**UNIVERSIDADE FEDERAL DO PARANÁ**

BRUNO CARABELLI

# EFEITO ANTIDEPRESSIVO DO ÓLEO DE PEIXE: POSSÍVEL PAPEL DOS RECEPTORES $5HT_{1A}$ PÓS-SINAPTICOS HIPOCAMPAIS?

CURITIBA, 2013

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Dissertação apresentada como requisito para a obtenção do grau de mestre em Fisiologia, curso de Pós-graduação em Fisiologia, Setor de Ciências Biológicas, Universidade Federal do Paraná.

**Orientadora**: Profa. Dra. Anete Curte Ferraz
**Co-orientador**: Prof. Dr. Marcelo M. S. Lima

CURITIBA, 2013

FOLHA DE APROVAÇÃO

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Setor de Ciências Biológicas
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Programa de Pós-Graduação em Fisiologia

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Curitiba, 29 de abril de 2013.

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## RESUMO

A fisiopatologia da depressão ainda não é completamente conhecida, mas estudos apontam para uma disfunção no sistema serotoninérgico como possível causa. Estudos mostram que a suplementação, em ratos, com Óleo de Peixe (OP) rico em Ácido Docosahexaenóico (DHA) e Ácido Eicosapentaenóico (EPA), durante períodos críticos do desenvolvimento, produz efeito antidepressivo através de um aumento da neurotransmissão serotoninérgica, particularmente no hipocampo. Considerando este efeito, que também é mediado pela ativação dos receptores 5-$HT_{1A}$ hipocampais, o presente estudo dedicou-se em investigar o impacto da suplementação com OP (na gestação e lactação) sobre os receptores 5-$HT_{1A}$ pós-sinápticos, bloqueando estes receptores com um antagonista seletivo, o WAY100635, administrado diretamente no hipocampo de ratos de 90 dias de idade.

As fêmeas foram suplementadas com OP durante a habituação, acasalamento, gestação e lactação. A prole, mantida sem suplementação até a vida adulta, foi submetida à testes comportamentais e análises neuroquímicas foram feitas para quantificar os níveis hipocampais de serotonina (5-HT) e seu metabólito, o Ácido 5-Hidroxiindolacético (5-HIAA). Além disso, a expressão dos receptores 5-$HT_{1A}$ hipocampais foi mensurada através da técnica de Western Blot.

No Teste da Natação Forçada Modificado (TNFM) os resultados mostram que os animais suplementados com OP apresentaram menor comportamento depressivo, refletido por frequência diminuída de imobilidade e aumentada de natação, mais ainda, este efeito antidepressivo foi revertido pela administração do antagonista 5-$HT_{1A}$ no hipocampo, o que ressalta o envolvimento destes receptores no efeito antidepressivo da suplementação, apesar de não ter sido observada nenhuma alteração na expressão destes receptores no hipocampo, através da análise por Western Blot. Em relação à análise de neurotransmissores, foi encontrado um aumento dos níveis de 5-HT no hipocampo de ratos suplementados. Os presentes achados sugerem envolvimento dos receptores $5HT_{1A}$ hipocampais no efeito antidepressivo da suplementação com óleo de peixe. Os presentes achados sugerem envolvimento dos receptores $5HT_{1A}$ hipocampais no efeito antidepressivo da suplementação com óleo de peixe.

**Palavras-chave**: Óleo de peixe, depressão, serotonina, receptores 5-$HT_{1A.}$

ABSTRACT

The physiopatology of depression is not completely known, but some studies point to serotonergic dysfunction as possible cause. The supplementation with fish oil rich in docosahexaenoic acid (DHA) and eiccosapentaenoic acid (EPA) during critical periods of development produces antidepressant effects by an increase in serotonergic neurotransmission, particularly in the hippocampus. Considering this antidepressant effect promoted by fish oil supplementation, that is mediated by activation of hippocampal $5HT_{1A}$ receptors, the present study was devoted to investigate the effects of fish oil supplementation (from conception to weaning) on $5HT_{1A}$ post-synaptic receptors blocking these receptors with the selective antagonist WAY100635 administrated directly in the hippocampus of 3-month old rats. Female rats were supplemented with FO during habituation, mating, gestation and lactation. The adult offspring was subjected to behavioral tests and neurochemical analysis was carried out in order to quantify hippocampal serotonin (5-HT) and its metabolite, 5-hydroxyindoleacetic (5-HIAA). Also, the $5HT_{1A}$ receptors expression in hippocampus was measured by western blot analysis. In the modified forced swim test the results demonstrated that fish oil-supplemented offspring displayed less depressive-like behaviors reflected by decreased immobility and increased swimming, meanwhile the analysis of western blotting reveled that there is no difference in expression of hippocampal 5-$HT_{1A}$ receptors among the groups. Concerning to monoamines analysis the results showed an increased 5-HT hippocampal levels. In summary, the present findings confirm the antidepressant effects of ω-3 PUFA supplementation, likely related to increased hippocampal serotonergic neurotransmission. Nevertheless, more studies are necessary to understand the serotonergic effect considering pre/pos-synaptic 5-$HT_{1A}$ receptors

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# 1 INTRODUÇÃO

## 1.1 DEPRESSÃO

Os Transtornos do Humor estão entre as formas mais prevalentes de doenças mentais, afetando grande parte da população mundial. Dentre eles estão a Bipolaridade, a Depressão Maior e a Distimia. Estes transtornos são recorrentes e apresentam grandes riscos devido à possibilidade de suicídio e compreendem a maior causa de morbidade no mundo (BLAZER, 2000).

A distimia é marcada por um humor deprimido crônico, ocorrendo na maior parte do tempo por pelo menos 2 anos, juntamente com um sintoma adicional (distúrbios do sono, fadiga; American Psychiatric Association - APA 1994). Ela é menos prevalente que a depressão maior, mas também causa sérios danos à saúde e prejuízos à vida do paciente (LASSER et al., 2000; ZIEDONES et al., 2008).

A bipolaridade ou “depressão bipolar” é caracterizada por recorrentes mudanças de humor. Existem subtipos, sendo os mais comuns a bipolaridade tipo I e a bipolaridade tipo II. O primeiro tipo consiste em ciclos de mania (sentimentos de grandeza, euforia, alegria intensa não justificada) e o segundo em ciclos de hipomania (sintomas mais brandos da mania) e depressão. Com uma rápida ciclagem (quatro ou mais episódios de depressão ou mania/hipomania ou episódios mistos dentro de um ano) esta doença tem o pior prognóstico dentre os Transtornos de Humor (BELMAKER, 2004; Manual Diagnóstico e Estatístico de Transtornos Mentais, DSM-IV, 1994). Na bipolaridade tipo I, que aflige cerca de 1,2% a 1,5% da população norte americana (NARROW et al., 2002) a depressão é 3 vezes mais comum que a mania (JUDD et al., 2002)

A depressão maior, comumente chamada apenas de “depressão” é o mais comum dentre os distúrbios psiquiátricos, com uma elevada taxa de prevalência durante a vida, e duas vezes mais comum em mulheres do que em homens (ALBERT *et al*., 2010). A Organização Mundial de Saúde (OMS) estima que em 2020 a depressão será a segunda maior causa de

incapacitação atrás das doenças cardiovasculares, e será a primeira em regiões em desenvolvimento (LIN *et al*., 2010).

Desde 1960, é diagnosticada de acordo com os critérios sintomáticos do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM) - quarta edição revisada em 2000 (DSM-IV-TR, 2000). De acordo com o DSM-IV, a depressão maior caracteriza-se por um ou mais episódios depressivos, com pelo menos 2 semanas de humor deprimido ou perda de interesse na maior parte das atividades acompanhados de ao menos quatro sintomas adicionais de depressão, que incluem sentimentos de desesperança, desvalia, culpa, desamparo, associados a alterações de apetite e sono, fadiga, retardo ou agitação psicomotora, diminuição do desempenho sexual, dificuldade de concentração e raciocínio e pensamentos recorrentes sobre morte, com ou sem tentativas de suicídio (DSM-IV-TR, 2000).

Tentativas de classificar a depressão em subtipos foram feitas, baseadas em certos conjuntos de sintomas (AKISKAL, 2000; BLAZER, 2000). Porém esses subtipos são baseados somente em diferenças sintomáticas e não há evidências que reflitam diferentes estados de doenças subjacentes (NESTLER *et al*., 2002).

Existem diferentes hipóteses para explicar a causa da depressão, porém nenhuma com total abrangência e consistência. Estudos epidemiológicos mostram que aproximadamente 40%-50% do risco de desenvolver depressão é genético. Fatores não-genéticos, como estresse e traumas emocionais, também são importantes, mas ainda não se sabe, com absoluta certeza, a fisiopatologia desta doença (NESTLER *et al*, 2002).

Está claro que a depressão pode ser resultado ou resultar em mudanças neuroanatômicas e neurofisiológicas no encéfalo (SELINE *et al*., 1996; DUMAN et al., 1997; SEMINOWICZ *et al*., 2004; DREVETS *et al*., 2008). Muitos estudos pré-clínicos e clínicos indicam que distúrbio no sistema serotoninérgico tenha papel importante no desencadeamento da doença, no entanto outros neurotransmissores, como a noradrenalina (NA) e dopamina (DA) também estão envolvidos (HARRO & ORELAND, 2001; NESTLER *et al*., 2002; NESTLER & CARLEZON, 2006). Esta é a “hipótese monoaminérgica da depressão”, sendo a mais aceita atualmente em termos neurobiológicos. A associação do distúrbio à deficiência das monoaminas é fundamentada em

resultados de exames bioquímicos que detectaram baixos níveis de NA e serotonina (5-HT) no sangue, na urina e no líquor de pacientes com depressão. Outro fator que corrobora essa teoria é que o tratamento, com inibidores da enzima monoaminooxidase (IMAOs) e com drogas que inibem a recaptação desses neurotransmissores, como os antidepressivos tricíclicos e os inibidores seletivos da recaptação da serotonina (ISRS) e/ou da noradrenalina (ISRN) são eficazes no tratamento da doença (CARLSON, 2001; SADOCK e SADOCK, 2007).

Além disso, existe uma forte associação entre baixos níveis do ácido 5-Hidroxiindolacético (5-HIAA), metabólito da serotonina, com a tendência ao suicídio na depressão (CARLSON, 2002).

Complementando esta hipótese, há o fato de que a menor expressão de receptores serotoninérgicos 5-$HT_{1A}$ pós-sinápticos, encontrados em áreas como hipocampo, córtex pré-frontal e córtex entorrinal, relaciona-se à sintomas depressivos e risco de suicídio. Estes receptores também se apresentam em menor número e apresentam uma menor afinidade em testes de ligação ao agonista (*binding*), em regiões como hipocampo e amígdala em vítimas de suicídio (CHEETHAM *et al*., 1990). Outros estudos *post-mortem* também evidenciaram uma redução na expressão do gene para o 5-$HT_{1A,}$ na afinidade e/ou número destes receptores no córtex pré-frontal (BOWEN *et al*., 1989). O hipocampo contêm um grande número de receptores $5HT_{1A}$, onde estes têm importante papel na regulação do comportamento e resposta ao estresse (CHALMERS & WATSON, 1991; PALACIOS *et al.,* 1990). Esta estrutura têm sido associada à adaptação ao estresse, permitindo que os animais se tornem tolerantes a estímulos aversivos crônicos (DEAKIN, GRAEFF & GUIMARÃES, 1992).

Estudos clínicos evidenciam que redução na função dos receptores $5HT_{1A}$ está associada à depressão e encéfalos de vítimas de suicídio apresentam redução do número de receptores $5HT_{1A}$ hipocampais e um aumento do número dos auto-receptores inibitórios (STOCKMEIER et al., 1998). A maioria dos antidepressivos aumentam, progressivamente, a função destes receptores no hipocampo (BLIER & DE MONTIGNY, 1994; HADDJERI, BLIER & DE MONTIGNY, 1998).

Muito dos efeitos destas drogas são somente observados com sua administração crônica, em especial dos ISRS. Estes efeitos podem ser resultantes de alterações plásticas, como uma reorganização funcional dos receptores serotoninérgicos, com dessensibilização dos auto-receptores somatodendríticos, localizados nos núcleos da rafe, que são inibitórios, exercendo importante papel na regulação da liberação da 5-HT para outras regiões do encéfalo (BLIER & de MONTIGNY, 1994; HERVAS *et al.,* 2001; MANSARI *et al*., 2005). Devido a esta latência temporal para o início dos efeitos terapêuticos, o uso dos antidepressivos deve ser monitorado, principalmente no início do tratamento, devido ao risco de suicídio e a falta de aderência ao próprio tratamento, sendo esta a maior crítica em relação ao uso destes medicamentos (RUSH *et al*., 2006, 2011; TRIVEDI *et al*., 2006). Devido a estas e muitas outras limitações envolvendo o tratamento farmacológico da depressão, alternativas com menos efeitos colaterais e de fácil acesso são investigadas, entre elas a ingestão de Gorduras Poli-insaturadas, principalmente ácidos graxos da família ômega-3.

## 1.2 ÁCIDOS GRAXOS POLI-INSATURADOS E DEPRESSÃO

Os mamíferos requerem obrigatoriamente (devido à incapacidade de síntese) na sua dieta a presença de alguns ácidos graxos poli-insaturados (AGPI's), como o Ácido Linoléico (LA, 18:2ω-6) e o Ácido α-Linolênico (ALA, 18:3 ω -3). Tais gorduras são consideradas, portanto, essenciais e são encontradas principalmente em algumas plantas e peixes de águas frias e profundas (HORROCKS e FAROOQUI, 2004).

O LA ω -6 dá origem aos ácidos graxos poli-insaturados de cadeia longa da família ômega-6 (AGPIs ω-6), dentre eles o Ácido Araquidônico (AA, 20:4 ω -6) e o Ácido Docosapentaenóico (DPA, 22:5 ω -6). O ALA ω-3, por sua vez, é precursor dos ácidos graxos poli-insaturados de cadeia longa da família ômega-3 (AGPIs ω-3), representados pelos Ácidos Eicosapentaenóico (EPA, 20:5 ω-3) e Docosahexaenóico (DHA,22:6 ω -3) (CARRIE *et al*., 2000; ATTAR-BASHI e SINCLAIR, 2004; OZIAS *et al*., 2007). A figura a seguir mostra as etapas bioquímicas necessárias para a síntese de um elemento da família de ambos os subtipos de ácidos graxos.

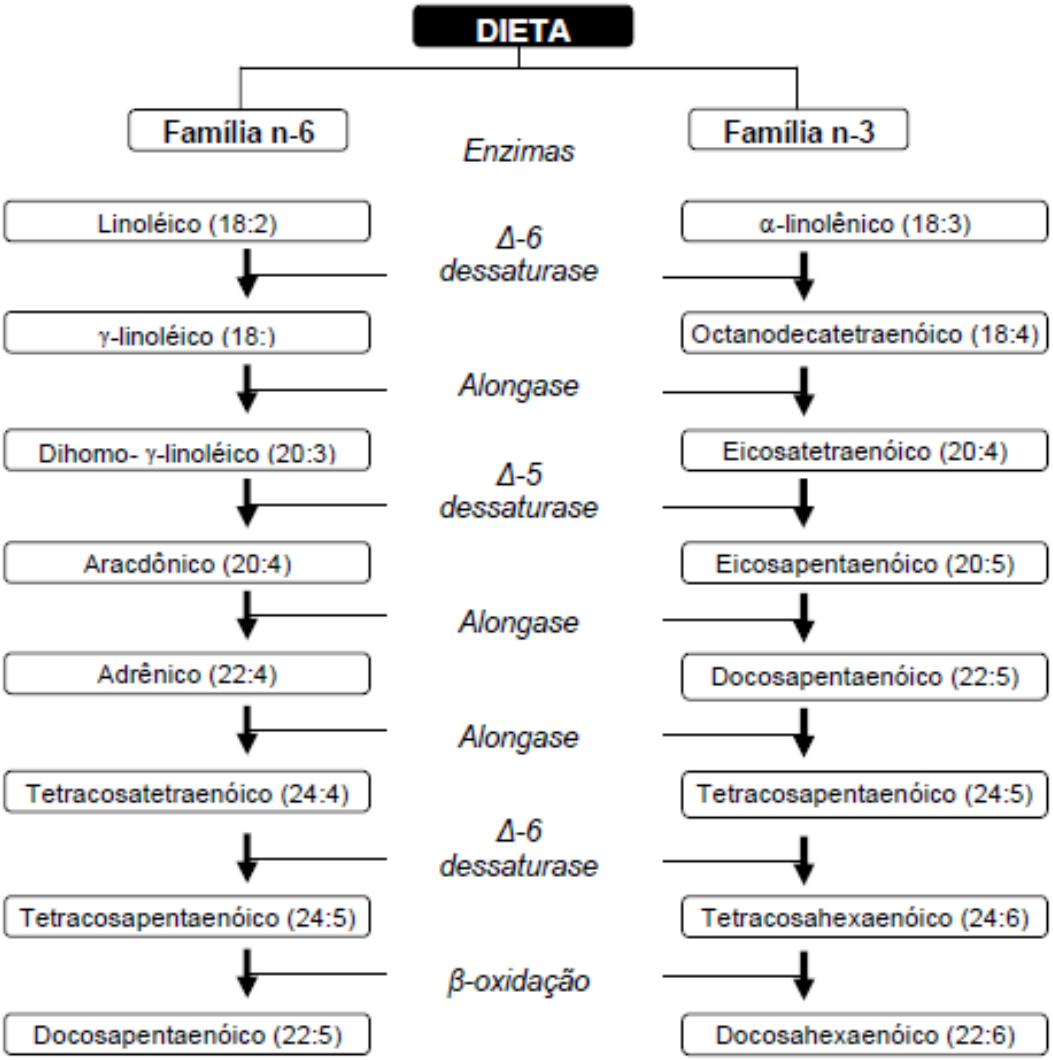


**Figura 1. Etapas bioquímicas para síntese dos ácidos graxos.**

**Fonte:** LAURITZEN et al., (2001).

O encéfalo possui altas concentrações de lipídeos complexos, os quais determinam propriedades estruturais e funcionais das membranas das células e das organelas (FAROOQUI *et al*., 2000; DAS, 2003). O DHA e o AA são os AGPI's mais importantes nas membranas neuronais, mas geralmente o DHA está em maior concentração (BROADHURST *et al*., 2002). O DHA tem um papel importante no metabolismo encefálico, influenciando o desempenho cognitivo, a acuidade visual, metabolismo de neurotransmissores e desenvolvimento neural (JIMÉNEZ *et al*., 1997; CHALON *et al*., 2001).

Uma deficiência em longo prazo de ALA leva a mudança na composição dos ácidos graxos do encéfalo, causando perda de habilidades de aprendizagem (INNIS, 2000) e durante o desenvolvimento neural pode ser responsável pelo comprometimento da memória ou por doenças neurológicas na vida adulta (MORIGUCHI *et al*., 2000; YOUDIM *et al*., 2000).

Se o perfil lipídico da dieta não é adequado para manter as necessidades cerebrais, as células irão substituir o DHA por outros AGPIs, causando alterações nas funções cerebrais (FERNSTROM, 1999; CARLSON, 2001). Logo, o balanço correto entre ômega-3 (ALA,18:3) e ômega-6 (LA, 18:2) dentre os fosfolipídios é, em última análise, essencial para o funcionamento neural normal.

O envolvimento dos ácidos graxos poli-insaturados na epidemiologia da depressão tem sido sugerido por diversos autores (HIBBELN, 1998; de VRIESE *et al.,* 2003). Evidências na literatura indicam uma estreita relação entre a ingestão de AGPIs ω-3 derivados de peixe e depressão (COLANGELO *et al*., 2009; GRENYER *et al.*, 2007), e estudos epidemiológicos têm demonstrado que uma dieta rica em ácidos graxos poderia auxiliar na prevenção da depressão (BOURRE, 2007; MORRIS, 2005; HIBBELN, 2002).

Ao se avaliar o efeito da suplementação com ácidos graxos ω-3 sobre déficits cognitivos e desordens psiquiátricas, estudos mostram que pacientes depressivos apresentam diminuição de DHA nas membranas de eritrócitos, o que indica o envolvimento destes ácidos graxos com o possível surgimento da depressão (SU *et al*., 2003; LEVANT, 2006). Outros autores também sugerem que a depressão clínica possa estar inversamente relacionada com a concentração de AGPIs ω-3 no sangue (SIDHU, 2003; SHIEPERS *et al*., 2009). Vários estudos observacionais também indicam que a depressão clínica, a

depressão pós-parto e o estado depressivo podem estar acompanhados por baixos níveis de AGPIs ω-3 medidos em amostras de sangue ou de tecido adiposo. Estes mesmos pesquisadores sugerem ainda a interação entre baixos níveis de ácidos graxos da família ômega-3 e aumento dos índices de depressão pós-parto, e que a suplementação alimentar com ômega-3 (mais especificamente o DHA) durante a gestação, poderia atuar como método profilático na depressão pós-parto (BODNAR e WISNER, 2005; de VRIESE *et al.*, 2002; REES *et al*., 2009). Em conjunto, estes resultados suportam a hipotética relação entre AGPIs ω-3 e depressão, e é provável que baixas concentrações de AGPIs ω-3, causadas principalmente por ingestão reduzida dos mesmos, contribua, de alguma maneira, para a susceptibilidade à depressão (SONTROP e CAMPBELL, 2006).

Diversos estudos têm demonstrado diferenças nos níveis de ácidos graxos da família ω-3 em pacientes com transtornos psiquiátricos quando comparados a grupos controle, principalmente nos níveis de DHA (GREEN, 2006). Uma das hipóteses para o efeito dos AGPIs ω-3 na depressão é a de que os níveis de DHA possam normalizar as alterações na microestrutura das membranas e dos neurotransmissores nos pacientes com depressão (PARKER, 2006). O metabolismo de serotonina e dopamina parece ser afetado pelo DHA, assim como pela razão entre os ácidos graxos ω-3 e ω-6 (WILLIAMS *et al.*, 2006, ROSS, 2007). Talvez por aumentarem a fluidez das membranas neuronais, altas concentrações de DHA cerebral melhoram a sensibilidade dos receptores de serotonina (BODNAR e WISNER, 2005).

Alguns pesquisadores acreditam que a redução nas concentrações de AGPIs ω-3 possa ser consequência de mudanças significativas na dieta, as quais ocorreram juntamente com o processo de industrialização nos últimos 150 anos (SIMOPOULOS, 2006). Neste intervalo de tempo houve grande aumento na ingestão de gorduras saturadas, óleos vegetais ricos em ácido linoléico (ω-6) e redução dos alimentos ricos em AGPIs ω -3, tais como peixes de águas frias e profundas (como a sardinha e o salmão) e vegetais de coloração verde escuro.

## 1.3 ÁCIDOS GRAXOS ω -3 x DEPRESSÃO, GESTAÇÃO e LACTAÇÃO

A ingestão de DHA e EPA durante os períodos críticos do desenvolvimento do sistema nervoso central (SNC) é essencial para a maturação cortical e mielinização neuronal, representando um risco diminuído para o desenvolvimento de psicopatologias em jovens adultos (McNAMARA e CARLSON, 2006; EILANDER *et al*., 2007; BORSONELO e GALDUROZ, 2008). Neste sentido, diversos trabalhos em modelos animais têm se dedicado ao estudo do efeito da suplementação com óleo de peixe, como por exemplo, aqueles que utilizam a suplementação em ratos nas fases precoces do desenvolvimento do SNC, especialmente durante a gestação e a lactação ou também no período do pós-desmame até a idade adulta do animal (90 dias em ratos). Estes estudos evidenciaram que ratos suplementados com óleo de peixe em ambas as fases (gestação e lactação ou pós-desmame até a vida adulta), quando submetidos ao teste da natação forçada (uma das principais ferramentas experimentais para a avaliação da atividade antidepressiva de drogas), exibiram um comportamento tipo antidepressivo, representado por um menor tempo de imobilidade (FERRAZ *et al*., 2008). O teste da natação forçada tem sido usado como modelo animal de depressão, ou seja, o animal, ao aumentar o tempo de imobilidade neste teste, expressa um comportamento depressivo. Drogas antidepressivas reduzem o tempo de imobilidade expresso pelos animais no dia do teste (PORSOLT *et al.*, 1977). Posteriormente, o teste da natação forçada foi substituído pelo teste da natação forçada modificado (adaptado de PORSOLT et *al*., 1977). No teste da natação forçada modificado, a análise dos comportamentos de natação e escalada nos sugere um envolvimento serotoninérgico ou noradrenérgico, respectivamente, do composto que está sendo estudado (CONSONI *et al.*, 2006). Ferraz *et al*., (2011) mostraram que o tratamento com óleo de peixe é capaz de causar aumento do comportamento de natação em ratos, sugerindo que o efeito antidepressivo deste óleo envolva principalmente o sistema serotoninérgico.

Um trabalho recente indica o envolvimento dos receptores serotoninérgicos, particularmente os do subtipo 5-$HT_{1A,}$ nos efeitos antidepressivos observados com a suplementação com óleo de peixe durante a gestação e lactação. Neste mesmo estudo, os resultados mostraram que o

tratamento com Way100135 (um antagonista 5-$HT_{1A}$) foi capaz de reverter o efeito antidepressivo produzido pela suplementação com óleo de peixe (Vines et al., 2012). Estes dados corroboram a idéia de que a suplementação com óleo de peixe pode produzir efeito antidepressivo mediado pelo aumento na neurotransmissão serotoninérgica, particularmente no hipocampo.

Entretanto, no estudo citado acima o Way 100135 foi administrado sistemicamente, possivelmente bloqueando tanto os receptores 5-$HT_{1A}$ pós-sinapticos, quanto os auto-receptores (pré-sinápticos) presentes nos núcleos da rafe. A partir dos resultados encontrados neste trabalho, pode-se esperar que a suplementação crônica com óleo de peixe aumente a atividade dos receptores 5-$HT_{1A}$ no hipocampo e/ou diminua esta atividade nos núcleos da rafe.

Portanto, decidimos então investigar a hipótese do envolvimento dos receptores 5-$HT_{1A}$ pós-sinápticos hipocampais. Por isso, escolhemos administrar um antagonista mais seletivo para o receptor 5-$HT_{1A,}$ o Way 100635, diretamente no hipocampo de animais suplementados (ou não) com óleo de peixe e submetidos ao teste de natação forçada modificado.

## 2. JUSTIFICATIVA

Devido a fatores como a grande prevalência da depressão afetando parcela significativa da população mundial, latência temporal longa para o aparecimento dos efeitos terapêuticos dos antidepressivos, além de seus efeitos colaterais e risco de suicídio nas primeiras semanas de tratamento, justifica-se a necessidade de pesquisa por compostos que possam ajudar no tratamento da depressão, potencializando o efeito dos antidepressivos ou ainda melhorando o perfil de efeitos colaterais por esses apresentados. Entre esses compostos, as gorduras poli-insaturadas da família ômega-3 (ω-3), que apresentam um efeito antidepressivo associado ao sistema serotoninérgico.

Portanto, este trabalho dedicou-se a ampliar a investigação do envolvimento serotoninérgico no efeito antidepressivo da suplementação com óleo de peixe durante as fases de gestação e lactação. Sendo assim, a investigação teve como alvo o estudo dos receptores 5-$HT_{1A,}$ pós-sinápticos hipocampais.

# 3. OBJETIVOS

## 3.1 OBJETIVO GERAL

Investigar o envolvimento do sistema serotoninérgico (receptores $5HT_{1A}$ pós-sinápticos) sobre o efeito antidepressivo da suplementação com óleo de peixe durante as fases de gestação e lactação.

## 3.2 OBJETIVOS ESPECÍFICOS

Investigar a função dos receptores $5\text{-}HT_{1A}$ localizados pós-sinapticamente no comportamento antidepressivo provocado pelo óleo de peixe no teste de natação forçada modificado sob efeito do antagonista $5\text{-}HT_{1A}$ Way 100635;

Dosar os níveis hipocampais de serotonina e seu metabólito em ratos suplementados ou não com óleo de peixe sob efeito do antagonista $5\text{-}HT_{1A}$ Way 100635 injetado no hipocampo;

Avaliar, através do teste do campo aberto, possíveis implicações comportamentais do citado tratamento sobre a motricidade dos animais;

Verificar a expressão dos receptores $5\text{-}HT_{1A}$ no hipocampo através da técnica de Western Blott.

4. MANUSCRITO

# THE ANTIDEPRESSANT FISH OIL EFFECT: A POSSIBLE ROLE OF HIPPOCAMPAL 5-HT$_{1A}$ POST-SYNAPTIC RECEPTOR ?

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Keywords: Polyunsaturated fatty acids, Fish Oil, Depression, BDNF, Serotonin, 5HT$_{1A}$ receptors.

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**Abstract**

The physiopatology of depression is not completely known, but some studies point to serotonergic dysfunction as possible cause. The supplementation with fish oil rich in docosahexaenoic acid (DHA) and eiccosapentaenoic acid (EPA) during critical periods of development produces antidepressant effects by an increase in serotonergic neurotransmission, particularly in the hippocampus. Considering this antidepressant effect promoted by fish oil supplementation, that is mediated by activation of hippocampal $5HT_{1A}$ receptors, the present study was devoted to investigate the effects of fish oil supplementation (from conception to weaning) on $5HT_{1A}$ post-synaptic receptors blocking these receptors with the selective antagonist WAY100635 administrated directly in the hippocampus of 3-month old rats. Female rats were supplemented with FO during habituation, mating, gestation and lactation. The adult offspring was subjected to behavioral tests and neurochemical analysis was carried out in order to quantify hippocampal serotonin (5-HT) and its metabolite, 5-hydroxyindoleacetic Acid (5-HIAA). Also, the $5HT_{1A}$ receptors expression in hippocampus was measured by western blot analysis. In the modified forced swim test the results demonstrated that fish oil-supplemented offspring displayed less depressive-like behaviors reflected by decreased immobility and increased swimming, meanwhile the analysis of western blotting reveled that there is no difference in expression of hippocampal $5\text{-}HT_{1A}$ receptors among the groups. Concerning to monoamines analysis the results showed an increased 5-HT hippocampal levels. In summary, the present findings confirm the antidepressant effects of ω-3 PUFA supplementation, likely related to increased hippocampal serotonergic neurotransmission. Nevertheless, more studies are necessary to understand the serotonergic effect considering pre/pos-synaptic $5\text{-}HT_{1A}$ receptors

## 1. Introduction

Depression is a severe psychiatric syndrome with high prevalence and socioeconomic impact (Andlin-Sobocki et al., 2005; Kessler et al., 2005; Smith 2011).

Abnormalities in serotonergic function have been believed to be a common factor in several mental illnesses since the 1950's (Woolley & Shaw, 1954, Nestler et al., 2002). Studies have shown reduced cerebrospinal fluid concentrations of the serotonin major metabolite – 5 – hydroxyindolacetic acid (5-HIAA) – in drug-free depressed patients (Asberg et al., 1976; Roy et al., 1989) as well as reduced concentrations of serotonin (5-HT) and 5-HIAA in the postmortem brain tissue of depressed and/or suicidal patients (Carlson, 2002; Hoyer et al., 1994; Savitz et al., 2009; Klein, 2010; Albert et al., 2011).

From the 5-$HT_1$ family, the 5-$HT_{1A}$ subtype plays a key role in the pathogenesis of depression, and not only contributes to the dynamic modulation of serotonergic activity impacting diverse functions such as cognition and emotion, but is thought to play a crucial role in the neuronal migration, neurite outgrowth and synapse formation inherent to the neuro-developemental process (Whitaker-Azmitia et al, 1996; Barnes & Sharp, 1999; Savitz et al., 2009).

Postmortem and neuroimaging studies suggest an increased density of the 5-$HT_{1A}$ autoreceptors in major depressive patients compared with control subjects (for review, see Artigas, 2012). So, evidence from human and rodent studies suggests that 5-$HT_{1A}$ receptors are implicated in a variety of phenotypes, among them affective disorders (for review, see Altieri et al.,2013).

The chronic antidepressant treatment, with the selective serotonin reuptake inhibitors (SSRIs) for example, leads to a serotonin-induced desensitization of raphe 5-$HT_{1A}$ inhibitory autoreceptors, increasing the amount of serotonin released in postsynaptic areas, such as hippocampus and prefrontal cortex (Blier & de Montigny, 1994; Hervas et al., 2001; Albert and Le François, 2010).

It has been postulated that deficiency of omega-3 polyunsaturated fatty acids (ω-3 PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), could play a role in the pathophysiology of a wide range of

psychiatric disorders as mood and hyperactivity disorders (McNamara and Carlson, 2006; Borsonelo and Galduroz, 2008; Colangelo et al., 2009). Chronic ω-3 PUFA dietary deficiency reduces central serotonin synthesis, suggesting that provision of essential ω-3 fatty acids in early stages of cerebral development affects brain function permanently (Kodas et al., 2002; McNamara et al., 2009).

According to previous results from our laboratory the fish oil supplementation during pregnancy and lactation promoted an antidepressant effect that is possibly related to a concomitant induction of brain derived neurotrophic factor (BDNF) expression and increased hippocampal and cortical serotonin neurotransmission (Vines, et al. 2012). In addition, the results also showed that blockade of $5HT_{1A}$ receptors with WAY 100135 reversed the antidepressant effects promoted by fish oil.

Considering this antidepressant effect, that is mediated by activation of hippocampal $5HT_{1A}$ receptors, possibly altering the number and/or sensitivity of $5HT_{1A}$ post-synaptic receptors, the present study was devoted to investigate these receptors in hippocampus, an area involved with etiology of depression. To this end, we investigated the effects of fish oil supplementation (from conception to weaning) on $5HT_{1A}$ post-synaptic receptors blocking these receptors with the selective antagonist WAY100635 administrated directly in the hippocampi of 3-month old rats and evaluated the behavior of these animals in the open field test (OF) and modified forced swim test (MFST). After the behavioral tests, neurochemical analysis was carried out in 102 day-old offspring in order to quantify hippocampal serotonin (5-HT) and its metabolite, 5-hydroxyindoleacetic Acid (5-HIAA). Also, the $5HT_{1A}$ receptors expression in hippocampus was measured by western blot analysis.

## 2. Methods

### *2.1. Animals*

Male and female Wistar rats were kept under a 12 h light /12 h dark cycle (lights on at 07:00 am) in a controlled temperature room (21 ± 2 °C), with food (rat chow, NuvitalNuvilab CR1- NuvitalNutrientes S/A, Colombo, Paraná,

Brazil) and water *ad libitum*. All experiments were approved by the Animal Experimentation Ethics Committee of the Universidade Federal do Paraná (# 512) and were performed according to the Guide for the Care and Use of Experimental Animals (Canadian Council on Animal Care).

### *2.2 Experimental Design*

For this experiment, 10 week-old virgin female Wistar rats were used as matrices to obtain the male offspring for subsequent tests. The female rats were randomly distributed in 2 experimental groups: control group (C, n=20) and fish oil supplemented group (FO, n=20). Females in the FO group were fed with regular chow, and a daily supplementation of 3.0 g/kg of fish oil containing 12% of EPA and 18% of DHA (kindly donated by Laboratório Herbarium Botânico S/A, Colombo, Paraná, Brazil), administered orally (by gavage), whereas those in the C group received only the regular chow diet and the same volume of water as previously described, also by gavage. The fatty acid composition of chow diet was the same as that presented in a recent report from our group (Ferraz et al. 2011). The FO group was supplemented during an adaptation period (14 days), mating (8 days), pregnancy (21 days) and nursing (21 days). The adaptation period was used to avoid possible stress generated by the gavage method. The males of the offspring were kept in the animal facility, under the same environmental conditions as described above, until adulthood (90 days), and was not subjected to further supplementation by any means. To avoid intragroup interference, only two pups of each matrice were used, being distributed among sham or operated group, for both supplemented as for control group. These animals were randomly redistributed in another two groups: animals that received WAY100635 infusion in the Ventral Hippocampus and the group that received saline in the same structure, generating four groups: Control (CS: non-supplemented, with infusion of saline, n=15), Control Fish Oil (FOS: supplemented with fish oil, infusion of saline, n=15), Control Way (CW: non-supplemented, with infusion of the drug WAY100635), Fish Oil Way (FOW: supplemented with fish oil, infusion of way100635, n=15). All groups were assessed for depressive-like and motor behaviors by the Modified Forced Swim Test and Open Field, respectively, after 10 minutes of the bilateral

infusion of WAY100635. After the behavioral tests, rats were decapitated and the hippocampi were dissected, to investigate long-term effects of ω-3 PUFA on monoamines content and their metabolites by High-performance liquid chromatography (HPLC), and to verify the expression of the $5HT_{1A}$ receptor by Western Blot. For more details of the complete experimental design see Figure 1.

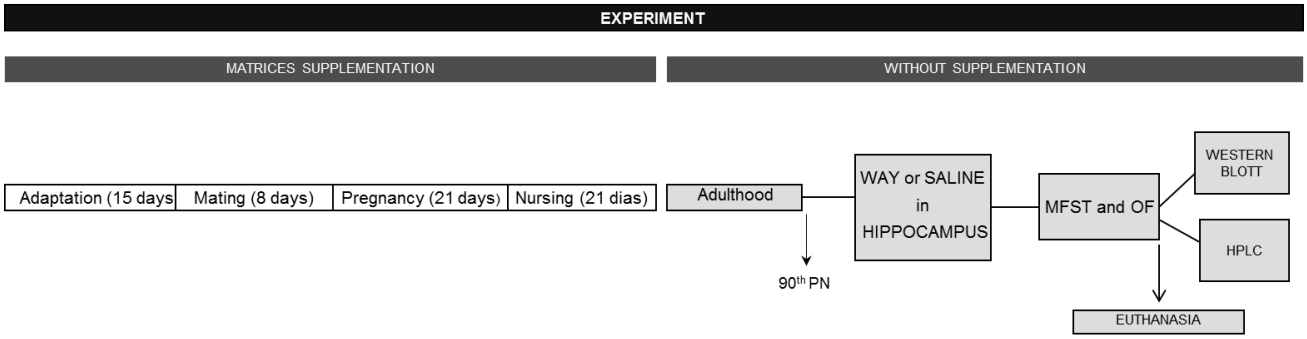


**Figure 1 - Time line showing experimental design.** WAY: WAY100635; MFST: Modified Forced Swimming Test; OF: Open Field; HPLC: High-performance liquid chromatography.

### *2.3 Surgery and Intra-hippocampal administration*

Animals were previously anesthetized with Xilazine (60 mg/kg, i.m.; *Syntec do Brasil Ltda*) and Ketamine (4mg/kg, i.m.; *Syntec do Brasil Ltda*) and fixed in a stereotaxic frame. All groups received bilateral guide cannula implanted 2.0 mm above the ventral hippocampus according to the following coordinates: anteroposterior (AP): −5.2 mm from bregma; mediolateral (ML): ±5.0 mm from midline; dorsoventral (DV): −5.0 mm from skull (Paxinos and Watson, 2005; Peleg-Raibstein et al., 2005). A stiletto inside the guide cannulas prevented its obstruction.

Seven days after the cannula implantation, the animals were manipulated in order to perform the WAY 100635 ($5HT_{1A}$ antagonist) infusion. This procedure was performed with a 27-gauge stainless injection needle

introduced through the guide cannula for the administration. For the drug's injection a microsyringe (Hamilton, USA) connected to needle through a polyethylene catheter (PE 10) was used. The control of the volume and flow of the injections were made by using an electronic pump (Insight BI 2000) at the rate of 0.5 µL/min for 1 min, followed by 2 min with the needle in the injection site to avoid reflux. During the infusion procedure the animal was free to move around inside the polypropylene home cage.

When the brains were removed, it was verified if the guide cannulas were exactly in the coordinates described above, at the Ventral Hippocampus.

### *2.4 Drugs*

The selective $5HT_{1A}$ antagonist WAY100635 (60 nmol /0.5µL; Santa Cruz, USA) was dissolved in sterile isotonic saline, and administered in a dose of 0.5 µL in each hippocampus 10 minutes before the behavioral tests (Joca et al., 2003).

### *2.5 Open Field Test*

The open-field (OF) test was performed in a circular arena (1 m diameter) limited by a 40 cm-high wall and illuminated by four 60 W lamps (Broadhurst, 1960). The arena's floor was black, with no apparent divisions. The subjects were individually placed in the central area, and allowed to freely explore the arena for 5 minutes. During this period, the Smart® Junior System (Panlab, Harvard Apparatus, Spain) was used to measure the subject's locomotion behavior, by analyzing distance run and velocity, and time spent in each area of the OF (central, middle and periphery). The open-field was cleaned with a 5% water-ethanol solution before each behavioral testing to eliminate possible bias due to odors left by previous rats.

### *2.6 Modified Forced Swim Test*

This is a modified version of the Porsolt test and was carried out as previously described (Cryan et al., 2002). Briefly, rats were placed, individually, in an opaque plastic cylinder (diameter 20 cm; height 50 cm) containing water up to 30 cm (24 ±1ºC); on day 1 the rats remained in the cylinder for 15 min (training session) and 24 h later they were placed back and tested for 5 min (test session). The test session was video recorded via a camera positioned above the cylinder for subsequent analysis. The behaviors assessed during the test session were: immobility (when the rat stopped all active behaviors and remained floating in the water with minimal movements, with its head just above the water), swimming (movements throughout the swim cylinder) and climbing (upward directed movements of the forepaws along the cylinder walls). During the 5 min session, the predominant behavior within each 5-s interval was recorded. The water was changed and the cylinder rinsed with clean water after each session. After the training and the test sessions, the animals were dried and placed in their home cages.

### *2.7 Western Blot*

The hippocampi were homogenized in lysis buffer (1% Triton X-100; 0.5% sodium deoxycholate; 100 mM Tris–HCl, pH 8.3; 150 mM NaCl; 10 mM EDTA; 0.1% SDS; 10% glycerol; 1% and protease and phosphatase inhibitors cocktails[Halt™, Thermo Scientific™]). Part of homogenate was 10x diluted in saline and assayed to total protein estimation (Bicinchoninic acid method, Micro-BCA® Pierce Chemical™, USA). The samples were diluted in equal volume of sample buffer (4% SDS, 125 mM Tris HCl pH 6.8, 20% Glycerol, 0.8 mg/ml bromophenol blue and 5% β-mercaptoetanol) and adjusted to 100µg/ml protein concentration. The samples were boiled to 95$^{o}$C for 5 min., and then loaded on 10% SDS-polyacrylamide gels, separated by electrophoresis and then transferred to nitrocellulose membranes (Amersham GE, Little Chalfont, UK). The membranes were blocked with 5% nonfat milk for 1 h and then incubated with primary antibodies overnight (at 4$^{o}$C, 1:500, 5HT-1A rabbit

polyclonal antibody; Santa Cruz, USA). After 5 washes of 5 min. with TBS (plus 1% Tweem 20), membranes were incubated with secondary antibody for 45 min with Alexa-680-conjugated anti-rabbit IgG (1:10,000, Invitrogen, Carlsbad, CA, USA). Again, after 5 washes of 5 min (TBS and 1% Tween20), digital images of the membranes were acquired and quantified (arbitrary unities) using the Odyssey Infrared Image System (LI-COR, Baltimore, MD, USA). Control and treatment values were corrected for blank values, normalized to their respective β-actin band (37KDa) intensity.

*2.8 Neurochemical quantification (HPLC)*

The animals were decapitated and their brains were removed and the hippocampi were dissected on a cold surface. The tissue samples were weighed individually and homogenized by sonication in 500 mL of extraction solution (0.1 M perchloric acid containing 0.4 mM sodium metabissulfite and 0.2 mMethylenediaminetetraacetic acid). The homogenates were centrifuged at 20,000 x *g* for 10 min, then filtered through 0.22mm membrane and stored at -80°C for further analysis. Precipitates were dissolved in 0.1 N NaOH and assayed for protein estimation (Bicinchoninic acid method, Pierce Chemical, Rockford, IL). Supernatants were submitted to fast isocratic separation through a C18 HPLC reversed-phase column system (Spheri-5, C18, ODS, 5 mm, 25 cm, 4.6 mm column; linked to a New-Guard Cartridge Column, RP-18,7 mm pre-column; Perkin Elmer Brownlee Columns, Shelton, CT) and electrochemically detected using an amperometric detector (L-ECD-6A, Shimadzu, Japan), by oxidation on glass carbon electrode at +850 mV in relation to an Ag-AgCl reference electrode (Machado et al. 2008). The mobile phase consisted of 0.163 M citric acid, 0.06 M sodium phosphate dibasic anhydrous, 0.69 mMoctyl sodium sulfate, 12 mMethylenediaminetetraacetic acid, acetonitrile 4%, tetrahydrofuran 1.7% and orthophosphoric acid sufficient to bring the pH to 2.85, diluted in double distilled water. The mobile phase was filtered through a 0.2 mm filter membrane, degassed under vacuum and delivered at a flow rate of 1.2 mL/min (HITACHI Pump System L-7100). Each sample was analyzed in duplicate for concentrations of serotonin (5-HT) and its

non-conjugated metabolite 5-hydroxyindoleacetic acid (5-HIAA). The recovery of the analytes was determined by adding a fixed concentration of internal standard DHBA (dihydroxybenzylamine) before tissue homogenization. An automatic injector (HITACHI L-7250, cut injection method) was utilized to improve the reproducibility of injections. All standards and salts were purchased from Sigma (USA) and the solvents (HPLC grade) were purchased from J.T. Baker (USA).

### *2.9. Statistical analysis*

Differences among groups in the behavioral and biochemical tests were analyzed by two-way analysis of variance (ANOVA) – with supplementation as the between-subjects factor and Infusion of WAY100635 as the within-subjects factor - followed by Duncan's test. The results are reported as mean ± S.E.M. Differences were considered statistically significant when $p \leq 0.05$.

## 3. Results

### 3.1 Open Field Test

Figure 2 shows total distance (A), central distance (B), peripheral distance (C), time in the center (D) and time in periphery (E) in the Open Field Test. All the measures were not significant between any groups. Two-way ANOVA did not show any difference for supplementation in total distance [F(1,40) = 0.42; n.s], central distance [F(1,40) = 0.07; n.s], peripheral distance [F(1,40) = 0.47; n.s], time in center of arena [F(1,40) = 0.41 ; n.s] and time in the periphery [F(1,40) = 0.41; n.s], for WAY infusion: in total distance [F(1,40) = 2.18; n.s], central distance [F(1,40) = 0.59; n.s], peripheral distance [F(1,40) = 1.68; n.s], time in center of arena [F(1,40) = 0.44 ; n.s] and time in the periphery [F(1,40) = 0.44; n.s], or for interaction between these two factors in the same measures respectively: [F(1,40) = 0.20; n.s], [F(1,40) = 0.61.; n.s], [F(1,40) = 0.34; n.s], [F(1,40) = 1.59; n.s] and [F(1,40) = 1.59; n.s].

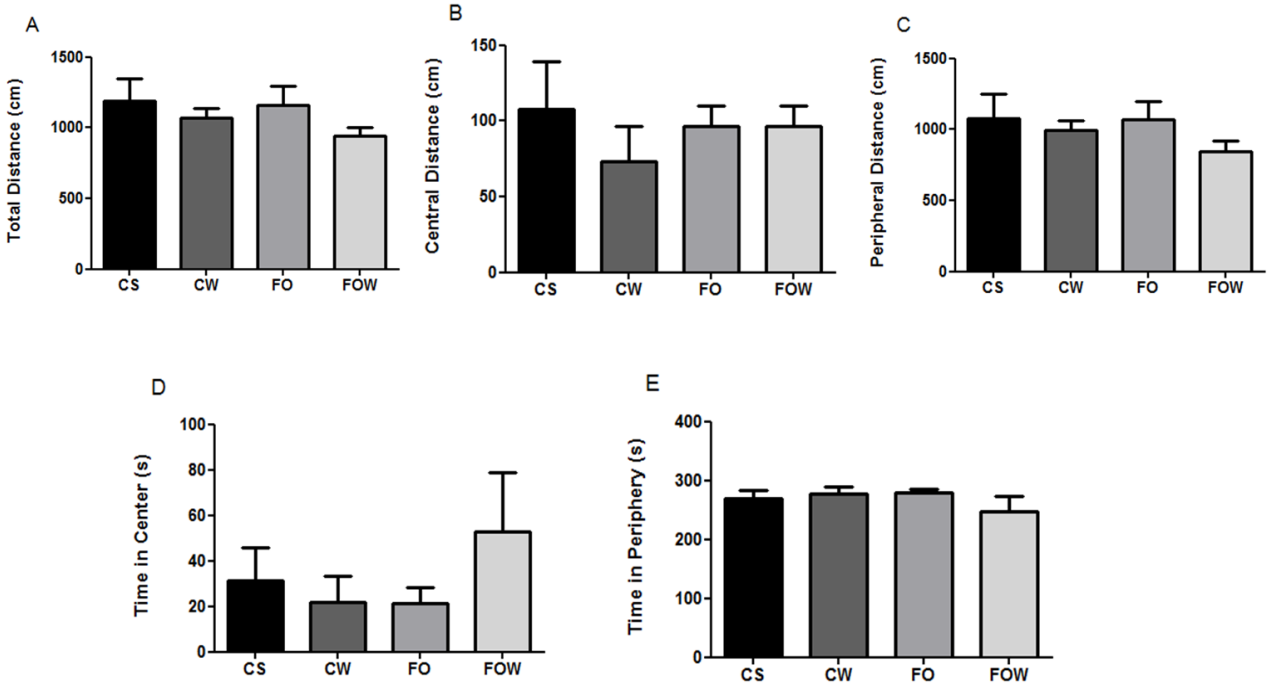


**Figure 2 – Open Field Test. (A) Total distance; (B) Central distance; (C) Peripheral distance; (D) Time in center and (E) Time in periphery.** Control-Saline (CS) n = 15; Control-Way (CW) n = 15; Fish Oil (FO) n = 14; Fish oil-Way (FOW) n = 15. Two-way ANOVA. Values are expressed as mean ± S.E.M

3.2 Modified Forced Swim Test

In this test we analyzed three parameters: immobility, swimming and climbing (Fig. 3). Regarding swimming (Fig. 3A), there was an effect of the fish oil supplementation [(F( 1,55) =38.00; p≤0,0001], an effect of way treatment [F(1,55) =14.98; p≤0,0001] and an interaction between these factors [F(1,55)=12.87; p≤0,0001]. Post hoc test showed that the fish oil group (FO) increased the swimming frequency compared to the other groups (p≤ 0,001). WAY treatment prevented the effect of fish oil, presenting similar frequency compared to the non-supplemented groups.

In the Immobility parameter (Fig. 3B), there was an effect of supplementation [F(1,55) = 23,02; p≤0,0001], an effect of way treatment [F(1,55) = 4.33; p≤0,05] and an interaction between these factors [F(1,55) = 5.49; p≤0,05]. Post hoc test showed that the fish oil group decreased frequency of immobility (p≤0,05), while the supplemented group that received WAY100635 infusion (FOW) increased this parameter reaching similar results to the control groups.

Differences in the climbing behavior were not observed among any group. There were no effects of supplementation, WAY infusion or interaction between these factors [F (1,55) = 2.26, n.s.]; [F (1,55) = 0.00014, n.s.]; [F(1,55) = 0.16, n.s.]; respectively (Fig. 3C).

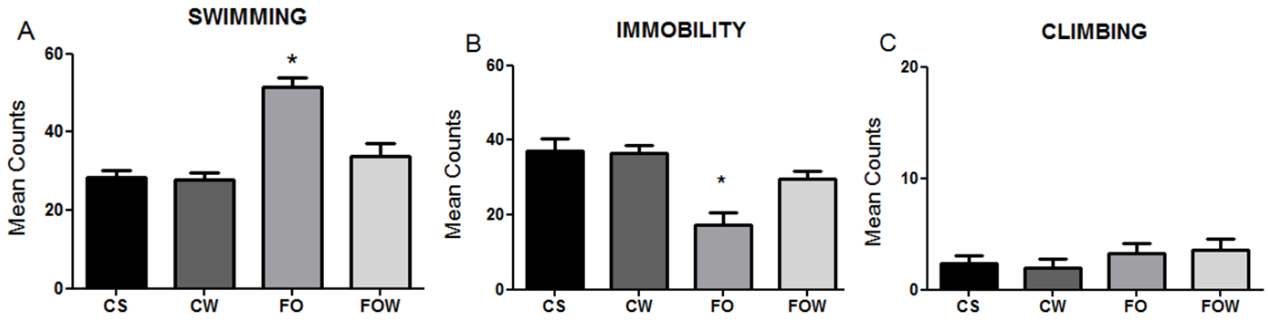


**Figure 3 – Results of Modified Forced Swim test. (A) Immobility, (B) Swimming and (C) Climbing.** Control-Saline (CS) n = 15; Control-Way (CW) n = 15; Fish Oil (FO) n = 14; Fish oil-Way (FOW) n = 15. Two-way ANOVA followed by Duncan's post hoc test. Values are expressed as mean ± S.E.M. *p<0,0001 comparing FO with all other groups.

3.3 Western Blot

Figure 4 shows the Western Blot analysis in the Hippocampus of adult animals. Two-way ANOVA did not revealed any differences in density of $5HT_{1A}$ receptors for the Fish Oil group [F(1,23) = 0.005 ;n.s], WAY [F(1,23) = 1.77; n.s] and no interaction between these two factors [F(1,23) = 1.71; n.s], in this brain structure.

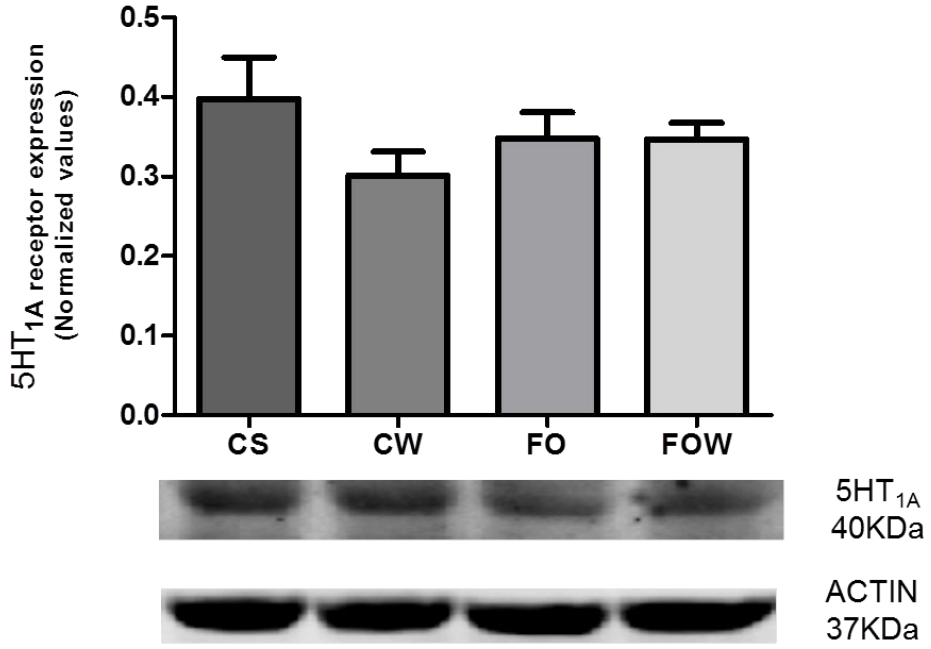


**Figure 4 – Western Blotting analysis: effect of fish oil supplementation on $5HT_{1A}$ receptor in Hippocampus.** Control-Saline (CS) n = 7; Control-Way (CW) n = 5; Fish Oil (FO) n = 7; Fish oil-Way (FOW) n = 8. . Two-way ANOVA. Values are expressed as mean ± S.E.M.

3.4 Neurochemichal data *(HPLC)*

Figure 5 shows neurochemical quantification in the hippocampus of adult animals. Analysis of hippocampal 5-HT content is depicted in figure 5 A; two-way ANOVA revealed effect of fish oil supplementation [F (1,26) = 26,6; p<0,001]. Duncan's post hoc test showed that fish oil increased 5-HT in both supplemented groups: the one that received infusion of saline (FO) (p<0,005) and the one that received infusion of WAY100625 (FOW) (p<0,005). Figures 5 B and C shows the 5-HIAA content and the Turnover (5-HIAA/5-HT) respectively; two-way ANOVA did not revealed any differences between the groups, for 5-HIAA [F(1,26) = 0.68; n.s] and Turnover [F(1,26) = 0.13; n.s].

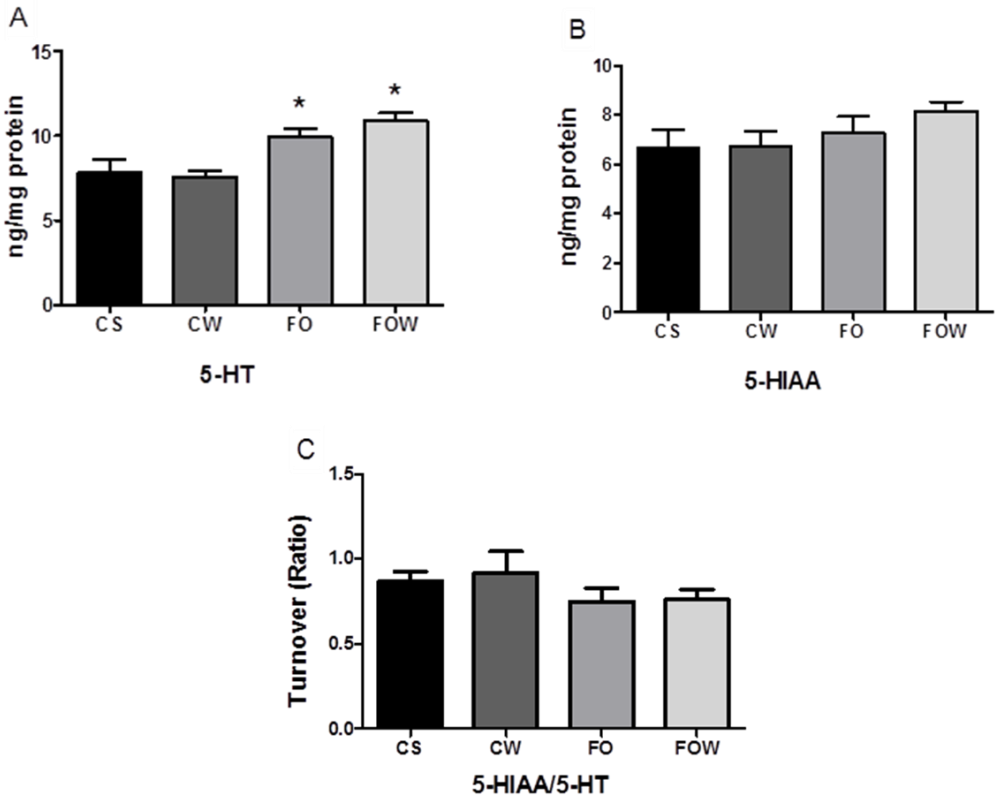


**Figure 5 – Hippocampal neurochemical data. (A) Neurotransmitter (5-HT); (B) Metabolite (5-HIAA) and (C) Turnover ratios.** Control-Saline (CS) n = 7; Control-Way (CW) n = 8; Fish Oil (FO) n = 7; Fish oil-Way (FOW) n = 8. Two-way ANOVA followed by Duncan's post hoc test. Values are expressed as mean ± S.E.M. *p< 0,005

## 4. Discussion

In a previous study, we hypothesized that blockade of the $5HT_{1A}$ receptor would prevent the antidepressant effects of fish oil supplementation during pregnancy and lactation periods, since the use of WAY 100135, a $5HT_{1A}$ antagonist and also a $5HT_{1B}$ and $5HT_{1D}$ parcial agonist reversed the fish oil effects (Vines et al., 2012).

The systemic administration of WAY 100135 presumably antagonizes both post-synaptic $5HT_{1A}$ receptors located in neurons in the hippocampus, septum, amygdale and corticolimbic areas and $5HT_{1A}$ autoreceptors present in neurons of the midbrain raphe nuclei. So, a possibly explanation for the results described above was that chronic fish oil supplementation could increase $5HT_{1A}$ receptor activity within the hippocampus and decrease it in the raphe nucleus.

So, the present study was performed to investigate the impact of fish oil supplementation during pre- and postnatal brain developmental periods on hippocampal 5-$HT_{1A}$ post-synaptic receptors and investigate the role of these receptors on antidepressant fish oil effect. For that, we administrated WAY 100635, a selective $5HT_{1A}$ antagonist directly in the ventral hippocampus of 3-month rats and evaluated the behavior of these animals in the OF and MFST.

In the open field test, there was no effect of fish oil or WAY 100635 on motor and anxiety-like behaviors. Concerning to fish oil-supplemented animals, these results are in agreement with previous studies from our group, using supplementation during pregnancy and lactation or post-weaning phases (Ferraz et al., 2011; Ferraz et al., 2008; Naliwaiko et al., 2004; Vines et al., 2012). Interestingly, there was no effect of WAY 100635 administration in hippocampus *per se* on hyperactivity or anxiety-like behavior. These data together indicate the absence of modifications of motor and anxiety-like behaviors on MFST results.

In the MFST the results demonstrated that fish oil-supplemented offspring displayed less depressive-like behaviors reflected by decreased immobility and increased swimming. The hippocampal administration of WAY 100635 reversed this antidepressant fish oil effect considering the above cited parameters reproducing the results presented in a previous study in which the WAY administration was systemic (Vines et al., 2012).

Since the use of the $5HT_{1A}$ agonist, F15599, has been shown to reduce immobility parameter in forced swim test through activation of $5HT_{1A}$ heteroreceptors localized in the frontal cortex (Assie et al., 2010; Newman-Tancredi et al., 2009), these findings support the idea that besides the $5HT_{1A}$ autoreceptors desensitization following antidepressant treatment, increased $5HT_{1A}$ heteroreceptors activity is necessary to promote antidepressant efficacy (Bortolozzi et al., 2012; Ferres-Coy et al., 2012). Moreover, reduced post-synaptic $5HT_{1A}$ receptors reduce behavioral response to serotonine (see for review Albert and Le François, 2010) and when $5HT_{1A}$ heteroreceptors were selective inactivated, an increased depression-like behavior was observed in the forced swim test (see Altieri et al., 2013)

In our study the animals were supplemented with 3.0 g/kg of fish oil containing 12% of EPA and 18% of DHA for approximately 2 months, so we believe that the results obtained in these two behavioral tests indicated that they are associated to hippocampal ω-3 PUFA incorporation. Considering the lipid analysis of neuronal membranes in the hippocampus, a recent study from our laboratory (Pudell et al. 2013, not published) showed an increase of DHA hippocampal content induced by chronic fish oil supplementation (rich in DHA and EPA) during pregnancy and lactation periods in 21, but not 102 day-old offspring, when the animals were tested. As the animals were no longer supplemented after weaning, we believe that the DHA incorporated to membranes was degraded. The results obtained with treatment protocol (gestation and lactation) has been adopted in our studies and suggest a long-term antidepressant effect of fish oil (Vines et al., 2012).

The idea that direct activation of $5\text{-}HT_{1A}$ post-synaptic receptors ameliorates anti-depressive therapy was confirmed by Savitz et al., 2009. Interestingly, in our study, analysis of western blot revealed that there is no difference in expression of hippocampal $5\text{-}HT_{1A}$ receptors among the groups; although the results in MFST reveal that there is a fish oil antidepressant effect.

It is known that the addition of the selective $5\text{-}HT_{1A}$ receptor antagonist DU125530 to fluoxetine treatment did not accelerate nor enhance the efficacy of this antidepressive drug. As this antagonist has equal potency and occupancy of pre- and postsynaptic $5\text{-}HT_{1A}$ receptors, the blockade of postsynaptic receptors could be responsible for the cancellation of benefits obtained by

DU125530 binding on serotonergic presynaptic receptors (see for review Artigas, 2012). Moreover, Bhagwagar et al., (2004) showed that postsynaptic receptors are unaltered or reduced in depressed patients and this alteration is not sensitive to antidepressant treatment.

On the other hand the prolonged antidepressant treatments result in a tonic activation of 5-$HT_{1A}$ receptors in the dorsal hippocampus (CA3 region) and that activation of these receptors in the dentate gyrus increases hippocampal neurogenesis (see for review Artigas, 2012).

Besides, a positive correlation between PUFA supplementation, over-expression of brain derived neurotrophic factor (BDNF) mRNA and protein in the hippocampus and antidepressant-like effects in the MFST has been previously shown (Venna et al., 2009). Utilizing the same protocol of fish oil supplementation, Vines et al., (2012) showed that the fish oil antidepressant effect is possibly related to the induction of BDNF expression and increased 5-HT neurotransmission in the hippocampus. The link between 5-HT and BDNF expression or function has been established, as BDNF promotes the development, survival and plasticity of serotonergic neurons during hippocampal development and adulthood, and this may be related to its role in depression (Yu and Chen, 2011).

Concerning to monoamines analysis the actual results showed an increased 5-HT hippocampal levels which is in agreement with the idea of Vines et al., (2012) that the antidepressant effect of fish oil supplementation was due to increased 5-HT. One hypothesis for the explanation of serotonergic antidepressant ω-3 effect would be the reuptake inhibition of this neurotransmitter or the inhibition of monoamine oxidase enzyme, thus levels of 5-HT would remain high. Another hypothesis would be to increase the levels and / or activity of the serotonin synthesis enzyme tryptophane hidroxylase, or increase the activity and/or the expression of the 5-$HT_{1A}$ receptors into hippocampus and/or decrease the expression or activity of the raphe dorsal nucleus.

Considering these results, instead of the absence of results showing increased expression of 5-$HT_{1A}$ receptors in hippocampus, we cannot discard the possibility of increased activity of these receptors, since we do not performed binding analysis of these receptors in hippocampus. So, the complex

organization and function of $5HT_{1A}$ receptor needs to be discovered enabling pharmacological strategies for mood and anxiety disorders by targeting these receptors.

Taken together, the current results suggest that fish oil supplementation during pre- and postnatal brain developmental periods prevented depressive-like behaviors in rats adults subjected to MFST test. In summary, the present findings confirm the antidepressant effects of ω-3 PUFA supplementation, likely related to increased hippocampal serotonergic neurotransmission. Nevertheless, more studies are necessary to understand the serotonergic effect considering pre/pos-synaptic $5\text{-}HT_{1A}$ receptors.

# 5 DISCUSSÃO

Em estudo anterior de nosso laboratório, formulamos a hipótese de que o bloqueio do receptor 5-$HT_{1A}$ reverteria os efeitos antidepressivos do Óleo de Peixe, em animais suplementados durante a gestação e lactação.

Para isso, foi usado o WAY100135, uma antagonista 5-$HT_{1A,}$ mas também agonista parcial 5-$HT_{1B}$ e 5-$HT_{1D}$, revertendo os efeitos do óleo de peixe (VINES e et al., 2012).

Uma vez que a administração desta droga foi sistêmica, possivelmente bloqueou tanto os receptores 5-$HT_{1A}$ pós-sinápticos, localizados em neurônios do hipocampo, septo, amígdala e áreas córtico-límbicas, como auto-receptores presentes em neurônios dos Núcleos da Rafe.

Uma possível explicação para estes resultados é que a suplementação crônica com óleo de peixe aumentaria a atividade e/ou expressão dos receptores 5-$HT_{1A}$ no hipocampo e diminuiria nos núcleos da rafe.

Deste modo, o presente estudo investigou o impacto da suplementação com óleo de peixe durante as fases de gestação e lactação, críticas para o desenvolvimento encefálico, sobre os receptores 5-$HT_{1A}$ no hipocampo e o papel destes no efeito antidepressivo do óleo de peixe.

Para isso, administramos WAY100635, um antagonista seletivo para receptores 5-$HT_{1A,}$ diretamente no Hipocampo Ventral de ratos com 90 dias de idade, avaliando o comportamento destes animais no teste do Campo Aberto (CA) e no Teste da Natação Forçada Modificado (TNFM).

Os resultados demonstram que a prole suplementada apresentou menor comportamento depressivo, traduzido por uma menor frequência de imobilidade e maior frequência de natação, reproduzindo os resultados de nosso estudo anterior (VINES et al., 2012).

No estudo atual, a administração do WAY100635, reverteu o efeito antidepressivo do óleo de peixe no Teste da Natação Forçada Modificado, considerando os parâmetros de imobilidade e natação.

No teste do Campo Aberto, não houve efeito do óleo de peixe ou do WAY100635 na motricidade ou no comportamento de ansiedade desses animais. Estes dados estão de acordo com estudos anteriores de nosso grupo, no protocolo de suplementação durante a gestação e lactação ou pós-

desmame (FERRAZ et al., 2011; FERRAZ et al., 2008; NALIWAIKO et al., 2004; VINES et al., 2012).

Interessantemente, não houve efeito *per se* da administração do WAY no hipocampo, sobre os comportamentos de hiperatividade ou ansiedade. Em conjunto, esses dados indicam a ausência de interferências relacionadas à motricidade e ansiedade nos resultados do TNFM.

Em relação ao Teste da Natação Forçada Modificado, os resultados apontam que a prole suplementada com óleo de peixe apresentou menor comportamento depressivo, refletido por uma diminuição na frequência de imobilidade e aumento da natação.

A administração intra-hipocampal do WAY100635 reverteu este efeito antidepressivo, reproduzindo os resultados obtidos em trabalho anterior, no qual a administração, neste do WAY100135, foi sistêmica (VINES et al., 2012).

Uma vez que o uso do agonista 5-$HT_{1A,}$ F1599, mostrou-se eficaz em reduzir o parâmetro de imobilidade no teste da natação forçada, através da ativação dos heteroreceptores 5-$HT_{1A}$ localizados no córtex frontal (ASSIE et al., 2010; NEWMAN-TANCREDI et al., 2009), tais resultados suportam a idéia de que, apesar de ocorrer a dessensibilização dos autorreceptores 5-$HT_{1A,}$ resultante do tratamento crônico com antidepressivos, o aumento da atividade dos heteroreceptores 5-$HT_{1A}$ é necessária para uma maior eficácia do tratamento (BORTOLOZZI et al., 2012; FERRES-COY et al., 2012).

Além disso, uma diminuição dos receptores pós-sinápticos reduz a resposta comportamental à serotonina (para revisão ver ALBERT e LE FRANÇOIS, 2010) e quando os heteroreceptores 5-$HT_{1A}$ são seletivamente inativados, observa-se um aumento no comportamento depressivo visto no Teste da Natação Forçada (ver ALTIERI et al., 2013).

Em nosso estudo, os animais foram suplementados com 3.0g/Kg de óleo de peixe contendo 12% de Ácido Eicosapentaenóico (EPA) e 18% de Ácido Docosahexaenóico (DHA) por aproximadamente 2 meses, portanto acreditamos que esses resultados obtidos nos dois testes comportamentais citados indicam que estes estão associados à incorporação de Ômega-3 (ω-3) nas membranas neuronais do hipocampo. Considerando a análise do perfil lipídico das membranas neuronais do hipocampo, estudo recente de nosso laboratório (PUDELL et al., 2013, dados não publicados) mostrou aumento de

DHA no hipocampo, induzido pela suplementação crônica com óleo de peixe (rico em DHA e EPA) durante as fases de gestação e lactação em animais de 21, mas não em animais de 102 dias de idade, quando estes foram submetidos aos testes.

Como os animais não foram suplementados após o desmame, acreditamos que o DHA incorporado às membranas foi degradado.

Os dados obtidos com esse protocolo de tratamento (gestação e lactação) foram adotados em nossos estudos e sugerem um efeito antidepressivo de longa duração, promovido pelo óleo de peixe (VINES et al., 2012).

A idéia de que a ativação direta dos receptores 5-$HT_{1A}$ pós-sinápticos melhora a terapia antidepressiva foi confirmada por Savitz et al., 2009.

Curiosamente, em nosso estudo, a análise através da técnica de Western Blot, não mostrou diferenças entre os grupos na expressão dos receptores 5-$HT_{1A}$ hipocampais, no entanto os resultados do Teste da Natação Forçada Modificado evidenciam o efeito antidepressivo do óleo de peixe.

Sabe-se que o uso combinado do antagonista seletivo para 5-$HT_{1A,}$ DU125530, ào tratamento com fluoxetina, não acelerou ou melhorou a eficácia deste antidepressivo. Como este antagonista tem igual preferência de ocupação entre os receptores pré- e pós-sinápticos, o bloqueio dos receptores pós-sinápticos poderia ser o responsável pela reversão dos benefícios obtidos com o bloqueio dos autorreceptores pelo DU125530 (ver ARTIGAS, 2012).

No entanto, Bhagwagar et al (2004) mostrou que estes receptores (pós-sinápticos) não estão alterados ou reduzidos em pacientes deprimidos e tal alteração não é sensível ao tratamento com antidepressivos.

Por outro lado, o tratamento crônico com antidepressivos resulta numa ativação tônica dos receptores 5-$HT_{1A}$ no hipocampo dorsal (área CA3) e a ativação destes receptores no Giro Denteado aumenta a neurogênese hipocampal (ver ARTIGAS, 2012).

Uma correlação positiva entre suplementação com Ácidos Graxos Poli-insaturados, expressão aumentada de BDNF e de seu RNAm no hipocampo e os efeitos antidepressivos no teste da natação foraçada modificado já foi demonstrada (VENNA et al., 2009).

Utilizando o mesmo protocolo de suplementação com óleo de peixe, Vines et al (2012) mostraram que o efeito antidepressivo do óleo de peixe possivelmente está relacionado à indução da expressão de BDNF e aumento da neurotransmissão serotoninérgica no hipocampo. A relação entre serotonina e expressão e/ou função do BDNF está estabelecida, uma vez que o BDNF promove o desenvolvimento, a sobrevivência e a plasticidade dos neurônios serotoninérgicos durante o desenvolvimento do hipocampo e também na vida adulta, e isto possivelmente está relacionado ao seu papel na fisiopatologia da depressão (YU e CHEN, 2011).

Em relação à análise das monoaminas, os resultados mostram aumento dos níveis hipocampais de serotonina, o que está de acordo com a idéia de Vines et al (2012), de que o efeito antidepressivo da suplementação com óleo de peixe é devido ao aumento de serotonina.

Uma hipótese para explicar este efeito do ω-3, através do sistema serotoninérgico, seria a inibição da recaptação da serotonina ou inibição da enzima Monoamino-oxidase (MAO), o que aumentaria os níveis de serotonina. Outra hipótese é a de que ocorra um aumento dos níveis e/ou atividade da enzima que sintetiza serotonina, a Triptofano Hidroxilase, ou ainda aumento na atividade e/ou expressão dos receptores 5-$HT_{1A}$ no hipocampo e/ou diminuição da expressão ou atividade destes nos núcleos da rafe.

Considerando estes dados, devido à ausência de resultados mostrando aumento na expressão dos receptores 5-$HT_{1A}$ no hipocampo, não podemos descartar a possibilidade do aumento da atividade destes receptores, uma vez que não realizamos a técnica de *binding.* Logo, a complexa organização e função dos receptores 5-$HT_{1A}$ precisa ser melhor elucidada através de estratégias farmacológicas, na investigação dos transtornos do humor e ansiedade, tendo como alvo os receptores 5-$HT_{1A.}$

Em conjunto, estes resultados sugerem que a suplementação com óleo de peixe durante períodos pré- e pós-natais do desenvolvimento encefálico, reverte/previne a ocorrência do comportamento depressivo em ratos adultos submetidos ao teste da natação forçada modificado. Em resumo, os presentes achados confirmam os efeitos antidepressivos da suplementação com Ácidos Graxos Poli-insaturados ω-3, relacionados ào aumento da neurotransmissão serotoninérgica no hipocampo.

No entanto mais estudos são necessários para um maior entendimento deste efeito sobre o sistema serotoninérgico tendo como foco os receptores 5-$HT_{1A}$ pré- e pós-sinápticos.

# 6 CONCLUSÃO

Através dos dados do presente estudo, confirma-se o efeito antidepressivo do óleo de peixe, agindo através do sistema serotoninérgico, e seus efeitos comportamentais que permanecem até a vida adulta, em animais que foram suplementados nas fases de gestação e lactação e submetidos ao teste da natação forçada modificado. No entanto, no que se refere ao papel dos receptores 5-$HT_{1A}$ pós-sinápticos, sua função neste efeito permanece pouco conhecida e mais estudos visando verificar a atividade destes receptores e também dos receptores pre-sinápticos localizados nos núcleos da rafe são necessários

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ANEXOS

## 8.1 ARTIGO 1

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# Motor and Non-Motor Features of Parkinson's Disease – A Review of Clinical and Experimental Studies

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**Abstract:** Classically, Parkinson's disease (PD) is considered to be a motor system affliction and its diagnosis is based on the presence of a set of cardinal motor signs (e.g. rigidity, bradykinesia, rest tremor and postural reflex disturbance). However, there is considerable evidence showing that non-motor alterations (e.g. anxiety, depression, sleep, gastrointestinal and cognitive functions) precede the classical motor symptoms seen in PD. The management of these non-motor symptoms remains a challenge. A pattern of regional neurodegeneration that varies considerably depending upon the neuronal population affected may explain the different symptoms. In fact, differential mechanisms of neuronal vulnerability within the substantia nigra pars compacta (SNpc) suggests that factors other than location contribute to the susceptibility of these neurons. In this review we discuss how these factors interact to ultimately target the SNpc. Remarkably, this region consists of approximately 95% of the tyrosine hydroxylase (TH)-immunoreactive neurons in both human and rat brains, and consequently this implicates elevated levels of dopamine metabolites, free radicals and other hazard species in these neurons. An understanding of how these factors promote neuronal death may be useful for the development of novel neuroprotective and/or neurorestorative strategies for PD.

**Keywords:** Tyrosine hydroxylase, dopamine, animal models, nigrostriatal pathway, neurodegeneration.

## INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease afflicting about 1% of people over 65 years old and 4-5% of people over 85 years [1, 2]. Major clinical features at presentation include the asymmetric onset of bradykinesia, rigidity, rest tremor and disturbances in balance. These are the result of the degeneration of the neurons of the substantia nigra pars compacta (SNpc) which leads to subsequent reduction of dopaminergic input to the striatum (Fig. **1**). Moreover, there is a degeneration of neurons of selected brain stem nuclei (locus coeruleus, raphe nuclei, dorsal motor nucleus of the vagus), cortical neurons (particularly within the cingulated gyrus and the entorhinal cortex), the nucleus basalis of Meynert and preganglionic sympathetic and parasympathetic neurons [3]. These, apparently, non-motor features progress and come to dominate the later onset of PD producing symptoms that include cognitive decline, depression, gastrointestinal and genitourinary disturbances, as well as sleep abnormalities [4]. Nevertheless, the dopamine (DA)-containing neurons seems to be the key players in PD.

Several mechanisms are implicated in the degeneration of nigrostriatal neurons such as oxidative stress, mitochondrial dysfunction, protein misfolding, disturbances of intracellular calcium and iron homeostasis besides polymorphisms in genes regulating DA metabolism and transport, neuroinflammation and necrosis/apoptosis [5-7]. It has been recently discussed that clinical deficits in PD are strongly associated with the location, rather than mechanism, of brain cell death. The regional selectivity of brain cell loss in different neurodegenerative disorders is reflected in the overlapping motor and non-motor impairments associated to the neurodegeneration [8]. Particularly in PD, this idea is strengthened by the Braak´s hypothesis that describes the existence of six neuropathological stages. Each of these stages is marked by the continual development of distinctive inclusion bodies that present in the form of spindle-like or thread-like and, in part, branching Lewy neurites (LNs) within cellular processes and as granular aggregations and spherical pale bodies and/or Lewy bodies (LBs) in the somata of the involved nerve cells [9].

It is postulated that only a few of the many nerve cell types within the human nervous system are prone to develop the abnormal proteinaceous aggregations. Other neuronal types, even when they are located directly next to involved nerve cells, maintain their morphological and functional integrity. This means that neuronal damage in the brain during PD is not random but obeys certain rules, thereby leaving a distinctive lesional distribution pattern (see Table **1**) [10-12]. The reasons for the marked vulnerability of some neuronal types and the decided resistance of others are still not adequately understood. Although, growing evidence originated from clinical studies, autopsy materials, and *in vitro* and *in vivo* experimental models of PD are available [13-17]. Recent studies discuss that the dopaminergic nature of SNpc neurons, and the subsequent consequences of this neurochemical phenotype for oxidative stress, has long been

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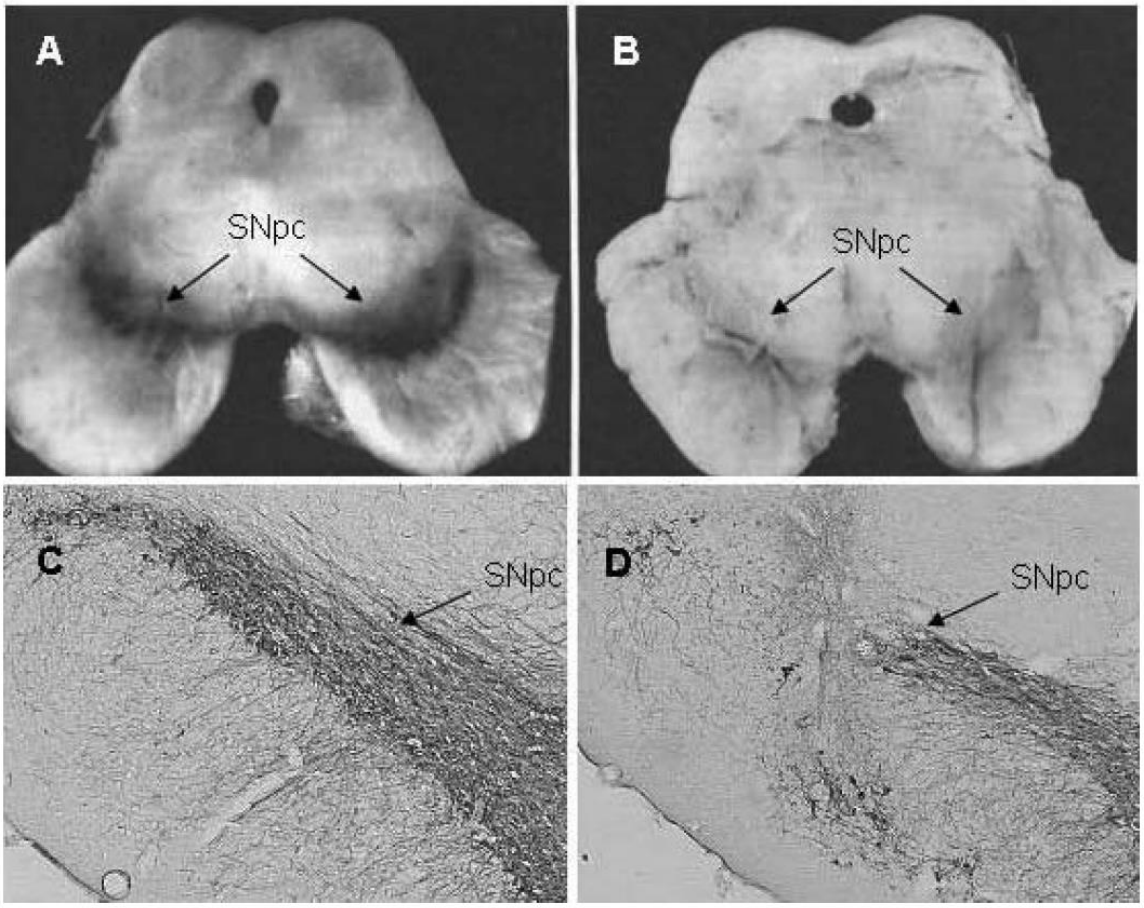


**Fig. (1).** Massive loss of midbrain dopaminergic neurons in the substantia nigra pars compacta (SNpc). Panels **A** and **B** represent the human postmortem bilateral midbrain of normal and Parkinsonian subjects, respectively. **C** and **D** show the unilateral equivalent of the rat midbrain after vehicle (**C**) or MPTP (**D**) intranigral injection. Arrows indicate the tyrosine hydroxylase immunoreactivity present within the SNpc.

suspected to contribute to their relative vulnerability. The most visually apparent feature of these cells, neuromelanin, is thought to form as a consequence of DA metabolism, and DA levels and/or the rate of DA metabolism within the neuron may vary due to the differential expression of tyrosine hydroxylase (TH) [18].

Modifications in this neuronal function as a result of PD, may contribute to dysfunctional dopaminergic circuitry and thus to the occurrence of non-motor and motor signs and symptoms. This review discusses how these factors interact, lead to mechanisms of selective dopaminergic neurodegeneration and correlate with PD symptoms in both clinical and experimental research. An understanding of these mechanisms will be helpful for the development of novel strategies to improve the survival of targeted neurons in PD.

## MOTOR IMPLICATIONS

Regional vulnerability in PD includes specific neurons other than those present within the SNpc. For example, significant neurodegeneration is observed in autonomic ganglia [19]; spinal cord [20]; brainstem [21]; basal forebrain [22]; limbic lobe [23] and the neocortex [24]. It is expected to observe a large variety of motor and non-motor disorders, however, the former is mainly associated to the overwhelming amount of damage of the SNpc neurons, impairing the projections to the caudate/putamen (nigrostriatal pathway) and consequently the DA release [25-27]. The common clinical syndrome associated with PD and other kinds of Parkinsonism includes motor problems related with slowing of movements (bradykinesia) [17, 28, 29], weakness [17, 28], rigidity [17, 28], tremors [30], postural instability [17] and fatigue (Table **1**) [31]. Here, it will be described the relationships of the selective dopaminergic neurodegeneration with some of the major motor implications and their impacts in the functional independence.

Bradykinesia is considered one of the cardinal features observed in PD [28]. This symptom describes the slowness of a voluntary movement, also referring to poverty of spontaneous and associated movements commonly

**Table 1. The Clinical-Pathological History of PD**

| Stage | Neuropathological Findings | Clinical Manifestations |
|---|---|---|
| 1-2 (pre-clinical) | • LNs and LBs inclusions in the anterior olfactory structures and regions of the medulla oblongata (in the dorsal motor nucleus of the vagus) and pontine tegmentum (including the noradrenergic neurons of the locus coeruleus and the serotonergic neurons of the caudal raphe nuclei) | • Olfactory disturbance<br>• Depression, anxiety<br>• Sleep disturbance<br>• Gastrointestinal dysfunction |
| 3 (early) | • LNs and LBs inclusions in the cholinergic nucleus basalis of Meynert | • Cognitive decline ($^1/_3$ PD patients)<br>• Depression, anxiety |
| 4 (late) | • LNs and LBs inclusions in the temporal mesocortex<br>• Considerable loss of dopaminergic neurons in the SNpc. | • Rest tremor, muscle rigidity and bradykinesia<br>• Postural instability<br>• Depression, anxiety, psychosis<br>• Cognitive decline ($^2/_3$ PD patients) |
| 5-6 (late) | • LNs and LBs inclusions in the neocortex | • Dementia<br>• Psychosis, depression, anxiety<br>• Cognitive decline (90% PD patients) |

Abbreviations: Lewy bodies (LBs); Lewy neurites (LNs); Parkinson´s disease (PD); substantia nigra pars compacta (SNpc).

manifested as freezing and the prolonged time it takes to initiate a movement (reaction time) [17, 28, 30, 31]. Central mechanisms of bradykinesia and how they relate to the basal ganglia dysfunction is the core of the motor deficits in PD, however there are additional secondary factors that can potentially contribute to bradykinesia: e.g. muscle weakness, rigidity, tremor, movement variability and slowing of thought [28].

Intrinsic muscle properties could not have changed when the strength of PD patients presented reduction, compared to controls [17, 30-33]. These results showed that weakness was likely due to an inability to activate the muscle voluntarily. In all studies, patients appeared to be readily motivated to produce strong contractions, so that lack of volitional drive was not thought to be a related factor, reinforcing an involvement of the central nervous system pathways related to voluntary muscle recruitment and avoiding an involvement of motivational factors. Other support that indicates that patients lack some part of the normal volitional input to lower motor centers is the existence of a physiological difference between PD and non-PD subjects in the electromyography activity suggesting disorganized voluntary drive to contract muscles in the same way as usual [34]. Rest and action tremor as well as secondary factors could also contribute to the bradykinesia, because they prolong reaction times and slow the initiation of any voluntary movement [17, 28, 29, 31]. All mentioned factors contribute to generate balance-related problems, interfering in many daily activities such as walking or standing up from a chair promoting fear of falling [17].

Recordings before the onset of movements revealed that bradykinesia could be due to slowness in activating the circuitry responsible to perform the voluntary movement. These studies include reaction time tests, electroencephalographic and magnetic encephalographic records, as well as records of the neuronal circuits activity at different times preceding a voluntary action [35, 36]. One reason for that could be explained by results showing that the increase in the threshold level of the motor excitability observed previous the movement or even during electromyography activity in PD patients is reached more slowly than the normal [28]. Pre-movement studies attempted to understand which areas of the motor system are functioning abnormally in bradykinesia and a general finding from these studies is that supplementary motor cortex and nearby areas (midline cortical motor areas) present reduced activity, perhaps coupled with extra activation of the lateral pre-motor area [28].

Decreased activity may be related to difficulties in preparing instructions to move and the increased activation may be an active process of compensation to improve performance that can be observed when external cues are available in the environment or they are given by physical therapists during rehabilitation programs to guide movement of PD patients [28, 37]. Evidence suggests that, although secondary factors such as weakness, rigidity, tremors, postural instability and fatigue may contribute to deficits in the functional independence, the main factor is probably related to the insufficient recruitment of muscle fibers by the descendent pathways during the initiation of movement [28, 29].

Despite a lot of primary and secondary factors that may contribute to clinical motor implications, characteristics of motor recruitment observed in PD can distinguish two features of Parkinsonian bradykinesia: (1) an undervalued muscle force resulted from an underscaling of commands in internally generated movements and (2) an ameliorated performance when external cues are given to guide movement [28]. These features have important therapeutic value and a wide impact in the functional independence of the PD patients.

## NON-MOTOR IMPLICATIONS

Remarkably, systematic reviews have indicated non-motor symptoms (e.g. depression, anxiety, cognitive and sleep disturbances) as major factors in determining health-related quality of life, progression of disability, and nursing home placement in PD patients (Table **1**) [38, 39]. Moreover, non-motor features of PD usually do not respond to dopaminergic therapy and probably is the major current

challenge faced in the clinical management [40]. Due to the novelty of this field the predominance of data that have been obtained recently is from animal models of Parkinsonism. In view of that, a selected core of topics regarding the non-motor implications of PD is scrutinized in the following sections.

## ANXIETY

Anxiety disorders are the second most common affective disturbance in PD and are present in up to 40% of patients [41]. Anxiety disorders include generalized anxiety disorder, agoraphobia, panic disorder, and social phobia, and frequently co-occur with depression in PD. Although some of these symptoms such as apprehension, fear, or worry are under-recognized and under-treated, others can be improved with available treatments [41]. According to Braak staging of PD pathology, noradrenalin (NA) dysfunction likely occurs prior to significant degeneration of dopamine (DA) neurons [9].

In view of the neurotoxin-related animal models of PD, that has been used extensively to investigate the motor deficits of the nigrostriatal degeneration, also can reveal different features of anxiety and depression. In view of that, it is described that bilateral nigrostriatal damage inflicted by 6-hydroxidopamine (6-OHDA) lesions induced symptoms of depression and anxiety characterized by several behavioral measures (depression: forced swim test, sucrose consumption; anxiety: elevated plus maze) [42]. Furthermore, other studies described the occurrence of depressive-like behaviors promoted by different neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), lipopolysaccharide (LPS) and rotenone [43-45]. Although, so far few studies have investigated the effects of MPTP administration in non-human primates on affect, whereas, MPTP-treated mice show profound increases in immobility in the tail-suspension test [46]. In addition, an increased anxiety-like behavior in open-field and light-dark box in Parkin null mice has been described [47]. However, transgenic mice over-expressing alpha-synuclein A53T exhibited reduced anxiety-like behavior by spending markedly greater amounts of time on the open arms of the plus maze and by a higher proportion of entries to the open arms [48]. In the open field test, these transgenic mice showed a trend toward reduced locomotor habituation and increased thigmotaxis [48]. Moreover, in a novel vesicular monoamine transporter-2 (VMAT-2) deficient mouse model of PD, mice displayed enhanced anxiety and depressive-like behaviors, which became more severe with advancing age [49]. Indeed, the contributions of the studies with animal models do corroborate the premise of different phases of the neurodegeneration in PD, and show the limitations of investigating these phases separately.

Several studies have noted that the onset of affective disorders predates the emergence of motor symptoms [50, 51], since motor symptoms typically do not manifest until about 70% of nigral DA neurons have been lost. In this sense it is observed that affective behaviors may be more sensitive to such depletion. In fact, it is reported an association between decreased binding to DA transporters in the left ventral striatum and depressive and anxious symptoms [52, 53]. DA-depleted rats manifest enhanced learned helplessness behaviors that were only partially alleviated by 3,4-dihydroxyphenylalanine (L-DOPA) [54]. Thus, it is suggested that such inability is associated with a supraphysiological release of DA into the prefrontal cortex and hippocampus [55].

## DEPRESSION

Depression has been found to be important determinant of PD patients in Europe [56-59]. In India female gender, presence of depression and low degree of independence had the most detrimental impact in patients with PD [60]. The etiology of depression in PD is complex and may result from changed 5-HT brain chemistry that is related with the central dopaminergic deficiency associated with PD motor symptoms [61, 62].

The basal ganglia receive DA input from the SNpc, which is known to be impaired in PD patients. Thus, observations of pathological features in the SNpc of depressed PD patients though only trending toward significance appears relevant and bolsters the notion that the nigrostriatal circuit is implicated in the depression of PD [63]. In addition, the nigral neuronal loss was seven times greater in post-mortem brains of PD patients with depression compared to non-depressed PD patients (Frisina *et al.*, 2009), suggesting that depression may be the result of more severe DA depletion.

There are pathophysiological evidence of serotonin (5-HT) alterations in patients with PD-associated depression [64], and a hypothesis concerning 5-HT has even been proposed for depression in PD [62]. This hypothesis considers a 5-HT-induced DA release in the nucleus accumbens which is down-regulated by $5\text{-HT}_{2C}$ receptors [65]. As a result, reductions in the 5-HT content or increases in the $5\text{-HT}_{2C}$ inhibitory activity could be associated to a decline on dopaminergic neurotransmission in PD patients and subsequent worsening of mood symptoms. However, noradrenergic anti-depressants, such as nortriptyline have recently demonstrated to be more effective than selective 5-HT reuptake inhibitors in PD patients with depression, and may suggest a more prominent role for NA [66].

Upon chronic medication, the loss of efficacy of L-DOPA that commonly leads to increasing L-DOPA dose regimen [67] may reflect a lower capability of 5-HT neurons to promote DA effects of L-DOPA. It is not known whether the lesions of serotoninergic neurons reported in Parkinsonian patients [68-70] result from the pathophysiological process of the disease or the chronic use of L-DOPA. Chronic treatment with an excessive dose of L-DOPA in normal rats has been shown to decrease striatal 5-HT tissue content [71]. Moreover, a single intranigral infusion of L-DOPA could promote a significant lesion in TH-ir neurons associated to decreased levels of striatal DA and motor impairment in the open field [72].

However, the impact of chronic L-DOPA treatment at therapeutically relevant dose in Parkinsonian rats on the integrity of 5-HT neurons is presently unknown although it could lead to aberrant *in vivo* 5-HT and DA releases in the brain. It was recently reported the occurrence of a strong *in vivo* evidence for a deleterious impact of chronic L-DOPA on 5-HT neuron function associated with a heterogeneous

loss of efficacy of L-DOPA to maintain DA transmission in the Parkinsonian brain [73]. This report demonstrated that chronic L-DOPA treatment results in two distinct inhibitory effects on 5-HT transmission. First, chronic L-DOPA treatment homogeneously decreases basal release of 5-HT in the brain *in vivo* and second, chronic L-DOPA treatment heterogeneously affects the ability of L-DOPA to inhibit 5-HT release [73]. Overall, depression in PD patients may exacerbate existing anatomical and functional abnormalities of the 5-HT and DA systems resulting in decompensated neurotransmitter systems that lead to vulnerability to depressive symptoms.

## COGNITIVE DEFICITS AND DEMENTIA

Many studies of PD describe elevated incidence of cognitive deficits associated with dementia, ranging from 20% to 80% [74]. This massive variation among studies is probably due to differences in the application of methods for cognitive assessment, dementia definition and data collection [75]. Cognitive dysfunction in patients with PD can be classified as domain-specific cognitive impairments, and dementia, a very common non-motor feature in PD, has important clinical consequences for the patients in terms of excess disability, risk for psychosis, reduced quality of life and increased mortality [75, 76]. TH enzyme and DA transporter (DAT) expressions are classic indicators of dopaminergic neuronal death and subsequent denervation in PD. These proteins are decreased in the striatum of PD patients with or without dementia compared to control patients i.e. 44 and 50%, respectively [77, 78]. On the other hand, neurochemical studies in postmortem inferior frontal gyrus from patients with PD and dementia appears also to sustain greater loss (40%) of both dopaminergic neurons and nerve terminals comparing to PD without dementia (20%) [77]. Significant loss of striatal dopaminergic terminals and DAT have been described either in PD patients with or without dementia, however, a greater loss of these markers is observed within the striatum and inferior frontal gyrus of PD patients with dementia [79].

Clinically, the cognitive decline comprises impairment of executive functions or memory deficits including working memory, long-term, visuospatial and procedural memories as well [75, 80-83]. The impairment of executive functions is the main feature in demented PD patients, including impairment in concept formation and rule finding, problem solving, set elaborating and planning, set shifting and set maintenance.

Several longitudinal studies have confirmed that patients with more severe and advanced Parkinsonism have a higher risk for dementia than those with less advanced PD. Motor symptoms such as rigidity, postural instability and gait disturbance predict more rapid cognitive decline and time to dementia [76, 84, 85]. Many authors considered the age factor as a potential risk to the development of dementia in PD [74-76]. On the other hand it is very difficult to distinguish the importance of age, duration of disease and age at onset of PD as the individual risk factor for dementia in PD, because all these parameters are highly correlated in PD cohorts. The previous existence of mild cognitive impairment in PD patients, mainly detected in tests that evaluated memory and executive function, is determinant in predicting a shorter time to develop dementia [86-89].

## SLEEP DISORDERS

The neurobiology of sleep has developed rapidly in the recent years, with remarkable neurophysiological and molecular progress of knowledge, about its mechanisms [90]. An overview of the literature demonstrates that studies concerning a role played by DA in the sleep regulation have becoming more numerous, especially after 1990 [91].

Sleep disturbances and daytime sleepiness are well-known phenomena in PD and were reported in the original description by James Parkinson (insert reference). Sleep disorders have a complex etiology related not only to the underlying neurodegenerative process, but also to the motor and non-motor features of PD and to dopaminergic therapy. A community-based study revealed that nearly two-thirds of PD patients reported sleep disturbances, which is significantly more frequently than observed in patients with diabetes and healthy control subjects [92]. Furthermore, about a third of PD patients rated their overall night-time problems as moderate to severe. Virtually all patients with PD suffer from various sleep disruptions [93]. In a prospective study in PD patients, cabergoline (a $D_1$ and $D_2$ agonist) treatment increased arousals, stage shifts, and awakenings, although quantitative electrophysiological measures of sleep were maintained, and subjective measures of sleep quality even improved [94].

The prevention of sleep and enhancement of waking by DA reuptake inhibitors and DA receptors agonists are the basis for their use in the treatment of other conditions such as narcolepsy and somnolence associated with hypodopaminergic states [95]. Moreover, PD patients present several disruptions of sleep, which are deteriorated by the use of dopaminergic $D_2$ receptor agonists [96-98]. In contrast, treatment with the $D_2$ antagonist and antipsychotic agent haloperidol attenuates hippocampal theta and gamma oscillations which is characteristic of rapid eye movement (REM) sleep [99]. The basal ganglia have been related to a variety of cognitive functions in addition to motor control. Experimental evidence has suggested a role for these nuclei in attention, time perception and learning and memory, while clinical data has contributed by pointing out the cognitive impairments in neurodegenerative disorders involving the basal ganglia, such as Huntington´s disease and PD. Impaired basal ganglia function has also been correlated with other pathologies such as obsessive-compulsive disorder, attention-deficit disorder, autism and schizophrenia [100].

## GASTROINTESTINAL DISTURBANCES

Gastrointestinal symptoms are common in PD and many such as dysphagia, dribbling of saliva and esophageal dysmotility can occur in advanced PD [101]. These symptoms contribute directly to the morbidity and complicate the disease's clinical management [102]. Constipation may precede development of PD [103]. A prospective study followed the bowel habits of 7,000 men for 24 years reported that those with initial constipation (< 1 bowel movement/day) had a threefold risk of developing PD after a mean interval of 10 years from initial constipation

[103]. Large projection cells of the dorsal motor nucleus of the vagal nerve generate long unmyelinated preganglionic fibers that connect the central nervous system with the postganglionic nerve cells of the enteric nervous system [104, 105]. Patients with PD present abnormal electrogastroenterography motility parameters which are similar to the acute stage of vagotomized patients [106]. It is postulated that the participation of dorsal motor nucleus of the vagal nerve is critical in Braak stage 1 [9]. At this stage, cases exhibit no more than a few isolated LB and LN-like inclusions in the dorsal motor nucleus and in the adjacent intermediate reticular zone and few spindle-shaped LN-like inclusions also may appear in the preganlionic axons of the vagal nerve [9, 107]. In addition, it is observed that similar forms of inclusion bodies as those found in the central nervous system occur in select neuronal types within the enteric nervous system [9].

However, a recent report evidence that there is comparable involvement of the dorsal vagal nucleus in multiple system atrophy (a condition associated with impaired control of gastrointestinal function) and different stages of LB disorders although, the relationship of dorsal vagal nucleus involvement and gastrointestinal symptoms is uncertain in PD [108]. A study analyzing eight patients with PD by use of defaecography and anorectal manometrics reported that after apomorphine treatment, defaecographic abnormalities were normalized in one of three patients, and all five individuals who underwent repeated anorectal manometry showed substantial improvements in manometric parameters [109]. The authors concluded that apomorphine can correct anorectal dysfunction in PD and suggested that abnormalities of defaecation and anorectal function occur as a consequence, at least in part, of dopamine deficiency secondary to the pathological changes of PD [110]. Data from a multi-centre, open-label observational study that used intrajejunal L-DOPA/carbidopa gel infusion in patients with non-motor symptoms and with advanced-stage PD demonstrated a significant beneficial effect in the majority of non-motor symptom domains, with paralleling improvement of motor symptoms [111]. According to the authors, this study was the first demonstration that a L-DOPA-based continuous stimulation was beneficial for non-motor symptoms and health-related quality of life of the patients, in addition to the reduction of motor fluctuations and dyskinesias [111].

## MECHANISMS OF NEURODEGENERATION IN PARKINSON'S DISEASE - CONTRIBUTION OF THE ANIMAL MODELS

In rodents a profusion of studies based on intracerebral injections of neurotoxins such as 6-OHDA, MPTP, rotenone and LPS (Fig. **1**) have been published [26, 42, 43, 112-116]. The neurotoxicity of 6-OHDA is attributed to its ability to generate reactive oxygen species (ROS) and quinones [117]; however, evidence also suggest that 6-OHDA can act directly by inhibiting the mitochondrial respiratory chain at the level of complex I [118]. In a translational perspective it is important to note that depending on the location of the injection site and dose of neurotoxin, the animal model can present different time-courses of progression and severity of lesion. Middle forebrain bundle or intranigral infusions of 6-OHDA induce an almost complete depletion on neuronal density of tyrosine hydroxilase immunorreative (THir) neurons (80-90% loss) similar to advanced stage of PD [119, 120]. Accompanying this marked degeneration several studies evaluated the efficacy of anti-dyskinetic treatments in unilateral 6-OHDA-lesioned-rats after repeated administration of either L-DOPA or dopamine agonists such as apomorphine [116, 121]. When the focus is to reproduce early stages of PD the 6-OHDA can be administrated into the striatum once the degeneration of the nigrostriatal system is protracted, and induces cell death in a retrograde fashion [122].

Considering a more prolonged time frame (approximately two weeks) to observe the maximal loss of THir neurons [123] and consequently the time for the agent tested to act, the 6-OHDA model is widely employed to investigate neuroprotective treatments [112]. In addition, this model reproduces non-motor symptoms including mild emotional dysfunction, depression, anxiety and cognitive decline that may be of equal or greater significance than that motor symptoms [42, 43, 124].

Many animal species including rats, mice, monkeys and non-human primates are commonly used to reproduce Parkinsonism induced by the MPTP [125-127]. For some researchers it is considered the best experimental model of PD, especially in the context of studies designed to explore molecular mechanisms involved in the dopaminergic neurons degeneration [128]. Once inside the dopaminergic neurons the 1-methyl-4-phenylpyridinium ($MPP^+$) (metabolite generated from monoamine oxidase-B conversion of MPTP) impairs mitochondrial electron transport by inhibiting of complex I, reducing ATP generation and causing increased production of ROS [129]. The toxin MPTP can be given by several different routes, including systemic (s.c; i.v; i.p; i.m.) or intracarotid artery injection, oral administration and intracerebral sterotaxic injection [130]. Comparatively this toxin reproduces an early phase of PD, since animals that received 6-OHDA intranigral bilaterally exhibited more intense loss of THir neurons and striatal dopamine depletion than MPTP-lesioned animals [113, 120]. Nevertheless, still challenging to reproduce the dopaminergic neurodegeneration chronic and progressively, analogous to patient with DP.

Repeated intranigral MPTP injection in rodent resulted in loss in TH protein expression and THir neurons in the SNpc 24h after the first neurotoxin administration, however during the time frame tested the MPTP effect was not progressive or cumulative [131]. Recently was reported that MPTP-induced a dose-dependent progressive loss in the SNpc THir neurons. These data suggest that an intermittent washout period (10 days) between each increased dose of MPTP is responsible to continued cell death; this finding is important for therapeutic interventions to be applied at any of several stages during progressive neurodegeneration [132]. In addition to these classically known toxins, in recent years the pesticide rotenone has shown promise results as an effective model of PD. Highly lipophilic, it easily crosses the blood brain barrier - without coupling to DAT - and diffuses into neurons where, in a manner similar to MPTP, accumulates within mitochondria and inhibits the complex I [129].

Because rotenone can freely enter all cells there remain critical issues regarding the translatability of this model [133]. Though, different routes of administration such intraperitoneal injection [134], intranigral [135], chronic oral administration [136] and chronic intravenous or subcutaneous administration [137] have been demonstrated success in to mimic the loss of THir neurons and the decrease of striatal DA. On the other hand, are reported consistent disadvantages in consequence of systemic administration of rotenone, such high mortality and systemic organ toxicity [138, 139]. Considering these drawbacks, there are growing interest in to investigate the intracerebral rotenona administration. A single bilateral intranigral injection of rotenone produced an intense reduction in THir neurons and severe depletion in striatal DA and apparently proved an ideal model to promote cognitive deficits that mimics the presyntomatic state of PD [135].

It has been shown that cyclooxygenase-2 (COX-2) induction produced by LPS and by other neurotoxins suggest an increase in microglial activation in the SNpc which, by itself, can be interpreted as a manifestation of damage in the dopaminergic system [113]. Conspicuously, the inflammatory stimuli and ROS imbricates in one activation of NF-κB in microglial cells, oligodendrocytes and neurons to promote the transcription of inflammatory cytokines (IL-1β, IL-6, interferon-γ, TNF-α), apoptosis-promoting factors (p53, Bax), COX-2 and inducible nitric oxide synthase. Considering these findings, it is suggested that intranigral LPS could be considered a neuroinflammatory model of PD in terms of the of COX-2 up-regulation and neurochemical alterations. Otherwise, LPS was surprisingly unable to replicate the motor impairment, which is the strongest characteristic of this disease, despite the TH content depletion achieved after MPTP the intranigral injection [113].

## CONCLUSION - TRANSLATIONAL PERSPECTIVE

Despite the focus on translational medicine and an upsurge in interest and funding [13, 14, 140-146], the idea of translated basic research into clinical treatments is not a novelty in PD. Indeed it yielded the Nobel Prize for Arvid Carlsson shared in Physiology and Medicine in 2000 along with Eric Kandel and Paul Greengard [147]. In this review, we have highlighted the varying aspects of both experimental research and clinical non-motor and motor features of PD. Intense efforts have been made toward developing improved PD models to better understand the etiology and pathogenesis of PD, and to identify new drug targets. However, neither neurotoxin induced nor transgenic animal model of PD, perfectly recapitulates all human symptoms. The various above findings provided compelling evidence of the different characteristics of the disease, such as motor deficit, neuroinflammation, oxidative stress and all the plethora of non-motor alterations. We envisage that the road ahead for detecting non-motor signs before the occurrence of motor ones is perhaps the brightest way to provide a window for more effective preventive/restorative treatments for PD.

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## CONFLICT OF INTEREST

The authors have declared that no conflict of interests exists.

## ABBREVIATIONS

| | | |
|---|---|---|
| PD | = | Parkinson´s disease |
| DA | = | Dopamine |
| LNs | = | Lewy neurites |
| LBs | = | Lewy bodies |
| TH | = | Tyrosine hydroxylase |
| SNpc | = | Substantia nigra pars compacta |
| NA | = | Noradrenalin |
| VMAT-2 | = | Transporter-2 |
| L-DOPA | = | 3,4-dihydroxyphenylalanine |
| 5-HT | = | Serotonin |
| BDNF | = | Brain derived neurotrophic factor |
| DAT | = | DA transporter |
| s.c. | = | Subcutaneous |
| i.v. | = | Intravenous |
| i.p. | = | Intraperitoneal |
| i.m. | = | Intramuscular |
| 6-OHDA | = | 6-hydroxydopamine |
| MPTP | = | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| LPS | = | Lipopolysaccharide |
| ROS | = | Reactive oxygen species |
| MFB | = | Middle forebrain bundle |
| THir | = | Tyrosine hydroxilase immunorreative |
| COX-2 | = | Cyclooxygenase-2 |

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8.3 ARTIGO 2 (SUBMETIDO)

# Multiple intranigral unilateral LPS infusion protocol generates a persistent cognitive impairment without cumulative dopaminergic impairment

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**Abstract**

Inflammation in Parkinson's disease (PD) is a continuous process and might be implicated in the progression of neuronal degeneration. Taking this into account, we proposed a new protocol with multiple and consecutive intranigral lipopolysaccharide (LPS) administration in order to analyze its effects on cognitive behavior. Additionally, striatal concentrations of neurotransmitters as dopamine (DA) and serotonin and their respective metabolites were assessed in four different time-points in order to analyze the consecutive and cumulative effects of LPS infusions. We demonstrated that from minimum dose applied there was stabilization of neuronal damage as revealed by absence of synergic effect on DA concentration. Although the DA decrease (-40%) generates an animal model of early phase of PD, without apparent motor impairment, the LPS group exhibited deficit in episodic-like memory behavior from the first time-point until the last one, indicating disturbances in memory-recognition responses that persisted even 30 days after the insult. These findings provide evidence that multiple intranigral LPS infusions are not sufficient to cause cumulative and progressive damage to dopaminergic neurons, but confirm that the LPS model can be adopted as a useful tool providing insight about the cognitive impairment observed in pre-motor phase of PD.

**Keywords**

Parkinson's disease; Lipopolysaccharide; Cognitive impairment; Novel-object recognition test.

## 8.4 ARTIGO 3 (SUBMETIDO)

# Fish oil improves anxiety-, depressive-like and cognitive behaviors in olfactory bulbectomized rats

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**Abstract**

Depression is increasingly present in the population, and its pathophysiology and treatment have been disclosed by several animal models, including olfactory bulbectomy (Obx). Fish oil (FO) supplementation during the pre- and postnatal periods decreases depression- and anxiety-like behaviors. The present study evaluated the effect of FO supplementation on Obx-induced depressive-like behavior and cognitive impairment. Female rats were supplemented with FO during habituation, mating, gestation and lactation and their pups were submitted to Obx, in adulthood; after the recovery period, the adult offspring was subjected to behavioral tests and determination of brain derived neurotrophic factor (BDNF), serotonin (5-HT) and the metabolite 5-hydroxyindoleacetic (5-HIAA) hippocampal content. Obx led to increased anxiety and depressive-like behaviors, and impairment in the object location task. All behavioral changes were reversed by FO supplementation. Obx caused reduction of hippocampal BDNF and 5-HT, whilst FO supplementation restored these parameters to normal values. In control rats, FO increased hippocampal content of 5-HT and reduced that of 5-HIAA, indicating low 5-HT metabolism in this brain region. The present results indicate that FO supplementation during critical periods of brain development attenuated anxiety and depressive-like behaviors and cognitive dysfunction induced by olfactory bulbectomy. These results may be explained by increased levels of hippocampal BDNF and 5-HT, two major regulators of neuronal survival and long-term plasticity in this brain structure.