (Wise, 2005; Tadori et al., 2011).

Os antipsicóticos são subdivididos em duas classes: os típicos e os atípicos. Os típicos exercem seus efeitos, preferencialmente, bloqueando receptores D_2 de dopamina e são caracterizados por induzirem os efeitos adversos extrapiramidais. Já os fármacos atípicos são mais recentes e caracterizam-se farmacologicamente pela sua baixa afinidade por receptores dopaminérgicos D_2 e pela alta afinidade por receptores de outras vias neuronais, como os serotoninérgicos (5-HT_{2A} e 5-HT_6), histamínicos (H1), adrenérgicos (α1) e colinérgicos (M1), não induzindo efeitos adversos extrapiramidais (Miyamoto et al., 2005; Jones et al., 2008).

Dentre os antipsicóticos podemos citar o haloperidol, que é um fármaco típico, do grupo das butirofenonas, o qual atua farmacologicamente como um antagonista dopaminérgico, especialmente em receptores D_2. Além disso, como outros antipsicóticos típicos, esse fármaco produz efeitos adversos extrapiramidais (Heusler et al., 2008). A sulpirida, por sua vez, é um antipsicótico atípico da classe das benzamidas, que atua principalmente sobre os receptores de dopamina D_2 e D_3, mas com menor potência do que

as butirofenonas, não induzindo efeitos adversos extrapiramidais (Jaworski et al., 2001; Tadori et al., 2011).

1.5 Peixe-zebra (*Danio rerio*)

O peixe-zebra (*Danio rerio*) é um pequeno teleósteo (3-4 cm) de água-doce, pertencente à família Cyprinidae, consolidado como modelo experimental em diversas áreas da ciência (Figura 3). Ele tem sido utilizado, por exemplo, como modelo animal em estudos toxicológicos (Yang et al., 2009), genéticos, teratológicos (Becker e Becker, 2008), farmacológicos (Bencan et al., 2009; Egan et al., 2009) e neurocomportamentais (Mathur e Guo, 2010; Sison e Gerlai, 2011).

Figura 3. O Peixe-zebra (*Danio rerio*)
(http://aquamundi.com.br/tag/zebrafish)

Esse peixe configura-se como um excelente modelo experimental para o estudo de doenças metabólicas, como as hiperprolinemias, pois pode ser continuamente exposto a diferentes concentrações de aminoácidos por longos períodos. Já em modelos utilizando roedores, as altas doses dos aminoácidos administradas são rapidamente metabolizadas (Moreira et al.,

1989). Além disso, sabe-se que o peixe-zebra apresenta uma fácil absorção e distribuição interna de substâncias (como os aminoácidos), as quais podem ser absorvidas pelos vasos sanguíneos das brânquias ou pela pele, sendo rapidamente difundidas através da circulação sistêmica atingindo diversos orgãos, como, por exemplo, o cérebro (Lele e Krone, 1996; Parng et al., 2002; Kari et al., 2007; Rosemberg et al., 2011a).

O peixe-zebra apresenta, ainda, outras características favoráveis que complementam os modelos experimentais existentes, tais como: pequeno custo e espaço requerido para manutenção, rápido desenvolvimento e ciclo biológico, grande prole e embriões translúcidos e suscetíveis à manipulação e microinjeção (Lele e Krone, 1996). Além disso, essa espécie apresenta um alto grau de similaridade com os genes de humanos e camundongos, quando comparados em sua sequência (Barbazuk et al., 2000).

Atualmente, o peixe-zebra vem destacando-se como um importante modelo animal para o estudo dos mecanismos envolvidos na fisiopatologia de diversas doenças neurológicas, bem como dos fenótipos comportamentais associados a essas doenças (Guo, 2004). Diferentes sistemas de neurotransmissão já foram identificados nesta espécie, tais como: glutamatérgico (Edwards e Michel, 2002), colinérgico (Behra et al., 2002), dopaminérgico (Boehmler et al., 2004), serotoninérgico (Rink e Guo, 2004), histaminérgico (Kaslin e Panula, 2001), gabaérgico (Kim et al., 2004) e purinérgico (Kucenas et al., 2003). Recentemente, estudos avaliando características comportamentais do peixe-zebra também foram desenvolvidos, como, por exemplo: comportamento social, atividade locomotora (Fontaine et al., 2008; Seibt et al., 2010), atividade exploratória

(Rosemberg et al., 2011b), ansiedade (Egan et al., 2009), estresse (Champagne et al., 2010; Piato et al., 2011) e aprendizado/memória (Pather et al., 2009; Blank et al., 2009). Além disso, os efeitos de fármacos psicoativos sobre parâmetros comportamentais nesta espécie também têm sido avaliados (Seibt et al., 2010; Norton e Bally-Cuif, 2010). Assim, esse teleósteo apresenta-se como um ótimo modelo experimental para o desenvolvimento de estudos neuroquímicos, moleculares e comportamentais na área de doenças geneticamente herdadas que afetam o sistema nervoso (Ganser e Dallman 2009).

1.6 Sinalização Colinérgica

A sinalização colinérgica é uma das mais importantes vias de modulação do SNC, desempenhando um papel fundamental em várias funções vitais como: na cognição, no processamento das funções sensoriais, na organização cortical do movimento e no controle do fluxo sanguíneo cerebral (Scremin et al., 1997; Mesulam et al., 2002; Sarter e Bruno, 2004).

A acetilcolina (ACh) é um neurotransmissor clássico que exerce seus efeitos pela ativação de receptores colinérgicos nicotínicos e muscarínicos, desencadeando diversas respostas celulares (Burgen, 1995). Dependendo do tipo de célula, esses receptores podem abrir ou fechar canais de K^{+}, Ca^{+2} ou Cl^{-}. Além disso, sabe-se que a ativação dos receptores muscarínicos pode induzir a despolarização ou hiperpolarização da membrana e, também, pode inibir a enzima adenilato ciclase e ativar a enzima fosfolipase C (Cooper, 1991).

A acetilcolina é sintetizada numa reação catalisada pela colina acetiltransferase (ChAT; EC 2.3.1.6) a partir de acetil-CoA, formada durante o metabolismo celular mitocondrial, e da colina, um importante produto do metabolismo dos lipídios. A colina usada na síntese de ACh pode ser obtida apenas por dois mecanismos distintos: a partir da fosfatidilcolina ou diretamente da reciclagem da ACh, que é hidrolisada pela acetilcolinesterase (AChE; EC 3.1.1.7) na fenda sináptica, uma vez que a colina presente no plasma não ultrapassa a barreira hemato-encefálica (Taylor e Brown, 1994). Portanto, na sinalização colinérgica, as colinesterases são importantes enzimas que hidrolisam a ACh na fenda sináptica, finalizando a neurotransmissão colinérgica (Zimmerman e Soreq, 2006). Existem dois tipos de colinesterases que diferem em suas propriedades catalíticas, especificidade por inibidores e distribuição nos tecidos. A AChE, uma serina hidrolase pertencente à família α/β hidrolase, é encontrada principalmente nas sinapses do SNC, SNP parassimpático e junção neuromuscular, enquanto que a butirilcolinesterase (E.C. 3.1.1.8, BuChE) é encontrada no sangue, no intestino e em outros tecidos (Massoulié e Bom, 1982; Soreq e Seidman, 2001; Zimmerman e Soreq, 2006).

O sistema colinérgico já foi estudado em cérebro de peixe-zebra através de análise histoquímica e imuno-histoquímica (Clemente et al., 2004). Zirger e colaboradores (2003) demonstraram que subunidades de receptores muscarínicos são expressas nessa espécie. Além disso, o gene da AChE já foi clonado e sequenciado e sua atividade enzimática já foi detectada no cérebro desse teleósteo. Entretanto, não foi identificado nessa espécie um gene que codifique a butirilcolinesterase, o que mostra que a

hidrólise de acetilcolina é essencialmente realizada pela AChE em peixe-zebra (Bertrand, et al., 2001).

Sabe-se que alterações no sistema colinérgico são eventos importantes associados à fisiopatologia de alguns distúrbios neurodegenerativos. Nesse sentido, estudos em peixe-zebra têm demonstrado que a AChE pode ser utilizada como um importante biomarcador de neurotoxicidade, uma vez que mudanças na atividade dessa enzima podem indicar alterações na disponibilidade de ACh e do nível de seus receptores colinérgicos (Fernandes e Hodges-Savola, 1992; Rico et al., 2006; Levin et al. 2006; Park et al., 2008; Seibt et al., 2009; Siebel et al., 2010). Assim, a AChE possui importante papel na transmissão colinérgica e estudos recentes demonstraram que altas concentrações de prolina podem alterar a atividade dessa enzima em cérebro de ratos (Delwing, et al., 2005b), sugerindo que alterações na atividade da AChE podem contribuir, pelo menos em parte, para as disfunções neurológicas características das hiperprolinemias.

1.7 Sinalização Purinérgica

A sinalização purinérgica está envolvida em diversas situações fisiológicas e patológicas em diferentes tecidos. É amplamente descrito que os nucleotídeos e nucleosídeos modulam, através dos receptores purinérgicos, diversos mecanismos neuronais e não neuronais, incluindo respostas imunes, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (Burnstock, 2004).

O ATP é um importante neurotransmissor excitatório que pode ser armazenado em vesículas pré-sinápticas e liberado para o meio extracelular juntamente com acetilcolina, glutamato, noradrenalina, serotonina e GABA (Burnstock e Knight, 2004). Esse nucleotídeo exerce seus efeitos através da ativação de receptores purinérgicos do tipo P2, os quais são divididos em duas subclasses: P2X e P2Y. A família P2X, ligada a um canal iônico, está envolvida na transmissão excitatória rápida e a família P2Y é composta por receptores metabotrópicos acoplados à proteína G. Dentre os subtipos de receptores, sete da família P2X ($P2X_{1-7}$) e oito subtipos de receptores P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) são farmacologicamente distintos e possuem suas respostas funcionais descritas (Ralevic e Burnstock, 1998; Burnstock, 2004). Em peixe-zebra, a clonagem e a caracterização molecular dos subtipos dos receptores P2X já foram realizadas (Diaz-Hernandez et al., 2002; Norton et al., 2000). A análise da sequência de nove genes sugere que cinco deles são ortólogos a genes dos receptores P2X de mamíferos, dois são parálogos e um ainda precisa ser devidamente classificado (Kucenas et al., 2003).

O ATP e seus respectivos nucleotídeos podem ser hidrolisados por uma variedade de enzimas denominadas ectonucleotidases, que estão localizadas na superfície celular (Zimmermann, 2001) (Figura 4). Assim, as ectonucleotidases desempenham um importante papel no controle da homeostasia dos níveis de nucleotídeos e nucleosídeos extracelulares. Dentre estas, pode-se destacar as E-NTPases (ecto-nucleosídeo trifosfato difosfoidrolases), as E-NPPs (ecto-nucleotídeo pirofosfatase/fosfodiesterase) e a ecto-5´-nucleotidase. Em peixe-zebra, estudos demonstraram a

presença das E-NTPDases e da ecto-5'-nucleotidase em membranas cerebrais (Rico et al., 2003; Senger et al., 2004; Rosemberg et al. 2010).

As E-NTPDases catalisam a hidrólise de nucleotídeos trifosfatados e difosfatados aos seus respectivos nucleotídeos monofosfatados (Zimmermann, 2001). Essa família de enzimas é composta por oito membros (E-NTPDases 1-8), sendo que quatro estão localizados na superfície das células, com um sítio catalítico voltado para o meio extracelular (E-NTPDase 1, 2, 3 e 8) e outros quatro são encontrados no meio intracelular (E-NTPDases 4-7) (Zimmermann, 2001; Zimmermann, 2006; Yegutkin, 2008).

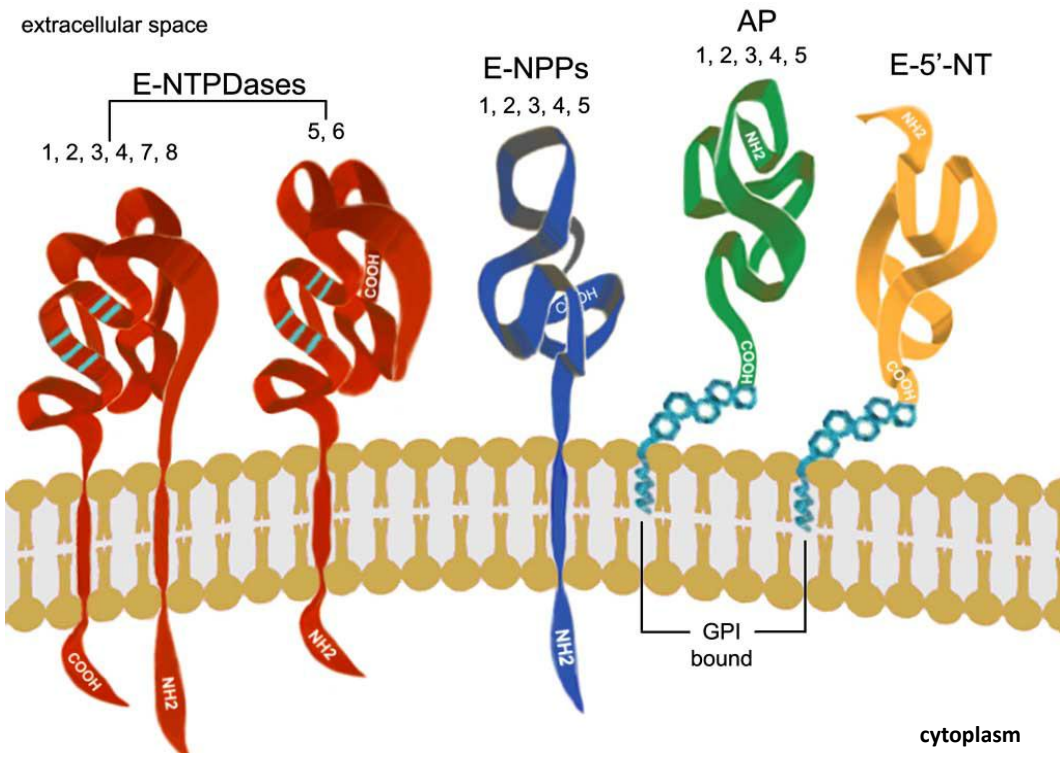

Figura 4: Enzimas que degradam nucleotídeos extracelulares. E-NPTDases e E-NPPs são enzimas integrais de membrana, enquanto que as fosfatases alcalinas (AP) e a ecto-5'-nucleotidase (5'-NT) estão ancoradas à membrana por um resíduo de glicosilfostafitidilinositol (GPI) (adaptado de Cognato e Bonan, 2010).

A adenosina, produto final da hidrólise do ATP, é um nucleosídeo abundante em todas as células. Ela participa de vias metabólicas essenciais como o metabolismo de nucleotídeos e nucleosídeos, metabolismo de aminoácidos, reações de transmetilação e metabolismo da amônia (Cunha, 2005). Esse nucleosídeo é considerado um importante neuromodulador e apresenta ações neuroprotetoras. Suas ações são exercidas através de um grupo de receptores acoplados a proteínas G, que estão divididos em quatro subtipos: A_1, A_{2A}, A_{2B} e A_3 (Fredholm et al., 2001).

Os receptores de adenosina transmitem seu sinal, principalmente, via proteínas G heterotriméricas, que podem tanto estimular (Gs) quanto inibir (Gi) a atividade da adenilato ciclase. Os receptores de alta e baixa afinidade, A_{2A} e A_{2B}, respectivamente, ativam a adenilato ciclase. Por outro lado, os receptores do tipo A_1 e A_3 são receptores de alta e baixa afinidade para adenosina, respectivamente, e ambos inibem a adenilato ciclase. Além de regular a atividade da adenilato ciclase, os subtipos de receptores de adenosina são também acoplados a distintas proteínas G, atuando em outros sistemas efetores, incluindo canais de cálcio e potássio, fosfolipase C, -D, -A2, GMP cíclico, fosfodiesterases e proteínas quinases ativadas por mitógenos, as quais podem modular diferentes funções celulares (Stone et al. 2009; Gomes et al. 2011).

2. OBJETIVOS

2.1 Objetivo geral

Considerando-se que: (i) o peixe-zebra é um modelo promissor para diversas doenças humanas, (ii) as sinalizações purinérgica e colinérgica são importantes sistemas de neurotransmissão relacionados com situações patológicas (iii) que os mecanismos pelos quais a hiperprolinemia causa alterações cerebrais são pouco compreendidos, o presente estudo teve como objetivo avaliar parâmetros comportamentais, bioquímicos e moleculares após a exposição aguda e crônica a diferentes concentrações de prolina em peixe-zebra.

2.2 Objetivos específicos

- Avaliar o efeito agudo (1 hora) e crônico (7 dias) de diferentes concentrações de prolina (1,5 mM e 3,0 mM) sobre parâmetros comportamentais em peixe-zebra, como atividade locomotora e interação social;
- Verificar a possível influência de fármacos antipsicóticos sobre as alterações comportamentais induzidas pela exposição a altas concentrações de prolina em peixe-zebra;
- Verificar o efeito *in vitro* de diferentes concentrações de prolina sobre a atividade da acetilcolinesterase em cérebro de peixe-zebra;

- Avaliar o efeito agudo (1 hora) e crônico (7 dias) de diferentes concentrações de prolina (1,5 mM e 3,0 mM) na atividade da acetilcolinesterase em cérebro de peixe-zebra;
- Verificar a possível influência de antipsicóticos sobre as alterações na atividade da acetilcolinesterase induzidas pela exposição à prolina;
- Verificar o efeito *in vitro* de diferentes concentrações de prolina sobre a atividade das E-NTPDases e ecto-5'-nucleotidase em cérebro de peixe-zebra;
- Avaliar o efeito agudo (1 hora) e crônico (7 dias) de diferentes concentrações de prolina (1,5 mM e 3,0 mM) sobre a atividade e expressão gênica das E-NTPDases e da ecto-5'-nucleotidase em cérebro de peixe-zebra.

3. RESULTADOS

Capítulo I

BEHAVIORAL CHANGES INDUCED BY LONG-TERM PROLINE EXPOSURE ARE REVERSED BY ANTIPSYCHOTICS IN ZEBRAFISH

Luiz Eduardo Baggio Savio, Fernanda Cenci Vuaden, Angelo L. Piato, Carla Denise Bonan, Angela T. S. Wyse

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Behavioral changes induced by long-term proline exposure are reversed by antipsychotics in zebrafish

Luiz Eduardo Baggio Savio [a], Fernanda Cenci Vuaden [a], Angelo L. Piato [b,c], Carla Denise Bonan [b,d], Angela T.S. Wyse [a,*]

[a] Laboratório de Neuroproteção e Doenças Metabólicas, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil
[b] Laboratório de Neuroquímica e Psicofarmacologia, Departamento Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil
[c] Programa de Pós-Graduação em Medicina e Ciências da Saúde, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6690, 90610-000, Porto Alegre, RS, Brazil
[d] Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003, Porto Alegre, RS, Brazil

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ABSTRACT

Hyperprolinemia is an inherited disorder of proline metabolism and patients affected by this disease may present neurological manifestations, including seizures and cognitive dysfunctions. Moreover, an association between adulthood schizoaffective disorders and moderate hyperprolinemia has been reported. However, the mechanisms underlying these behavioral phenotypes still remain unclear. In the present study, we investigated the effect of proline treatments on behavioral parameters in zebrafish, such as locomotor activity, anxiety, and social interaction. Adult zebrafish (*Danio rerio*) were exposed to proline (1.5 and 3.0 mM) during 1 h or 7 days (short- or long-term treatments, respectively). Short-term proline exposure did not promote significant changes on the behavioral parameters observed. Long-term exposure at 1.5 mM proline significantly increased the number of line crossing (47%), the total distance (29%), and the mean speed (33%) when compared to control group. A significant increase in the time spent in the upper portion of the test tank was also observed after this treatment (91%), which may be interpreted as an indicator of anxiolytic behavior. Proline at 1.5 mM also induced social interaction impairment (78%), when compared to the untreated group after long-term treatment. Moreover, these proline-induced behavioral changes in zebrafish were completely reversed by acute administration of an atypical antipsychotic drug (sulpiride), but not by a typical (haloperidol). These findings demonstrate that proline is able to induce schizophrenia-like symptoms in zebrafish, which reinforce the use of this species as a complementary vertebrate model for studying behavioral phenotypes associated with neurological dysfunctions characteristic of metabolic diseases.

1. Introduction

Hyperprolinemia is a metabolic disease that may be caused by two distinct inherited disorders of proline metabolism. The hyperprolinemia type I (HPI) is provoked by a deficiency of proline oxidase (POX; EC 1.5.1.2) activity. This enzyme is encoded by proline dehydrogenase (PRODH) gene located in the 22q11 chromosomal region. In the hyperprolinemia type II (HPII), the enzyme defect involves Δ^1-pyrroline-5-carboxylic acid dehydrogenase (P5CDh; EC 1.5.1.12) due to mutation in the P5CDH gene (Phang et al., 2001). Studies demonstrated that siblings affected by these diseases may present neurological manifestations, including seizures and cognitive dysfunctions (Di Rosa et al., 2008; Flynn et al., 1989; Phang et al., 2001). Moreover, it has been reported an association between proline metabolism and neuropsychiatric disorders, such as schizophrenia (Jacquet et al., 2005; Oresic et al., 2011). However, the mechanisms related to these behavioral phenotypes still remain unclear.

Evidence supports an influence of proline on excitatory neurotransmission in central nervous system (CNS). A brain-specific high-affinity proline transporter has been identified exclusively in a subset of glutamatergic neurons (Fremeau et al., 1992; Renick et al., 1999). It has also been shown that high proline concentrations activate NMDA and AMPA receptors, suggesting that proline may potentiate the glutamatergic neurotransmission (Cohen and Nadler, 1997; Nadler,

Abbreviations: HPI, Hyperprolinemia type I; POX, Proline oxidase; PRODH, Proline dehydrogenase gene; HPII, Hyperprolinemia type II; P5CDh, Δ^1-pyrroline-5-carboxylic acid dehydrogenase; P5CDH, Δ^1-pyrroline-5-carboxylic acid dehydrogenase gene; NMDA, N-Methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; UFRGS, Federal University of Rio Grande do Sul; COBEA, Brazilian Collegium of Animal Experimentation; CCAC, Canadian Council for Animal Care; DMSO, polymerase chain, dimethylsulfoxide; Pro, Proline; MK-801, dizocilpine; PCP, phencyclidine; DA, dopamine.

* Corresponding author at: Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5573; fax: +55 51 3308 5535.
E-mail address: wyse@ufrgs.br (A.T.S. Wyse).

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1987; Nadler et al., 1992). This hypothesis is supported by findings that demonstrate that higher proline and glutamate levels could be found in cerebrospinal fluid of hyperprolinemic patients (Phang et al., 2001; Van Harreveld and Fifkova, 1974). Moreover, studies also demonstrated that proline impairs memory (Bavaresco et al., 2005; Delwing et al., 2006) and decreases glutamate uptake in rat brain, as well as the Na^+, K^+-ATPase, creatine kinase, and acetylcholinesterase activities, which are crucial enzymes for normal brain function (Delwing et al., 2005, 2007; Kessler et al., 2003; Pontes et al., 1999, 2001). These reports propose that high proline levels have a detrimental effect on neuronal integrity inducing changes in different neurotransmitter systems. In this sense, the identification of an effective treatment for this disease is going to require a better understanding of the proline-induced physiological and behavioral responses (Wyse and Netto, 2011).

The zebrafish have emerged as an excellent vertebrate model for assessing neurobehavioral phenotypes associated with metabolic diseases, such as hyperprolinemia. Firstly, this species is a well-established model system used in developmental biology and genetic studies because of its known biological features (Zon and Peterson, 2005). Secondly, since zebrafish have optimal absorption and internal distribution of substances added in its tank water, this small teleost is considered also as one of the most cost-effective vertebrates that can be used for high throughput screening and toxicological studies (Kari et al., 2007; Lele and Krone, 1996; Parng et al., 2002). Thus, it can be easily and continuously exposed to different concentrations of amino acids for long periods, whereas in rats the doses administered are rapidly metabolized (Moreira et al., 1989). Furthermore, this species exhibits genetic and anatomic conservation in relation to both mice and humans and a high degree of genetic homology, which is an additional attractive feature for studying genetic basis of human neurological disorders (Barbazuk et al., 2000; Ganser and Dallman, 2009; Guo, 2004; Kabashi et al., 2010). Finally, recent studies have also examined behavioral phenotypes in zebrafish including, social behavior, locomotor activity (Fontaine et al., 2008; Seibt et al., 2010), exploratory activity (Rosemberg et al., 2011), anxiety (Egan et al., 2009), stress (Champagne et al., 2010; Piato et al., 2011), and learning and memory (Blank et al., 2009; Pather and Gerlai, 2009). The effects of neuroactive drugs on behavioral parameters in zebrafish have also been evaluated (Norton and Bally-Cuif, 2010; Seibt et al., 2010; Stewart et al., 2011).

Considering that: (i) the development of novel animal models that can simulate, at least in part, human diseases is a field of growing interest, (ii) new models can contribute to a better understanding of the relevant pathways and mechanisms to the development of clinical treatments for those diseases, (iii) the zebrafish has become a promising model to many human diseases and, finally, (iv) recent studies suggest a relationship between the proline metabolism and psychiatric diseases, we sought to investigate the effects of short- and long-term proline exposure on behavioral parameters in zebrafish, such as locomotion, anxiety, and social interaction. Since antipsychotic drugs are effective in treating neuropsychiatric symptoms, we also verified the effects of typical and atypical antipsychotic drugs on proline-induced behavioral changes in the zebrafish.

2. Materials and methods

2.1. Animals

Adult males and females (approximately in the ratio 1:1) of the "wild type" (short fin – SF) zebrafish (*Danio rerio*) strain (6–8-months-old) were obtained from a commercial supplier (Redfish, RS, Brazil). Animals were kept in 50 L housing tanks with tap water previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mgO_2/L) at 28 ± 2 °C, under a 14–10 h light/dark photoperiod in at a density of up to five animals per liter (Westerfield, 2007). Animals were acclimated for at least 2 weeks before the experiments and fed three times a day to satiety with TetraMin Tropical Flake Fish®. All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (UFRGS) under license number 19636 and followed Brazilian legislation, the guidelines of the Brazilian Collegium of Animal Experimentation (COBEA), and the Canadian Council for Animal Care (CCAC) Guide on the care and use of fish in research, teaching, and testing.

2.2. Chemicals

Proline, haloperidol, sulpiride, and dimethylsulfoxide (DMSO) were used. The antipsychotics used were from clinical grade/suppliers, while proline and DMSO were purchased from Sigma-Aldrich (St. Louis, USA). Tank water was used as the vehicle for haloperidol and tank water with 5% DMSO was used as the vehicle to sulpiride.

2.3. Experimental protocols

Animals were exposed to two proline concentrations (1.5 and 3.0 mM). For the short-term proline exposure, animals were exposed to treatments for 1 h, while the long-term proline exposure lasted 7 days. The tank water was replaced daily, and behavioral tests were performed immediately after the period of exposure.

In order to verify the effects of antipsychotics on proline-induced behavioral changes, fish were exposed to proline (1.5 mM) during 7 days (long-term exposure). Afterwards, the following acute treatments were performed in a beaker for 15 min: (i) a control group (exposed to water); (ii) a proline group; (iii) a proline group plus DMSO (5%); (iv) a proline group plus sulpiride (250 μM); and (v) a proline group plus haloperidol (9 μM).

The short-term (1 h) and long-term (7 days) proline exposures were performed as previously described in studies with rats and also based on plasma proline levels verified in human hyperprolinemic patients (Delwing et al., 2005; Phang et al., 2001). The antipsychotic concentrations and time of exposure were chosen based on previous studies with zebrafish (Seibt et al., 2010; 2011).

2.4. Behavioral assessment

2.4.1. Locomotion and anxiety

Behavioral testing of drug effects took place during the light phase between 10:00 a.m. and 1:00 p.m. Animals were individually placed in the test tank (30 cm × 15 cm × 10 cm, length × height × width, Fig. 1) immediately after the pharmacological manipulation and kept for 30 s before the video recording as previously described (Gerlai et al., 2000). There was no drug exposure during behavioral experiments.

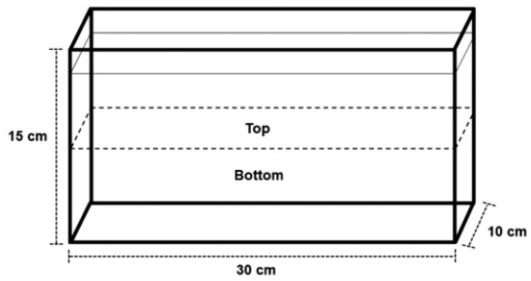

Fig. 1. The apparatus consisted in a rectangular glass tank with the specific dimensions described above and virtual divisions were used to evaluation of zebrafish swimming activity in the novel tank test, with two vertical areas (bottom and top) and eight horizontal sections with 4 sections per area.

The locomotor activity was recorded on video for 5 min after the habituation period and simultaneously analyzed using the 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: number of line crossings (vertical and horizontal lines), distance traveled and mean speed. The time spent in each tank position (bottom vs. upper levels) was considered as the index of anxiety. This task exploits the natural tendency for zebrafish to spend most of the time at the bottom when introduced into a novel environment and then gradually to 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 heightened anxiety (Levin et al., 2007). Visual observations throughout the experimental periods allow the documentation of erratic movements, defined as sharp changes in direction or velocity and repeated rapid darting behaviors (Levin et al., 2007). In addition, these movements may be manifested by bouts of vertical swimming or sideways swimming, suggesting a problem with coordination (Giacomini et al., 2006).

2.4.2. Social interaction

The zebrafish is a schooling fish that may exhibit preference for its conspecifics under certain circumstances. The rationale behind using a group of five fish as subjects is that this social setting biases behavior toward schooling. Fish were placed in groups of five in a small experimental tank (30 × 15 × 10 cm, length × height × width, Fig. 1). On one side of the test tank an empty tank was placed, and on the other side, a tank of identical size held 15 zebrafish, designed as "stimulus fish". The experimental fish were kept in the experimental tank for a 30 s period, after which their behavior was video recorded during 10 s. In order to quantify their preference between the "stimulus fish" side of their tank in detriment of the empty tank, the experimental fish tank was divided in two equal sections and the amount of time the five experimental fish spent on the side of the tank closer to that the conspecific school was measured using an event recorder program (Gerlai et al., 2000).

2.5. Statistical analysis

Results were expressed as mean ± standard error of mean (S.E.M.). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test. Statistically significant differences between groups were considered for a $p<0.05$.

3. Results

3.1. Effects of proline on behavioral parameters in zebrafish

The effects of proline were evaluated on behavioral parameters in zebrafish after short- (1 h) or long-term (7 days) treatments. Short-term proline exposure did not promote significant changes on behavioral parameters examined in this study. However, after long-term exposure, proline at 1.5 mM induced significant changes on parameters of zebrafish swimming activity. As indicated by the number of line crossings, locomotor activity increased (47%; $F(2,18)=10$; $p<0.01$) when compared with the control group (215.8 ± 10.4 line crossings) (Fig. 2A). Long-term proline exposure at 1.5 mM also increased the distance traveled (29%; $F(2,18)=6.2$; $p<0.05$) and the mean speed (33%; $F(2,18)=5.2$; $p<0.05$) in relation to control group (17.8 ± 0.9 m; 0.059 ± 0.003 m/s) (Fig. 2B and C). We also observed a significant increase in the time spent in the upper portion of the test tank (91%; $F(2,18)=7.9$; $p<0.05$) when compared with the control group (97.3 ± 22.2 s) (Fig. 2D), which may be interpreted as an indicator of anxiolytic behavior. The long-term proline exposure

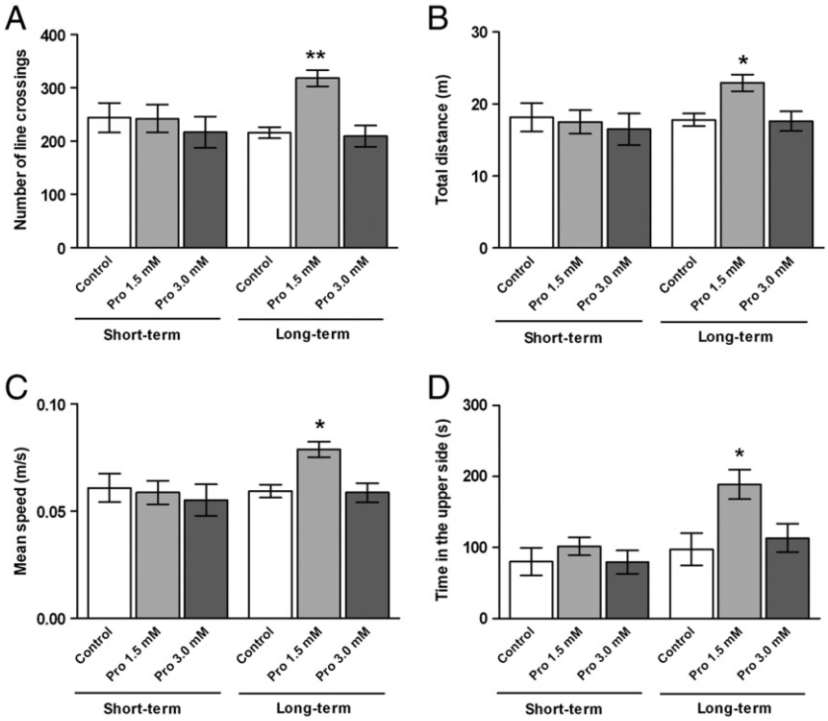

Fig. 2. Effect of short-term (1 h) and long-term (7 days) proline exposure on the number of line crossings (A), distance traveled (B), mean speed (C), and time spent in the upper zone (D) in zebrafish determined during 5 min of videorecording in the tank diving behavioral test. Fishes were exposed at two proline concentrations (1.5 and 3.0 mM) during 1 h or 7 days (short- or long-term treatments, respectively). Data were expressed as mean ± S.E.M. of at least 6 animals for each group and were analyzed by one-way ANOVA followed

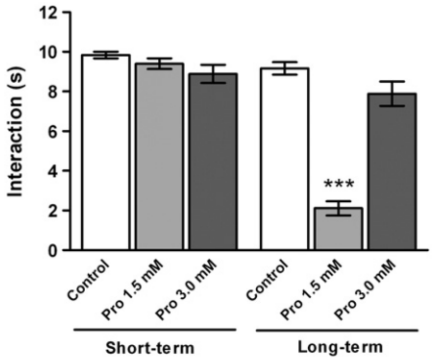

Fig. 3. Effect of short-term (1 h) and long-term (7 days) proline exposure on social behavior in zebrafish. Fishes were exposed at two proline concentrations (1.5 and 3.0 mM) during 1 h or 7 days (short- or long-term treatments, respectively). Data were expressed as mean ± S.E.M. of at least 6 animals for each group and were analyzed by one-way ANOVA followed by followed by Tukey test as post-hoc test. The asterisks represent $p<0.001$ (***).

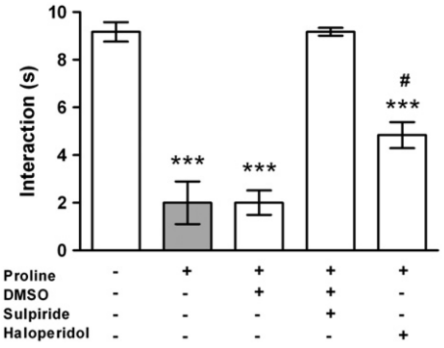

Fig. 5. Effects of haloperidol and sulpiride on proline-induced social interaction deficits in zebrafish. Fishes were exposed to proline (1.5 mM) during 7 days (long-term exposure). Afterwards, the following acute treatments were performed in a beaker for 15 min: (i) a control group (exposed to water); (ii) a proline group; (iii) a proline group plus DMSO (5%); (iv) a proline group plus sulpiride (250 μM); and (v) a proline group plus haloperidol (9 μM). Data were expressed as mean ± S.E.M. of at least 6 animals for each group and were analyzed by one-way ANOVA followed by followed by Tukey test as post-hoc test. The asterisks represent $p<0.001$ (***) compared to untreated group; and # $p<0.05$ when compared to proline group.

at 3.0 mM did not promote significant changes on parameters of zebrafish swimming activity when compared to the untreated group. Moreover, we also examined the effects of proline on social interaction after short- and long-term exposures. The results demonstrated that only long-term proline exposure at 1.5 mM induced social interaction impairment (78%; $F(2,21)=62.10$; $p<0.001$) in zebrafish when compared to the untreated group (9.2 ± 0.3 s) (Fig. 3).

3.2. Antipsychotic drugs reverse behavioral changes induced by long-term proline exposure

Since long-term proline exposure at concentration of 1.5 mM induced hyperlocomotor behavior and social interaction impairment, we also verified whether typical (haloperidol) and atypical (sulpiride) antipsychotic drugs are able to reverse these proline-induced behavioral changes. The results confirmed that long-term proline exposure significantly increased the number of line crossings ($F(4,27)=16.8$; $p<0.001$), the distance traveled ($F(4,27)=17.22$; $p<0.001$), the mean speed ($F(4,27)=13.18$; $p<0.001$), and the time spent in the upper portion ($F(4,27)=18.88$; $p<0.001$). The post-hoc test showed that only sulpiride was able to reverse these proline-induced effects compared to the untreated group ($p>0.05$) (Fig. 4A, B, C and D). Fig. 5 shows that long-term proline exposure induced social interaction impairment ($F(4,25)=41.78$; $p<0.001$), post-hoc analysis showed that sulpiride reversed the social impairment ($p>0.05$), while haloperidol was able to partially reversed this effect as compared to proline group ($p<0.05$).

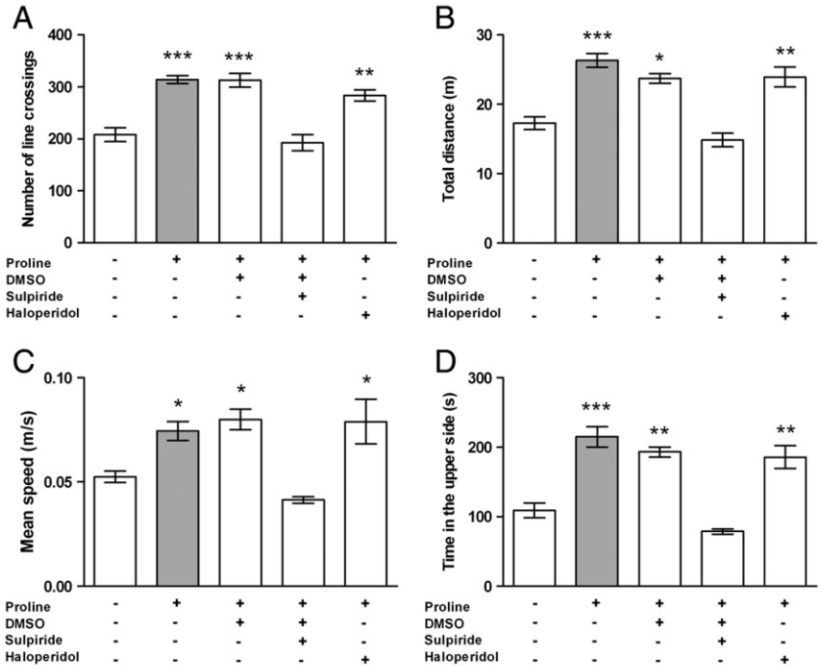

Fig. 4. Effects of haloperidol and sulpiride on proline-induced increased number of line crossings (A), distance traveled (B), mean speed (C), and time spent in the upper zone (D) in zebrafish determined during 5 min of videorecording in the tank diving behavioral test. Fishes were exposed to proline (1.5 mM) during 7 days (long-term exposure). Afterwards, the following acute treatments were performed in a beaker for 15 min: (i) a control group (exposed to water); (ii) a proline group; (iii) a proline group plus DMSO (5%); (iv) a proline group plus sulpiride (250 μM); and (v) a proline group plus haloperidol (9 μM). Data were expressed as mean ± S.E.M. of at least 6 animals for each group and were analyzed by one-way ANOVA followed by followed by Tukey test as post-hoc test. The asterisks represent $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

4. Discussion

Hyperprolinemic patients usually present neurological manifestations and the proline metabolism seems to be specifically related to psychotic disorders, such as schizophrenia (Jacquet et al., 2005; Oresic et al., 2011; Phang et al., 2001). Although the mechanisms that lead to abnormal brain function in these patients remain unclear, there is evidence that at least part of the pathology and symptomatology of hyperprolinemia results from a dysfunction of the glutamatergic neurotransmission (Vorstman et al., 2009). Nevertheless, there is no effective treatment for this disease and few studies have been conducted to identify potential therapeutic mechanisms to minimize the impact of the symptoms on patient's quality of life (Mitsubuchi et al., 2008; Wyse and Netto, 2011). Therefore, in this study, we characterized the effects of proline exposure on behavioral parameters in zebrafish, a promising vertebrate model for studying the mechanisms underlying human diseases and pharmacological treatments. Furthermore, we demonstrated that proline-induced behavioral changes were completely reversed by acute administration of an atypical antipsychotic drug (sulpiride), but not by a typical (haloperidol).

The influence of proline on glutamatergic system has become more evident over the last few years. Several studies showed that high proline concentrations activate NMDA and AMPA receptors, suggesting that proline may potentiate the glutamatergic neurotransmission and increase glutamate release (Cohen and Nadler, 1997; Nadler, 1987; Nadler et al., 1992). Interestingly, NMDA receptor antagonists, such as dizocilpine (MK-801) and phencyclidine (PCP), psychomimetic drugs used as pharmacological model of schizophrenia, also increase the glutamate release and this glutamatergic dysfunction may lead to secondary dopamine (DA) release in the prefrontal cortex (Moghaddam, 2002; Moghaddam and Adams, 1998). A putative modulatory effect of proline on glutamatergic transmission inducing DA release in the brain has been proposed (Paterlini et al., 2005; Vorstman et al., 2009). In agreement with this hypothesis, we showed that long-term proline exposure at concentration of 1.5 mM induces hyperlocomotion and social interaction impairment (schizophrenia-like symptoms) in zebrafish. Likewise, studies demonstrated that MK-801 (a NMDA receptor antagonist) induced hyperlocomotion and social deficits in zebrafish (Seibt et al., 2010, 2011).

We also demonstrated that proline at 3.0 mM was not able to induce these schizophrenia-like symptoms. Authors have reported that increased levels of DA result in a dose dependent response, which resembles an inverted U curve (Lavergne and Jay, 2010; Vijayraghavan et al., 2007). The DA inverted U dose–response curve of NMDA receptor effects seems to provide a regulatory mechanism that may be protective for the neuron, preventing toxic responses (Lavergne and Jay, 2010; Skolnick et al., 2009). Therefore, the potential effect of proline at 3.0 mM on the glutamatergic system could induce a higher dopamine release at non-responsive levels. As a result, we did not observe behavioral changes at this proline concentration.

Antipsychotic drugs are widely used for the treatment of neuropsychiatric disorders, including schizophrenia. Previous studies showed that antipsychotic drugs are able to reverse the MK-801-induced hyperlocomotion and social interaction deficits in zebrafish. In addition, the authors demonstrated that these drugs per se did not alter these behavioral parameters (Seibt et al., 2010, 2011). As we already reported, we showed in our experiment that an atypical antipsychotic drug completely reversed the proline-induced hyperlocomotion and social deficits while a typical antipsychotic was only able to attenuate the social impairment.

Several mechanisms may be involved in the inhibitory effect of antipsychotic drugs on proline-induced hyperlocomotion and social interaction deficits. Typical antipsychotics, such as haloperidol, act preferentially via dopamine D_2 receptor blockade and induce severe motor side effects (Heusler et al., 2008). Atypical antipsychotics, while less potent than their typical counterparts in blocking central D_2 receptors, have affinity for a wide range of other receptors including dopaminergic D_1 and D_4, serotonergic 5-HT2A and 5-HT6, adrenergic α1, histaminergic H1, and muscarinic M1 (Jones et al., 2008). Sulpiride, an atypical antipsychotic, acts preferentially via D_2 and D_3 dopamine receptor blockade (Jaworski et al., 2001; Tadori et al., 2011). In agreement to our data, studies have shown that atypical antipsychotics are more potent than typical antipsychotic drugs in inhibiting the locomotor activity and social impairment induced by NMDA receptor antagonists (Geyer et al., 2001; Jentsch and Roth, 1999; Seibt et al., 2010, 2011). Boulay et al. (2004) reported that haloperidol did not reverse acute NMDA antagonist-induced deficits in social investigation. On the other hand, Linck et al. (2008) showed that sulpiride completely prevented the MK-801-induced social deficits. Therefore, it is possible that sulpiride completely reverses the proline-induced hyperlocomotion and social deficit through a similar mechanism.

At last, a proline-induced anxiolytic-like effect was also observed in our study after long-term exposure at 1.5 mM. This data is in agreement with a previous study in zebrafish, in which MK-801 induced anxiolytic-like behavior (Seibt et al., 2010). Other studies conducted in mammalian models also reported that animals treated with MK-801 and submitted to the elevated plus-maze test presented an increase in time spent in the open arms, indicative of an anxiolytic-like effect (Bertoglio and Carobrez, 2003; Dunn et al., 1989). Seibt et al. (2010) also reported that antipsychotic drugs failed to reverse the anxiolytic-like effect of MK-801 in zebrafish. However, we showed that the proline-induced anxiolytic-like effect was reversed only by sulpiride, as we also verified in the other behavioral parameters analyzed in this study.

5. Conclusion

In summary, our findings demonstrated that long-term proline exposure induced schizophrenia-like behaviors in zebrafish, suggesting an influence of this amino acid on glutamatergic and dopaminergic systems. Moreover, these behavioral changes were completely reversed by acute administration of an atypical antipsychotic drug, but not by a typical antipsychotic. These data may contribute to a better understanding of the pathophysiological mechanisms that increase the susceptibility to psychotic disease in hyperprolinemic patients. In addition, such findings might facilitate the use of zebrafish as a complementary vertebrate model for studying inborn errors of metabolism and pharmacological treatments as well as for assessing behavioral phenotypes associated with these diseases.

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Capítulo II

LONG-TERM PROLINE EXPOSURE ALTERS ACETYLCHOLINESTERASE ACTIVITY IN ZEBRAFISH BRAIN: REVERSAL BY ANTIPSYCHOTIC DRUGS

Luiz Eduardo Baggio Savio, Fernanda C. Vuaden, Denis B. Rosemberg, Maurício Reis Bogo, Carla Denise Bonan, Angela T. S. Wyse

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Long-term proline exposure alters acetylcholinesterase activity in zebrafish brain: reversal by antipsychotic drugs

Luiz Eduardo Baggio Savio [a,b], Fernanda Cenci Vuaden [a,b], Denis B. Rosemberg [a], Maurício R. Bogo [d], Carla D. Bonan [c,e], Angela T. S. Wyse [a,b*]

[a] Programa de Pós-Graduação em Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil.

[b] Laboratório de Neuroproteção e Doenças Metabólicas, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil.

[c] Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil.

[d] Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil;

[e] Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003, Porto Alegre, RS, Brazil

*Corresponding Author: Angela T. S. Wyse, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil.

Phone: +55 51 3308 5573; Fax: +55 51 3308 5535

E-mail: wyse@ufrgs.br

Abstract

Hyperprolinemia is an inherited disorder of proline metabolism and hyperprolinemic patients can present neurological manifestations, such as seizures and cognitive dysfunctions. However, the mechanisms related to these symptoms are still unclear. In the present study, we evaluated the *in vivo* and *in vitro* effects of proline on acetylcholinesterase (AChE) activity in zebrafish brain. For the *in vivo* studies, animals were exposed at two proline concentrations (1.5 and 3.0 mM) during 1 hour or 7 days (short- or long-term treatments, respectively). For the *in vitro* assays, different proline concentrations (ranging from 3.0 μM to 1000 μM) were tested. Long-term proline exposures significantly increased AChE activity for both treated groups when compared to the control group (34% and 39%). Moreover, the proline-induced increase in AChE activity was completely reversed by acute administration of antipsychotic drugs. When assessed *in vitro*, proline did not promote significant changes on AChE activity. Altogether, these data indicate that the enzyme responsible for the control of acetylcholine levels might be altered after proline exposure in adult zebrafish. These findings contribute for the better understanding of the pathophysiology of hyperprolinemia and might contribute for the use of zebrafish as a complementary vertebrate model for studying inborn errors of amino acid metabolism.

Keywords: zebrafish; acetylcholine; acetylcholinesterase; proline; inherited diseases; hyperprolinemia.

Introduction

Hyperprolinemia can be caused by two distinct inherited disorders of proline metabolism. Hyperprolinemia type I (HPI) occurs due to the deficiency of proline oxidase (POX; EC 1.5.1.2). The other type of hyperprolinemia, type II (HPII), is caused by deficiency of Δ^1-pyrroline-5-carboxylic acid dehydrogenase (P5CDh; EC 1.5.1.12) activity. These enzymatic defects cause proline accumulation in blood and others tissues, such as brain (Phang et al. 2001). As a result, some hyperprolinemic patients can present epilepsy and cognitive dysfunctions whereas others are asymptomatic (Flynn et al. 1989; Phang et al. 2001; Di Rosa et al. 2008). Although proline metabolism seems to be specifically related to psychotic disorders, such as schizophrenia (Phang et al. 2001; Jacquet et al 2005; Oresic et al. 2011), the mechanisms underlying these neurological manifestations still remain poorly understood.

Several reports propose that high proline levels have a detrimental effect on neuronal integrity, inducing changes in different neurotransmitter systems. Studies showed that proline may activate NMDA and AMPA receptors, suggesting that it potentiates the glutamatergic neurotransmission (Nadler 1987; Nadler et al. 1992; Cohen and Nadler 1997). Moreover, it has been demonstrated that high proline levels decrease glutamate uptake in rat brain, as well as the Na^+, K^+-ATPase and creatine kinase activities, which are crucial enzymes for normal brain function (Pontes et al. 1999; 2001; Kessler et al. 2003; Delwing et al. 2007). Proline also impairs memory (Bavaresco et

al. 2005; Delwing et al. 2006) and alters the acetylcholinesterase activity in rat brain (Delwing et al. 2005; Ferreira et al. 2011).

It is currently accepted that the cholinergic neurotransmission plays an important role in the CNS by regulating many biological processes, such as learning, memory, sensory perception, and cortical organization of movement (Mesulam et al. 2002; Sarter and Bruno 2004). At synaptic cleft, acetylcholine triggers muscarinic (metabotropic) and nicotinic (ionotropic) acetylcholine receptors. The inactivation of cholinergic signaling is promoted by the cholinesterases, which cleave acetylcholine into choline and acetate. Two different types of cholinesterases hydrolyze acetylcholine: acetylcholinesterase (AChE) (E.C.3.1.1.7) and butyrylcholinesterase (BuChE) (E.C.3.1.1.8) (Soreq and Seidman 2001).

Zebrafish (*Danio rerio*) has been used as an emergent organism for neurobehavioral studies (Guo 2004; Egan et al. 2009; Blaser et al. 2010). This species has several features that complement the existing mammalian models, such as low maintenance, translucent embryos, rapid development, and high fecundity (Gerlai et al. 2006; Igham 2009). Since zebrafish has also optimal absorption and internal distribution of substances mixed in its tank water, this small vertebrate has been used for drug screening and toxicological assays (Parng et al. 2002; Kari et al. 2007). In this sense, it can be easily and continuously exposed to different concentrations of amino acids for long periods. Contrastingly, in rats, the doses administered via intraperitoneal injections are rapidly metabolized (Moreira et al 1989). Furthermore, zebrafish genes present a high degree of conservation sharing a 70–80% homology with human genes, which is an additional attractive

feature to study genetic and biochemical mechanisms of neurological diseases (Barbazuk et al. 2000; Dooley and Zon 2000; Best and Alderton 2008). Parameters of cholinergic signaling have already been characterized in zebrafish brain (Clemente et al. 2004; Rico et al. 2006). It has been shown that acetylcholinesterase is encoded by a single gene, while butyrylcholinesterase was not detected in zebrafish genome (Clemente et al. 2004; Ninkovic et al. 2006). Thus, the effects of high amino acids concentrations on the gene expression and neurochemical changes can be evaluated in this species, as well as several parameters of neurotoxicity during development, including teratogenicity, cell death, and selected neuronal subtypes (Ton et al. 2006; Parng et al. 2007; David and Pancharatna 2009; Long et al. 2011; Pan et al. 2011). Previous study from our group has already characterized the effects of proline exposure on behavioral parameters in zebrafish (Savio et al. 2012). It was demonstrated that proline-induced behavioral changes were reversed by acute administration antipsychotic drugs in this species. However, there is no evidence regarding the neurochemical mechanisms that may contribute to these behavioral responses.

Considering that: (i) the hyperprolinemic patients can present neurological dysfunctions, (ii) the cholinergic system is associated with several neurological disorders, (iii) recent studies suggest an influence of proline on cholinergic neurotransmission, and, finally, (iv) zebrafish has become a prominent vertebrate to study neurological disorders related to human inherited diseases, here, we sought to investigate the effects of short- and long-term proline exposure on AChE activity in zebrafish brain.

Furthermore, we also verified the effects of typical and atypical antipsychotic drugs on proline-induced changes in AChE activity.

Materials and methods

Animals

Adult (6-8-months-old) males and females (approximately in the ratio of 1:1) wild type (short fin - SF) zebrafish (*Danio rerio*) were obtained from a commercial supplier (Redfish, RS, Brazil). Animals were kept in 50L housing tanks with tap water previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mgO_2/L) at 28 ± 2ºC, under a 14-10 h light/dark photoperiod. Fish were kept at a density of up to five animals per liter (Westerfield 2007). Animals were acclimated for at least 2 weeks before the experiments and fed three times a day to satiety with TetraMin Tropical Flake Fish®. All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (UFRGS) under license number 19636 and followed Brazilian legislation, the guidelines of the Brazilian Collegium of Animal Experimentation (COBEA), and the Canadian Council for Animal Care (CCAC) Guide on the care and use of fish in research, teaching, and testing.

Chemicals

L-Proline, Trizma Base, ethylenedioxy–diethylene–dinitrilo–tetraacetic acid (EDTA), ethylene glycol bis(betaaminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), sodium citrate, Coomassie Blue, bovine serum albumin, acetylthiocholine, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All reagents used were of analytical grade.

In vivo treatments

For the *in vivo* studies, animals were exposed to proline (1.5 and 3.0 mM). For the short-term proline exposure, animals were treated for 1 h, while the long-term proline exposure lasted 7 days. To ensure a similar amount of amino acid present in the aquarium, the tank water was replaced daily (Savio et al., 2012). Immediately after the treatments, fish were cryoanaesthetized and further euthanized by decapitation. Whole brains were dissected and the homogenates were prepared.

In order to verify the effects of antipsychotics on proline-induced effects on AChE activity, fish were exposed to proline (1.5 mM and 3.0 mM) during 7 days (long-term exposure) or water (control group). Afterwards, the following acute treatments were performed in a beaker for 15 min: (i) a control group plus water; (ii) a control group plus DMSO (5%); (iii) a control group plus haloperidol (9 µM); (iv) a control group plus sulpiride (250 µM); (v) a proline group (1.5 mM) plus water; (vi) a proline group (3.0 mM) plus water; (vii) a proline group (1.5 mM) plus haloperidol (9 µM); (viii) a proline group (3.0 mM)

plus haloperidol (9 µM); (ix) a proline (1.5 mM) plus sulpiride (250 µM); and (x) a proline (3.0 mM) plus sulpiride (250 µM).

The short-term (1 h) and long-term (7 days) proline concentrations, as well as the antipsychotic concentrations were determined based on previously used concentrations for zebrafish and mammalian models (Delwing et al. 2007; Savio et al. 2012).

In vitro treatments

For the *in vitro* assays, proline (final concentrations of 3, 30, 500, and 1000 µM) were added directly to the reaction medium (described below), preincubated with the brain samples and maintained throughout the enzyme assay. For the control group, the experiments were performed in the absence of proline (no drug added in the reaction medium). The proline concentrations were chosen based on cerebrospinal fluid reported in hyperprolinemic patients (Phang et al. 2001).

Determination of AChE activity

A pool of three whole zebrafish brains was used to prepare each homogenate fraction. The brains were gently homogenized on ice in 60 volumes (v/w) of Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) using a Potter–Elvehjen-type glass homogenizer. AChE activity was measured according to Ellman and colleagues (1961). AChE activity in the homogenate was measured by determining the rate of

hydrolysis of acetylthiocholine iodide (0.88 mM) in 300 µL, with 33 µL of 100 mM phosphate buffer, pH 7.5 mixed to 33 µL of 2.0 mM 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB). Briefly, samples containing 5 µg protein and the reaction medium mentioned above were preincubated for 10 min at 25 °C. The hydrolysis of acetylthiocholine iodide was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. Importantly, the linearity of absorbance towards time and protein concentration was previously determined. All reactions were performed in quadruplicate. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein (µmol thiocholine. h^{-1}. mg $protein^{-1}$).

Protein Determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard (Bradford, 1976).

Statistical analysis

Results are expressed as means ± standard error of mean (S.E.M). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test. Statistically significant differences between groups were considered for a $p< 0.05$.

Results

The *in vivo* effect of 1.5 and 3.0 mM proline was evaluated on AChE activity of zebrafish brain. After the short-term proline exposure (1 h), we did not observe changes in AChE activity [$F(2,15)=1.297$; $p > 0.05$] (Fig. 1). However, after long-term exposure (7 days), proline significantly increased AChE activity (34% and 39%) in both treated groups when compared to control [$F(9,40)=14.64$; $p < 0.001$] (Fig. 2).

Since long-term proline exposures increased AChE activity, we also verified whether typical (haloperidol) and atypical (sulpiride) antipsychotic drugs were able to reverse this effect. Our data showed that only sulpiride reversed the effect promoted by the two proline concentrations on AChE activity as compared to the untreated group ($p > 0.05$). On the other hand, haloperidol reversed the increase in AChE activity only at 3.0 mM proline ($p > 0.05$).

Furthermore, in order to evaluate whether proline could act directly on AChE activity, we tested the *in vitro* effect of different proline concentrations (ranging from 3.0 µM to 1000 µM). The results showed no significant changes on AChE activity [$F(4,15)=1.414$; $p > 0.05$] when compared to the control group (Fig. 3).

Discussion

The present report showed, for the first time, that long-term, but not short-term proline exposure increased AChE activity in zebrafish brain. We also demonstrated that the proline-induced increase AChE activity was reversed by acute administration of antipsychotic drugs. When proline was added directly to the reaction medium, it did not promote significant changes on AChE activity, suggesting that it may act indirectly in zebrafish brain, through distinct cell signaling pathways.

Although the mechanisms which lead to abnormal brain function in hyperprolinemic patients still remain poorly understood, studies have demonstrated that hyperprolinemia induces neurochemical and behavioral changes mainly affecting the glutamatergic neurotransmission (Vorstman et al. 2009; Wyse and Netto 2011). Authors have reported that high proline concentrations activate NMDA and AMPA receptors, suggesting that proline may potentiate the glutamatergic neurotransmission, consequently, increasing glutamate release (Nadler, 1987; Nadler et al., 1992; Cohen and Nadler, 1997). The increased glutamate levels induced by proline seem to lead to secondary dopamine (DA) release, inducing schizophrenia-like symptoms in animal models. Therefore, high proline concentrations appear to mimic the neurobehavioral effects induced by NMDA receptor antagonists, such as dizocilpine (MK-801) and phencyclidine (PCP) (Paterlini et al. 2005; Vorstman et al., 2009, Savio et al. 2012). Interestingly, studies have demonstrated that the blockade of NMDA receptors increases the extracellular dopamine and acetylcholine concentrations in the brain as well as motor activity (Del Arco and Mora, 2005; Del Arco et al., 2008). Considering the effects of proline on ionotropic glutamate receptors, is

possible that high concentrations of this amino acid increase the acetylcholine release, inducing behavioral changes, such as the hyperlocomotion (Savio et al. 2012). In accordance to this hypothesis, we showed a significant increase on AChE activity in zebrafish brain after long-term proline exposure (at 1.5 and 3.0 mM). Our data are in agreement with a previous study in rats, which reported that chronic proline administration significantly increased hippocampal AChE activity (Ferreira et al. 2011). Therefore, these results could be related to a compensatory response decreasing the acetylcholine levels in the synaptic cleft in order to minimize the effects of increased levels of this neurotransmitter on normal brain function.

Antipsychotic drugs are widely used for the treatment of neuropsychiatric disorders. In a previous study we showed that an atypical antipsychotic drug (sulpiride) completely reversed the proline-induced hyperlocomotion and social deficits while a typical antipsychotic (haloperidol) has only attenuated the social interaction impairment (Savio et al, 2011). Here we demonstrated that the proline-induced increase in AChE activity was completely reversed by sulpiride. However, haloperidol kept the AChE activity at control levels only at 3.0 mM proline. These data are in agreement with our previous study, which showed that haloperidol failed to reverse the hyperlocomotion induced by long-term proline exposure (at 1.5 mM) in zebrafish (Savio et al., 2012).

Several neuronal pathways could be involved in the in the effects of antipsychotic drugs on proline-induced increase in AChE activity. Ichikawa and colleagues (2002) reported that olanzapine, rispiridone, and ziprasidone

increased acetylcholine release in rat medial prefrontal cortex, whereas haloperidol and sulpiride were unable to induce such effect. Moreover, it has been shown that the administration of D_1 and D_2 antagonists reduced the motor effects induced by the blockade of NMDA receptors in the prefrontal cortex (Del Arco et al 2008). Thus, considering that the haloperidol acts via dopamine D_2 receptor blockade (Heusler et al., 2008) and sulpiride acts preferentially via D_2 and D_3 dopamine receptor blockade (Jaworski et al., 2001; Tadori et al., 2011) it is possible that these drugs reverse the proline-induced enhancement on AChE activity by a similar mechanism, reducing the acetylcholine availability. However, it is also important to emphasize that atypical antipsychotics have affinity for a wide range of other receptors, such as serotonergic 5-HT_{2A} and 5-HT_6, adrenergic α1, histaminergic H1, and muscarinic M1 (Jones et al., 2008). Thus, the involvement of these mechanisms in the antipsychotics and proline-induced effects in zebrafish brain cannot be ruled out and further studies are still required to elucidate the contribution of dopaminergic and serotoninergic systems.

In conclusion, our findings demonstrate that long-term proline exposure alters AChE activity in zebrafish brain. Furthermore, the proline-induced increase in AChE activity was completely reversed by acute administration of antipsychotic drugs. These findings might facilitate the use of zebrafish for studying metabolic diseases and contribute for better understanding the mechanisms underlying cognitive and psychiatry dysfunctions observed in hyperprolinemic patients.

Conflict of Interest

The authors declare that no conflict of interest exists.

Acknowledgments

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Legend to Figures:

Figure 1. Effect of short-term proline exposure (1.5 and 3.0 mM) on AChE activity in zebrafish brain. Data are expressed as mean ± S.E.M of five independent experiments performed in quadruplicate and were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test.

Figure 2. Effects of haloperidol and sulpiride on proline-induced increase in AChE activity. Fish were exposed to proline (1.5 and 3.0 mM) during 7 days (long-term exposure). Afterwards, acute treatments with antipsychotic drugs (haloperidol - 9 µM and sulpiride - 250 µM) were performed in a beaker for 15 min. Data are expressed as mean ± S.E.M of five independent experiments performed in quadruplicate and were analyzed by one-way ANOVA followed by followed by Tukey test as post-hoc test. The asterisks represent $p < 0.001$(*) compared to untreated group; and # $p < 0.05$ when compared to proline groups.

Figure 3. *In vitro* effect of proline on AChE activity from zebrafish brain. Data are expressed as mean ± S.E.M for four independent experiments

performed in quadruplicate. Data were analyzed statistically by one-way ANOVA.

Figure 1

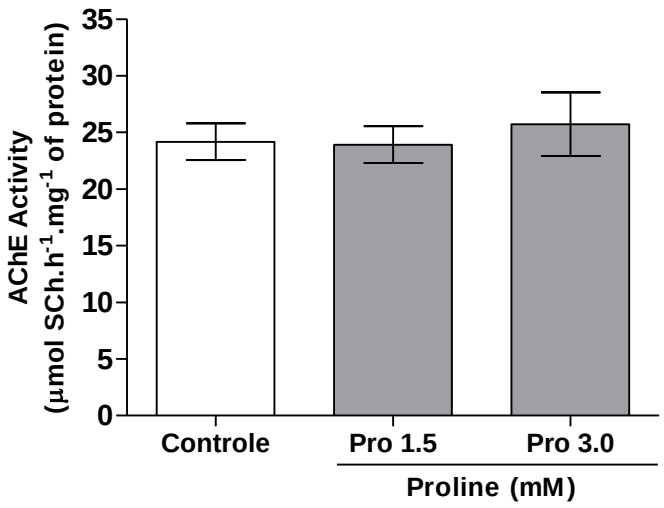
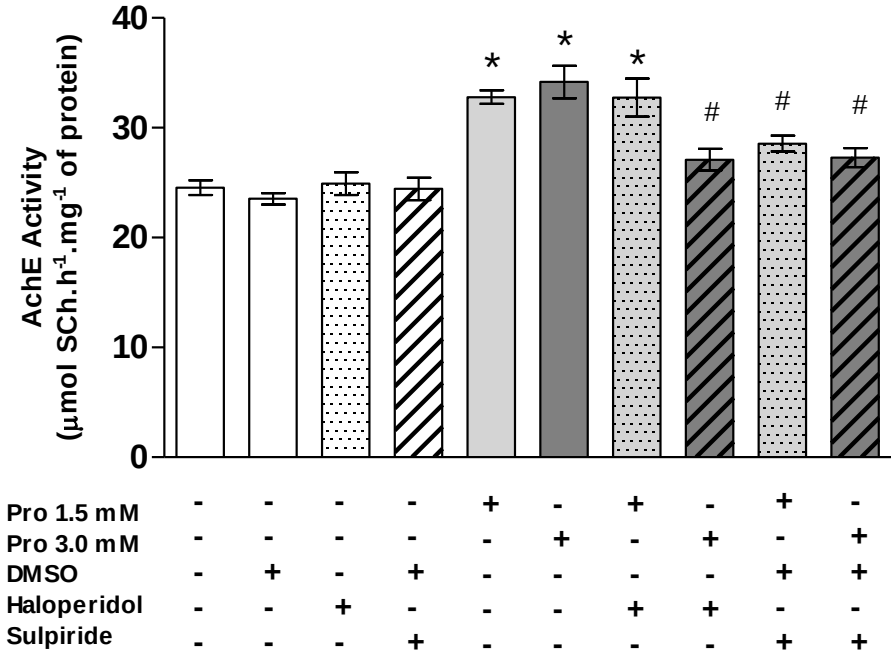


Figure 2


AchE Activity
(µmol SCh.h-1.mg-1 of protein)
40
30
20
10
0
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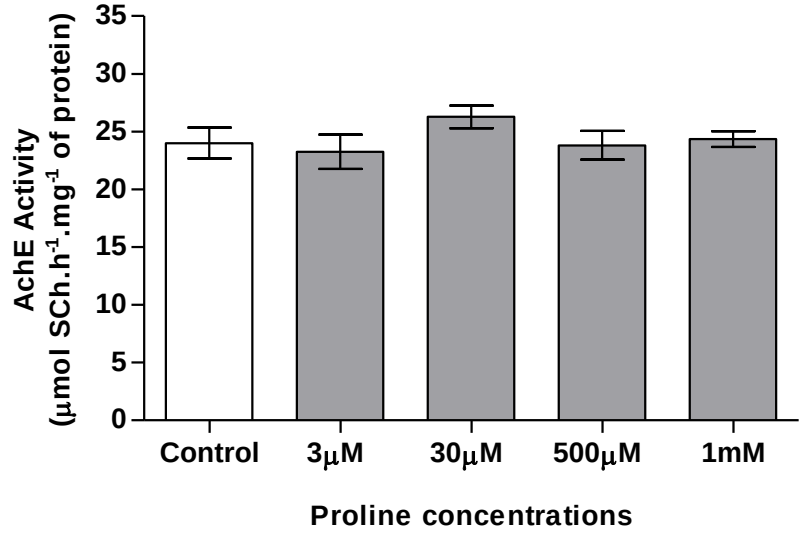
Pro 1.5 mM - - - - + - + - + -
Pro 3.0 mM - - - - - + - + - +
DMSO - + - + - - - - + +
Haloperidol - - + - - - + + - -
Sulpiride - - - + - - - - + +

Figure 3


AchE Activity
(µmol SCh.h-1.mg-1 of protein)
35
30
25
20
15
10
5
0
Control
3µM
30µM
500µM
1mM
Proline concentrations

Capítulo III

LONG-TERM PROLINE EXPOSURE ALTERS NUCLEOTIDE CATABOLISM AND ECTONUCLEOTIDASE GENE EXPRESSION IN ZEBRAFISH BRAIN

Luiz Eduardo Baggio Savio, Fernanda C. Vuaden, Denis B. Rosemberg, Maurício Reis Bogo, Carla Denise Bonan, Angela T. S. Wyse

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Long-term proline exposure alters nucleotide catabolism and ectonucleotidase gene expression in zebrafish brain

Luiz Eduardo Baggio Savio [a,b], Fernanda Cenci Vuaden [a,b], Denis B. Rosemberg [a], Maurício R. Bogo [d], Carla D. Bonan [c,e], Angela T. S. Wyse [a,b*]

[a] Programa de Pós-Graduação em Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil.

[b] Laboratório de Neuroproteção e Doenças Metabólicas, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil.

[c] Laboratório de Neuroquímica e Psicofarmacologia, Departamento deBiologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil.

[d] Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil;

[e] Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003, Porto Alegre, RS, Brazil

*Corresponding Author: Angela T. S. Wyse, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil.

Phone: +55 51 3308 5573; Fax: +55 51 3308 5535

E-mail: wyse@ufrgs.br

Abstract

Hyperprolinemia is an inherited disorder of proline metabolism and hyperprolinemic patients can present neurological manifestations, such as seizures cognitive dysfunctions, and psychotic disorders. However, the underlying mechanisms of these symptoms are still unclear. Since adenine nucleotides play crucial roles in neurotransmission and neuromodulation, we evaluated the *in vivo* and *in vitro* effects of proline on ectonucleotidase activities and gene expression in zebrafish brain. For *the in vivo* studies, animals were exposed at two proline concentrations (1.5 and 3.0 mM) during 1 hour or 7 days (short- or long-term treatments, respectively). For the *in vitro* assays, different proline concentrations (ranging from 3.0 to 1000 μM) were tested. Short-term proline exposure did not promote significant changes on the ectonucleotidase activities and gene expression. Long-term proline exposure significantly increased ATP catabolism in both concentrations tested (14% and 22%, respectively), whereas ADP and AMP hydrolysis were increased only at 3.0 mM proline (21% and 17%, respectively) when compared to control. Moreover, the relative gene expression of *enpd3* increased in both treated groups after long-term proline, whereas *enptd1* increased only at 3.0 mM proline. Proline *in vitro* did not promote significant changes on ectonucleotidase activities. Altogether, these data indicate that the enzymes responsible for the control of extracellular nucleotides levels might be altered after proline exposure in zebrafish, contributing to better understand the pathophysiology of this disease. Moreover, such findings might facilitate the use of the zebrafish as a complementary vertebrate model for studying inborn errors of amino acid metabolism.

keywords: zebrafish; adenosine; ATP; proline; inherited diseases; hyperprolinemia.

Introduction

Hyperprolinemia may be caused by two distinct inherited disorders of proline metabolism. Hyperprolinemia type I (HPI) occurs by a deficiency in proline oxidase (POX; EC 1.5.1.2), a mitochondrial enzyme, which catalyzes the first step of the proline degradation pathway, while the Hyperprolinemia type II (HPII) is caused by an absence of Δ^1-pyrroline-5-carboxylic acid dehydrogenase (P5CDh; EC 1.5.1.12) activity. These enzyme defects cause proline accumulation in the blood (>500 μM) and in others tissues, such as brain (Phang et al. 2001). Studies have showed that some hyperprolinemic patients can present epilepsy and cognitive dysfunctions, whereas others are asymptomatic (Flynn et al. 1989; Phang et al. 2001; Di Rosa et al. 2008). Moreover, increased proline levels seem to be specifically related to psychotic disorders (Phang et al. 2001; Jacquet et al 2005; Oresic et al. 2011). Nevertheless, the mechanisms involved in these neurological symptoms still remain unclear.

A role for the proline in excitatory neurotransmission has become more accepted over the last few years. Studies showed that high proline concentrations activate NMDA and AMPA receptors, suggesting that this amino acid may potentiate the glutamatergic neurotransmission (Nadler 1987; Nadler et al. 1992; Cohen and Nadler 1997). Moreover, it has been demonstrated that hyperprolinemia provokes memory deficit (Bavaresco et al. 2005; Delwing et al. 2006) and decreases glutamate uptake in rat brain, as well as the Na^+, K^+-ATPase and creatine kinase activities, which are

important enzymes for normal brain function (Pontes et al. 1999; 2001; Kessler et al. 2003; Delwing et al. 2007a). Proline also impairs energy metabolism (Ferreira et al. 2010), inhibits ATP breakdown (Delwing et al. 2007b), and alters acetylcholinesterase activity in cerebral cortex of rats (Delwing et al. 2003; 2005). Therefore, high proline levels seem to be neurotoxic or even predispose to brain damage, inducing changes in different neurotransmitter systems (Wyse and Netto 2011).

The purinergic signaling is involved in several pathological conditions, such as seizures, ischemia, and neurodegenerative and neuropsychiatric diseases. Besides, extracellular nucleotides and nucleosides play important role in the central nervous system (Burnstock 2008; Abbracchio et al. 2009; Cognato et al. 2011). ATP is a well-known co-transmitter, which acts as a neurotransmitter and/or a neuromodulator via ionotropic (P2X) or metabotropic (P2Y) receptors (Ralevic and Burnstock 1998, Burnstock 2004). The levels of ATP and other extracellular nucleotides are controlled by the action of cell surface-locates enzymes named ectonucleotidases, such as the ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), and the ecto-5'-nucleotidase (Zimmermann 2006, Yegutkin 2008). E-NTPDases hydrolyze extracellular tri- and diphosphonucleosides to monophosphonucleosides and the ecto-5'-nucleotidase is the enzyme responsible for AMP hydrolysis, generating adenosine, the final product of ATP breakdown (Zimmermann 1996; 2001). Adenosine is an important neuromodulator operating G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3), which can inhibit (A_1 and A_3) or facilitate (A_{2A} and A_{2B}) neuronal communication. Moreover, this nucleoside has been described as an

endogenous neuroprotective agent (Fredholm 2001, Cunha 2005, Stone et al. 2007).

The teleost *Danio rerio*, popularly known as zebrafish, is a promising vertebrate model for studying the mechanisms underlying human neurological diseases and clinical treatments (Guo 2004; Ganser and Dallman 2009, Kabashi et al. 2010). This species possesses numerous advantages as a model organism, such as low maintenance, translucent embryos, rapid development, and high fecundity as compared to mammalian models (Gerlai et al. 2006; Igham 2009). Zebrafish genes present a high degree of conservation, sharing a 70–80% homology with human genes, which is an additional attractive feature to study genetic and biochemical mechanisms of neurological diseases (Barbazuk et al. 2000; Dooley and Zon 2000; Best and Alderton 2008). In zebrafish brain, parameters related to puringergic signaling have already been characterized, including the nucleotide hydrolysis and the E-NTPDase family gene expression (Kucenas et al 2003; Rico et al. 2003, Senger et al. 2004, Boehmler et al. 2009, Rosemberg et al. 2010). Furthermore, reports have also described the use of zebrafish to drug screening and toxicological assays, because it has optimal absorption and internal distribution of substances mixed to the tank water (Parng et al. 2002; Kari et al. 2007). Therefore, considering chemical manipulations, it can be easily and continuously exposed to different concentrations of amino acids for long periods, while in rats the doses administered are rapidly metabolized (Moreira et al 1989). In this context, we have already demonstrated that long-term proline exposure induces behavioral changes in zebrafish (Savio et al. 2012). However, there is no

evidence regarding the mechanisms underlying these proline-induced behaviors.

Since adenine nucleotides play crucial roles in neurotransmission and neuromodulation, the aim of the current study was to investigate the effects of short- and long-term proline exposure on nucleotide catabolism promoted by ectonucleotidases in zebrafish brain, as well as to investigate the gene expression pattern of E-NTPDase1, 2, and 3 and ecto-5'-nucleotidase in order to better understand the detrimental effects of high proline concentrations in the central nervous system.

Materials and methods

Animals

Adult (6-8-months-old) males and females (approximately in the ratio of 1:1) wild type (short fin - SF) zebrafish (*Danio rerio*) were obtained from a commercial supplier (Redfish, RS, Brazil). Animals were kept in 50L housing tanks with tap water previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mgO_2/l) at 28 ± 2ºC, under a 14-10 h light/dark photoperiod in at a density of up to five animals per liter (Westerfield 2007). Animals were acclimated for at least 2 weeks before the experiments and fed three times a day to satiety with TetraMin Tropical Flake Fish®. All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (UFRGS) under license number 19636 and

followed Brazilian legislation, the guidelines of the Brazilian Collegium of Animal Experimentation (COBEA), and the Canadian Council for Animal Care (CCAC) Guide on the care and use of fish in research, teaching, and testing.

Chemicals

L-proline, Coomassie Blue, nucleotides (ATP, ADP, and AMP), Malachite Green, and Trizma Base were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trizol® Reagent, dNTPs, oligonucleotides, Taq polymerase, Low DNA Mass Ladder, and SuperScript™ III First-Strand Synthesis SuperMix were purchased from Invitrogen (Carlsbad, CA, USA). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA) and GelRed™ was purchased from Biotium (Hayward, CA, USA). All reagents used were of analytical grade.

In vivo treatments

For the *in vivo* studies, animals were introduced to the test aquariums (4 L) and exposed at two proline concentrations (1.5 and 3.0 mM). For the short-term proline treatment, animals were maintained in the test aquarium during 1 h whereas the long-term proline exposure was performed during 7 days, replacing the water of the fish treatment tanks daily. Immediately after the treatments, the fish were cryoanaesthetized and further euthanized by decapitation. The whole brains were dissected and the brain membranes

were prepared. The short-term (1 h) and long-term (7 days) proline exposures were performed as previously described for zebrafish and mammalian models (Delwing et al. 2005; Savio et al. 2012).

In vitro treatments

For the *in vitro* assays, proline (final concentrations of 3, 30, 500, and 1000 µM) was directly added to reaction medium (described below), preincubated with the brain membrane samples and maintained throughout the enzyme assay. For the control group, the experiments were performed in the absence of proline (no drug added in the reaction medium). The in vitro assays were performed based on the cerebrospinal fluid proline concentration verified in hyperprolinemic patients (Phang et al. 2001).

Membrane preparation

Brain membranes were prepared as described previously (Barnes et al. 1993). Zebrafish brains were removed and briefly homogenized in 60 volumes (v/w) of chilled Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor driven Teflon-glass homogenizer. The samples were centrifuged at 1000 x g for 10 min and the pellet was discarded. The supernatant was then centrifuged for 25 min at 40,000 x g. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris–citrate buffer, and centrifuged for 20 min at 40,000 x g. This freeze–thaw–wash procedure was used to ensure the lysis of the brain membranes.

The final pellet was resuspended and used in the enzyme assays. All samples were maintained at 2–4 ºC throughout preparation.

Assays of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-5'-nucleotidase activities

The conditions for the E-NTPDase and ecto-5’-nucleotidase assays have been described previously (Rico et al. 2003; Senger et al. 2004). Briefly, zebrafish 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 NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for ecto-5’-nucleotidase activity) in a final volume of 200 µL. The samples were preincubated for 10 min at 37 ºC and the reaction was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min by the addition of trichloroacetic acid in a final concentration of 5% and the samples were chilled on ice for 10 min. The inorganic phosphate (Pi) release was determined by adding 1 mL of a mixture containing 2.3% polyvinyl alcohol, 5.7% ammonium molybdate and 0.08% malachite green (Chan et al. 1986). Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct for nonenzymatic hydrolysis of the substrates. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity was expressed as nanomoles of Pi released per minute per milligram of protein. All enzyme assays were run in triplicate.

Protein Determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard (Bradford 1976).

Analysis of gene expression by semi-quantitative RT-PCR

The expressions of E-NTPDase1 (*entpd1*), 2 (*entpd2*), 3 (*enptd3*), and ecto-5'-nucleotidase (*nt5e*) were analyzed by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. The optimal conditions for primer annealing for each sequence were determined from information on GenBank and data previously published in the literature (Senger et al. 2006; Rico et al. 2008). RT-PCR conditions for *entpd3* were optimized before the experiments and the *β-actin* primers were designed as described previously (Chen et al. 2004) (see Table 1). After short- and long-term proline exposures, zebrafish brains were isolated for total RNA extraction using the TRIzol® reagent (Invitrogen) in accordance with the manufacturer's instructions. cDNA species were synthesized with the SuperScript™ First-Strand (Synthesis System for RT-PCR) Invitrogen Kit® following the suppliers' instructions. PCR reactions for different *entpd2*, *enptd3*, *nt5e*, and *β-actin* genes were performed in a total volume of 20 µL, containing 0.1 µM primers (Table 1), 0.2 mM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA Polymerase® (Invitrogen). The PCR conditions for *entpd1* were similar to those described above, except that 1.5 mM $MgCl_2$ was employed.

The following conditions were used for the PCR reactions: 1 min at 94 ºC, 1 min at the annealing temperature (Table 1), and 1 min at 72 ºC for 35 cycles. Post-extension at 72 ºC was performed for 10 min. For each set of PCR reactions a negative control was included. In a previous study, the PCR conditions were pre-optimized by performing a curve with distinct concentrations of $MgCl_2$. PCR products were analyzed on a 1% agarose gel containing GelRed® (Biotium) 10 x, and visualized with ultraviolet light. The band intensities were measured by optical densitometry using the freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the band intensities of ectonucleotidase genes compared to *β-actin* (enzyme/β-actin). Each experiment was repeated four times using RNA isolated from independent extractions and run in a single gel. The expression analysis was performed in quadruplicate and representative data are shown.

Statistical analysis

Results are expressed as means ± standard error of mean (S.E.M). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test. Statistically significant differences between groups were considered for a $p < 0.05$.

Results

In vivo and *in vitro* effects proline on ectonucleotidase activities in zebrafish brain

The *in vivo* effects of two proline concentrations (1.5 and 3.0 mM) were evaluated on adenine nucleotide catabolism in zebrafish brain membranes. Short-term treatments (1 h) did not induce significant changes on ATP [$F(2,15) = 0.65$; $p > 0.05$], ADP [$F(2,12) = 0.20$; $p > 0.05$], and AMP [$F(2,12) = 0.02$; $p < 0.01$] hydrolysis in zebrafish brain when compared to the control group (Fig. 1A, B, and C). However, after long-term treatment, we observed that proline increases ATP catabolism in both concentrations (1.5 and 3.0 mM) tested (14 % and 22%, respectively) [$F(2,15)=11.00$; $p < 0.05$] (Fig. 1A), whereas the ADP and AMP hydrolysis were increased only at 3.0 mM proline (21 % [$F(2,12) = 4.4$; $p < 0.05$] and 17 % [$F(2,12) = 8.29$; $p < 0.01$], respectively) when compared to control group(Fig,1B and C).

In order to evaluate whether proline could act directly on ectonucleotidase activities from zebrafish brain, we tested the in vitro effect of different proline concentrations (ranging from 3.0 µM to 1.0 mM). As reported in Fig 2, proline in vitro did not promote significant changes on ectonucleotidase activities [$F(4,15)=1.414$; $p > 0.05$] in comparison to the control group.

Effects of proline on ectonucleotidase gene expression in zebrafish brain

We also verified the effects of proline treatments on ectonucleotidase gene expression in zebrafish brain. Short-term proline exposure did not alter the ectonucleotidase gene expression (data not shown). In contrast, as demonstrated in Fig. 3 the relative amount of *entpd3* transcripts significantly

increased after long-term exposure in both treated groups [$F(2,9) = 10.93$; $p < 0.01$], whereas for the *enptd1* we observed a significant increase only at 3.0 mM proline compared to the untreated group [$F(2,9) = 14.13$; $p < 0.01$]. The other *entpd* genes did not reveal significant changes in their expression profile after long-term treatment (data not show).

Discussion

Hyperprolinemia is an inherited disorder of proline metabolism and patients affected by this disease may present neurological symptoms, such as seizures and cognitive deficits (Phang et al. 2001; Di Rosa et al. 2008). Moreover, an association between psychotic disorders and moderate hyperprolinemia has been reported (Jacquet et al. 2005; Oresic et al. 2011). Nevertheless, the mechanisms that lead to neurological dysfunction in these patients remain poorly understood and few studies have been conducted to identify potential therapeutic mechanisms for this metabolic disease (Mitsubuchi et al. 2008; Wyse and Netto 2011). Since adenine nucleotides are signaling molecules that perform crucial roles in neurotransmission and neuromodulation, in this study we demonstrated that long-term proline exposure increases the adenine nucleotide catabolism, as well as the ectonucleotidase gene expression in zebrafish brain. Conversely, when proline was directly added to the enzyme assays, we did not observe significant changes on ectonucleotidase activities. Such result could be related to the fact that the *in vitro* experiments evaluate the direct effect of the

drug on the enzyme without the influence of other mechanisms, such as cell signaling pathways.

Purine nucleotides and nucleosides, such as adenosine 5′-triphosphate (ATP) and adenosine exert important roles in the central nervous system mainly controlling excitatory glutamatergic synapses (Burnstock et al. 2011). Interestingly, authors have proposed that at least part of the pathophysiology of hyperprolinemia results from a dysfunction of the glutamate homeostasis (Paterlini et al. 2002; Vorstman et al. 2009). Studies showed that high proline concentrations activate NMDA and AMPA receptors, suggesting that proline may potentiate the glutamatergic neurotransmission and increase glutamate release (Nadler 1987; Nadler et al. 1992; Cohen and Nadler 1997). Moreover, it has been reported that hyperprolinemia reduces glutamate uptake, as well as the Na^+, K^+-ATPase activity and intracellular ATP levels in rat brain (Delwing et al. 2007; Ferreira et al. 2011).

It is currently accepted that ATP is released as a co-transmitter together with classical transmitters, such as glutamate. Studies demonstrated that ATP co-released with glutamate in the brain plays a modulatory effect on glutamatergic mechanisms (Mori et al. 2001; Illes et al. 2001). Moreover, Fujii et al. (2004) reported an interaction between the extracellular ATP and NMDA receptors in the induction of long-term potentiation (LTP) in hippocampal neurons. Therefore, is possible that high proline levels increase glutamate release, as well as the extracellular ATP, which is hydrolyzed to ADP, AMP, and adenosine by the action of ectonucleotidases (E-NTPDases and ecto-5'-nucleotidase) (Zimmermann 2001), producing adenosine. In agreement with this hypothesis, we showed a significant increase on ATP

hydrolysis in brain membrane preparations during long-term proline exposure in both concentrations tested (1.5 and 3.0mM). For ADP and AMP hydrolysis, we verified an increase only at 3.0 mM proline, while 1.5 mM proline did not show asignificant effect a trend towards an increase, but it was not statistically significant. Since the increased extracellular ATP induces excitotoxicity and cell death via activation of $P2X_7$ receptor in neuronal cells (Le Feuvre et al. 2002), our results could be related to a compensatory response leading to a decrease in ATP availability, reducing its detrimental effects on normal brain excitability, and, consequently, contributing to the production of extracellular adenosine.

The neuromodulatory role of adenosine occurs from a balanced activation of inhibitory (A_1 and A_3) or excitatory (A_{2A} and A_{2B}) P1 receptors (Gomes et al. 2011). Studies have reported that adenosine homeostasis is altered in many neurological dysfunctions, including epilepsy and psychotic diseases (Lara et al. 2006, Rosim et al. 2011; Gomes et al. 2011). Adenosine acts as a neuromodulator in the central nervous system mostly controlling excitatory glutamatergic neurotransmission (Gomes et al. 2011). Nishizaki et al. (2004) showed that adenosine stimulates glutamate release from astrocytes via adenosine A_{2A} receptors. Furthermore, Dunwiddie and Masino (2001) showed that adenosine A_1 receptors presynaptically inhibit the glutamate release. On the other hand, postsynaptically adenosine A_1 receptors reduce the function of NMDA receptors (de Mendonça et al. 1995). Thus, the control of adenosine levels promoted by ectonucleotidases can influence the effects induced by hyperprolinemia on glutamatergic neurotransmission.

In a previous study, we have already demonstrated that long-term proline exposure induced schizophrenia-like behaviors in zebrafish and these behavioral changes were completely reversed by acute administration of an atypical antipsychotic drug (dopamine receptor antagonist) (Savio et al. 2012), suggesting an influence of this amino acid on glutamatergic and dopaminergic systems. Interestingly, we showed that only 1.5 mM proline induced behavioral changes, while at 3.0 mM proline we did not observe any significant effects. In contrast, we demonstrated that AMP hydrolysis was increased only at proline 3.0 mM, but not at 1.5 mM. This in an important point because adenosine agonists induce behavioral effects similar to those of antipsychotic drugs (dopamine antagonists) (Ferre 1997; Rimondin et al. 1997; Andersen et al. 2002). Therefore, the increased AMP hydrolysis after 3.0 mM proline exposure lead us to suggest that the production of extracellular adenosine via AMP in the brain was increased, which could contribute to minimize the proline-induced neurobehavioral changes.

At last, in order to evaluate whether the proline treatments could also alter the ectonucleotidase gene expression, we performed RT-PCR assays. The gene expression pattern of ectonucleotidases presented an increase in mRNA levels in both groups treated with proline for *entpd3*. The *enpd1* expression increased only at 3.0 mM proline. This augmented expression of *entpd1* and *entpd3* could contribute to the enhancement observed in ATP and ADP hydrolysis in brain membranes of proline-treated zebrafish. However, these enzymes hydrolyze tri- and diphosphonucleosides with different preferences. In mammals, E-NTPDase 1 hydrolyzes ATP and ADP to a similar extent, whereas E-NTPDase 3 hydrolyzes preferentially ATP than

ADP in a ratio of 3:1 (Zimmermann 2001). Therefore, the increase observed in ATP hydrolysis after long-term proline treatment at 1.5 mM may be related to increase in *entdp3* mRNA levels. For other enzyme mRNA transcript levels analyzed, the differences between treated groups and control group were not so evident. Nevertheless, gene expression is regulated by various factors involving cell machinery and signal transduction pathways and enzyme activity cannot be directly correlated with the gene expression pattern or with protein levels due to the existence of several post-translational events (Nedeljkovic et al. 2005).

In summary, our data demonstrate that long-term proline exposure increases the ectonucleotidase activities and gene expression in zebrafish brain, controlling the extracellular nucleotide levels, and, consequently, the purinergic signaling. These results may contribute to a better understanding of the pathophysiological mechanisms that increase the susceptibility to neurological symptoms in hyperprolinemic patients. In addition, such findings might facilitate the use of zebrafish as a complementary vertebrate model for studying metabolic diseases and pharmacological treatments.

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Conflict of Interest

The authors declare that no conflict of interest exists.

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Table 1: PCR primers sequences

Enzyme		Sequence (5'-3')	Annealing temperature (ºC)	PCR product (bp)
entpd1	Sense	CCCATGGCACAGGCCGGTTG	54	380
entpd1	Antisense	GCAGTCTCATGCCAGCCGTG		
entpd2_mg[a]	Sense	GGAAGTGTTTGACTCGCCTTGCACG	64	554
entpd2_mg[a]	Antisense	CAGGACACAAGCCCTTCCGGATC		
entpd2_mq[a]	Sense	CCAGCGGATTTAGAGCACGCTG	64	313
entpd2_mq[a]	Antisense	GAAGAACGGCGGCACGCCAC		
entpd2_mv[a]	Sense	GCTCATTTAGAGGACGCTGCTCGTG	64	263
enptd2_mv[a]	Antisense	GCAACGTTTTCGGCAGGCAGC		
entpd3	Sense	TACTTTCTTTGGACAGAGCAACCCTG	62	424
entpd3	Antisense	AAGCATATAGCCCAGGGACCAGG		
nt5e	Sense	ACCTCCGAGGAGTGTCGCTTTCG	54	433
nt5e	Antisense	CCTTGTTGGGGACCAGCGGTTC		
β-actin	Sense	GTCCCTGTACGCCTCTGGTCG	54	678
β-actin	Antisense	GCCGGACTCATCGTACTCCTG		

[a] Correspond to the two first amino acids residues of the protein sequence.

Legend to Figures

Figure 1: *In vivo* effects of short-term (1 h) and long-term (7 days) proline exposure on ATP (A), ADP (B), and AMP (C) hydrolysis in zebrafish brain membranes. The data represent mean ± S.E.M (n=5 at least). Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test. The asterisks represent $p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***), respectively.

Figure 2: *In vitro* effects of different proline concentrations on ATP (A), ADP (B), and AMP (C) hydrolysis in zebrafish brain membranes. The data represent mean ± S.E.M of four independent experiments. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test.

Figure 3: Effects of long-term (7 days) proline exposures (1.5 and 3.0 mM) on *enptd1* and *entpd3* gene expression in zebrafish brain. Data are expressed as mean ± S.E.M of four independent experiments. The relative mRNA levels were determined by optical densitometry analysis using the enzyme/β-actin ratio. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test. The asterisks represent $p < 0.05$(*) and $p < 0.01$(**), respectively.

Figure 1

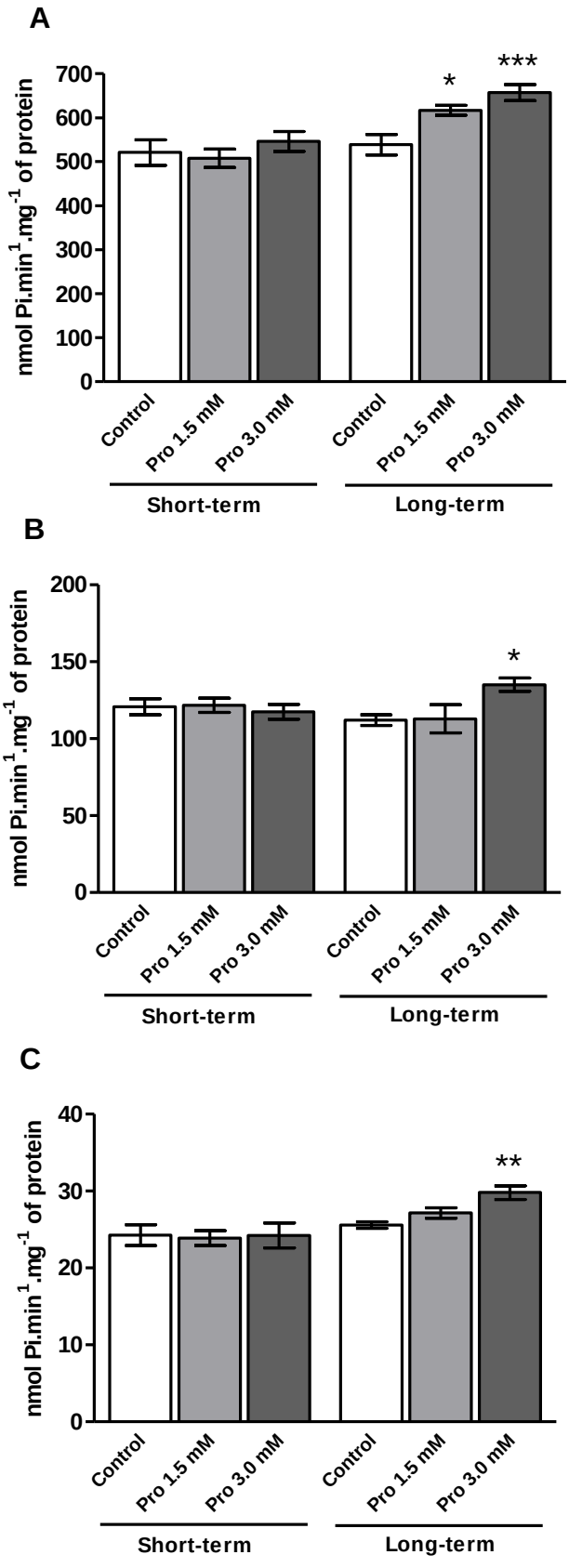

A
nmol Pi.min-1.mg-1 of protein
700
600
500
400
300
200
100
0
*
***
Control
Pro 1.5 mM
Pro 3.0 mM
Control
Pro 1.5 mM
Pro 3.0 mM
Short-term
Long-term
B
nmol Pi.min-1.mg-1 of protein
200
150
100
50
0
*
Control
Pro 1.5 mM
Pro 3.0 mM
Control
Pro 1.5 mM
Pro 3.0 mM
Short-term
Long-term
C
nmol Pi.min-1.mg-1 of protein
40
30
20
10
0
**
Control
Pro 1.5 mM
Pro 3.0 mM
Control
Pro 1.5 mM
Pro 3.0 mM
Short-term
Long-term

Figure 2

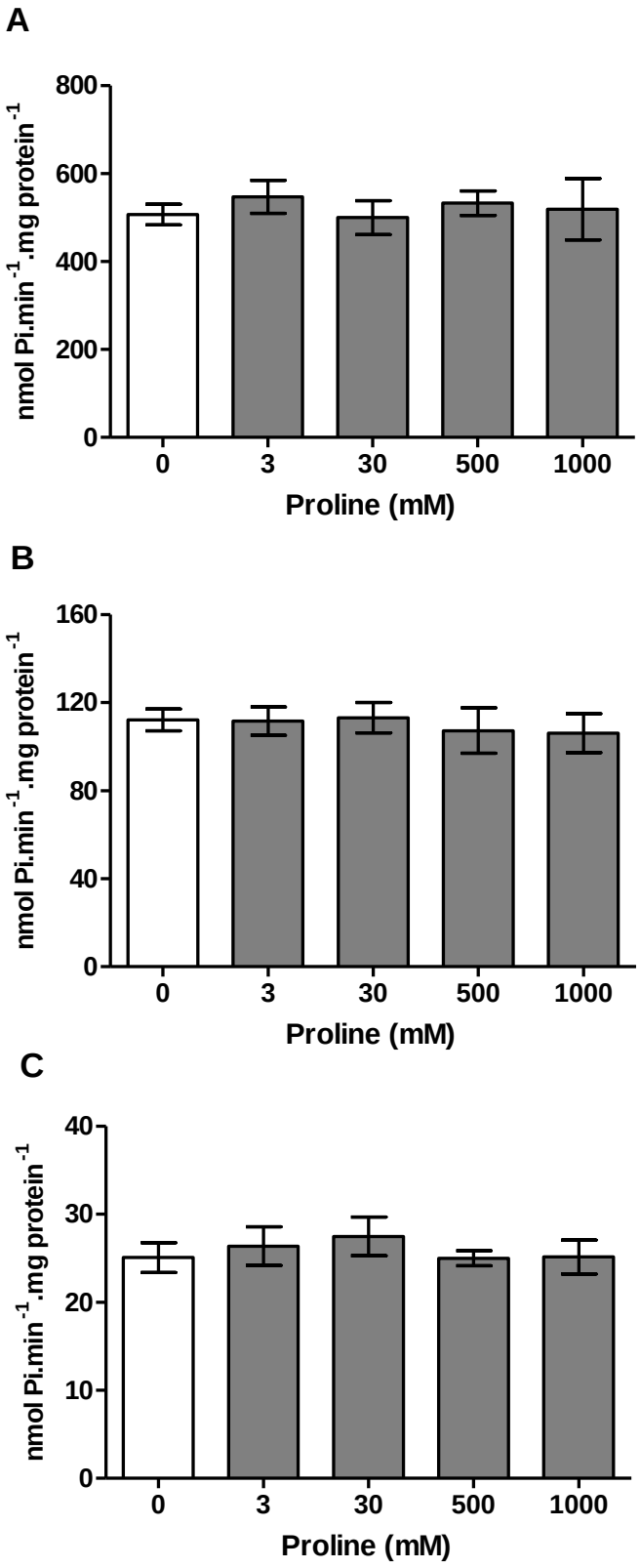
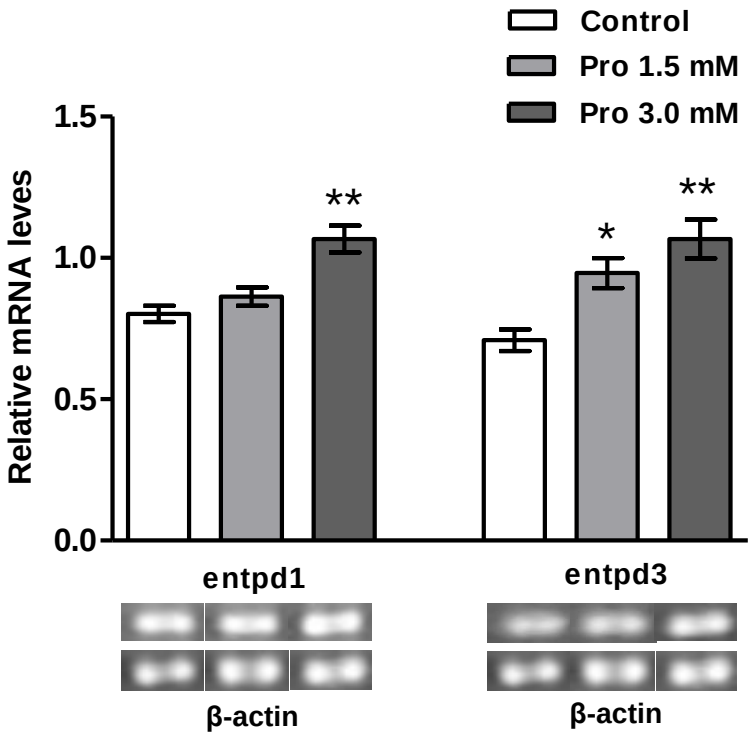

A
800
600
400
200
0
nmol Pi.min-1.mg protein-1
0
3
30
500
1000
Proline (mM)
B
160
120
80
40
0
nmol Pi.min-1.mg protein-1
0
3
30
500
1000
Proline (mM)
C
40
30
20
10
0
nmol Pi.min-1.mg protein-1
0
3
30
500
1000
Proline (mM)

Figure 3


Control
Pro 1.5 mM
Pro 3.0 mM
Relative mRNA leves
1.5
1.0
0.5
0.0
**
*
**
entpd1
entpd3
β-actin
β-actin

4. DISCUSSÃO

As hiperprolinemias podem ser causadas por dois distintos erros inatos do metabolismo da prolina. A hiperprolinemia tipo I (HPI) é provocada por uma deficiência na atividade da enzima prolina oxidase e a hiperprolinemia tipo II (HPII) ocorre devido a um defeito enzimático na enzima Δ^1-pirrolino-5-carboxilato desidrogenase (Phang et al., 2001). Os pacientes hiperprolinêmicos podem apresentar distúrbios neurológicos, como convulsões e retardo mental (Phang et al, 2001; Di Rosa et al, 2008). Além disso, estudos têm demonstrado que pacientes com hiperprolimenia moderada apresentam uma maior suceptibilidade a doenças neuropsiquiátricas, como a esquizofrenia (Jacquet et al, 2005; Oresic et al, 2011). Embora ainda não se tenha um completo entendimento acerca dos mecanismos relacionados a estas manifestações neurológicas, existem evidências de que pelo menos parte da sintomatologia da hiperprolinemia deve-se a disfunções na neurotransmissão glutamatérgica (Vorstman et al., 2009). Entretanto, até o momento, parece não existir um tratamento eficaz para esta doença e poucos estudos têm sido realizados para identificar potenciais mecanismos terapêuticos, buscando minimizar o impacto dos sintomas na qualidade de vida dos pacientes (Mitsubuchi et al, 2008; Wyse e Netto, 2011).

Portanto, neste estudo, primeiramente, avaliamos o efeito da exposição aguda e crônica à prolina sobre parâmetros comportamentais em peixe-zebra, um promissor modelo experimental para o estudo de doenças neurológicas. Constatamos que a exposição crônica à prolina induziu

alterações comportamentais no peixe-zebra, como a hiperlocomoção e o déficit de interação social. Além disso, observamos que essas alterações comportamentais foram completamente revertidas pela administração aguda de um antipsicótico atípico: a sulpirida.

Diversos mecanismos podem estar envolvidos nas respostas comportamentais induzidas pela prolina no peixe-zebra. Entretanto, os efeitos deste aminoácido sobre o sistema glutamatérgico tornaram-se mais evidentes ao longo dos últimos anos. Estudos têm demonstrado que altas concentrações de prolina ativam receptores NMDA e AMPA, sugerindo que a prolina pode potencializar a neurotransmissão glutamatérgica e, consequentemente, aumentar a liberação de glutamato (Nadler, 1987; Nadler et al, 1992; Cohen e Nadler, 1997). Sabe-se que antagonistas de receptores NMDA, como a dizocilpina (MK-801) e a fenciclidina (PCP), que mimetizam alguns sintomas da esquizofrenia, também podem aumentar a liberação de glutamato atuando inicialmente via ativação secundária de receptores não-NMDA e essa disfunção glutamatérgica pode gerar um aumento na liberação de dopamina (DA) no córtex pré-frontal (Moghaddam e Adams, 1998; Moghaddam, 2002).

Nesse contexto, tem sido proposto que a prolina poderia exercer um feito modulatório semelhante na neurotransmissão glutamatérgica, induzindo um aumento na liberação de DA (Paterlini et al 2005; Vorstman et al, 2009). De acordo com essa hipótese, nós demonstramos que a exposição crônica à prolina na concentração de 1,5 mM induziu hiperlocomoção e provocou um déficit de interação social no peixe-zebra, comportamentos verificados em pacientes esquizofrênicos. Corroborando com os dados desta pesquisa,

estudos demonstraram que o MK-801, um antagonista do receptor NMDA, também induziu as mesmas respostas comportamentais no peixe-zebra (Seibt et al, 2010; 2011).

A exposição crônica à prolina na concentração de 3,0 mM não induziu alterações comportamentais no peixe-zebra. Esse resultado pode ser explicado pelo fato de que a curva de dose-resposta para DA assemelha-se a uma curva de U invertido (Vijayraghavan et al, 2007; Lavergne e Jay, 2010). Alguns autores propõem que a curva de dose-resposta em U invertido seria um mecanismo regulatório de proteção neuronal, evitando respostas tóxicas (Skolnick et al, 2009; Lavergne e Jay, 2010). Portanto, é possível que a prolina na concentração de 3,0 mM provoque uma maior liberação de dopamina a níveis não responsivos. Dessa forma, não foram observadas alterações comportamentais nesta concentração de prolina testada.

Os fármacos antipsicóticos são amplamente utilizados para o tratamento de transtornos neuropsiquiátricos. Estudos prévios demonstraram que os fármacos antipsicóticos são capazes de reverter a hiperlocomoção e o déficit de interação social induzidos pelo MK-801 em peixe-zebra (Seibt et al, 2010; 2011). Neste estudo, constatamos que a sulpirida, um antipsicótico atípico, reverteu completamente as alterações comportamentais induzidas pela prolina no peixe-zebra, enquanto que o haloperidol, um fármaco típico, foi capaz apenas de atenuar o déficit de interação social.

Vários mecanismos podem estar envolvidos nos efeitos benéficos do tratamento com fármacos antipsicóticos sobre alterações comportamentais induzidas pela exposição crônica à prolina. Os antipsicóticos típicos, como o haloperidol, agem preferencialmente bloqueando receptores D_2 de dopamina

(Heusler et al., 2008). Já os antipsicóticos atípicos, embora menos potentes do que os típicos em bloquear os receptores D_2, têm afinidade por outros receptores, incluindo os D_1 e D_4 de dopamina, serotoninérgicos (5-HT_{2A} e 5-HT_6), adrenérgicos (α1), histaminérgicos (H1) e muscarínicos (M1) (Jones et al., 2008). A sulpirida, um antipsicótico atípico, age preferencialmente via bloqueio dos receptores D_2 e D_3 de dopamina (Jaworski et al., 2001; Tadori et al., 2011.)

Numerosos estudos têm demonstrado que os antipsicóticos atípicos são mais potentes do que os típicos para reverter as alterações induzidas por antagonistas do receptor NMDA sobre parâmetros locomotores e no comportamento social (Jentsch e Roth, 1999; Geyer et al, 2001; Seibt et al. , 2010; 2011). Boulay e colaboradores (2004) constataram que o haloperidol não foi capaz de reverter o déficit de interação social induzido por um antagonista do receptor NMDA. Já Linck e colaboradores (2008) demonstraram que a sulpirida reverteu completamente o déficit social induzido pelo MK-801. Portanto, é possível que a sulpirida tenha revertido completamente as alterações locomotoras e o déficit de interação social induzidos pela prolina através de um mecanismo semelhante.

A exposição crônica à prolina na concentração de 1,5 mM também aumentou o tempo que os animais permaneceram na parte superior do aquário, o que pode ser interpretado como um comportamento ansiolítico. Esse resultado está de acordo com um estudo anterior, o qual demonstra que o MK-801 induz um comportamento ansiolítico no peixe-zebra (Seibt et al., 2010). Outros estudos realizados em mamíferos também observaram que animais tratados com MK-801 e submetidos ao teste do labirinto em

cruz elevada apresentaram um aumento no tempo gasto nos braços abertos, o que é considerado um comportamento ansiolítico (Dunn et al., 1989; Bertoglio e Carobrez, 2003). Seibt e colaboradores (2010) relataram, ainda, que os fármacos antipsicóticos não foram capazes de reverter o efeito ansiolítico do MK-801 no peixe-zebra. No entanto, no presente estudo, o efeito ansiolítico induzido pela prolina foi revertido pela sulpirida, como verificado nos outros parâmetros comportamentais analisados.

Esses resultados demostraram que a exposição crônica à prolina foi capaz de induzir alterações comportamentais semelhantes àquelas verificadas em modelos experimentais que mimetizam sintomas esquizofrênicos. Além disso, demostramos que os fármacos antipsicóticos foram efetivos em reverter essas alterações. Porém, não se tem um completo entendimento sobre os mecanismos neuroquímicos relacionados a essas alterações comportamentais.

Estudos têm demonstrado que o bloqueio farmacológico dos receptores NMDA provoca um aumento não só nos níveis cerebrais de dopamina, mas também nos níveis de acetilcolina, consequentemente, provocando um aumento na atividade locomotora (Del Arco e Mora, 2005; Del Arco et al., 2008). Dessa forma, considerando-se os efeitos da prolina sobre os receptores ionotrópicos de glutamato, é possível que altas concentrações desse aminoácido aumentem a liberação de acetilcolina. Nesse sentido, um aumento nos níveis de acetilcolina pode estar relacionado, pelo menos em parte, às alterações comportamentais verificadas neste estudo, como, por exemplo, a hiperlocomoção (Savio et al., 2012). Portanto, no segundo capítulo desta dissertação, avaliou-se o efeito

da exposição aguda e crônica à prolina sobre a atividade da AChE em cérebro de peixe-zebra. Nós verificamos um aumento significativo na atividade da AChE em cérebro de peixe-zebra após exposição crônica à prolina em ambas as concentrações testadas (1,5 e 3,0 mM). Esses achados estão de acordo com um estudo prévio, o qual demonstra que a administração crônica de prolina aumenta significativamente a atividade da AChE em hipocampo de ratos adultos (Ferreira et al., 2011). Portanto, esses resultados podem estar relacionados a uma resposta compensatória a fim de reduzir os níveis aumentados de acetilcolina na fenda sináptica.

O aumento na atividade da AChE induzido pela prolina foi completamente revertido pelo tratamento agudo com a sulpirida. No entanto, o haloperidol somente reverteu o aumento da atividade da AChE induzido pela prolina na concentração de 3,0 mM, enquanto que na concentração de 1,5 mM de prolina esse fármaco não teve qualquer efeito. Esses resultados corroboram com nossos estudos comportamentais, nos quais verificamos que o haloperidol não foi capaz de reverter hiperlocomoção induzida pela exposição crônica à prolina na concentração de 1,5 mM (Savio et al., 2012).

Del Arco e colaboadores (2008) demonstraram que a administração de antagonistas dos receptores D_1 e D_2 de dopamina reduziu as alterações motoras induzidas pelo bloqueio de receptores NMDA no córtex pré-frontal. Assim, considerando que o haloperidol age principalmente através do bloqueio de receptores D_2 de dopamina (Heusler et al., 2008) e a sulpirida desempenha suas ações preferencialmente bloqueando os receptores D_2 e D_3 de dopamina (Jaworski et al., 2001; Tadori et al., 2011), é possível que esses fármacos revertam o aumento na atividade da AChE induzido pela

prolina por um mecanismo semelhante, reduzindo a liberação de acetilcolina. Entretanto, como relatamos anteriormente, os antipsicóticos atípicos têm afinidade com outros receptores, como os serotoninérgicos (5-HT_{2A} e 5-$HT_{6)}$, os adrenérgicos (α1), os histaminérgicos (H1) e muscarínicos (M1) (Jones et al., 2008). Assim, outros mecanismos podem estar envolvidos nos efeitos benéficos dos antipsicóticos sobre as alterações induzidas pela exposição à prolina em cérebro de peixe-zebra. Estudos posteriores ainda são necessários para elucidar a contribuição de outros sistemas de neurotrasmissão como, por exemplo, do sistema serotoninérgico.

O ATP e a adenosina exercem importantes papéis no sistema nervoso central, principalmente controlando sinapses glutamatérgicas (Burnstock et al., 2011). O ATP é co-liberado com uma série de outros neurotransmissores, tais como a acetilcolina e o glutamato. Estudos demonstraram que o ATP co-liberado com glutamato desempenha um papel modulatório sobre os mecanismos glutamatérgicos (Mori et al., 2001; Illes et al., 2001). Portanto, é possível que altos níveis de prolina aumentem a liberação de glutamato, bem como de ATP extracelular, o qual pode ser hidrolisado à ADP e AMP pela ação de ectonucleotidases (E-NTPDases e ecto-5'-nucleotidase), gerando adenosina (Zimmermann, 2001). De acordo com essa hipótese, no terceiro capítulo desta dissertação, nós demonstramos um aumento significativo na hidrólise de ATP em cérebro de peixe-zebra após a exposição crônica à prolina em ambas as concentrações testadas (1,5 mM e 3,0 mm). Além disso, verificamos um aumento significativo na hidrólise de ADP e AMP, porém apenas na concentração de 3,0 mM. Dessa forma, considerando que um aumento nos níveis de ATP

extracelular pode induzir excitotoxicidade e provocar morte neuronal via ativação de receptores $P2X_7$ (Le Feuvre et al., 2002), o aumento no catabolismo de nucleotídeos pode estar relacionado a uma resposta compensatória levando a uma diminuição nos níveis extracelulares de ATP, reduzindo, assim, seus efeitos neurotóxicos e, contribuindo para o aumento nos níveis de adenosina.

Estudos têm demonstrado que a homeostase da adenosina pode estar alterada em diversas doenças neurológicas, como na epilepsia e na esquizofrenia (Lara et al., 2006; Rosim et al., 2011; Gomes et al., 2011). A adenosina atua como um neuromodulador no sistema nervoso central, principalmente, controlando a neurotransmissão glutamatérgica (Gomes et al. 2011). Nishizaki e colaboradores (2004) demonstraram que a adenosina estimula a liberação de glutamato de astrócitos através da ativação de receptores de adenosina A_{2A}. Além disso, Dunwiddie e Masino (2001) relataram que os receptores A_1 de adenosina inibem a liberação de glutamato em neurônios pré-sinápticos. Por outro lado, em neurônios pós-sinápticos, os receptores A_1 reduzem a atividade dos receptores NMDA (de Mendonça et al., 1995). Assim, o controle dos níveis de adenosina promovido pela ectonucleotidases pode modular os efeitos induzidos pela hiperprolinemia na neurotransmissão glutamatérgica.

Estudos demonstram, ainda, que a adenosina pode induzir respostas comportamentais semelhantes às de fármacos antipsicóticos (antagonistas de dopamina) (Ferre, 1997; Rimondin et al., 1997; Andersen et al., 2002). Nossos dados corroboram com essa hipótese, pois demonstramos que apenas a exposição crônica à prolina na concentração de 1,5 mM foi capaz

de induzir alterações comportamentais no peixe-zebra, enquanto que na concentração de 3,0 mM não observamos alterações significativas. Por outro lado, a hidrólise de AMP foi aumentada apenas após a exposição crônica à prolina na concentração de 3,0 mM e nenhum efeito foi observado na concentração de 1,5 mM. Portanto, o aumento na hidrólise de AMP poderia estar contribuindo para produção extracelular de adenosina e esta, por sua vez, poderia estar modulando as respostas comportamentais induzidas pela exposição a altas concentrações de prolina.

Com relação à expressão gênica das ectonucleotidases, observamos um aumento nos níveis de RNA mensageiro para os transcritos da E-NTPase3 em ambos os grupos tratados com prolina. Já a E-NTPase1 apresentou um aumento nos níveis de transcritos apenas na concentração de 3,0 mM. Assim, o fato de termos observado um aumento somente na hidrólise de ATP após a exposição crônica à prolina na concentração de 1,5 mM pode ter ocorrido devido ao aumento na expressão gênica da enzima E-NTPDase3, pois é sabido que, pelo menos em mamíferos, a E-NTPDase1 hidrolisa ATP e ADP igualmente, enquanto que a E-NTPDase3 prefere o ATP em relação ao ADP numa razão de hidrólise de aproximadamente 3:1 (Zimmermann, 2001). Dessa forma, o aumento na expressão gênica dessas enzimas pode estar relacionado com a hidrólise aumentada de ATP e ADP em cérebro de peixe-zebra após a exposição à prolina.

Verificamos, ainda, que o perfil de expressão gênica ecto’5-nucleotidase não foi alterado após a exposição crônica à prolina na concentração de 3,0 mM, apesar deste tratamento ter provocado um aumento na hidrólise de AMP. Esse resultado pode ser explicado pelo fato

de que a expressão gênica pode ser regulada por diversos fatores, como, por exemplo, diferentes vias de transdução de sinal e, portanto, a atividade da enzima, muitas vezes, pode não estar diretamente relacionada ao seu o perfil de expressão gênica ou aos seus níveis de proteína devido à existência de vários eventos pós-transcricionais, como eventos de fosforilação (Nedeljkovic et al., 2005). Dessa forma, novos estudos são necessários para que se possa elucidar os possíveis mecanismos responsáveis pelo aumento da hidrólise de AMP após a exposição crônica à prolina.

Nos estudos *in vitro*, não observamos alterações significativas nas atividades ectonucleotidásicas e da AChE em cérebro de peixe-zebra. Esses resultados podem estar relacionados ao fato de que os experimentos *in vitro* avaliam somente o efeito direto do aminoácido sobre a enzima, sem a influência de mecanismos externos, como, por exemplo, as vias de sinalização celular.

Os resultados deste estudo demonstram que a exposição crônica à prolina induz alterações neuroquímicas e comportamentais no peixe-zebra. Verificou-se, também, que essas alterações podem ser completamente revertidas pela administração aguda de fármacos antipsicóticos. Esses achados podem contribuir, pelo menos em parte, para uma melhor compreensão dos mecanismos relacionados às manifestações neurológicas verificadas em pacientes hiperprolinêmicos, como os transtornos psicóticos e cognitivos. Além disso, este estudo contribui para a consolidação do peixe-zebra como modelo experimental para o estudo de erros inatos do metabolismo que afetam o sistema nervoso central.

5. CONCLUSÕES

- A exposição crônica (7 dias) à prolina na concentração de 1,5 mM provocou alterações comportamentais no peixe-zebra, tais como aumento na atividade locomotora, déficit de interação social e comportamento ansiolítico;
- As alterações comportamentais induzidas pela exposição à prolina no peixe-zebra foram completamente revertidas pelo tratamento agudo com a sulpirida, um antipsicótico atípico. Entretanto, o haloperidol, um antipsicótico típico, foi apenas capaz de atenuar o déficit de interação social observado no peixe-zebra;
- A exposição aguda (1 hora) à prolina nas concentrações testadas (1,5 e 3,0 mM) não induziu alterações comportamentais, bioquímicas ou moleculares no peixe-zebra;
- A exposição crônica à prolina (1,5 e 3,0 mM) provocou um aumento na atividade da acetilcolinesterase em cérebro de peixe-zebra e os fármacos antipsicóticos foram capazes de reverter esse aumento;
- A prolina *in vitro* não provocou alterações significativas na atividade das E-NTPDases, ecto-5'-nucleotidase e acetilcolinesterase em preparações de cérebro do peixe-zebra;
- A exposição crônica à prolina (1,5 e 3,0 mM) aumentou a atividade das ectonucleotidases em cérebro de peixe-zebra, bem como a expressão gênica das enzimas E-NPTDase1 e 3.

6. PERSPECTIVAS

- Verificar o efeito da exposição crônica à prolina (1,5 e 3,0 mM) sobre a atividade e padrão de expressão gênica da adenosina deaminase em cérebro de peixe-zebra;
- Avaliar o efeito da exposição crônica à prolina (1,5 e 3,0 mM) na captação de glutamato e expressão gênica de seus transportadores em cérebro de peixe-zebra;
- Avaliar o efeito da exposição crônica à prolina (1,5 e 3,0 mM) no padrão de expressão gênica dos receptores P1 de adenosina em cérebro de peixe-zebra;
- Analisar parâmetros de estresse oxidativo em cérebro de peixe-zebra após a exposição crônica à prolina.

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