**FACULTAD DE CIENCIAS DEL MAR**
**UNIVERSIDAD CATÓLICA DEL NORTE**
**DOCTORADO EN BIOLOGÍA Y ECOLOGÍA APLICADA**

¨EFECTOS DE LA SUPLEMENTACIÓN CON ÁCIDOS GRASOS OMEGA-3 SOBRE LAS ALTERACIONES INDUCIDAS POR EL ESTRÉS CRÓNICO EN EL APRENDIZAJE, MEMORIA Y PLASTICIDAD SINÁPTICA”,

Miguel Ángel Pérez Lizama

Profesor Guía: Dr. Alexies Dagnino Subiabre

COQUIMBO, 2013

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Actividad de Titulación presentada
para optar al Título de Doctor en
Biología y Ecología Aplicada

Miguel Ángel Pérez Lizama

Coquimbo, agosto de 2013

*Dedicada con todo cariño a mi padres, hermanos*

*y en especial a mis grandes amores, Mildred y Gustavito.*

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**Lista de abreviaciones**

| | |
|---|---|
| **AA** | Ácido araquidónico |
| **ACTH** | Hormona adrenocorticotropa |
| **AD** | Enfermedad de Alzheimer |
| **AGO** | Alimento elaborado en base a gónadas de ostión |
| **ANOVA** | Análisis de varianzas |
| **AV** | Vasopresina |
| **BDNF** | Factor neurotrófico derivado del cerebro |
| **BLA** | Amígdala basolateral |
| **CA1** | Cornu ammonis 1 |
| **CA3** | Cornu ammonis 3 |
| **CB1** | Receptor de canabinoide |
| **CI** | Colículo inferior |
| **CS** | Colículo superior |
| **cm** | Centímetros |
| **COXs** | Cicloxigenasa |
| **CORT** | Corticosterona |
| **CRH** | Hormona liberadora de corticotropina |
| **CS** | Estímulo condicionado |
| **dB** | Decibeles |
| **DHA** | Ácido docosahexaenoico |
| **DPA** | Ácido docosapentaenoico |
| **DRN** | Núcleo dorsal de rafe |
| **eCB** | Endocanabinoide |
| **EPA** | Ácido eicosapentaenoico |
| **FC** | Fosfatidil-colina |
| **FE** | Fosfatidil-etanolamina |
| **FI** | Fosfatidil-inositol |
| **FLs** | Fosfolípidos |
| **FS** | Fosfatidil-serina |

| | |
|---|---|
| **GABA** | Ácido aminobutírico |
| **GRs** | Receptores de glucocorticoides |
| **Hz** | Hercios |
| **HPA** | Eje hipotalámico-pituitario-adrenal |
| **LA** | Amígdala lateral |
| **LG** | Núcleo geniculado lateral |
| **LL** | Lemnisco lateral |
| **LNA** | Ácido linolénico (ω-3) |
| **LN** | Ácido linoleico (ω-6) |
| **LOXs** | Lipoxigenasa |
| **LTP** | Potenciación de largo plazo |
| **mA** | Miliamperio |
| **mg** | Miligramo |
| **MG** | Núcleo Geniculado Medial |
| **mL** | Mililitros |
| **MRs** | Receptores de mineralocorticoides |
| **ms** | Milisegundos |
| **ng/mL** | Nanogramos/ Mililitros |
| **NCAM** | Molécula de adhesión celular neuronal |
| **NMDA** | N-metil D-aspartato |
| **PS** | Plasticidad sináptica |
| **PUFAs** | Ácidos grasos poli-insaturados de cadena larga |
| **sIPSC** | Corrientes postsinápticas inhibitorias espontáneas |
| **sEPSC** | Corrientes postsinápticas inhibitorias espontáneas |
| **US** | Estímulo no condicionado |
| **µL** | Microlitros |
| **µM** | Micromolar |
| **ω-3** | Ácidos grasos se la serie omega-3 |
| **ω-6** | Ácidos grasos se la serie omega-6 |
| **2-AG** | 2-araquidonilglicerol |
| **5-HT** | Serotonina |

**Resumen**

El estrés crónico induce cambios morfológicos en las dendritas los cuales afectan funcionalmente a regiones del cerebro relacionadas con el aprendizaje y la memoria. Estas funciones cognitivas son dependientes de cambios en el balance entre la excitación e inhibición y por cambios a corto y largo plazo en la eficacia sináptica. El estrés crónico aumenta la transmisión GABAérgica en el hipocampo, un área del cerebro que regula la consolidación de memorias, mientras que la disminuye en el complejo amigdaloide, involucrado en el procesamiento de las emociones. Varios estudios han demostrado que la deficiencia de los ácidos grasos poli-insaturados (PUFAs) del tipo *ω-3* afecta a las neuronas, aumentando la prevalencia de enfermedades con alto impacto social y económico en el mundo, tales como los trastornos depresivos, Alzheimer y esquizofrenia. Contrariamente, la suplementación con PUFAs *ω-3* tiene un efecto protector sobre la memoria y el aprendizaje de ratas *Sprague-Dawley* en ratas sometidas a estrés crónico.

En esta tesis doctoral se estudió el efecto anti-estrés de los PUFAs *ω*-3, además de sus efectos sobre el aprendizaje, memoria y la transmisión sináptica. Ratas *Sprague-Dawley* fueron sometidas a estrés por restricción para determinar el efecto de la suplementación con PUFAs *ω*-3 sobre el aprendizaje auditivo, concentración plasmática de corticosterona (CORT, la hormona del estrés), ansiedad, memoria y transmisión sináptica. El estrés crónico deterioró el aprendizaje auditivo y aumentó los principales marcadores del estrés (ansiedad y CORT). La suplementación con PUFAs *ω*-3 mejoró estas alteraciones. A través de estudios de morfometría neuronal se observa que el efecto positivo de la suplementación con PUFAs *ω*-3, esta correlacionado con un aumento del tamaño dendrítico y de la eficacia sináptica GABAérgica en neuronas hipocampales de ratas estresadas, mientras que a nivel conductual se observó un aumento de la memoria espacial. Estos resultados demuestran que la suplementación con PUFAs *ω*-3 tiene un efecto terapéutico sobre el deterioro que induce el estrés crónico sobre el aprendizaje y la memoria. Además observamos que el estrés crónico disminuyó la atención auditiva y la eficacia de la transmisión GABAérgica en la corteza auditiva. También se encontró que la CORT afecta el aprendizaje y a las neuronas del mesencéfalo auditivo. Estos resultados serán de gran ayuda para orientar a nuevas investigaciones sobre el uso de suplementos con PUFAs *ω*-3 en modelos animales y humanos, los cuales pueden ser utilizados como un agente anti-estrés y potenciador de la memoria.

**Abstract**

Chronic stress induces morphologic changes in dendrites that affect brain areas related with learning and memory. Synaptic plasticity as well as the excitatory and inhibitory balance in the brain regulates these cognitive functions. Chronic stress increases the GABAergic transmission in the hippocampus, a brain area that regulates memory consolidation, while decreases in the amygdaloid complex, involved in the emotional processing. Several studies have shown that deficiency of polyunsaturated fatty acids (PUFAs) ω-3 affects the neuronal functions, which increase the prevalence of chronic diseases with high social and economic impact worldwide, such as depressive disorders, Alzheimer and schizophrenia. Conversely, a protective effect on learning and memory was found in Sprague-Dawley rats supplemented with PUFAs ω-3.

The anti-stress effect of PUFAs ω-3 was studied in this doctoral thesis, as well as their effects on learning, memory and synaptic transmission. Sprague-Dawley rats were subjected to restraint stress to determine the effect of PUFAs ω-3 supplementation on auditory learning, plasma CORT levels, anxiety, spatial memory and synaptic transmission. Chronic stress impaired the auditory learning and increased the main stress markers (anxiety and CORT). Supplementation with PUFAs ω-3 improved these alterations. The positive effect of PUFAs ω-3 supplementation was correlated with increases of dendrites length and GABAergic synaptic efficacy at the hippocampal neurons of stressed rats. These results were correlated with memory improvement. Overall, PUFAs ω-3 supplementation had a therapeutic effect in the stress-induced impairments on learning and memory. In addition, we found that stress decreased the auditory attention and the GABAergic transmission in the auditory cortex We also found that CORT affects learning and auditory midbrain neurons.

These results will help to guide further research related with the use of PUFAs ω-3 supplementation as anti-stress agent and memory enhancer in animal models and humans.

## Introducción

### ***El estrés***

El estrés es una compleja reacción biológica, común en todos los organismos, que permite la restauración de la homeostasis y adaptación a la presión del ambiente (estresores) (Selye, 1936; Goldstein y McEwen, 2002), en cuyo proceso participan varios sistemas neuroendocrinos (Aboitiz y Dagnino-Subiabre, 2007; McEwen, 2007). Existen dos tipos de estrés, uno positivo llamado euestrés, inducido cuando la fuente de estrés es breve, de corta duración y controlable por los organismos (Lazarus, 1974; Tafet y Barnandini, 2003), permitiendo la generación de una respuesta al estrés, posibilitando la adaptación y manutención en el tiempo. Otro tipo de estrés, corresponde al diestrés (conocido sólo como estrés), que corresponde a la forma negativa, que se origina cuando la fuente de estrés es fuerte y prolongada en el tiempo, impidiendo la capacidad de responder a los cambios ambientales, volviéndose éstos incontrolables para el individuo, generando una mala respuesta adaptativa, provocando efectos negativos (Gibbons et al., 2007; Tafet y Barnandini, 2003).

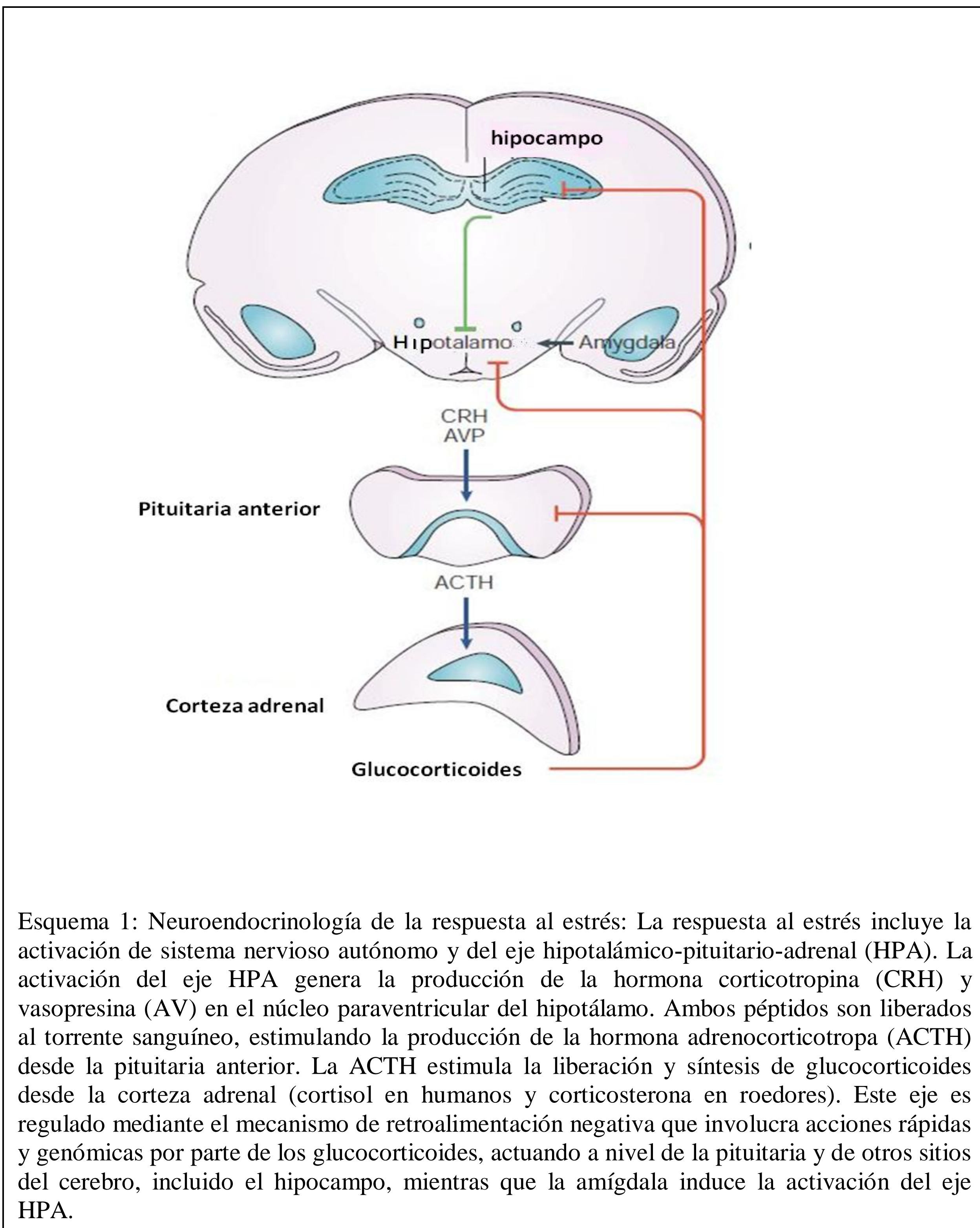
En humanos el estrés es llamado estrés psicosocial y es un factor de riesgo que predispone al desarrollo de diversas enfermedades crónicas que tienen un fuerte impacto social y económico, tal como los trastornos depresivos y ansiosos (Tafet y Bernardini, 2003; Tafet y Smolovich, 2004; van Praag, 2005). Estudios de neuroimágenes han demostrado que el estrés psicosocial en humanos produce una disminución del volumen del hipocampo (Pruessner et al., 2005, 2008) y alteraciones funcionales de la corteza prefrontal (Liston, 2009). En patologías psiquiátricas relacionadas con el estrés psicosocial los pacientes presentan alteraciones morfológicas similares, presentando atrofia hipocampal (Sheline et al., 1996, Manji et al., 2003; Colla et al., 2007), alteraciones funcionales de la corteza prefrontal (Johnstone et al., 2007) e hiperactivación del complejo amigdaloide (Zhong et al., 2011). Los efectos de los estresores cotidianos que amenazan la salud y la calidad de vida de los individuos han sido investigados intensamente a corto y largo plazo. Sin embargo, los aspectos neurobiológicos que subyacen al estrés psicosocial son poco conocidos. Por tal motivo, una alternativa para su estudio han sido los modelos animales. Estos modelos son útiles para conocer las bases neurobiológicas que subyacen al estrés, ansiedad y depresión, para determinar sus consecuencias y son útiles para determinar las acciones de distintos fármacos en el tratamiento clínico.

Los roedores han sido ampliamente utilizados como modelos animales de experimentación, debido principalmente a que los mecanismos fisiológicos y neurobiológicos son similares y pueden ser extrapolados a los mecanismos humanos para incrementar el conocimiento en la búsqueda de terapias para un amplio rango de enfermedades con el propósito de mejorar la salud humana. En el estudio del estrés, el modelo de estrés crónico más usado para reproducir e investigar las alteraciones funcionales y morfológicas observadas en humanos expuestos a estrés psicosocial caracterizado por la atrofia del hipocampo y el deterioro de la memoria es el modelo de restricción de movimiento (Watanabe et al., 1992a,b; Magarinos y McEwen, 1995; Conrad et al., 1999; Stewart et al., 2005). Este modelo consiste en reproducir lo que un sujeto vive diariamente en los medios de transporte de algunas de las grandes ciudades, como Santiago. Por ejemplo, un gran número de personas en Santiago se desplaza todos los días a través del metro o el transporte público. En las horas punta, dentro de los vagones o los buses las personas están sometidas por varias horas al día a una restricción de sus movimientos y al estresor psicosocial de no poder escaparse del tren o del bus durante el viaje. En el modelo de estrés por restricción de movimientos en ratas, el animal es introducido en un restrictor de acrílico, durante 6 horas diarias y por 21 días (Glavin et al., 1994; Servatius et al., 2000). Este modelo de estrés crónico produce la atrofia del hipocampo y el deterioro de la memoria. Además, es ampliamente utilizado para realizar estudios de biología molecular y de actividad sináptica en el hipocampo (Donahue et al., 2006; Ejchel-Cohen et al., 2006; Gao et al., 2006; McEwen, 1999; Stewart et al., 2005; Venero et al., 2002).

***Sistema de respuesta al estrés***

La respuesta al estrés es mediada por la activación de un sistema neuroendocrino llamado eje hipotalámico-pituitario-adrenal (HPA) (Esquema 1) que permite la comunicación entre el cerebro que percibe la señal nueva o amenazante, con el resto de los sistemas del cuerpo, tales como el sistema cardiovascular y muscular (McEwen, 2007). En el hipotálamo se induce la liberación de la hormona liberadora de corticotropina (CRH), que activa a su vez la liberación de la hormona adrenocorticotropa (ACTH) desde la pituitaria anterior. En la glándula suprarrenal, ACTH estimula la liberación de la *"hormona del estrés"* cortisol en humanos y corticosterona en roedores (CORT) (Ströhle y Holsboer, 2003). Los estresores de tipo incontrolables, incrementan significativamente la actividad del eje HPA y el nivel

plasmático de CORT en las ratas sometidas a estrés crónico en comparación con las ratas no sometidas a estrés, provocando en ellos una mala respuesta adaptativa (Tafet y Bernardini, 2003; Ferraz et al., 2011).




Esquema 1: Neuroendocrinología de la respuesta al estrés: La respuesta al estrés incluye la activación de sistema nervioso autónomo y del eje hipotalámico-pituitario-adrenal (HPA). La activación del eje HPA genera la producción de la hormona corticotropina (CRH) y vasopresina (AV) en el núcleo paraventricular del hipotálamo. Ambos péptidos son liberados al torrente sanguíneo, estimulando la producción de la hormona adrenocorticotropa (ACTH) desde la pituitaria anterior. La ACTH estimula la liberación y síntesis de glucocorticoides desde la corteza adrenal (cortisol en humanos y corticosterona en roedores). Este eje es regulado mediante el mecanismo de retroalimentación negativa que involucra acciones rápidas y genómicas por parte de los glucocorticoides, actuando a nivel de la pituitaria y de otros sitios del cerebro, incluido el hipocampo, mientras que la amígdala induce la activación del eje HPA.

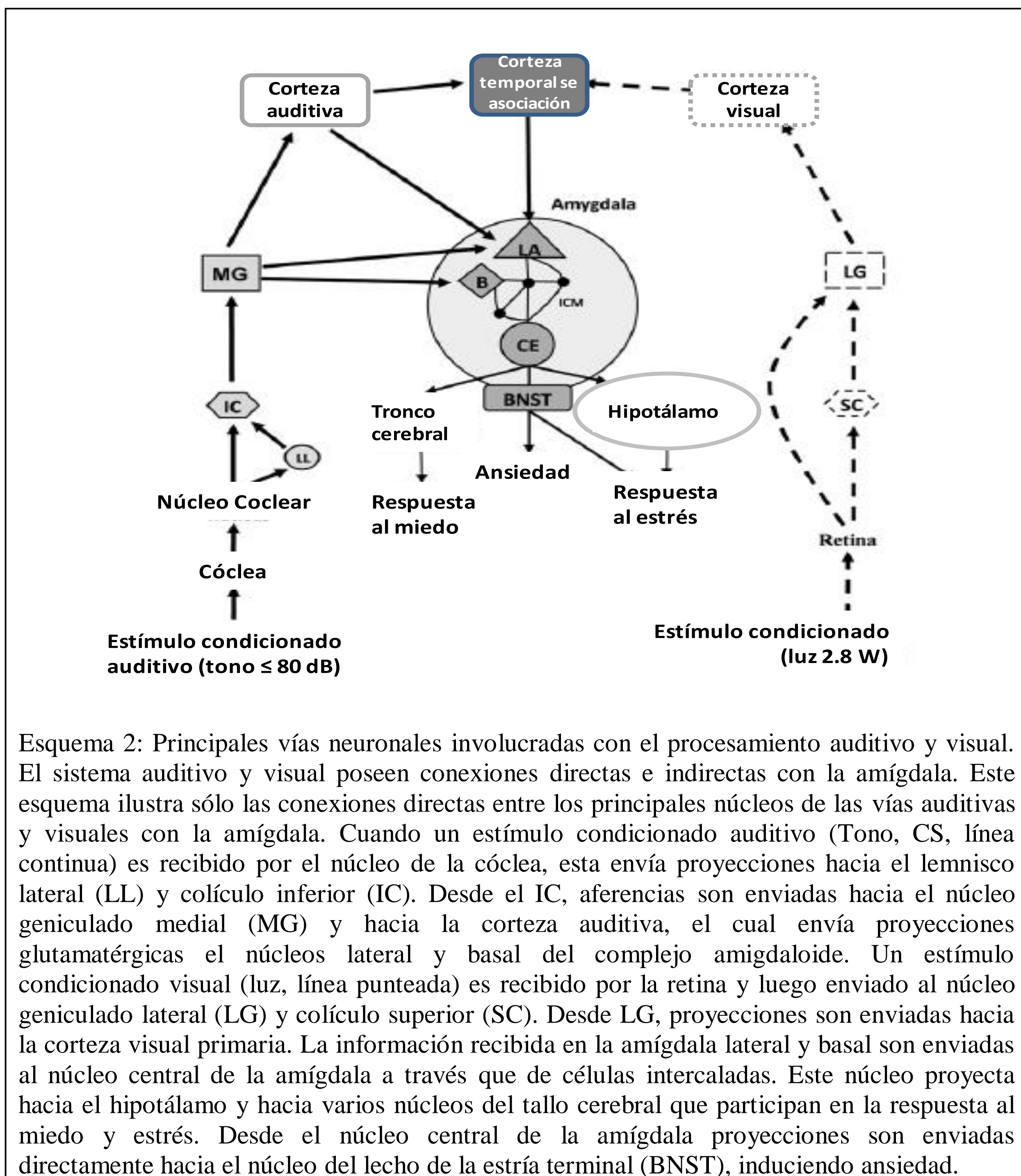
La CORT ejerce su función a través de la activación de los receptores de glucocorticoides (GRs) y los receptores de mineralocorticoides (MRs) (Herman et al., 1996; Ströhle y Holsboer, 2003; Smith y Vale, 2006). Ambos receptores difieren en su afinidad a los corticoides. Los GRs actúan principalmente cuando la secreción de corticoides aumenta y se mantiene prolongada en el tiempo (Ströhle y Holsboer, 2003). Esto permite la generación de una respuesta al estrés (Holsboer, 2000), permitiendo la transcripción de varios genes, así como la represión de otros involucrados en la respuesta inmune (Ströhle y Holsboer, 2003). Estructuras límbicas tales como el hipocampo, la amígdala y la corteza prefrontal medial poseen una alta expresión de GRs (Gray y Bingaman, 1996; Joels, 2001; Wellman, 2001). La administración crónica de glucocorticoides induce la atrofia del hipocampo (McEwen, 1992; Watanabe et al., 1992; Magariños et al., 1998) y de la corteza prefrontal medial (Magariños et al., 1998), mientras que el tratamiento agudo con CORT induce hipertrofia del núcleo basolateral de la amígdala (BLA), incrementando la ansiedad (Conrad et al., 2004; Cordero et al., 2008; Mitra y Sapolsky, 2008).

El estrés crónico y el tratamiento con CORT afectan la arquitectura dendrítica y la función de áreas del cerebro relacionadas con la memoria, aprendizaje y el procesamiento emocional (Watanabe et al., 1992; Magariños y McEwen, 1995; Magariños et al., 1998; Wellman, 2001; Vyas et al., 2002). Estas alteraciones producen un aumento de la ansiedad, la cual es una reacción adaptativa, inducida cuando un animal se enfrenta a una amenaza y/o peligro potencial. De hecho, la ansiedad posee una función bilógico-adaptativa clave altamente conservada durante la evolución (Ohl et al., 2008). La ansiedad es regulada principalmente por la amígdala basolateral (BLA) y por el núcleo del lecho de la estría terminal (BNST) (Pêgo et al., 2008; Davis et al., 2010). Niveles altos o patológicos de ansiedad inducen una mala respuesta adaptativa (Ohl et al., 2008). Múltiples protocolos de inducción del estrés, que producen un aumento de la ansiedad, se correlacionan con la hipertrofia dendrítica en BLA y BNST (Vyas et al., 2003, 2004).

***Efecto del estrés sobre el aprendizaje auditivo***

El aprendizaje condicionado en modelos animales involucra la asociación de una señal auditiva (estímulo condicionado, CS) con un estímulo aversivo no condicionado (US) (Esquema 2). Una vez adquirido el aprendizaje, el CS por si mismo provoca una respuesta de

tipo condicionada. Por ejemplo, el congelamiento “*freezing*” es una respuesta condicionada al miedo en la prueba de condicionamiento aversivo activo de 2 cámaras (2-AA) (Fig. 6, anexo). Las ratas son entrenadas para evitar el estímulo US “*foot shock*” indicado por una señal auditiva (Choi et al., 2010). El estrés crónico deteriora el aprendizaje en el 2-AA (Pérez et al., 2013) y la morfología neuronal del sistema auditivo (Dagnino-Subiabre et al., 2005).




Esquema 2: Principales vías neuronales involucradas con el procesamiento auditivo y visual. El sistema auditivo y visual poseen conexiones directas e indirectas con la amígdala. Este esquema ilustra sólo las conexiones directas entre los principales núcleos de las vías auditivas y visuales con la amígdala. Cuando un estímulo condicionado auditivo (Tono, CS, línea continua) es recibido por el núcleo de la cóclea, esta envía proyecciones hacia el lemnisco lateral (LL) y colículo inferior (IC). Desde el IC, aferencias son enviadas hacia el núcleo geniculado medial (MG) y hacia la corteza auditiva, el cual envía proyecciones glutamatérgicas el núcleos lateral y basal del complejo amigdaloide. Un estímulo condicionado visual (luz, línea punteada) es recibido por la retina y luego enviado al núcleo geniculado lateral (LG) y colículo superior (SC). Desde LG, proyecciones son enviadas hacia la corteza visual primaria. La información recibida en la amígdala lateral y basal son enviadas al núcleo central de la amígdala a través que de células intercaladas. Este núcleo proyecta hacia el hipotálamo y hacia varios núcleos del tallo cerebral que participan en la respuesta al miedo y estrés. Desde el núcleo central de la amígdala proyecciones son enviadas directamente hacia el núcleo del lecho de la estría terminal (BNST), induciendo ansiedad.

El estrés induce atrofia dendrítica en el colículo inferior (CI), la principal estructura del sistema auditivo, y no afecta la morfología neuronal del colículo superior, un área importante en el procesamiento de los estímulos visuales en el cerebro (Dagnino-Subiabre et al., 2005). Además, se ha demostrado previamente que el estrés por restricción induce atrofia dendrítica en el CI, en el geniculado medial (MG, tálamo auditivo) de ratas y en neuronas de las capas II/III y V/VI de la corteza auditiva primaria (A1) (Dagnino-Subiabre et al., 2005; 2009; Bose et al., 2010). Estos resultados se correlacionaron con una reducción significativa en la adquisición de una respuesta de evitación condicionada a estímulos auditivos a través del condicionamiento al miedo (Dagnino-Subiabre et al., 2009). Ambos tipos de alteraciones en el sistema auditivo, morfológicas y conductuales, fueron revertidas 15 días después del término del estrés, mostrando un alto grado de plasticidad neuronal en el CI, evidenciado por la recuperación del largo dendrítico y por el aumento del rendimiento en la respuesta de evitación condicionada alcanzando valores semejante a la condición pre-estrés (Dagnino-Subiabre et al., 2005). Estos resultados sugieren que, en ratas, el sistema auditivo es más susceptible al estrés que el sistema visual y que la atrofia en el CI puede afectar el condicionamiento al miedo y la ansiedad en el cerebro a través de alteraciones en el sistema aversivo. Estos cambios inducidos por el estrés en ratas han sido observados también en pacientes con trastornos depresivos, los cuales tienen deteriorado el procesamiento auditivo, condición que es revertida con el tratamiento con antidepresivos (Michael, 2004, Tollkötter et al., 2006, Christ et al., 2008).

En la red neuronal involucrada en el procesamiento emocional a estímulos auditivos, el CI, envía proyecciones hacia el MG. Parte de la información recibida por el MG es enviado directamente hacia la amígdala lateral (McDonald, 1998, Wilensky et al., 2006). La adquisición de memorias emocionales de la amígdala están asociadas con la plasticidad neuronal en la BLA y en el MG (Maren et al., 2001; Poremba & Gabriel, 2001). Estudios de lesión de la BLA muestran ausencia de *freezing* durante el condicionamiento al miedo, demostrando la dependencia de esta estructura para la memorias traumáticas (Maren et al., 2001).

***Arquitectura dendrítica y memoria espacial***

La remodelación dendrítica se ha correlacionado con el déficit de la memoria espacial

(Magariños y McEwen, 1995; Luine et al., 1996; Conrad et al., 1996; Wright y Conrad, 2005; Kleen et al., 2006; McLaughlin et al., 2007). Se ha observado que ratas sometidas a estrés crónico durante 6 horas diarias por 21 días, generan un déficit de la memoria espacial y manifiestan la atrofia dendrítica de las neuronas CA3 del hipocampo (Conrad et al., 1996). Sin embargo, 6 horas diarias de estrés crónico durante 7 y/o 14 días no induce un déficit en la memoria espacial y aprendizaje (Luine et al., 1996) y tampoco induce cambios en la arquitectura dendrítica (Luine et al., 1996). Además, ratas que presentan un deterioro en la memoria espacial, a las cuales se les ha permitido un tiempo de recuperación post-estrés durante 10 días, muestran una recuperación de la memoria espacial (Luine et al., 1994), con el correspondiente retorno del árbol dendrítico (remodelación dendrítica) a la condiciones pre-estrés (Conrad et al., 1999; Vyas et al., 2004). Por lo tanto, los cambios en la arquitectura dendrítica en las neuronas CA3 del hipocampo se correlacionan con el déficit de la función hipocampal, tal como el déficit en la memoria espacial.

***Plasticidad sináptica y funciones cognitivas***

El concepto de plasticidad sináptica implica la capacidad del sistema nervioso de modificar sus circuitos de acuerdo a los requerimientos del entorno. A nivel sináptico, se puede expresar como una variación de la eficacia con que se transmite la información entre neuronas. Numerosas evidencias han demostrado que cambios a corto y a largo plazo de la eficacia sináptica son fundamentales para muchas funciones cerebrales como el aprendizaje y la memoria (Dan y Poo, 2004). Aunque se ha determinado que el estrés crónico afecta la plasticidad sináptica, poco se conoce acerca de los mecanismos moleculares por el cual ocurriría esta alteración. Se ha determinado que el estrés puede bloquear la potenciación de largo plazo (LTP) en el hipocampo, inducida por estimulación eléctrica de alta frecuencia (Rowan et al., 1998; Hirata et al., 2008). En ratas, se ha observado que la aplicación de fármacos que bloquean la LTP, muestran un déficit en el aprendizaje y memoria (Baker y Kim, 2009; Mumby et al., 2005, 2010). Por otro parte, se ha demostrado que la activación de los GRs produce un deterioro en la LTP en el hipocampo, mientras que la activación de los MRs resulta en la facilitación de la LTP (Massaoudi et al., 2002; Korz y Frey, 2003). Se ha observado que la disminución en la LTP está acompañada de una disminución en la fluidez de la membrana, afectando a la liberación de neurotransmisores debido a que este proceso

requiere la fusión de las vesículas sinápticas con la membrana plasmática (Martin et al., 2002). Tal disminución, se debe principalmente a la disminución en la concentración de ácidos grasos poli-insaturados de cadena larga (PUFAs), tales como el ácido araquidónico (AA), el ácido docosahexaenoico (DHA) y el ácido eicosapentaenoico (EPA) (Nishikawa et al., 1994; Calderon y Kim, 2004).

***Efecto del estrés sobre la transmisión sináptica***

Un factor que no ha recibido mucha atención, es el efecto del estrés crónico sobre la transmisión sináptica glutamatérgica y GABAérgica. El balance entre la excitación e inhibición en el cerebro es esencial para la plasticidad sináptica y para el buen funcionamiento cognitivo (Buzsaki y Chrobak, 1995; Cobb et al., 2005). En el sistema nervioso central (SNC), el control de la transmisión excitatoria depende en gran parte de la eficacia de la sinapsis inhibitoria (Mohler, 2006; Kullmann y Lamsa, 2007). Debido a la estrecha relación anatómica y funcional entre estos dos tipos de sinapsis, cambios en la eficacia de la transmisión inhibitoria pueden modificar la plasticidad de las sinapsis excitatorias y viceversa (Chevaleyre y Castillo, 2004; Kullmann y Lamsa, 2007). Estudios de registros intracelulares *in vivo,* evaluando las corrientes excitatorias e inhibitorias en función de la frecuencia del tono (kHz) han mostrado un papel fundamental del efecto sinérgico entre la actividad excitatoria e inhibitoria sobre las neuronas de la región A1 (corteza auditiva primaria) para el procesamiento auditivo (Tan y Wehr, 2009). Del mismo modo, las interneuronas ejercen un fuerte control sobre el balance y sincronización de los circuitos neuronales (Lewis et al., 2005; Bartos et al., 2007, Wulff et al., 2008). Así, la modulación de la eficacia sináptica glutamatérgica y GABAérgica es el principal mecanismo de regulación para el aprendizaje, memoria y procesos cognitivos complejos (Oswald et al., 2006; Levy y Reyes, 2012). El estrés crónico y los glucocorticoides incrementan la transmisión sináptica GABAérgica en el hipocampo (Oswald et al., 2006; Hu et al., 2010; Levy y Reyes, 2012), mientras que en la amígdala lateral (LA), el estrés crónico disminuye la inhibición GABAérgica (Rodríguez et al., 2005; Reznikov et al., 2009; Roozendaal et al., 2009). A nivel cortical, el estrés disminuye la razón inhibición/excitación en la corteza temporal, provocando una disminución en la amplitud de las corrientes postsinápticas inhibitorias sin producir cambios sobre las corrientes postsinápticas excitatorias (Garcia-Oscos et al., 2012). Las diferencias encontradas en el

sistema GABAérgico se deben a variaciones locales. Por ejemplo, GABA puede estar alterado en la interneuronas del giro dentado sin cambiar en la región del hipocampo (Herman et al., 2003).

Alteraciones en la arquitectura dendrítica afectan la plasticidad sináptica. De hecho, las entradas sinápticas se correlacionan con la geometría dendrítica (Purves y Lichtman, 1985; Rall, 1964) y la estructura del árbol dendrítico modifica las propiedades de disparo de las neuronas (Schaefer et al., 2003). Además, la liberación de aminoácidos excitatorios participa en el proceso de retracción dendrítica mediada por el estrés crónico. La inyección de fenitoína, un antiepiléptico que interfiere con la liberación de aminoácidos excitatorios a ratas sometidas a estrés crónico (Crowder y Bradford, 1987), previene la retracción dendrítica de las neuronas de la región CA3 del hipocampo (Magariños et al., 1996; Watanabe et al., 1992). Las interneuronas inhibitorias de hipocampo también ejercen un fuerte control sobre la arquitectura neuronal (Feldblum et al., 2003). La activación de los receptores γ-aminobutírico (GABA) con adinazolam, un derivado de benzodiazepinas, bloquea la retracción dendrítica causada por el estrés crónico (Magariños et al., 1999). En un modelo animal de depresión, ratas con síntomas tipo anhedonia, muestran una disminución de la probabilidad de liberación de GABA en las células granulares del giro dentado (Holm et al., 2011).

## ***El DHA es un ácido graso importante en las neuronas***

La composición de las membranas celulares en el cerebro desempeña un papel fundamental para la correcta realización de todas las funciones biológicas y, por el contrario, cualquier alteración en ésta resultan en una pérdida de la homeostasis celular (Clandinin, 1999), pudiendo generar por ejemplo cambios en la actividad de enzimas ligadas a la membrana y que dan como resultado el deterioro de algunas funciones celulares (Farooqui et al., 1997). El cerebro está constituido por un 60% de lípidos, de los cuales cerca de un 35% corresponden a fosfolípidos (FLs) formados por PUFAs que resultan ser de vital importancia para su correcto funcionamiento (Head, 2008). Estos PUFAs son sintetizados a partir de sus precursores, tales como el ácido linoleico (LN; 18:2 *ω-6*) y el ácido linolénico (LNA; 18:3 *ω-3*), mediante sucesivas reacciones de desaturación y elongación (Yaqoob, 2003; Kaduce et al., 2008). Los PUFAs *ω-3* corresponden a un grupo de ácidos grasos esenciales, tales como el DHA (22:6 *ω-3*) y EPA (20:5 *ω-3*) que se encuentran en altas concentraciones en nuestro

cerebro, cumpliendo una función importante en su funcionamiento y que sólo son obtenidos a través de la dieta (Head, 2008), principalmente de los alimentos de origen marino (Tabla I, anexo). En la actualidad, existen diversos productos alimenticios enriquecidos con PUFAs ω-3 y suplementos en forma de capsulas que contienen principalmente aceites de pescado. Se ha estimado que los requerimientos diarios de PUFAs ω-3 pueden variar entre 100-600 mg para una persona normal, pero no existen antecedentes de si estos requerimientos diarios son suficientes en condiciones de estrés. Por tal motivo, uno de los objetivos de este trabajo es determinar el efecto de la ingesta de un alimento elaborado con gónadas de ostión sobre las alteraciones en el aprendizaje, memoria y trasmisión sináptica inducidas por el estrés crónico en ratas. Al respecto, el ostión del norte (*Argopecten purpuratus*), un bivalvo de gran importancia para el desarrollo de las regiones del norte de Chile, presenta en las gónadas concentraciones de PUFAs *ω-3,* del tipo DHA (14 mg/g por materia seca), preferentemente en la forma de FLs (Caers et al., 1999), convirtiéndolo en un candidato ideal para confeccionar dietas con un alto contenido de PUFAs *ω-3*.

Los PUFAs *ω-3* y *ω-6* compiten en varios pasos de la vía metabólica por la misma enzima, la Δ6-desaturasa, la cual ha sido considerada como la enzima limitante en la síntesis de AA y DHA a partir de sus precursores *ω-3* y *ω-6*, aunque los ácidos grasos del tipo *ω*-3 presentan mayor afinidad para su síntesis (Fig. 1, anexo). La conversión de LNA a DHA ocurre en el hígado, y en un menor grado en los astrocitos y en células del endotelio vascular de la retina y cerebro (Burdge, 2006). Sin embargo, a pesar que el DHA puede ser convertido a partir de los precursores *ω-3*, la síntesis a través de esta vía no alcanza a suplir los requerimientos diarios de DHA, razón por la cual es de especial importancia la ingesta directa en la forma de DHA (Kaduce et al., 2008). Por lo tanto, otro de los objetivo de este trabajo es determinar el efecto de la suplementación directa mediante vía oral con PUFAs *ω-3* (DHA y EPA) sobre las alteraciones en el aprendizaje y memoria inducidas por el estrés crónico en ratas.

El DHA en las neuronas, se encuentra preferentemente en la forma de FLs. Estos, dependiendo del tipo celular, mantienen una proporción particular (Rapoport, 2001) que se debe mantener para no afectar a la fluidez de la membrana, como para no producir alteraciones de las funciones de muchas proteínas integrales, o asociadas a las membranas. La mayor parte del DHA es incorporado en la forma de fosfatidil-etanolamina (FE) y en menos cantidad en la

forma de fosfatidil-serina (FS). De forma similar, la gran mayoría del AA es incorporado en fosfatidil-inositol (FI) y FE, mientras que la gran mayoría del ácido oleico (18:1 $\omega$-9) es incorporado como fosfatidil-colina (FC) (Tabla II, anexo). La incorporación diferencial de AA y DHA en los diferentes fosfolípidos tales como FI, FS y FE comparado con su incorporación en FC, pueden contribuir a las diferencias funcionales de las neuronas. Por ejemplo, en la línea celular Neuro 2A, la suplementación con DHA (25 µM) induce un aumento en la acumulación de FS en la membranas, induciendo un efecto protector contra la apoptosis, evaluado a través de la actividad de la caspasa-3 (Akbar et al., 2005).

En las neuronas, el DHA también se encuentra en las dendritas, donde puede participar en la extensión y generación de la arborización dendrítica, proceso que ocurre durante la formación de la memoria (Marszalek y Lodish, 2005). Adicionalmente, el DHA puede ser importante para la eficiencia de regeneración de los axones y dendritas después de un daño en las neuronas (Marszalek y Lodish, 2005). Las membranas celulares, a nivel sináptico, se encuentran altamente enriquecidas con DHA, mejorando la eficiencia de fusión de las vesículas que liberan los neurotransmisores y favoreciendo la señalización (Marszalek y Lodish, 2005). En las membranas neuronales estos ácidos grasos no sólo alteran las propiedades fisicoquímicas, tales como la fluidez y la permeabilidad, sino que también modulan la expresión génica de muchas proteínas involucradas en la traducción de señales. Varios PUFAs incluido el DHA, son ligandos para el receptor X retinoide (RXR) en el cerebro, cuya activación es un paso obligatorio en la señalización hacia el núcleo (Sessler y Ntambi, 1998; Tanabe et al., 2004).

## ***DHA: Molécula de señalización***

La liberación de los PUFAs desde los FLs por acción de las fosfolipasas (PLAs), puede servir para generar directamente moléculas de señalización o pueden dar origen a otras moléculas de señalización, tales como las prostaglandinas, leucotrienos y tromboxanos (Tapiero et al., 2002; Bazan, 2003; Zamaria, 2004). Estos son conocidos, colectivamente, como eicosanoides (derivados de ácidos grasos de 20 carbonos) y los docosanoides (derivados de ácidos de 22 carbonos), tales como los docosatrienos y neuroprotectinas (Mukherjee et al., 2007). El AA y EPA y/o DHA son convertidos en potentes moléculas de señalización autocrina y paracrina por la acción de las enzimas cicloxigenasa (COXs), lipoxigenasa

(LOXs), y citocromo p450 monoxigenasa (Parker et al., 2006). En general, los metabolitos de los ácidos grasos *ω-3* y *ω-6* poseen efectos biológicos inversos. Por ejemplo, las prostaglandinas $E_2$, metabolitos sintetizados a partir de los ácidos grasos *ω-6* son pro-inflamatorios (Blok et al., 1996), mientras que la forma obtenida a partir de los ácidos grasos *ω-3* (prostaglandinas $E_3$) son anti-inflamatorios (Simopoulos, 2002; Lands, 2003; Calon et al., 2004; Bazan, 2009). En consecuencia, la relación de AA/(EPA, DHA) en la membrana posee un potencial para influenciar los procesos bioquímicos y las respuestas fisiológicas al estrés.

Existen evidencias que indican que la liberación de AA desde los FLs, es importante para la función normal del cerebro (Farooqui et al., 1997). Sin embargo, su aumento ha sido observado en condiciones neuropatológicas inflamatorias (Farooqui et al., 1997), y su liberación ha sido estimulada por compuestos oxidantes, tal como el peróxido de hidrógeno (Xu et al., 2003). Debido a que los ácidos grasos *ω-3* y *ω-6* compiten por la incorporación a los FLs de las membranas neuronales, incrementos en el nivel de DHA y/o EPA en las neuronas resulta en una disminución de AA en FLs, lo cual se traduce en una reducida síntesis de los derivados del AA. Adicionalmente el DHA, es convertido en docosanoides, los cuales protegen a las neuronas del estrés oxidativo (Bazan, 2009). El DHA y los docosanoides inhiben la conversión de AA en eicosanoides (Farooqui et al., 1997).

El incremento en el nivel de ácidos grasos *ω-3*, pueden alterar la actividad de las proteínas integrales, alterar la fluidez de la membrana y contrarrestar la función pro-inflamatorias de los eicosanoides derivados del acido AA. Múltiples evidencias sugieren que el incremento en la actividad de las enzimas COX-2 y fosfolipasa-2, que producen eicosanoides derivados del AA, son un componente importante de la etiología de la neurodegeneración que ocurre durante la progresión de la enfermedad de Parkison y de Alzheimer (Stephenson et al., 1996). Esto se produce, probablemente, a consecuencia de la producción de eicosanoides inflamatorios producidos a partir del AA por acción de la enzima fosfolipasa-2 en neuronas y glías (Stephenson et al., 1999).

### ***Deficiencia en el contenido de DHA***

Diversos estudios en humanos han demostrado que la deficiencia de los PUFAs *ω-3*, producen varias alteraciones neuronales. Al respecto, se ha demostrado que el estrés crónico reduce la cantidad PUFAs *ω-3* incorporados en la células neuronales (Hennebelle et al., 2012).

Estas alteraciones en los PUFAs *ω-3*, se relacionan con la prevalencia de enfermedades de alto impacto tales como las enfermedades cardiovasculares (Kris-Etheryton et al., 2010), cáncer, enfermedades neurodegenerativas y en enfermedades relacionadas con el estrés crónico, como los trastornos depresivos (Schachter, 2005; Riemer et al., 2010). La proporción de ácidos grasos *ω-6/ω-3* obtenida en la dieta moderna incrementa dramáticamente en las personas que consumen ácidos grasos saturados, en los cuales se ha determinado un incremento en la prevalencia de los desórdenes depresivos (Logan, 2004; Simopoulos, 2006). En pacientes depresivos se ha encontrado una reducción significativa en el contenido de PUFAs *ω-3* (EPA, DHA o PUFAs *ω-3* en total), pero no se han encontrado variaciones en los niveles de los ácidos grasos *ω-6* (De Vriese et al., 2003; Ross, 2007; Su, 2008; Riemer et al., 2010), lo que genera un aumento en la proporción *ω-6/ω-3* en éstos (Parker et al., 2006). Es más, el contenido de EPA en los FLs de los eritrocitos está negativamente correlacionado con el grado del trastorno depresivo, a su vez, la proporción de AA/EPA se correlaciona positivamente con los síntomas depresivos (Adams et al., 1996). De forma similar, se ha encontrado una reducción significativa en la cantidad de DHA en los FLs del hipocampo y en el plasma sanguíneo en pacientes con la enfermedad de Alzheimer (Soderberg et al., 1999) y en pacientes esquizofrénicos (Peet et al., 2001; Sethom et al., 2010), mientras que los niveles de ácidos grasos *ω-6* totales se encuentran aumentados (Conquer et al., 2000).

Los modelos animales de experimentación son útiles para determinar los efectos de una dieta deficiente o determinar el efecto de la suplementación con PUFAs sobre las funciones cognitivas, tales como la memoria y aprendizaje. Una dieta deficiente en PUFAs ω-3 produce una disminución significativa en el contenido de éstos en el cerebro (Fedorova et al., 2007), generando un incremento en la proporción ω-6/ω-3 en los FLs (Moriguchi et al., 2000). De forma similar, se ha encontrado que una dieta deficiente en LNA ω-3 en ratas, genera una modificación en la composición de PUFAs, tanto en células cerebrales (neuronas, oligodendrocitos y astroglías), organelos (mitocondrias y retículo endoplasmático) y membranas sinápticas del sistema nervioso, las cuales podrían reducir el aprendizaje (Yamamoto et al., 1987; Bourre et al., 1989). Esta reducción del aprendizaje, inducido por deficiencia de PUFAs *ω-3*, podría estar relacionada con la disminución en la liberación de neurotransmisores. Al respecto, Delion y colaboradores (1994) demostraron que ratas deficientes de PUFAs *ω-3*, presentan una disminución en el contenido de dopamina en el

cerebro, un neurotransmisor importante en la regulación de conductas motivacionales (Levant et al., 2003). En otros estudios se ha encontrado que deficiencias en el contenido de DHA, se relacionan con un pobre reciclaje de las vesículas sinápticas en el hipocampo, afectando de forma negativa el aprendizaje y la memoria espacial (Gamoh et al., 2001). De forma similar, se ha determinado que la ingesta de una dieta deficiente de PUFAs ω-3 produce una reducción en la memoria espacial comparado con las ratas que ingirieron una cantidad adecuada de PUFAs ω-3 (Moreguchi et al., 2000; Lim et al., 2005; Forodova et al., 2007).

***Suplementación con DHA***

Diversos estudios en humanos han sugerido una relación entre el consumo de PUFAs ω-3 y los trastornos depresivos. Por ejemplo, se ha encontrado una correlación positiva entre el consumo de pescado y la ausencia de trastornos depresivos (Hibbeln, 1998). En estudios clínicos, se ha demostrado que la suplementación con PUFAs ω-3 en pacientes con trastornos depresivos produce una mejora de los síntomas, tanto en los pacientes con trastornos unipolares como en los pacientes con trastornos bipolares, en comparación con los pacientes del grupo placebo (Stoll et al., 1999; Nemets et al., 2002). Sin embargo, cómo los ácidos grasos *ω-3* pueden reducir los síntomas de la depresión, aun no están claros.

La suplementación de cultivos neuronales con DHA o AA incrementa el crecimiento de neuritas (Marszalek et al., 2004). La suplementación con AA o DHA en un rango de concentración de 1.5-60 µM aumentan significativamente el crecimiento de neuritas en varios tipos celulares (PC12 y cultivos primarios de hipocampo). Sin embargo, existe un límite en la cantidad de PUFAs que pueden ser utilizados, debido a que la suplementación con AA a una concentración mayor a 60 µM produce un efecto citotóxico. Por el contrario, la administración crónica mediante vía oral de DHA (300 mg/kg por día), aumenta el rendimiento en tareas de aprendizaje espacial, tanto en ratas jóvenes como en ratas adultas (Gamoh et al., 2001). De forma similar, la suplementación con PUFAs *ω*-3 (180 mg/kg de EPA y 120mg/kg de DHA) en ratas deficientes en ácidos grasos produce un aumento en los niveles de DHA en el cerebro y un aumento en la memoria espacial (Chung et al., 2008), y disminuye la ansiedad en ratas sometidas al modelo de adicción a drogas (Buydens-Branchey et al., 2008). En otros estudios, la suplementación crónica con ácidos grados *ω*-3 (120 mg/kg de EPA y 180mg/kg de DHA) han generado un efecto neuroprotector en modelos animales de la enfermedad de Parkinson

(Delattre et al., 2010), mientras que en modelos animales de la enfermedad de Alzheimer (Calon et al., 2005), la administración de DHA en la dieta protege contra el deterioro que se produce en la memoria y aprendizaje (Hashimoto et al., 2002). Sin embargo, la suplementación con 600 mg/kg de DHA diario a ratas produce un déficit en el rendimiento de la memoria espacial (Pan et al., 2011).

Diversos estudios han demostrado que el estrés crónico y el tratamiento con CORT inducen atrofia neuronal y producen un deterioro de aéreas del cerebro relacionadas con la memoria y aprendizaje (Magariños y McEwen, 1995; Vyas et al., 2002). Además, se ha demostrado que el estrés crónico produce una alteración en el balance de la transmisión excitatoria e inhibitoria en el cerebro (Hu et al., 2010; Levy y Reyes, 2012), produciendo un déficit en el aprendizaje y memoria (Baker y Kim, 2009; Mumby et al., 2005, 2010). Se ha demostrado que el estrés crónico produce una disminución en el contenido de PUFAs $\omega$-3, mientras que dietas deficientes en PUFAs $\omega$-3 se relacionan con un pobre rendimiento cognitivo. Sin embargo, aún existen vacios en el conocimiento con respecto al efecto de la suplementación con PUFAs $\omega$-3 sobre las alteraciones inducidas por el estrés crónico sobre los principales marcadores del estrés y sobre el balance entre la transmisión glutamatérgica y GABAérgica en ratas.

## Hipótesis y Objetivos

En base a los antecedentes presentados anteriormente, se proponen la siguiente hipótesis de trabajo y objetivos.

### Hipótesis:

La suplementación con PUFAs del tipo omega-3 o la ingesta de un alimento elaborado con gónadas de ostión (AGO), disminuyen significativamente el deterioro que produce el estrés crónico en la memoria, aprendizaje auditivo y en la plasticidad sináptica de ratas *Sprague-Dawley*.

### Objetivo General:

Determinar el efecto de la suplementación con PUFAs del tipo omega-3 sobre el deterioro en el aprendizaje auditivo y memoria espacial inducido por el estrés crónico en ratas *Sprague-Dawley*.

### Objetivos específicos:

1.- *Determinar el efecto de la suplementación con PUFAs ω-3 sobre el deterioro inducido por estrés crónico en el aprendizaje auditivo, ansiedad y concentración plasmática de Corticosterona.*

2.- *Evaluar el efecto de suplementación con PUFAs ω-3 sobre el deterioro inducido por estrés crónico sobre la memoria espacial, la morfología dendrítica y en la plasticidad sináptica.*

3.- *Verificar el efecto de la ingesta de AGO sobre la ansiedad, morfología dendrítica y aprendizaje auditivo en ratas sometidas a estrés crónico.*

**Breve descripción del contenido de la tesis doctoral.**

En la primera sección, se realizará una descripción y determinación de cada uno de los objetivos propuestos en esta tesis doctoral, que serán abordados en los capítulos del I al III, respectivamente. Cada capítulo difiere en su forma de presentación, debido principalmente a que los resultados del Capítulo I, ya se encuentran publicados, por lo que se ha adjuntado el artículo, mientras que el capítulo II y III serán presentados a partir de la metodología. En la segunda sección, se ha adjuntado como material anexo artículos que han sido realizadas durante el desarrollo de esta tesis doctoral.

**Primera sección:**

**En el capítulo I**, se adjunta la publicación *"Long-Term ω-3 Fatty Acid supplementation Induces Anti-Stress Effects and Improves Learning in Rats"* correspondiente al objetivo N°1 y que actualmente se encuentra en prensa. Cuyo objetivo de estudio fue evaluar el efecto de la suplementación con PUFAS *ω-3* sobre el aprendizaje y sobre los principales marcadores del estrés. Se trabajo con ratas *Sprague-Dawley* machos los cuales fueron asignadas a 3 grupos experimentales: 1) control, 2) vehículo y 3) suplementados. Cada grupo experimental fue dividido en 2 subgrupos: uno de ellos no fueron sometidos al protocolo de estrés, mientras que el otro grupo fue sometido al protocolo de estrés crónico. Posteriormente el aprendizaje fue evaluado mediante el condicionamiento de evitación condicionada. Además, el nivel de CORT y la ansiedad fueron evaluados como marcadores de estrés mediante la técnica de ELISA y la prueba del laberinto elevado en forma de cruz, respectivamente. Estas alteraciones fueron prevenidas mediante la suplementación con PUFAS *ω-3*, por lo que nuestros resultados demuestran que la suplementación tuvo dos efectos beneficiosos en las ratas sometidas a estrés, un fuerte efecto anti-estrés y la mejora del aprendizaje.

**En el capítulo II**, se muestran los resultados del objetivo N°2, cuya publicación *"Long-Term ω-3 Fatty Acids Supplementation Impairs Spatial Memory in Healthy Rats, while Improves Memory in Stressed Rats: Synaptic Plasticity Evidences"* se encuentra en redacción. Cuyo objetivo de estudio fue evaluar el efecto de la suplementación con PUFAS *ω-3* sobre la memoria, morfología dendrítica, marcadores de estrés y sobre la transmisión sináptica en ratas

sometidas a estrés crónico. Se trabajo con ratas *Sprague-Dawley* machos los cuales fueron asignadas a 3 grupos experimentales: 1) control, 2) vehículo y 3) suplementados. Cada grupo experimental fue dividido en 2 subgrupos: uno de ellos no fueron sometidos al protocolo de estrés, mientras que el otro grupo fue sometido al protocolo de estrés crónico. Posteriormente la memoria espacial fue evaluada en la prueba del laberinto en forma de Y, mientras que el porcentaje de ganancia en peso de las ratas y la ansiedad fueron evaluados como marcadores de estrés. La morfología dendrítica fue evaluada a través de la técnica de tinción de Golgi y los estudios de transmisión sináptica fueron evaluadas a través de estudios de electrofisiología mediante la técnica de patch clamp en la modalidad de fijación de voltaje para el registro de corrientes post-sinápticas excitatorias e inhibitorias. El deterioro en la memoria, de la transmisión sináptica GABAérgica, aumento en la ansiedad y la atrofia dendrítica fueron prevenidas mediante la suplementación con PUFAS *ω-3* en la ratas sometidas a estrés, por lo que nuestros resultados demuestran que la suplementación podría mejorar la memoria en la ratas estresadas mediante la recuperación del tono GABAérgico y del largo dendrítico.

**En el capítulo III**, se muestran los resultados del objetivo N°3. Cuyo objetivo de estudio fue evaluar el efecto de la ingesta de un alimento elaborado con gónadas de ostión (AGO), que contiene una alta concentración de PUFAs *ω-3* sobre las alteraciones en el aprendizaje, memoria, morfología dendrítica y sobre los principales marcadores de estrés inducidos por el estrés crónico. Se trabajo con ratas *Sprague-Dawley* machos los cuales fueron asignadas a 2 grupos experimentales: 1) control, 2) AGO. Cada grupo experimental fue dividido en 2 subgrupos: uno de ellos no fueron sometidos al protocolo de estrés, mientras que el otro grupo fue sometido al protocolo de estrés crónico por restricción de movimiento. Posteriormente la memoria espacial fue evaluada en la prueba del laberinto en forma de Y, el aprendizaje evaluado mediante el condicionamiento de evitación condicionada y los marcadores del estrés y el largo dendrítico evaluados como han sido descritos anteriormente. El consumo de un alimento elaborado con gónadas de ostión no previno el deterioro inducido por el estrés crónico sobre la memoria, aprendizaje y sobre el largo dendrítico de las neuronas del hipocampo, por lo que nuestros resultados no demuestran un efecto benéfico en las ratas sometidas a estrés crónico alimentadas con una dieta en base a gónadas de ostión.

**Segunda sección:**

Se ha adjuntado como material anexo, las publicaciones que han sido realizadas durante el desarrollo de esta tesis doctoral. Dichas publicaciones describen como el estrés crónico o el tratamiento con glucocorticoides (CORT) producen alteraciones de los principales núcleos del sistema auditivo (corteza auditiva primaria y colículo inferior), generando un deterioro en el rendimiento en pruebas que dependen de tales núcleos, como la atención auditiva y el aprendizaje al miedo. Mi trabajo de tesis tiene como foco de estudio el estrés crónico y como afecta al aprendizaje auditivo, evaluado mediante el condicionamiento de evitación condicionada, por lo que estos trabajos se relacionan en como el estrés afecta a los principales núcleos del procesamiento auditivo.

**Publicación N°1**: *Repeated Restraint Stress Impairs Auditory Attention and GABAergic Synaptic Efficacy in the Rat Auditory Cortex,* cuyo objetivo fue determinar si el estrés crónico afecta la atención auditiva y la transmisión sináptica en la corteza auditiva primaria (A1). Ratas *Sprague-Dawley* machos fueron entrenados en la prueba de elección de 2 alternativas (2-ACT), una prueba de atención auditiva en ratas. Ratas que alcanzaron un 80% de ensayos correctos en 2-ACT fueron asignadas a dos grupos 1) control y 2) estrés. Mientras que otro grupo fue utilizado para evaluar la transmisión sináptica en A1. El estrés crónico disminuye el número de ensayos correctos en un 28% comparado al grupo control ($p < 0,001$). Además, el estrés reduce la frecuencia de las corrientes postsinápticas inhibitorias espontaneas (sIPSC) y en miniatura (mIPSC) en A1, mientras que la eficacia glutamatérgica no fue afectada. Estos resultados demuestran que el estrés crónico disminuye la atención auditiva y la eficacia sináptica GABAérgica en A1.

**Publicación N°2**: *Corticosterone Treatment Impairs Auditory Fear Learning and the Dendritic Morphology of the Rat Inferior Colliculus*, cuyo objetivo fue determinar el efecto de la administración crónica de corticosterona (CORT) en el condicionamiento al miedo auditivo y visual. Ratas *Sprague-Dawley* machos recibieron CORT (400mg/ml) en el agua durante 10 días consecutivos, produciendo un aumento en el nivel de CORT en el plasma. CORT produce un deterioro del condicionamiento al miedo y de la extinción del miedo en el condicionamiento al miedo auditivo. Además, se determinó el efecto de CORT en la

morfología neuronal en el colículo inferior (núcleo clave del cerebro para el procesamiento auditivo) y superior (núcleo relativo al procesamiento visual) a través de la técnica de tinción de Golgi. CORT disminuye la arborización dendrítica en el colículo inferior en un 50% y no afecta a las neuronas del colículo superior, por lo que estos resultados demuestran que CORT produce un efecto deletéreo en el procesamiento al miedo auditivo más que en el visual en el cerebro.

# Capítulos

***Capítulo I:*** *Objetivo N°1*

# *Publicación N•1*

Estado: In Press

Long-Term ω-3 Fatty Acid Supplementation Induces Anti-Stress Effects and Improves Learning in Rats

Behavioral and Brain Functions, 2013, 9:25 (ISI=2.1)

**Miguel Ángel Pérez**, Gonzalo Terreros y Alexies Dagnino-Subiabre

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# Long-term ω-3 fatty acid supplementation induces anti-stress effects and improves learning in rats

Miguel Á Pérez[1,2], Gonzalo Terreros[1] and Alexies Dagnino-Subiabre[1*]

**Abstract**

Chronic stress leads to secretion of the adrenal steroid hormone corticosterone, inducing hippocampal atrophy and dendritic hypertrophy in the rat amygdala. Both alterations have been correlated with memory impairment and increased anxiety. Supplementation with ω-3 fatty acids improves memory and learning in rats. The aim of this study was to evaluate the effects of ω-3 supplementation on learning and major biological and behavioral stress markers. Male Sprague–Dawley rats were randomly assigned to three experimental groups: 1) Control, 2) Vehicle, animals supplemented with water, and 3) ω-3, rats supplemented with ω-3 (100 mg of DHA+25 mg of EPA). Each experimental group was divided into two subgroups: one of which was not subjected to stress while the other was subjected to a restraint stress paradigm. Afterwards, learning was analyzed by avoidance conditioning. As well, plasma corticosterone levels and anxiety were evaluated as stress markers, respectively by ELISA and the plus-maze test. Restraint stress impaired learning and increased both corticosterone levels and the number of entries into the open-arm (elevated plus-maze). These alterations were prevented by ω-3 supplementation. Thus, our results demonstrate that ω-3 supplementation had two beneficial effects on the stressed rats, a strong anti-stress effect and improved learning.

**Keywords:** Stress, ω-3 polyunsaturated fatty acid, Anxiety, Learning

## Introduction

Stress is a complex biological reaction common to all living organisms that allows them to adapt to environmental pressure (i.e., stressors) [1,2]. Stress responses are mainly mediated by the activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to secretion of glucocorticoids from the adrenal gland; glucocorticoids are bound to glucocorticoid receptors in peripheral tissues and the brain to regulate stress responses [3-5]. Stressors increase the release of corticotrophin releasing factor (CRF) from the hypothamus, inducing adrenocorticotropic hormone release from the anterior pituitary, which in turn stimulates the secretion of corticosterone from the adrenal cortex [6]. Corticosterone is bound to glucocorticoid receptors (GRs) in peripheral tissues and the brain [3,5,7]. The hippocampus, amygdala and medial prefrontal cortex have high concentrations of GRs [8-10]. Chronic glucocorticoid treatment induces dendritic atrophy in the hippocampus [11-13] and medial prefrontal cortex [13], while acute corticosterone treatment induces dendritic hypertrophy in the basolateral amygdaloid nucleus and enhances anxiety [14-16].

Chronic stress and corticosterone treatment affect the dendritic morphology of limbic areas of the rat brain, such as the hippocampus, amygdaloid complex, and prefrontal cortex [17-19]. These alterations increase anxiety, and impair both memory and spatial learning [20-22]. Anxiety is an adaptive reaction induced when an animal is confronted with potential demands and dangers. Indeed, anxiety has a key biological-adaptive role, which is highly conserved during evolution [23]. Excessive or pathological levels of anxiety induce maladaptive responses [23]. In humans, chronic stress or psychosocial stress also produces hippocampal volume atrophy [24] and functional changes in the prefrontal cortex [25].

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Learning in animal models involves associating an auditory cue (conditioned stimulus, CS) with an aversive unconditioned stimulus (US). Once learned, the CS will by itself elicit a conditioned response. For instance, freezing is a conditioned response to fear in two-way signaled active avoidance conditioning (2-AA). Rats are trained in a shuttle box to avoid a foot shock signaled by an auditory cue [26]. Chronic restraint stress impaired the learning in the 2-AA and the neuronal morphology of the auditory system [27,28].

There is abundant evidence that chronic stress and/or diet affects the brain physiology in animal models as well as in humans. However, the interaction between stress and diet is poorly understood [29]. Docosahexaenoic acid (22:6 ω-3; DHA) is a predominant dietary ω-3 polyunsaturated fatty acid (PUFAs) that improves learning by animals in the 2-AA paradigm [30]. Conversely, rats subjected to maternal separation and a ω-3 PUFA-deficient diet are more anxious and fearful than control animals [31]. Long-term EPA and DHA supplementation decreases anger and anxiety in animal models of drug addiction [32,33]. In humans, PUFAs have positive effects on the pathophysiology of a wide range of stress-related disorders [32,34-37]. Patients suffering major depression, a stress-related disorder, have shown reductions in the plasma levels of ω-3 PUFAs, without any change in fatty acid ω-6 levels [38-41].

These findings suggest that long-term ω-3 supplementation of the diet of post-weaning animals has two effects on chronically stressed rats: preventing the stress-induced learning impairment in a 2-AA test and decreasing plasma corticosterone levels and anxiety. The objective of this study was to analyze the effects of a DHA and EPA mix on learning, corticosterone levels and anxiety of Sprague−Dawley rats subjected to a chronic restraint-stress paradigm.

## Materials and methods

### Animals

Male *Sprague–Dawley* rats (80–100 g, 21 days old at the start of the experiment) were housed in groups of three animals per cage, under a 12/12 light/dark cycle (lights on at 8:00 am). They were maintained in a temperature and humidity controlled room (20 ± 1°C, 55%) and weighed every day on a digital scale (Model WLC2/A1, Radwag, Poland). All procedures relating to animal experimentation were in strict accordance with animal care standards outlined in the National Institute of Health (USA) guidelines and approved by the Institutional Animal Ethics Committee of the Universidad de Valparaíso and Universidad Católica del Norte. Efforts were made to minimize the number of animals used and their suffering.

### Experimental design

Figure 1 shows a schematic drawing of the experimental design used in this study. Rats were maintained with *ad libitum* access to food (rat chow, Champion®, Santiago, Chile) and water during all experiments. Each rat ate between 15–30 g of rat chow per day; 0.34% of 1 g of rat chow was ω-3 fatty acids. Rats were randomly assigned into three experimental groups for supplementation: Control (*n* = 60), animals did not receive supplementation; Vehicle (*n* = 60), rats were supplemented daily with 2.5 ml of water by oral administration; and ω-3-supplemented animals (*n* = 60), which were supplemented daily with 2.5 ml of a mix of 100 mg of DHA and 25 mg of EPA per kg animal weight by oral administration (Knop Laboratories S.A. Santiago, Chile). Supplementation was applied once per day; the rat was picked up from its home cage and gently held in the hand of the experimenter for the oral administration of vehicle or ω-3. The procedure took approximately one minute. Animals from the vehicle and ω-3 groups received water or ω-3 fatty acids respectively between postnatal days (PND) 21 and 61 (Figure 1). Each experimental group was divided into two subgroups: one of which was not subjected to any type of stress [control + unstressed (U), C-U, *n* = 30; vehicle + unstressed, V-U, *n* = 30; ω-3 + unstressed, ω-3-U, *n* = 30], while the other one was subjected to a restraint-stress protocol (control + stress, C-S, *n* = 30; vehicle + stress, V-S, *n* = 30; ω-3 + stress, ω-3-S, *n* = 30). Stressed and unstressed animals were littermates and after weaning were housed in separate rooms. Unstressed rats were never exposed to stressed and the restraint stress was applied in different room. Table 1 shows the number of animals used in each experiment.

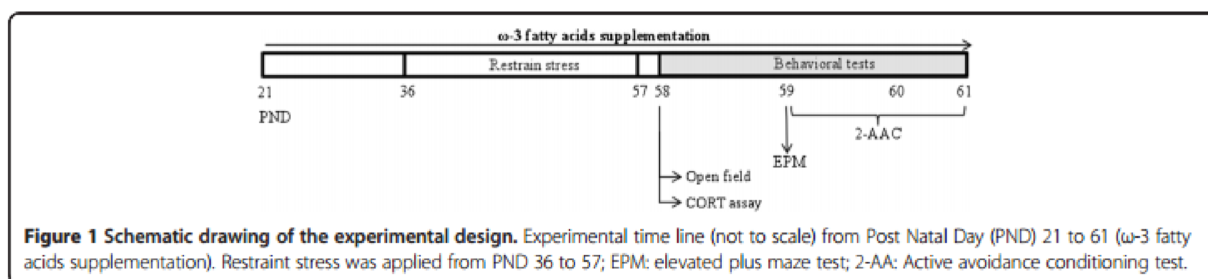


**Figure 1 Schematic drawing of the experimental design.** Experimental time line (not to scale) from Post Natal Day (PND) 21 to 61 (ω-3 fatty acids supplementation). Restraint stress was applied from PND 36 to 57; EPM: elevated plus maze test; 2-AA: Active avoidance conditioning test.

**Table 1 Number of rats used in each experiment**

| | Unstressed rats | | | Stressed rats | | | |
|---|---|---|---|---|---|---|---|
| **Experiment** | **Control** | **Vehicle** | **ω-3** | **Control** | **Vehicle** | **ω-3** | **Total** |
| Locomotor activities and Anxiety | $n = 9$ | $n = 9$ | $n = 9$ | $n = 9$ | $n = 9$ | $n = 9$ | $n = 54$ |
| CORT levels in unstimulated rats | $n = 6$ | $n = 6$ | $n = 6$ | $n = 6$ | $n = 6$ | $n = 6$ | $n = 36$ |
| CORT levels after acute swim | $n = 6$ | $n = 6$ | $n = 6$ | $n = 6$ | $n = 6$ | $n = 6$ | $n = 36$ |
| Learning (active avoidance conditioning) | $n = 9$ | $n = 9$ | $n = 9$ | $n = 9$ | $n = 9$ | $n = 9$ | $n = 54$ |
| **Total** | 30 | 30 | 30 | 30 | 30 | 30 | $\mathbf{n = 180}$ |

### Handling procedure and restraint stress

Rats were removed every day by hand and transferred to another cage on the pan of a balance to be weighed. Different investigators did this procedure from those applying the restraint stress. All rats in every group were handled with the same procedures. Animals were placed in acrylic restrainers (6 cm wide × 12 cm long and then 6 cm wide × 20 cm long as the rats grew) in their home cages. They were subject to restriction for 6 h every day, beginning at 10 am, from the 36 to 57 PND (21 days of restraint stress). Restrainers were perforated at each end to allow ventilation and avoid overheating the animals. During the stress protocol, animals could breathe without difficulty and urinate and defecate without being in constant contact with their wastes. The following additional parameters were measured to monitor the overall effects of the stress protocol: percentage gain in body weight, plasma corticosterone levels, and anxiety (see below).

### Behavioral procedures

On day one and day two after the end of the stress protocol rats were analyzed individually in the open field and the EPM respectively. A separate set of rats was used for this experiment (C-U, $n = 9$; C-S, $n = 9$; V-U, $n = 9$; V-S, $n = 9$; ω-3-U, $n = 9$; ω-3-S, $n = 9$). Behavioral tests were carried out from 10 am to 2 pm in the test room. The activity of each rat was recorded by Internet Protocol (IP) cameras (VIVOTEK, Sunnyvale CA, USA) fixed above the behavioral apparatus and connected to a computer in another room. Videos were acquired by Nuuo software (Nuuo, Taipei, Taiwan) and analyzed with ANY-maze video tracking system (Stoelting Co., IL, USA). Mazes were wiped and cleaned with 5% ethanol solution after each trial. In all experiments, animals from control and stress groups were evaluated at the same time.

### Open field test

Behavior tests were conducted in a soundproof and temperature-controlled (21 ± 1°C) room. Each rat was placed in the center of a black Plexiglass cage (70 × 70 × 40 cm) for 5 min. The background noise level in the open field was 40 dB (Precision sound level meter, Model # 1100, Quest Technologies, Oconomowoc, WI) and the arena was illuminated by 300 ± 20 lux (measured by a digital lux meter, Model # LX-1010B, Weafo Instrument Co., Shanghai, China). The total distance travelled and average speed were determined from the video recordings and analyzed with the ANY-maze video tracking system (Stoelting Co., IL, USA).

### Elevated Plus-Maze (EPM)

Twenty-four hours after the open field test, we measured anxiety levels using an EPM test. Each rat was placed individually in an EPM, consisting of two open arms (60 × 15 cm each), two closed arms (60 × 15 × 20 cm each) and a central platform (15 × 15 cm) arranged so that the two arms of each type were opposite to each other. The maze was elevated 100 cm above the floor. The illumination was 300 ± 10 luxs in the open arms and 210 ± 10 luxs in the closed arms. At the beginning of each trial, animals were placed at the center of the maze, facing an open arm. During a 5-min test period we recorded the frequency of entries to the open and closed arms. The percentage of entries and the ratio of open to total arm entries (open/total×100) were used as measures of the anxiety level. Total arm entries were taken as an indicator of general locomotor activity. Entry into an arm was defined as having occurred when the animal placed all four limbs onto the arm.

### Active avoidance conditioning (2-AA)

#### *Apparatus*

Rats were trained in a shuttle box (50 × 25 × 25 cm3) that was divided into two identical stainless steel modular testing units by a black Plexiglas divider. The divider had a narrow passage (8 cm) opened between the sections. The grid floor had 38 stainless steel bars arranged parallel to the dividers (Panlab Instruments, Barcelona, Spain). A 3-kHz 80-dB tone was presented to subjects as a conditioned stimulus (CS) signaling the upcoming unconditioned stimulus (US) in the form of a foot shock (0.5 mA) delivered by a shocker (LE 100–26, Panlab S.L., Barcelona, Spain). The CS was delivered simultaneously by speakers located on opposite walls of the chamber (20 cm high). Both the CS and US stimuli were regulated by Shutavoid software (Panlab S.L., Barcelona,

Spain). The conditioning chamber was placed in a sound-attenuating box. The inside of the box was dimly illuminated with a 0.5-W LED bulb.

### Behavioral training

A separate set of rats was used for this experiment (C-U, $n$ = 9; C-S, $n$ = 9; V-U, $n$ = 9; V-S, $n$ = 9; ω-3-U, $n$ = 9; ω-3-S, $n$ = 9). The rats were placed in the shuttle box and trained individually. During the training sessions the rats were subjected to a 5-min stimulus-free acclimation period. On day 1, all rats were first exposed to a 5-min acclimation period, followed by the habituation trials (habituation) where rats received a CS tone for 20 sec, with an average inter-trial interval (ITI) of 30 s without presenting the US. Rats were then returned to their home cages. On day 2 (conditioning day 1), after an acclimation period, rats received 100 signaled avoidance trials with an average ITI of 30 sec. Each trial consisted of 20 sec of CS, the last 10 sec of which coincided with a 10-sec US. Shuttling action by the rat cut the tone immediately and prevented the foot shock. If there was no shuttling during the 20-sec tone, the foot shock was applied until the rat shuttled (escape response) or the shock continued for a maximum of 10 sec. Rats were then returned to their home cages for 24 h. On day 3 (conditioning day 2), rats were returned to the chamber and received 50 signaled avoidance trials with an average ITI of 30 sec, the last 10 sec of which overlapped with a 0.5-mA foot shock (maximum shock duration of 10 s) until the animal escaped to the opposite chamber.

### Behavioral measurement

All animal movements were recorded by IP cameras mounted inside the sound-attenuating box. Conditioned avoidance response (CR) was defined as the rat crossing to the opposite chamber within the first 10 s after the tone started. One hundred and fifty training trials were applied to all animals on days 2 and 3, which were divided into fifteen blocks of 10 trials each. The number of CRs was measured in each block of trials and the percentage of CRs (% CR) was calculated [(number of CR/10 training trials)×100]. All data were measured by Shutavoid software (Panlab S.L., Barcelona, Spain).

### Plasma corticosterone measurement by ELISA

This experiment analyzed whether restraint stress affects the stress levels of the rats one day after the stress had ended. The most conventional method to determine if animals are stressed is to measure the plasma levels of the stress hormone corticosterone. Stressed animals show an increase in HPA axis activity and plasma corticosterone levels compared to controls after exposure to an uncontrollable stressor, leading to maladaptive responses [42]. In this way, acute swim stress in a water maze increases plasma corticosterone levels of *Sprague–Dawley* rats [43]. Therefore, we measured the plasma corticosterone levels of the rats one day after of the last restraint session, when behavioral experiments were initially conducted.

Animals were subjected to a new stressor (swimming in a water maze) and corticosterone plasma levels were quantified before and after water maze exposure.

A separate set of animals was used to measure the concentration of corticosterone in plasma to avoid the stressfulness of blood collection being a contaminating factor in the behavioral experiments. One set of rats (C-U, $n$ = 6, C-S, $n$ = 6, V-U, $n$ = 6, V-S, $n$ = 6, ω-3-U, $n$ = 6, ω-3-S, $n$ = 6) was given a 60 s probe trial in a water maze at 11 am after which the animals were transferred to a heated holding cage for 10 minutes. Afterward, the animals were taken to a separate room (time used approximately 10 s) and quickly anesthetized with isoflurane (time used approximately 5 s) and immediately sacrificed via decapitation under deep anesthesia for blood collection. Animals were not exposed to other decapitated animals before being anesthetized. Another set of rats (C-U, $n$ = 6, C-S, $n$ = 6, V-U, $n$ = 6, V-S, $n$ = 6, ω-3-U, $n$ = 6, ω-3-S, $n$ = 6) was not disturbed and was sacrificed at 11:11 am under deep anesthesia. The Morris water maze consisted of a blue circular tank (183 cm diameter) in a room that was rich with spatial cues. The tank contained non-toxic colored water at 19°C (black non-toxic tempura paint).

Blood (1 ml) was collected in heparinized tubes, centrifuged at 3,000 rpm (Model # MiniSpin Plus; Eppendorf AG, Hamburg, Germany) for 20 min to obtain plasma, which was then stored at −70°C. Total corticosterone was determined by an Enzyme Immunoassay kit (CorticosteroneBioAssay™, Catalog. # C7903-30) purchased from US Biological (Swampscott, MA). Optical density values were measured at 450 nm using a microplate reader (Model # Anthos 2010 Microplate Reader, Biochrom Ltd, UK). Samples were diluted 1:10 and processed in duplicates. Averaged final values were represented as μg/dL.

### Statistical analysis

#### *Open field test and percentage of body weight gain*

Time, total distance travelled, and average speeds were analyzed with the Student's $t$-test. Percentage of body weight gain was analyzed using a two-way repeated-measures ANOVA [groups (control, stress) × days (1, 7, 14, 21)] followed by Bonferroni *post-hoc* comparison tests. A two-way ANOVA compared groups for anxiety levels in the open field test. The dependent variable for anxiety was the time spent in the center of the open field and the independent variables were restraint stress (unstressed and stressed) and the diet (control, vehicle and ω-3).

#### Anxiety and corticosterone levels

A two-way ANOVA compared groups for anxiety levels in the plus-maze. The dependent variable for anxiety was the percentage of open-arm entries and the independent variables were restraint stress (unstressed and stressed) and diet (control, vehicle and ω-3). The corticosterone levels were analyzed by a 3 × 2 factorial ANOVA.

#### Active avoidance

The CR percentage was analyzed with a two-way repeated-measures ANOVA [groups (control, stress)×trials (habituation, conditioning day 1, conditioning day 2)] followed by a Bonferroni *post-hoc* comparisons test. Data from the % CR were transformed to arcsine [(arcsine of square root of (% CR/100)] to satisfy requirements of the ANOVA model and then the slopes were analyzed by regression analysis.

## Results

#### Body weight gain and locomotor activity

The two-way repeated-measures ANOVA showed that chronic restraint stress significantly reduced body weight gain beginning on PND 21 ($F_{(1,48)}$ = 35.61, $p < 0.0001$) (Figure 2A). There was a main effect of diet and interaction on body weight gain, where the post-hoc test showed that rats subjected to restraint stress from both control and ω-3 diet groups had significantly decreased body weight gain compared to the respective unstressed controls ($F_{(1,48)}$ = 1559, $p < 0.0001$). Conversely, body weight gain of rats from the vehicle (diet) group subjected to restraint stress was not affected compared to unstressed rats from the vehicle group (Figure 2A). Figure 2B shows body weight gain at PND 21 (same data as in Figure 2A). There was a main effect of both stress and diet where the body weight gain of rats subjected to the stress protocol was significantly lower ($F_{(2,48)}$ = 35.612, $p < 0.0001$), while rats subjected to stress that received supplements (vehicle and ω-3 diet) had significantly lower body weight gain than rats on the control diet ($F_{(2,48)}$ = 48.701, $p < 0.0001$). There was a significant effect of the diet-stress interaction on body weight gain ($F_{(2,48)}$ = 3.46, $p < 0.05$). The post-hoc test showed that rats of the control and ω-3 groups that were subjected to restraint stress had significantly lower body weight gain than unstressed rats ($F_{(2,48)}$ = 3.46, $p < 0.001$). Conversely, restraint stress did not affect the body weight gain of animals in the vehicle group ($F_{(2,48)}$= 3.46, $p = 0.849$).

Restraint stress did not affect locomotor activity levels (total distance traveled and average speed) in any experimental group (Table 2). Restraint stress significantly reduced time spent in the center of open field among control and vehicle groups rats ($F_{(1,48)}$ = 9.06, $p < 0.01$), while stress did not affect ω-3 group rats. Animals of the vehicle group spent significantly more time in the center of open field than control group rats ($F_{(2,48)}$ = 4.93, $p < 0.01$). A two-way ANOVA did not show an interaction between restraint stress and diet (Table 2).

#### Effect of ω-3 fatty acids supplementation on anxiety and corticosterone plasma levels

Restraint stress and vehicle treatment significantly decreased the percentage of open-arm entries (control group: stressed rats: 32.42 ± 6.24 entries, unstressed rats: 67.58 ± 6.24 entries, $p = 0.002$; vehicle group: stressed rats: 39.44 ± 7.63 entries, unstressed rats: 60.56 ± 7.64 entries; $p > 0.150$). This effect was prevented with ω-3 supplementation (stressed rats: ω-3 supplementation: 62.20 ± 3.42 entries, vehicle-treated rats: 39.44 ± 7.63 entries, control rats: 32.42 ± 6.24 entries, ($F_{(1,48)}$ = 5.11, $p = 0.03$) (Figure 3A).

Figure 3B shows the level of circulating corticosterone in rats subjected to 60-s probe trials in the water maze and in animals that were not disturbed. There were no significant differences in the corticosterone levels between unstressed and stressed rats that were left undisturbed (Figure 3B, left side). Rats that were subjected to acute swim had significantly higher plasma corticosterone

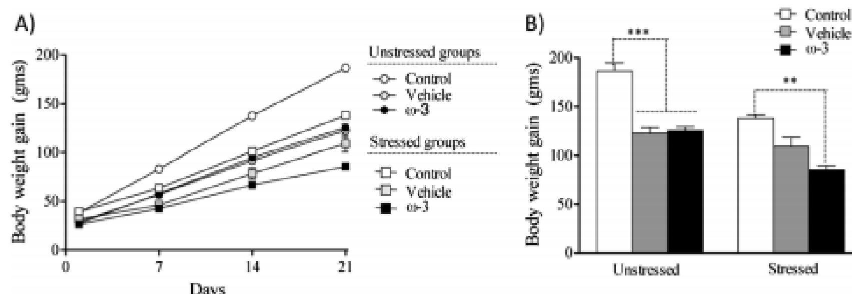


**Figure 2 Effect of chronic restraint stress on body weight gain.** Rats subjected to restraint stress failed to gain weight (**A** and **B**). Differences in body weight gain were observed from day 7 between stressed and unstressed rats from both control and ω-3 groups, and from day 14 for animals from the vehicle groups (**A**). In contrast, unstressed rats gained weight throughout the study. ω-3: ω-3 supplementation. Data are represented as means ± SEM. An asterisk (*) indicates significant differences.

**Table 2 Locomotor activity of the animals**

| Groups | Subjects | Total distance traveled (m) | Average speed (m) | Time spent in center of open field (sec) |
|---|---|---|---|---|
| *Unstressed groups* | | | | |
| Control | 9 | 23.1 ± 3.1 | 0.07 ± 0.01 | 19.0 ± 3.04 |
| Vehicle | 9 | 20.3 ± 1.8 | 0.07 ± 0.01 | 26.5 ± 2.88 |
| ω-3 | 9 | 24.1 ± 3.4 | 0.08 ± 0.01 | 16.0 ± 1.53 |
| *Stressed groups* | | | | |
| Control | 9 | 22.6 ± 3.6 | 0.07 ± 0.01 | 9.00 ± 2.22 |
| Vehicle | 9 | 18.4 ± 1.3 | 0.06 ± 0.004 | 16.8 ± 2.01 |
| ω-3 | 9 | 21.2 ± 1.9 | 0.07 ± 0.01 | 16.3 ± 4.48 |

For each experiment shows the means value ± SEM.

levels than rats that were not disturbed ($F_{(1,66)}$ = 107.98, $p < 0.001$) (Figure 3B, right side). In the control group, rats that were subjected to restraint stress and swimming for 60 s in the water maze had higher corticosterone levels than rats that were unstressed (stressed = 420.0 ± 30.2 ng/ml, n = 6, unstressed = 285.1 ± 16.9 ng/ml, n = 6, p < 0.01) (Figure 3B). This effect was prevented by ω-3 supplementation (acute swimming; ω-3 group: stressed = 321.5 ± 25.3 ng/ml, n = 6, unstressed = 247.5 ± 2.5 ng/ml, n = 6, p > 0.05). After acute swimming, vehicle treatment significantly increased the plasma corticosterone levels in the unstressed rats compared to that of unstressed rats of control group (unstressed rats of vehicle group: 383.6 ± 38.5 ng/ml, n = 6, unstressed rats of control group: 285.1 ± 16.9 ng/ml, n = 6, p > 0.05).

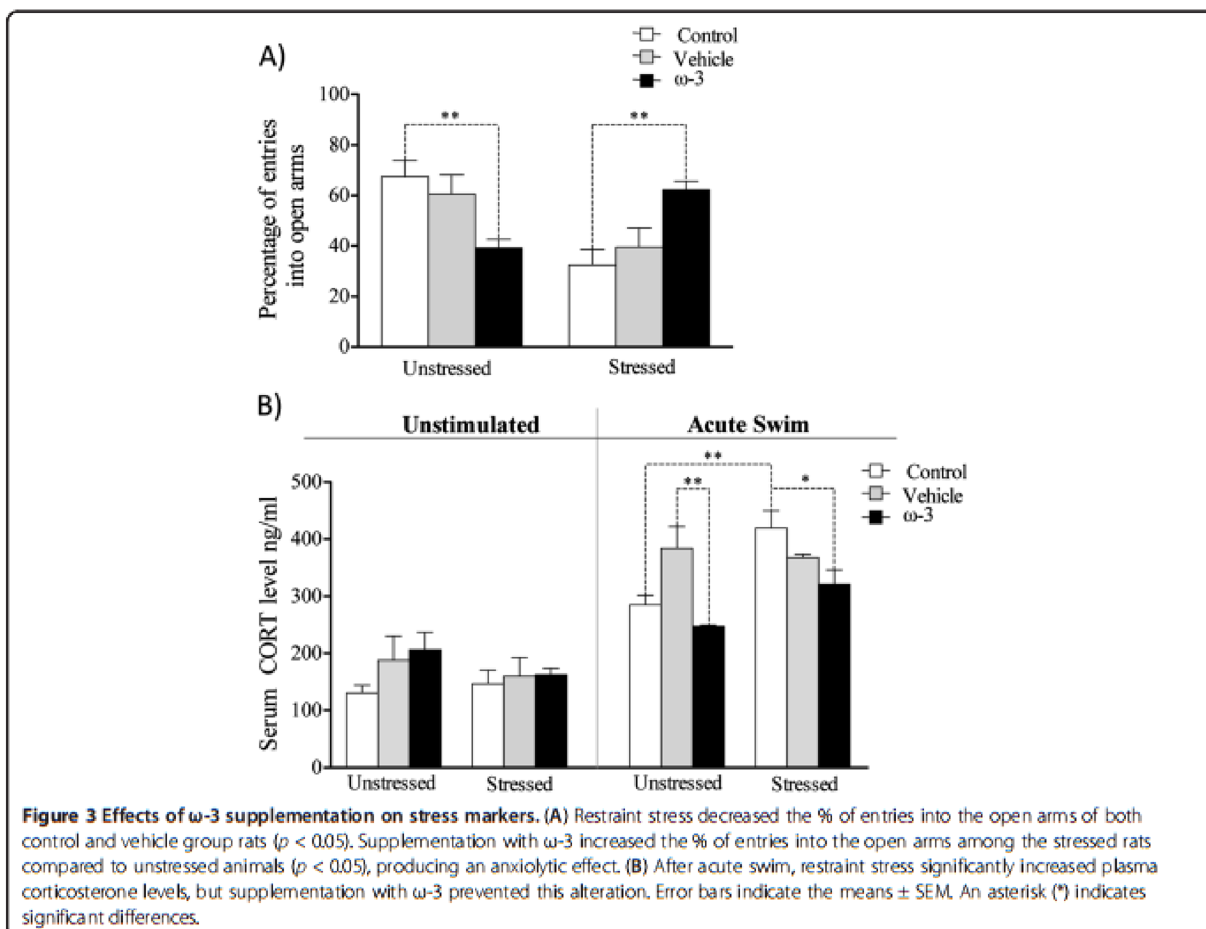


**Figure 3 Effects of ω-3 supplementation on stress markers.** **(A)** Restraint stress decreased the % of entries into the open arms of both control and vehicle group rats ($p < 0.05$). Supplementation with ω-3 increased the % of entries into the open arms among the stressed rats compared to unstressed animals ($p < 0.05$), producing an anxiolytic effect. **(B)** After acute swim, restraint stress significantly increased plasma corticosterone levels, but supplementation with ω-3 prevented this alteration. Error bars indicate the means ± SEM. An asterisk (*) indicates significant differences.

### Effects of ω-3 fatty acids supplementation on active avoidance conditioning

Figures 4A and B show the percentage of conditioned responses (% CR) during the avoidance conditioning to the tone under the three dietary regimes in unstressed and stressed conditions. The two-way repeated-measures ANOVA shows the main effects of the trials and interactions (trials* diet) on % CR of the unstressed rats (Figure 4A). There was no main effect of diet on % CR in rats of the unstressed group ($F_{(2,34)} = 2.87$, $p = 0.07$). Likewise, in the stressed groups, there was a main effect of trials and interaction (trials*diet) on % CR (Figure 4B). A 2 × 3 factorial ANOVA showed that chronic restraint stress significantly reduced the % CR on day 1 of conditioning ($F_{(1,48)} = 15.417$, $p < 0.01$) (Figure 4C). There was a main effect of diet on % CR ($F_{(2,48)} = 5.779$, $p < 0.01$), where the post-hoc test showed that the vehicle groups had significantly lower % CR compared to the control group. This effect was prevented by ω-3 supplementation (Figure 4C).

Figure 4D shows the % CR on day 2 of conditioning (Day 2). A 2 × 3 factorial ANOVA showed a main effect of chronic restraint stress on % CR ($F_{(1,48)} = 46.949$, $p < 0.001$), where the post-hoc test indicates that restraint stress significantly decreased the % CR compared to unstressed rats. There was a main effect of diet on % CR ($F_{(2,48)} = 13.671$, $p < 0.001$), where the post hoc test shows that the vehicle group had a significantly lower % CR than the control group. This effect was prevented by ω-3 supplementation (Figure 4D). Regression analysis to compare the slopes of stressed rats on day 2 of conditioning showed that animals from the ω-3 group had a steeper slope than rats of the other groups subjected to stress ($F_{(1,176)} = 2.9$, $p = 0.046$) (Figure 4E).

## Discussion

In this study we analyzed the effects of ω-3 supplementation on anxiety, plasma corticosterone levels, and learning of chronically stressed rats. First, we investigated whether our stress protocol was effective in triggering stress responses. Stressed rats of all experimental groups had less body weight gain than unstressed control group rats (Figure 2A,B). This demonstrates that the stress protocol used was effective and that ω-3 supplementation did not prevent this effect (Figure 2A,B). Comparable results have been reported using similar stress paradigms and corticosterone administration [44-46]. Diets enriched with PUFAs, in particular the ω-3 family, decreased both the adipose tissue mass and plasma leptin levels in rats [47]. Leptin is released from adipocytes and regulates food intake and body weight by binding to leptin receptors to the hypothalamus [48,49]. Thus, it is

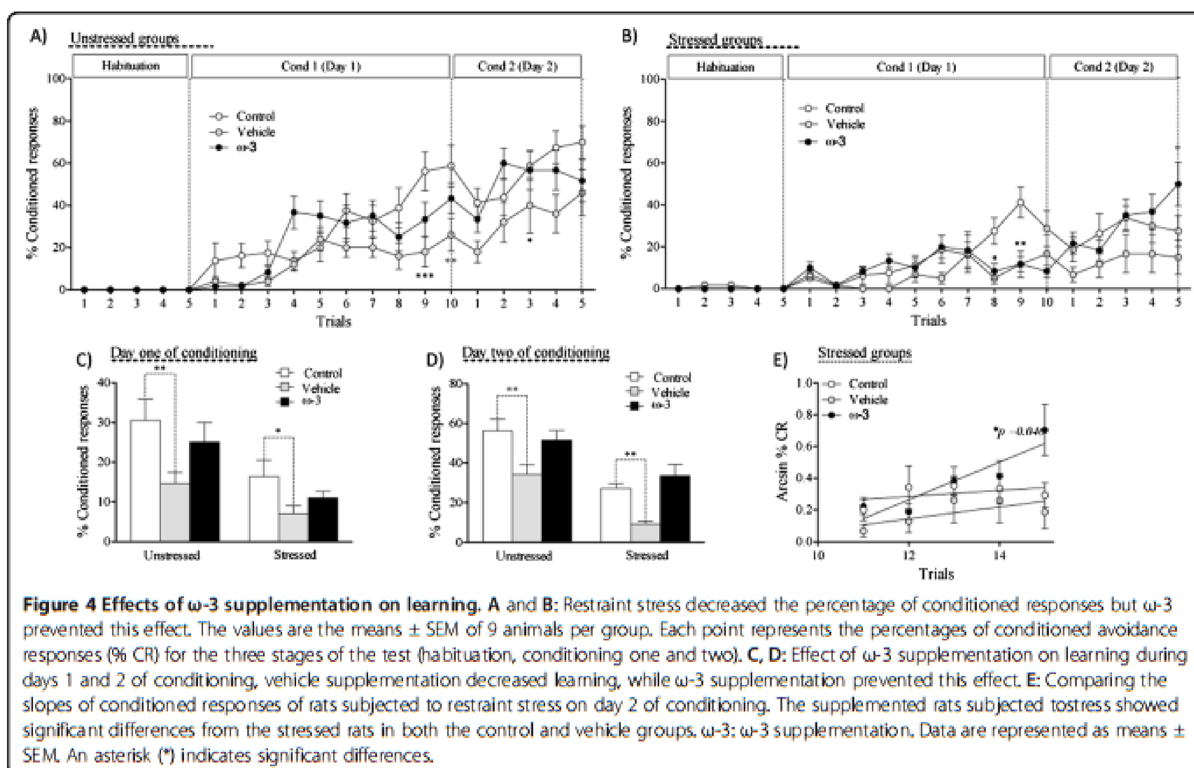


**Figure 4 Effects of ω-3 supplementation on learning. A** and **B:** Restraint stress decreased the percentage of conditioned responses but ω-3 prevented this effect. The values are the means ± SEM of 9 animals per group. Each point represents the percentages of conditioned avoidance responses (% CR) for the three stages of the test (habituation, conditioning one and two). **C, D:** Effect of ω-3 supplementation on learning during days 1 and 2 of conditioning, vehicle supplementation decreased learning, while ω-3 supplementation prevented this effect. **E:** Comparing the slopes of conditioned responses of rats subjected to restraint stress on day 2 of conditioning. The supplemented rats subjected tostress showed significant differences from the stressed rats in both the control and vehicle groups. ω-3: ω-3 supplementation. Data are represented as means ± SEM. An asterisk (*) indicates significant differences.

probable that the ω-3 supplementation used in our experiments decreased the body weight of the rats compared to that of controls (Figure 2A,B).

### Effects of restraint stress and ω-3 fatty acid on anxiety and corticosterone plasma levels

Stressed control and vehicle groups rats had significantly lower percentages of entries into the open arms of the EPM and spent less time in the center of the open field test than unstressed rats (Figure 3A, Table 2). These behaviors were related to an anxiogenic effect induced by restraint stress and vehicle treatment (Figure 3A). Interestingly, supplementation with ω-3 had an opposite effect on the stressed rats. Indeed, ω-3 supplementation increased the number of entries into the open arm of the EPM, which was related to an anxiolytic effect (Figure 3A) that was not associated with locomotor impairments due to restraint stress and vehicle (Table 2).

Comparable results were obtained using another chronic restraint stress paradigm and ω-3 supplementation [50]. Anxiety is mainly regulated by the basolateral amygdala and the bed nucleus of the stria terminalis (BNST) [51-53]. In some chronic stress paradigms, such as chronic unpredictable stress or immobilization, enhanced anxiety has been correlated with dendritic hypertrophy in the basolateral amygdala and BNST [18,54,55]. It is possible that the chronic stress protocol and vehicle treatment in our study induced hyperactivation of the basolateral amygdala and/or BNST by plastic neuronal changes, which significantly increased anxiety, HPA axis activity, and plasma corticosterone levels (Figure 3A,B). Another brain area that modulates BNST neuronal excitability is the dorsal raphé nucleus (DRN) [56]. Axons of the serotoninergic neurons located in the DRN are sent to the BNST and serotonin is released to the synaptic space, which in turn inhibits the neuronal excitability in the BNST by activation of the $5\text{-HT}_{1A}$ and $5\text{-HT}_{1B}$ receptors [56,57]. Serotonin levels are reduced in the brain of stressed rats [58,59], and thereby the $5\text{-HT}_{1A}$ and $5\text{-HT}_{1B}$ receptors in the BNST are not activated. This alteration may contribute to increasing neuronal excitability in the BNST and anxiety in the stressed rats (Figure 3A).

On the other hand, ω-3 supplementation significantly decreased corticosterone levels in the unstressed and stressed rats (Figure 3A, right side). This suggests that ω-3 supplementation prevents hyperactivation of the HPA axis induced by chronic stress, decreasing the effects of corticosterone on dendritic morphology and neuronal activity of the basolateral amygdala and BNST. Supplementation with ω-3 increases serotonin levels in the brain of stressed rats [60], which in turn may reduce BNST neuronal excitability by activation of the $5\text{-HT}_{1A}$ and $5\text{-HT}_{1B}$. Thus, ω-3 supplementation decreases anxiety of the stressed rats.

Figure 3A and Table 2 show that ω-3 supplementation had an anxiogenic effect on the unstressed rats and significantly reduced corticosterone levels compared to the level in unstressed rats treated with vehicle (Figure 3B). This was unexpected and could be explained by the effects of both ω-3 and serotonin on the HPA axis and neuronal activity in the BNST, respectively. Oral administration of vehicle was a stressor for the rats due because it increased corticosterone levels in the unstressed rats. However, this effect was prevented by ω-3 supplementation (Figure 3B, right side). It is possible that ω-3 prevents stress-induced dendritic hypertrophy in the amygdala of unstressed rats and this decreases corticosterone levels compared to that of unstressed rats treated with vehicle (Figure 3B, right side). Supplementation with ω-3 may increase serotonin levels in the brain of rats [60]. We suggest that to counteract this effect, the expression of the $5\text{-HT}_{1A}$ and $5\text{-HT}_{1B}$ receptors was down-regulated in the BNST of unstressed rats supplemented with ω-3. In this context, neuronal excitability in the BNST may increase because the inhibitory control of serotonin over the BNST is lost. As result, anxiety is enhanced in unstressed rats supplemented with ω-3 (Figure 3A).

Unstressed and stressed rats of control group that were not stimulated had similar corticosterone levels, suggesting that the rats adapted to 21 days of restraint stress (Figure 3B, left side). Previous studies have shown that 3 or 6 hours per day of restraint stress significantly increased corticosterone plasma levels during the first week, while in the second and third weeks of restraint stress the increases of corticosterone levels were less pronounced [61,62]. Therefore, if the effects of 21 days of restraint stress on HPA axis activity and corticosterone levels had been lost, the rats that were subjected to restraint stress and unstressed rats would have had comparable plasma corticosterone levels after exposure to a new uncontrollable stressor (acute swimming). However, control group rats subjected to restraint stress had significantly higher plasma corticosterone levels than unstressed rats following one minute of swimming (Figure 3B). This suggests that one day after the restraint stress ended, unstressed and stressed rats had similar HPA axis activity in an environment without stressors. On the other hand, stressed control group rats still showed higher levels of the HPA axis activity than unstressed rats exposed to a new uncontrollable stressor. This neuroendocrine alteration, which induces maladaptive responses to stressors, is characteristic of stressed animals [42,50].

Corticosterone plasma levels increased for approximately the first seven days of restraint stress [10]. However, the long-term impact of the chronic stress on the neuronal morphology of the lateral amygdala and on

anxiety levels were measured after twenty-one days of stress-free recovery [55]. Therefore, chronically stressed rats may have had enhanced anxiety and hyperactivity of the HPA axis at the same time as they were subjected to new stressors like the EPM and swimming in a water maze (Figure 3A,B).

In our experiments, supplementation was applied by oral administration and this method resulted in higher corticosterone levels after acute swimming in unstressed vehicle group rats than those of unstressed control group rats (Figure 3B, right side). This suggests that vehicle treatment, which was applied from weaning to the end of the stress period, was sufficient to induce short-term stress in the supplemented rats. Handing could be comparable to oral administration of vehicle applied before chronic restraint stress protocol. A longer period of handling has gradually less inhibitory effects on the HPA axis activity and significantly decreases the animal's sensitivity to the restraint stress [63], while a shorter period of handing before applying acute restraint stress results in significantly lower corticosterone and adrenocorticotropic hormone plasma levels than those of rats without handling [63].

The method used to apply the supplementation in our experiments could have induced more profound desensitization of the mechanisms involved in inducing the HPA axis response to restraint stress. This in turn could have resulted in lower corticosterone plasma levels in the stressed rats supplemented with vehicle than in animals that were not supplemented in the control group, after acute swimming (Figure 3B, right side). Desensitization of the HPA axis might involve the loss of CRH receptors in the anterior pituitary, which in turn may induce the corticotrophs to become refractory to CRH hypersecretion during restraint stress.

### The effects of restraint stress and ω-3 fatty acid supplementation on learning

Restraint stress and oral administration of vehicle were stressful for the rats given that the two treatments increased plasma corticosterone levels (Figure 3B). In addition, these treatments impaired learning during the conditioned trials (Figure 4C,D). Lesion studies in the main nuclei of the auditory system that regulates learning, the inferior colliculus (IC, auditory mesencephalon) and the medial geniculate nucleus (MG, auditory thalamus), have demonstrated that the two brain structures are key factors for acquiring aversive memories to auditory cues during fear conditioning in rats [64]. As well, restraint stress induces dendritic atrophy in the IC, MG, and primary auditory cortex, and affects auditory processing [27,65,66]. A recent study using micro Positron Emission Tomography supports these findings, in that chronic mild stress induced a significant decrease in glucose metabolism in the IC, but not in the superior colliculus (visual mesencephalon) [67]. In our study, learning impairment could have been due to stress-induced dendritic atrophy in the IC and/or MG. In support of this idea, unstressed and stressed rats supplemented with vehicle showed significant less learning than animals without supplementation. However, ω-3 supplementation prevented this effect (Figure 4C,D,E), possibly by preventing the stress-induced impairment in the IC and/or MG. In fact, ω-3 fatty acid deficiency impairs active avoidance and decreases the polyunsaturated fatty acid composition in the cellular and subcellular fractions [38]. As well, learning alterations associated with maternal deficiency of α-linolenic acid are prevented by α-linolenic acid supplementation after weaning [68]. Likewise, DHA supplementation prevents learning impairments in rats induced by ω-3 deficiency in rats [30].

Other brain nuclei that are key for acquiring and evoking auditory avoidance conditioned responses are the lateral (LA) and basal amygdala [26]. Therefore, another possible explanation for our results in the 2-AA is that restraint stress and ω-3 supplementation had opposite effects on these nuclei, that restraint stress induced dendritic hypertrophy in the LA and this dendritic change enhanced anxiety-like behaviors by the BNST [54]. On the other hand, ω-3 supplementation may have prevented these morphologic alterations and produced anxiolitic effects in stressed rats. This, in turn could have improved learning, as has been seen with anxiolitic drugs such as midazolam, which facilitates avoidance retrieval in rats [69].

### Possible cellular mechanisms underlying the anti-stress effects of ω-3 fatty acids supplementation

A growing body of evidence suggests that ω-3 PUFA levels in the brain modulate the reactivity and sensitivity to stress [70]. In addition, chronic stress reduces the DHA content in the brain phospholipids and prevents the incorporation of supplemental-DHA in the neuronal membranes [60,71]. We suggest that restraint stress decreases DHA content in the phosoholipid membranes of glutamatergic neurons at the amygdaloid complex, whereas it increases arachidonic acid (AA) content. In support of this idea, studies with monkeys have shown that chronic stress is associated with a higher phosphatidylethanolamine ω-6/ω-3 ratio, suggesting lower ω-3 fatty acid status in stressed animals [72]. AA is released from the phospholipid membranes to the cytoplasm by cytoplasmic phospholipase A2 ($cPLA_2$) activity and is transformed into endocannabinoid (eCb), which in turn inhibits GABA release from presynaptic neurons [73-75]. Through this mechanism, chronic stress may increase excitatory neuronal activity in the amygdala and enhance anxiety. On the other hand, ω-3 supplementation may

increase DHA content in the phospholipid membranes of excitatory neurons; which in turn decrease AA levels in the cytoplasm of neurons. Thus, inhibitory transmission could be reduced in the amygdala; decreasing anxiety and plasma corticosterone levels in the stressed rats (Figure 3A,B).

The anxiolytic effect of ω-3 supplementation may be related to increased serotonin levels in the brain of chronically stressed rats. Serotonin has a key role in the regulation of anxiety-like behaviors [76]. In the case of our study, ω-3 PUFA supplementation could have enhanced the serotonin level in the brain [76,77].

The positive effect of ω-3 supplementation on the learning could be related to a direct effect of ω-3 on the auditory brain nuclei that modulates fear learning, such as the MG and IC, which are affected in the stressed rats [65,67]. Chronic stress may have decreased proplastic protein levels in the brain nuclei that produce dendritic atrophy. Proplastic proteins are implicated in neurite extension, cell survival and synaptic plasticity [78]. Alternatively, ω-3 supplementation may increase the level of the proteins that prevent dendritic atrophy in the MG and IC of stressed rats. On the other hand, the positive effects of ω-3 fatty acids on learning may have been by a direct effect in the LA, a brain area key for fear learning [26]. Long-term potentiation studies show that auditory fear learning depends on AMPA receptor insertion in the plasmatic membrane of LA neurons [79]. Thus, chronic stress may impair this process in the LA, while ω-3 supplementation could prevent this effect. As well, a mixture of the two mechanisms may be associated with the positive effects of ω-3 fatty acids on the learning of stressed rats.

### Clinical impact of ω-3 fatty acid supplementation on stress-related disorders

Preclinical and clinical studies support the use of ω-3 supplementation in stress-related disorders such as depressive and anxiety disorders. For example, diets rich in ω-3 improve the effects of antidepressants in animal models of depressive-like behaviors [80,81], as well as in patients with major depression [82,83] and anxiety disorders [40]. In this context, we propose that due to its anxiolytic and anti-stress effects, ω-3 supplementation can improve the symptoms of patients with depressive and anxiety disorders. Conversely, diets poor in ω-3 could be a risk factor for developing depressive and anxiety disorders.

## Conclusions

The present findings demonstrate that ω-3 supplementation in an early phase of brain development has strong anti-stress effects, decreasing plasma corticosterone levels and anxiety. In addition, ω-3 supplementation can improve learning in stressed rats. Our results suggest that key brain areas for learning, such as the amygdala and auditory thalamus, could be targets for the positive effects of ω-3 supplementation to improve learning in stressed rats.

### Abbreviations

BNST: Bed nucleus of stria terminalis; CR: Conditioned avoidance response; CRH: Corticotrophin releasing factor; CS: Conditioned stimulus; dB: Decibel; DHA: Docosahexaenoic acid; DRN: dorsal raphé nucleus; EPA: Eicosapentaenoic acid; EPM: Elevated Plus Maze; g: Gram; GR: Glucocorticoid receptor; IC: Inferior colliculus; ITI: Intertrial interval; kHz: Kilohertz; LA: Lateral amygdale; mA: Milliampere; MG: Medial geniculate nucleus; ml: Millilitre; PND: Postnatal days; PUFA: Polyunsaturated fatty acid; sec: Second; μg/dL: Micrograme/Decilitre; US: Unconditioned stimulus; W: Watt; ω-3: Omega-3 fatty acid; ω-6: Omega-6 fatty acid; U: Unstressed; 2-AA: Two-active avoidance conditioning.

### Competing interests

The authors declare that they have no competing interest.

### Authors' contributions

MAP: Carried out the behavioral, hormonal, and supplementation studies. Participated in the data analyses and drafted the manuscript. GT: Participated in the behavioral studies. ADS: Participated in the data analyses and drafted the manuscript. All authors read and approved the final manuscript

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***Capítulo II:*** *Objetivo N°2*

# *Capítulo N•2*

Estado: Artículo en redacción

Long-Term ω-3 Fatty Acids Supplementation Impairs Spatial Memory in Healthy Rats, while Improves Memory in Stressed Rats: Synaptic Plasticity Evidences.

**Miguel Ángel Pérez**, Juan Ahumada, Marco Fuenzalida y Alexies Dagnino-Subiabre

**Experimental procedures**

***Animals***

Male *Sprague–Dawley* rats (80–100 g, 21 days old at the start of the experiment) were housed in groups of three animals per cage, under a 12/12 light/dark cycle (lights on at 8:00 A.M.). They were maintained in a temperature and humidity controlled room (20 ±1°C, 60%) and weighed every day on a digital scale (Model WLC2/A1, Radwag, Poland). All procedures relating to animal experimentation were in strict accordance with animal care standards outlined in the National Institute of Health (USA) guidelines and approved by the Institutional Animal Ethics Committee of the Universidad de Valparaíso and Universidad Católica del Norte. Efforts were made to minimize the number of animals used and their suffering.

***Experimental Design***

Figure 1 shows a schematic drawing of the experimental design used in this study. Rats were maintained with *ad libitum* access to food (rats chow, Champion®, Santiago, Chile) and water during all experiments. Each rat ate between 15 -30 g of rat chow per day; 0.34 % of 1 g of rat chow was ω-3 fatty acids. Rats were randomly assigned into three experimental groups for supplementation: Control (*n* = 60), animals did not receive supplementation; Vehicle (*n* = 60), rats were supplemented daily with 2.5 ml of water by oral administration; and ω-3-supplemented animals (*n* = 60), which were supplemented daily with 2.5 ml of a mix of 100 mg of DHA and 25 mg of EPA per kg animal weight by oral administration (Knop Laboratories S.A. Santiago, Chile). Supplementation was applied once per day; the rat was picked up from it´s home cage and gently held in the hand of the experimenter for the oral administration of vehicle or ω-3. The procedure took approximately one minute. Animals from the vehicle and ω-3 groups received water or ω-3 fatty acids respectively between postnatal days (PND) 21 and 61 (Fig. 1). Each experimental group was divided into two subgroups: one that was not subjected to any type of stress [control + unstressed(U), C-U, n= 30;vehicle + unstressed, V-U, n= 30; ω-3 + unstressed, ω-3-U, n= 30], and the other one was subjected to a restraint-stress protocol (control + stress, C-S, n= 30;vehicle + stress, V-S, n= 30; ω-3 + stress, ω-3-S, n= 30). Stressed and unstressed animals were littermates and after weaning were housed in separate rooms. Unstressed rats were never exposed to stressed rats and the restraint stress was applied in different room.

***Handling Procedure and Restraint Stress***

Rats were removed every day by hand and transferred to another cage on the pan of a balance to be weighed. Different investigators did this procedure from those applying the restraint stress. All rats in every group were handled with the same procedures. Animals were placed into acrylic restrainers (inner diameter: 6 cm) in their home cages (Fig 2, annex). Restriction was for 6 h every day, beginning at 10 A.M., from the 36 to 57 PND. Restrictors were perforated at their ends to allow ventilation and for avoiding the overheating of the animals. During the stress protocol, animals could breath without problem and urinate and defecate without being in constant contact with their wastes. The following additional parameters were measured to monitor the overall effects of the stress protocol: percentage gain in body weight and anxiety level as determined by performance in the elevated plus maze (see below).

***Behavioral Procedures***

On day one after the end of the stress protocol rats were analyzed individually in the open field and in Y-maze test. On day two after rats were analyzed EPM. A separate set of rats was used for this experiment (C-U, n= 9; C-S, n= 9; V-U, n= 9; V-S, n= 9; ω-3-U, n= 9; ω-3-S, n= 9). Behavioral tests were carried out from 10 am to 2 pm in the test room. The activity of each rat was recorded by Internet Protocol (IP) cameras (VIVOTEK, Sunnyvale CA, USA) fixed above the behavioral apparatus and connected to a computer in another room. Videos were acquired by Nuuo software (Nuuo, Taipei, Taiwan) and analyzed withANY-maze video tracking system (Stoelting Co., IL, USA). Mazes were wiped and cleaned with 5% ethanol solution after each trial. In all experiments, animals from control and stress groups were evaluated at the same time.

***Open Field Test***

Behavior tests were conducted in a soundproof and temperature-controlled (21 ±1 ºC) room. Each rat was placed in the center of a black Plexiglass cage (70 x 70 x 40 cm) for 5 min (Fig. 3, annex). The background noise level in the open field was 40 dB (Precision sound level meter, Model # 1100, Quest Technologies, Oconomowoc, WI) and the arena was illuminated by 300 ±20 lux (measured by a digital lux meter, Model # LX-1010B, Weafo Instrument Co.,

Shanghai, China). The total distance travelled and average speed were determined from the video recordings and analyzed with the ANY-maze video tracking system (Stoelting Co., IL, USA).

***Y-Maze***

Spatial memory was tested on the Y-maze twenty-four hours before completion the analysis of the elevated plus-maze. The Y-maze consisted of three equilaterally intersecting black Plexiglas arms (58 cm long x 19 cm wide x 38 cm high) and several extra-maze cues on the surrounding walls (Fig. 4, annex). The three arms were assigned as Novel, Start and Other, and were counterbalanced among rats. Control and stressed rats were tested at the same time and in separate Y-mazes. Through training, one arm (Novel) was blocked and the animals were placed on the Start to explore for 15 min both the Start and Other arms. After training, the Novel arm was unblocked and rats were returned to their home cages and room. Four hours later, rats were returned to the same start location used during training, and were allowed to freely explore all arms for 5 min. Rats tend to explore novel environments, consequently an intact spatial memory if the rats showed a preference for the Novel arm. Entry into an arm was defined when the animal placed all limbs onto the arm. Behavior was videotaped and entries were converted into percentages. Entries into all arms were counted (total entries) to determine whether locomotor activity levels were similar between experimental groups. To analyze the stress effect on spatial memory ability, a difference score (DS) was measured subtracting the percentage of entries in the novel arm from the percentage of entries in the alternate arm.

***Elevated Plus-Maze***

Twenty-four hours after the open field test, we measured anxiety levels using an EPM test. Each rat was placed individually in an EPM, consisting of two open arms (60 x 15 cm each), two closed arms (60 x 15 x 20 cm each) and a central platform (15 x 15 cm) arranged so that the two arms of each type were opposite to each other (Fig. 5, annex). The maze was elevated 100 cm above the floor. The illumination was 300 ±10 luxs in the open arms and 210 ±10 luxs in the closed arms. At the beginning of each trial, animals were placed at the center of the maze, facing an open arm. During a 5-min test period we recorded the frequency of

entries to the open and closed arms. The percentage of entries and the ratio of open to total arm entries (open/total×100) were used as measures of the anxiety level. Total arm entries were taken as an indicator of general locomotor activity. Entry into an arm was defined as having occurred when the animal placed all four limbs onto the arm.

***Morphometric Study***

A new set of rats (Nonstressed, n=24; stressed, n=24) were used to study the effects of DHA supplementation on dendritic length of CA3 hippocampal neurons. Immediately after completion of the stress protocol each rat was euthanized under deep anesthesia with isoflorane (Lab Baxter, USA). The brain was removed quickly and processed using FD Rapid GolgiStain™ kit (FD Neuro Technologies, Inc., Ellicott City, MD, USA). Both hemispheres were cut in the sagittal plane using a cryostat (Microm®, Walldorf, Model H525, Germany) and 150-µm-thick sections were obtained. Sections were collected serially, dehydrated in absolute alcohol, cleared in xylene, and coverslipped. Slides were coded before quantitative analysis, and the code was broken only after the analysis was completed. For proper Golgi analysis, Golgi-impregnated CA3 pyramidal neurons were chosen based on the following criteria: 1) location in the dorsal portion of the CA3 hippocampal field (Fig. 7, annex); (2) dark and consistent impregnation throughout the extent of all of the dendrites; (3) relative isolation from neighboring impregnated neurons to avoid overlap, and (4) presence of untruncated dendrites. For each brain, 4 CA3 pyramidal cells short-shaft type were selected (Fig. 8, annex). Each selected neuron was drawn at 500X using camera lucida tracings (BH-2, Olympus Co., Tokyo, Japan) and then scanned (eight-bit grayscale TIFF images with 1200 d.p.i. resolution, HP-Deskjet F-380) along with a calibrated scale for subsequent computerized image analysis. Scion image 1.6 software (Scion, Maryland, USA) was used for morphometric analysis of digitized images. In each selected neuron the dendritic length was determined.

***Electrophysiology***

A new set of rats (nonstressed, $n$ = 9, stressed, $n$ = 9) was used to study the chronic stress effects on the efficacy of the glutamatergic and GABAergic systems in CA1 (Fig 9, annex). After completion of the stress protocol each rat was decapitated under deep anesthesia with isoflurane. The brain was removed quickly and submerged in cold (~ 4°C) artificial

cerebrospinal fluid (in mM: 124.00 NaCl, 2.69 KCl, 1.25 KH2PO4, 2.00 MgSO4, 26.00 NaHCO3, 2.00 CaCl2, 10.00 glucose). The pH of the artificial cerebrospinal fluid was stabilized at 7.4 by bubbling carbogen (95% O2, 5% CO2).

Coronal brain slices (350-400 µm) were cut with a Vibratome (Campden Instruments, model MA752, England) and incubated in the artificial cerebrospinal fluid (>1 hour, at room temperature; 20-22° C). Slices were transferred to a 2 ml chamber fixed to an upright microscope stage (NIKON, model Eclipse FN1, Tokyo, Japan) equipped with infrared differential interference contrast (DIC) video microscopy and 40x water immersion objectives. Slices were superfused with carbogen-bubbled artificial cerebrospinal fluid (2 ml/minute) and maintained at room temperature (22-24 °C). 2-amino-5-phosphonopentanoic acid (D-AP5; 50 µM) and 7-nitro-2,3-dioxo-1,4-dihydroquinoxaline-6-carbonitrile (CNQX; 20 µM) were added to the artificial cerebrospinal fluid as needed.

Whole-cell recordings were performed from the soma of CA1 pyramidal neurons with patch pipettes (4-8 MΩ) filled with an internal solution that contained in mM: 100 Cs-Gluconate, 10 HEPES, 10 EGTA, 4 Na2-ATP, 10 TEA-Cl and 1 MgCl2-6H2O, buffered to pH 7.2 – 7.3 with CsOH. Recordings were performed in voltage-clamp modes using an EPC-7 patch-clamp amplifier (HEKA, Instruments). In voltage-clamp experiments the Vh was adjusted to –65 or 0 mV to recorder excitatory post-synaptic currents (EPSCs) and inhibitory post-synaptic currents (IPSCs), respectively. In the voltage-clamp configuration the series resistance was compensated to ~70% and neurons were accepted only when the seal resistance was > 1GΩ and the series resistance (7-14MΩ) did not change >10 % during the experiment. The liquid junction potential was measured (~6mV) but was not corrected. Voltage-clamp data were low-pass filtered at 3.0 kHz and sampled at rates between 6.0 and 10.0 kHz using an A/D converter (ITC-16, InstruTech) and stored with Pulse FIT software (Heka Instruments). The Pulse Fit program was used to generate stimulus timing signals and transmembrane current pulses. The recording analysis was made off-line with pClamp software (Clamp-fit, Molecular Devices). EPSCs and IPSCs were evoked by stimulation of Schaffer collateral (SCs) with concentric bipolar electrode (60 mm diameter, tip separation ~100mm (FHC Inc, ME), placed stratum radiatum close to recorded pyramidal neurons (µ100µm).

In average of IPSC (n = 10) was obtained under voltage clamp by repeated stimulation at 0.3 Hz. Chemicals were purchased from Sigma-Aldrich Chemistry (Santiago, Chile), and

Tocris (Bioscience, USA). The paired pulse ratio (PPR) in inhibitory transmission was calculated as 1-(R2/R1)*100, where R1 and R2 are the peak amplitudes of the first and second IPSCs, respectively. The excitatory transmission was calculated as (R2-R1)/R1 where R1 and R2 are the peak amplitudes of the first and second EPSCs respectively.

To determine whether chronic stress could simultaneously affect the glutamatergic and GABAergic pyramidal neuron synapses; we voltage-clamped CA1 PNs at the reversal potential for evoked excitatory or inhibitory synaptic currents (IPSCs or EPSCs, respectively). Values of the reversal potential of EPSCs and IPSCs were estimated from current-voltage relationships of EPSCs ($0.3 \pm 0.5$ mV; $n = 10$) and IPSCs ($-64.2 \pm 2.3$ mV; $n = 10$), respectively. Moreover, in some experiment the excitatory or inhibitory synaptic transmission were isolated after blocking GABAA with picrotoxin (10 µM) or NMDA and AMPA receptors with D-AP5 (50 µM) and CNQX (20 µM).

The spontaneous inhibitory and excitatory postsynaptic currents (sIPSC or sEPSC) were analyzed off-line using the Minianalysis software (Minianalysis; Synaptosoft), which allowed visual detection of events and selection for analysis of those that exceeded an arbitrary threshold.

***Statistical Analysis***

*Open Field Test and Percentage of Body Weight Gain:* Time, total distance travelled, and average speeds were analyzed with the Student's t-test. Percentage of body weight gain was analyzed using two-ways repeated-measures ANOVA [groups (control, stress) ×days (1, 7, 14, 21)] followed by Bonferroni post-hoc comparisons test. A two-way ANOVA compared groups for anxiety levels in the open field test. The dependent variable for anxiety was the time.

*Spatial memory*: The DS number of entries was analyzed by a two-way ANOVA. The dependent variable for memory was the DS of percentage of number of entries and the independent variables were restraint stress (unstressed and stressed) and diet (control, vehicle and ω-3).

*Anxiety:* A two-way ANOVA compared groups for anxiety levels in the plus-maze test. The dependent variable for anxiety was the percentage of open-arm entries and the independent variables were restraint stress (unstressed and stressed) and diet (control, vehicle

and ω-3). The corticosterone levels were analyzed by a 3 x 2 factorial ANOVA.

*Morphometric studies:* The total apical length of CA3 hippocampal neurons was analyzed by a 3 x 2 factorial ANOVA followed by a tukey post hoc comparisons test.

*Electrophysiological Studies*: data analysis and statistical evaluations were made with both the pClamp program (Molecular Devices Corporation, Chicago USA) and Origin 7.0 (Originlab Corporation, MA. USA). Results are presented as percentage of control. Statistical analysis was performed using Student's two-tailed t-test.

*Verification of assumptions of ANOVA:* The normality of the residuals was assessed through the Kolmogorov-Smirnov test, while the homogeneity of variances was evaluated through to Bartlett'sf or Levene test.

Results are presented as the mean ± SEM, for the electrophysiological studies $n$ = number of cells. A probability level of 0.05 or less was accepted as significant.

## Results

### *Chronic restraint stress induces a decreased body weight gain*

Body weight changes were measured at 1, 7, 14 and 21 days from the start the stress protocol. The two-ways repeated-measures ANOVA showed that chronic restraint stress significantly reduced body weight gain beginning on day 7 after stress protocol (Table I) in both control and ω-3 groups (C-S: $F(3,16) = 30.3$, $p < 0.0001$; ω-3-S: $F(3,16) = 101.8$, $p < 0.0001$), and on day 14 to vehicle group ($F(3,16) = 7.68$, $p < 0.0001$).

### *Effects of ω–3 supplementation on anxiety, memory and locomotor activity*

Restraint stress and vehicle diet significantly decreased the percentage of open-arm entries (control group: stressed rats: 32.42 ± 6.24 entries, nonstressed rats: 67.58 ± 6.24 entries, $p = 0.002$; vehicle group: stressed rats: 39.44 ± 7.63 entries, unstressed rats: 60.56 ± 7.64 entries; $p > 0.150$). This effect was prevented with ω-3 supplementation (stressed rats: ω-3 supplementation: 62.20 ± 3.42 entries, vehicle-treated rats: 39.44 ± 7.63 entries, control rats: 32.42 ± 6.24 entries, ($F(1,48) = 5.11$, $p = 0.03$) (Table 1).

Repeated restraint stress induces a not significant reduction on difference score compared to nonstressed rats on Y-maze (Fig. 1). However, ω-3 supplementation had an antagonistic effect on spatial memory. The two-way ANOVA analysis reveled that rats subject to restraint stress ω-3 supplemented improved significantly their performance on spatial memory, increasing the DS of number of entries compared to other groups subject to stress protocol ($F(2,26) = 3.6$, $p < 0.05$) (Fig. 1). Conversely, ω-3 supplementation to nonstressed rats had significantly lower DS of number of entries compared to controls diet nonstressed groups.

Both restraint stress and ω-3 supplementation did not induce any significant change in locomotor activity levels in open field (total distance traveled and average speed) when compared to controls (data no show).

**Experiment Nº 2**

***ω-3 supplementation induces an increase in total apical length in stressed rats.***

To compare the possible effects of ω-3 supplementation in dendritic arborization, we measured the total apical length of pyramidal CA3 neurons. Figure 2A shows in the upper portion the photomicrographs of representative neurons of CA3 region for all groups (nonstressed and stressed rat) and in the lower portion the tracing of Golgi-stained CA3 neurons. A two-way ANOVA analysis showed that chronic restraint stress protocol significantly reduced the total apical length of CA3 hippocampal neurons ($F(2,53) = 6.4$, $p < 0.0001$) (Fig. 2B). In contrast to stressed rats, there were no significant differences in total apical length between rats of nonstressed groups ($F(2,53) = 2.3$, $p > 0.05$).

**Experiment Nº 3**

***ω-3 supplementation prevents decreased probability of GABA release in stressed rats***

To determine whether ω-3 supplementation prevent the effect of restraint stress on the efficacy of the GABAergic on pyramidal neurons of CA1, we evoked isolated IPSC by paired-pulse protocol. Afterward, modifications of the paired pulse depression (PPD) were quantified by an index (1-(R2/R1)*100). Figure 3A shows comparisons of representative IPSC recording from CA1 Pyramidal neurons between both nonstressed and stressed rats .We observed that nonstressed rats (control and vehicle), the PPD was characterized by depression where the second (R2) IPSC was smaller than the first (R1), indicating that the group of inhibitory synapses stimulated had a high release probability. However, we observed that nonstressed ω-3 supplemented rats reduced the PPD (34.14 ± 4.46 %) compared to control (52.5 ± 3.40 %) (Fig. 3C). This could be suggesting that control ω-3 supplemented rats induced a depression of GABA release. Furthermore, we observed that both stressed and stressed vehicle rats show reduced PPD compared to controls, this reduction on percentage of probability of GABA release was reverted in stressed ω-3 supplemented rats (48.5 ± 5.98 Hz, n= 13 cells/ 6 rats) (Fig. 3C). In parallel experiments, we also analyzed the effect of restraint stress and ω-3 supplementation on probability of glutamatergic release, we electrically evoked isolated EPSC by paired-pulse protocol. Afterward, the paired pulse facilitation (PPF) were quantified by an

index (R2-R1)/R1. Figure 4D shows comparisons of representative EPSC recording in both nonstressed and stressed rats. We observed that in all treatments, the PPF was characterized by facilitation where the second (R2) EPSC was higher than the first (R1), indicating that the group of excitatory synapses stimulated had smaller release probability. Restraint stress did not affect the PPF compared to controls. However, the PPF in control nonstressed rats was 31.90 ± 5.3, while it reached 21.4 ± 2.1in vehicle nonstressed rats, a significant reduction ($p < 0.05$). In contrast to the changes on PPF between groups within nonstressed rats, there were no changes within the stressed rats (Fig. 4D).

***ω-3 supplementation prevents chronic restraint stress-impairment spontaneous GABAergic neurotransmission***

To analyze the possible effect of ω-3 supplementation on inhibitory GABA receptor-mediated neurotransmission, we recorded spontaneous inhibitory postsynaptic currents in the CA1 area of the hippocampus. The soma of CA1 pyramidal neurons were voltage clamped at 0 mV which is the reversal potencial of excitatory activity, then the spontaneous inhibitory postsynaptic currents (sIPSC) were recorded in nonstressed and stressed rats (Fig 5A,B). We observed that restraint stress decreased sIPSC in all treatment (Fig 5C, $p<0.05$). The average of sIPSC frequency of control nonstressed rat (C-Non) was 2.39 ± 0.57 Hz, n= 12 cells/ 6 rats, while it reached 1.09± 0.12 Hz, n= 13 cells/ 6 rats in stressed rats, a significant reduction ($P < 0.05$), this reduction on sIPSC frequency in stressed rats was reversed in stressed rats supplemented with ω-3 (ω-3-S), which reached 2.38 ± 0.12 Hz, n= 12 cells/ 6 rats (Fig. 5C). Moreover, control ω-3 supplemented rats reached 4.50 ± 0.65 Hz, n= 13 cells/ 6 rats on sIPSC frequency, a significant increase compared to control nonstressed rats. In contrast to the changes on sIPSC, the amplitude of IPSC was unchanged in all groups (Fig. 5D). In parallel experiments, we also analyzed the effect of restraint stress and ω-3 supplementation on glutamatergic synaptic transmission. Figure 6A,B shows a representative sEPSC recording from A1 Pyramidal neurons in both nonstressed and stressed rats. There were no significant differences on sEPSC frequency and amplitude within nonstressed rats (Fig. 6C,D). Restraints stress did not affect the sEPSC frequency and amplitude compared to controls. However, the frequency of sEPSP in stressed ω-3 supplemented rats was 0.9 ± 0.2 Hz, significant higher compared to stressed rats ($p < 0.05$) (Fig. 6C).

# *Figuras*
## *Capítulo II*
*Objetivo N°2*

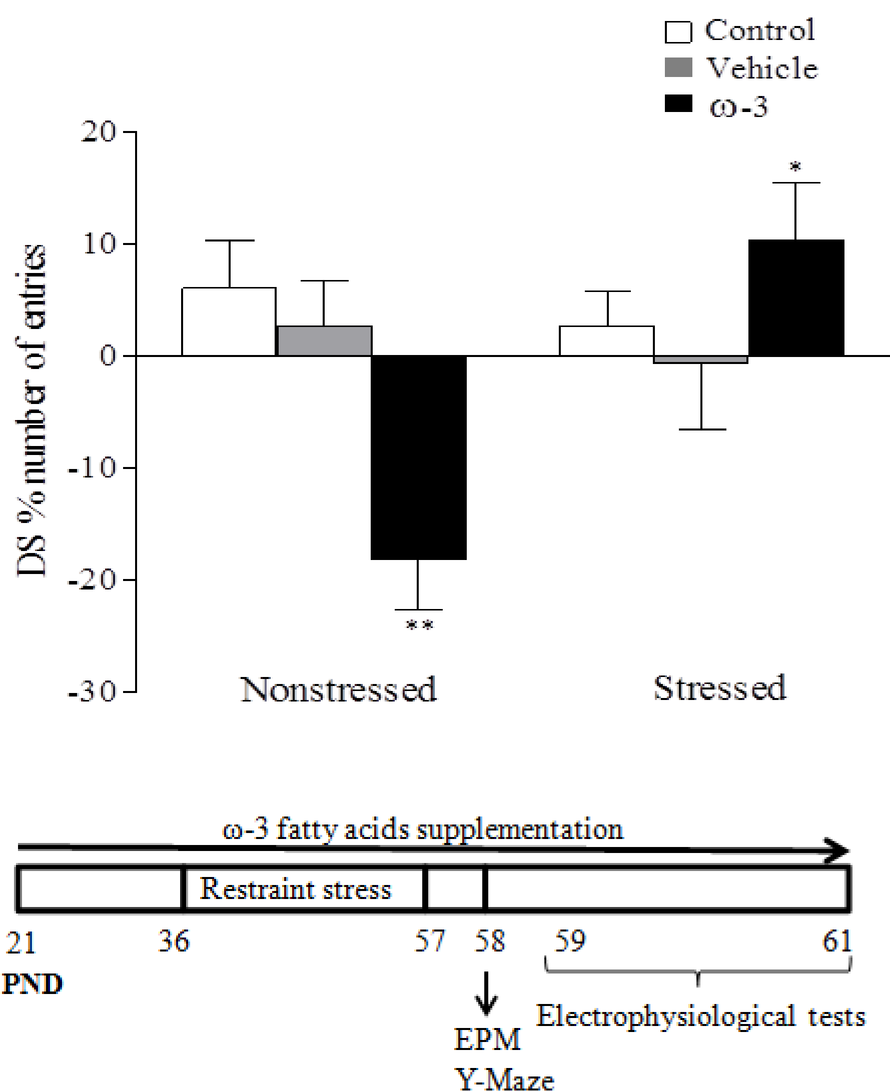


Fig. 1- Effect of ω-3 supplementation on spatial memory. The upper portion of the figure shows DS index of three treatments (control, vehicle and ω-3) under two experimental conditions (nonstressed and stressed). Data represent difference between percentage of number of entries to open arm and number of entries to other arm and expressed as mean ± SEM. *$P<0.005$ compared with control group, and the lower portion of the figure scheme of experimental time line (not to scale) from day 21 to 61 (ω-3 fatty acids supplementation). Restraint stress was from day 36 to 57; PND: post natal day; EPM: elevated plus maze test. Electrophysiological test from day 59 to 61.

**Table I. Stress Markers.** Body weight and anxiety.

| Groups | Subjects | Day 1 | Day 7 | Day 14 | Day 21 | Anxiety |
|---|---|---|---|---|---|---|
| C-Non | 9 | 38.2 ± 1.5 | 83.3 ± 4.2 | 137.8 ± 6.1 | 186.9 ± 8.0 | 27.3 ± 6.8 |
| C-S | 9 | 39.4 ± 1.0 | 63.6 ± 2.1** | 101.2 ± 2.8* | 138.3 ± 3.3** | 17.9 ± 3.1 |
| Veh-Non | 9 | 28.9 ± 1.7 | 57.1 ± 2.4 | 91.9 ± 3.5 | 122.7 ± 5.6 | 43.0 ± 3.1 |
| Veh-S | 9 | 31.8 ± 1.8 | 46.3 ± 3.1 | 78.5 ± 5.3* | 109.5 ± 8.3* | 38.4 ± 4.9 |
| ω-3-Non | 9 | 27.1 ± 1.5 | 57.9 ± 1.0 | 94.8 ± 2.9 | 125.8 ± 3.4 | 28.9 ± 3.7 |
| ω-3-S | 9 | 26.4 ± 2.2 | 42.7 ± 1.8** | 66.8 ± 2.8*** | 85.8 ± 3.8** | 43.5 ± 5.0* |

Comparisons of body weight gain during stress protocol, and levels of anxiety. The weight was registered on day 1, 7, 14 and 21, analyzed using two-ways repeated-measures ANOVA. Anxiety was evaluated in elevated plus maze on day 58 (scheme, Fig. 1 lower portion). The comparisons were made within each treatments (nonstressed and stressed) analyzed by one-way ANOVA test. Data as expressed as mean ± SEM. (*) represents significant differences.

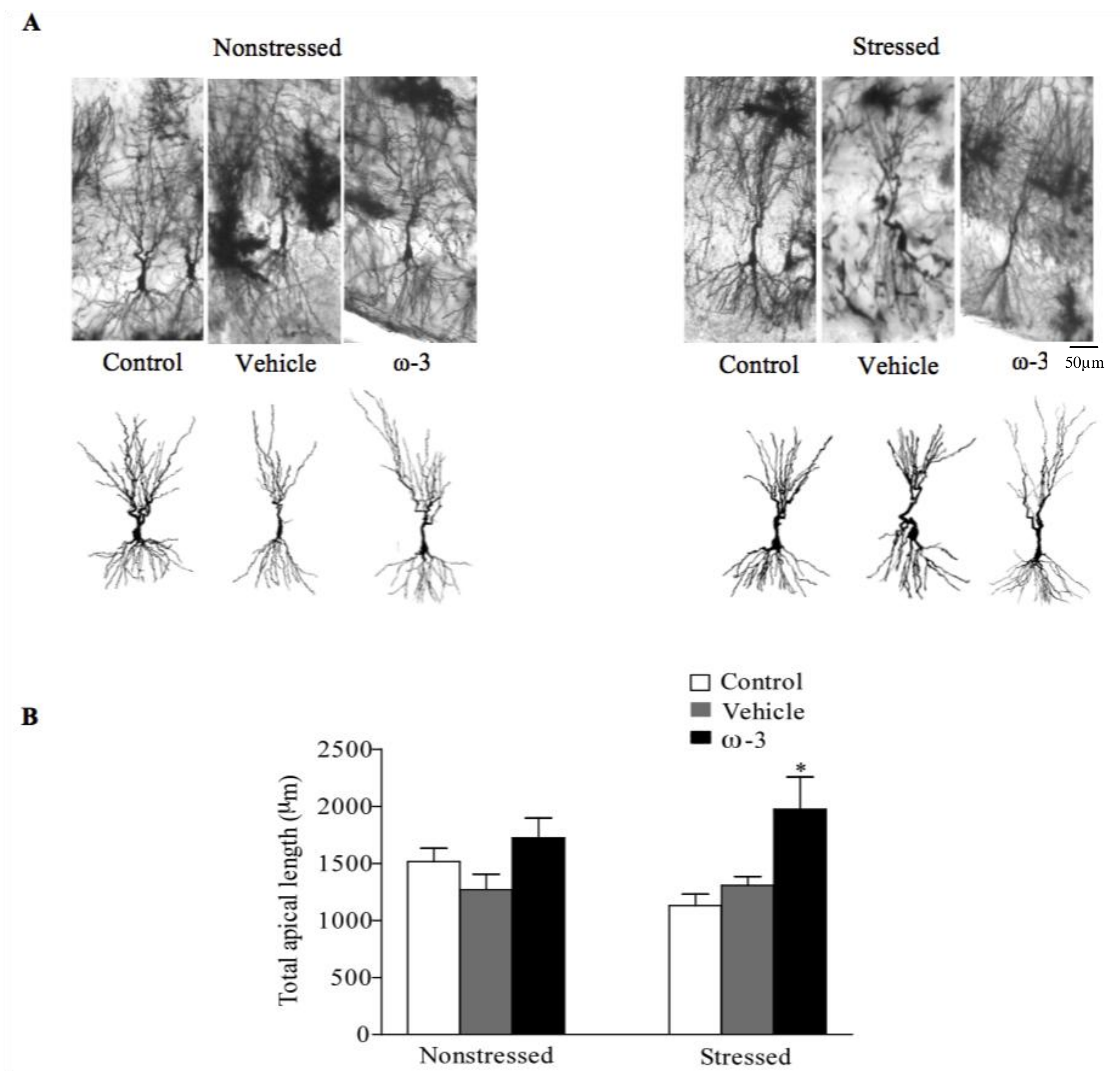


Fig. 2- Effect of ω-3 supplementation on dendritic length of pyramidal CA3 neurons. (A) Photomicrographs (upper portion) and camera lucida tracings (lower portion) of representative Golgi-stained CA3 neurons of all groups (Nonstressed and Stressed). Scale var, 50 µm. (B) Morphometric analysis of pyramidal CA3 neurons from nonstressed and stressed rats. Data are representative of three independent experiments and expressed as mean ± SEM from 18 to 20 neurons. *$P$<0.005 compared with control within a particular experimental conditions.

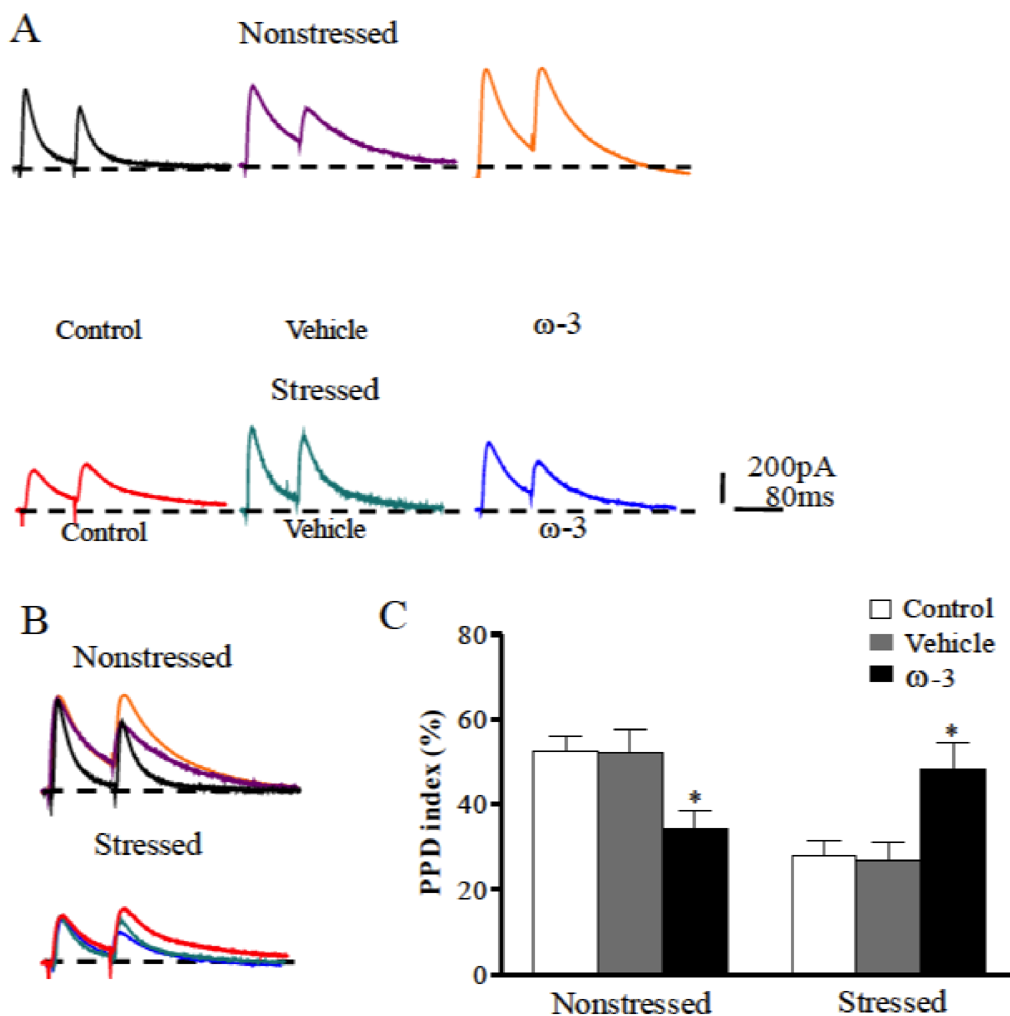


Fig. 3. Antagonist effect of ω-3 supplementation on probability of GABAergic release. A, representative averaged spontaneous inhibitory postsynaptic current recorded in nonstressed (control, vehicle, ω-3) and stressed (control, vehicle, ω-3) rats. *B*, At upper portion, superimposed representative averaged spontaneous inhibitory postsynaptic current recorded in nonstressed rats and lower portion averaged spontaneous inhibitory postsynaptic current recorded in stressed rats. *C*, Summary data showing the changes in PPD index between nonstressed and stressed rats.

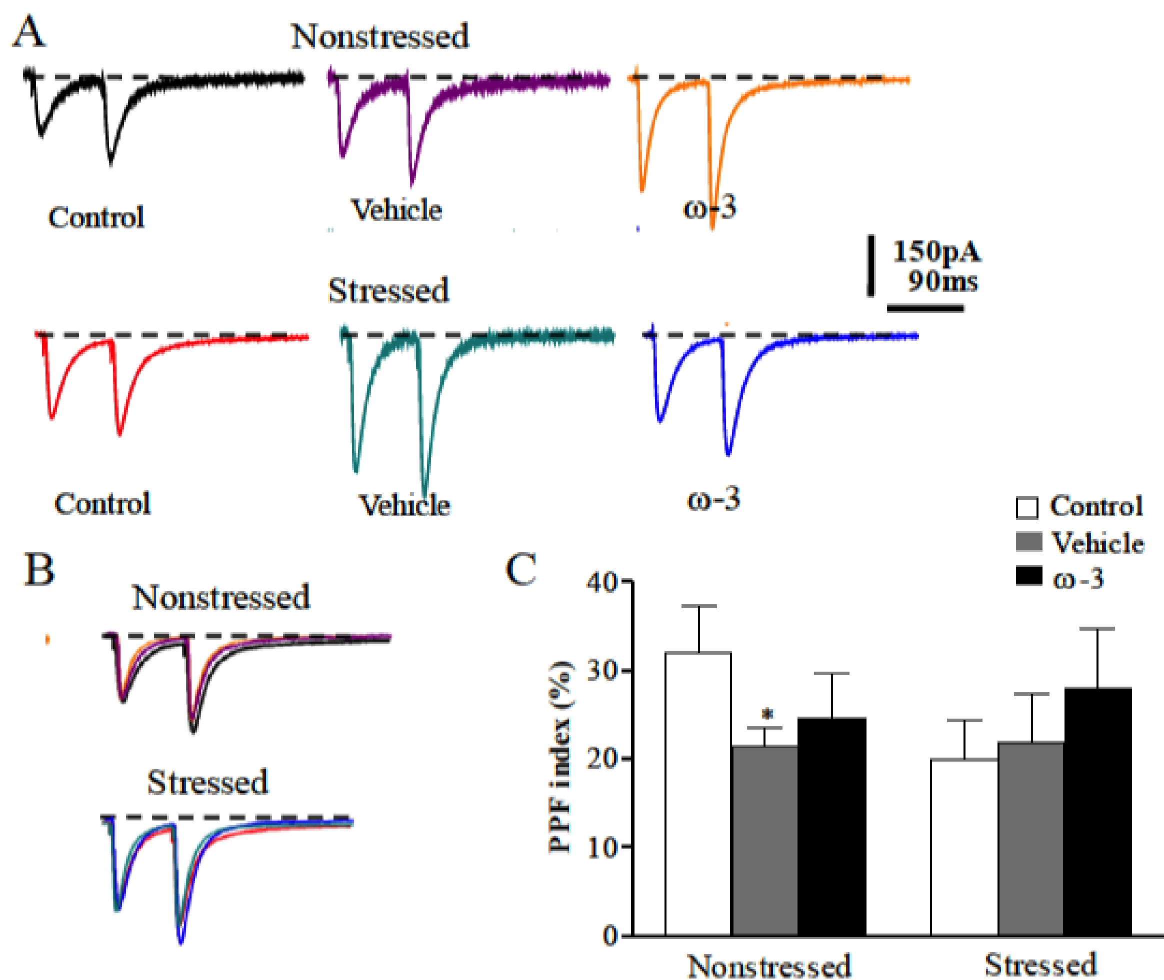


Fig. 4. Restraint stress did not affect the probability of glutamatergic release. A, representative averaged spontaneous excitatory postsynaptic current recorded in nonstressed (control, vehicle, ω-3) and stressed (control, vehicle, ω-3) rats. *B*, At upper portion, superimposed representative averaged spontaneous excitatory postsynaptic current recorded in nonstressed rats and lower portion averaged spontaneous excitatory postsynaptic current recorded in stressed rats. *C*, Summary data showing the changes in PPF index between nonstressed and stressed rats.

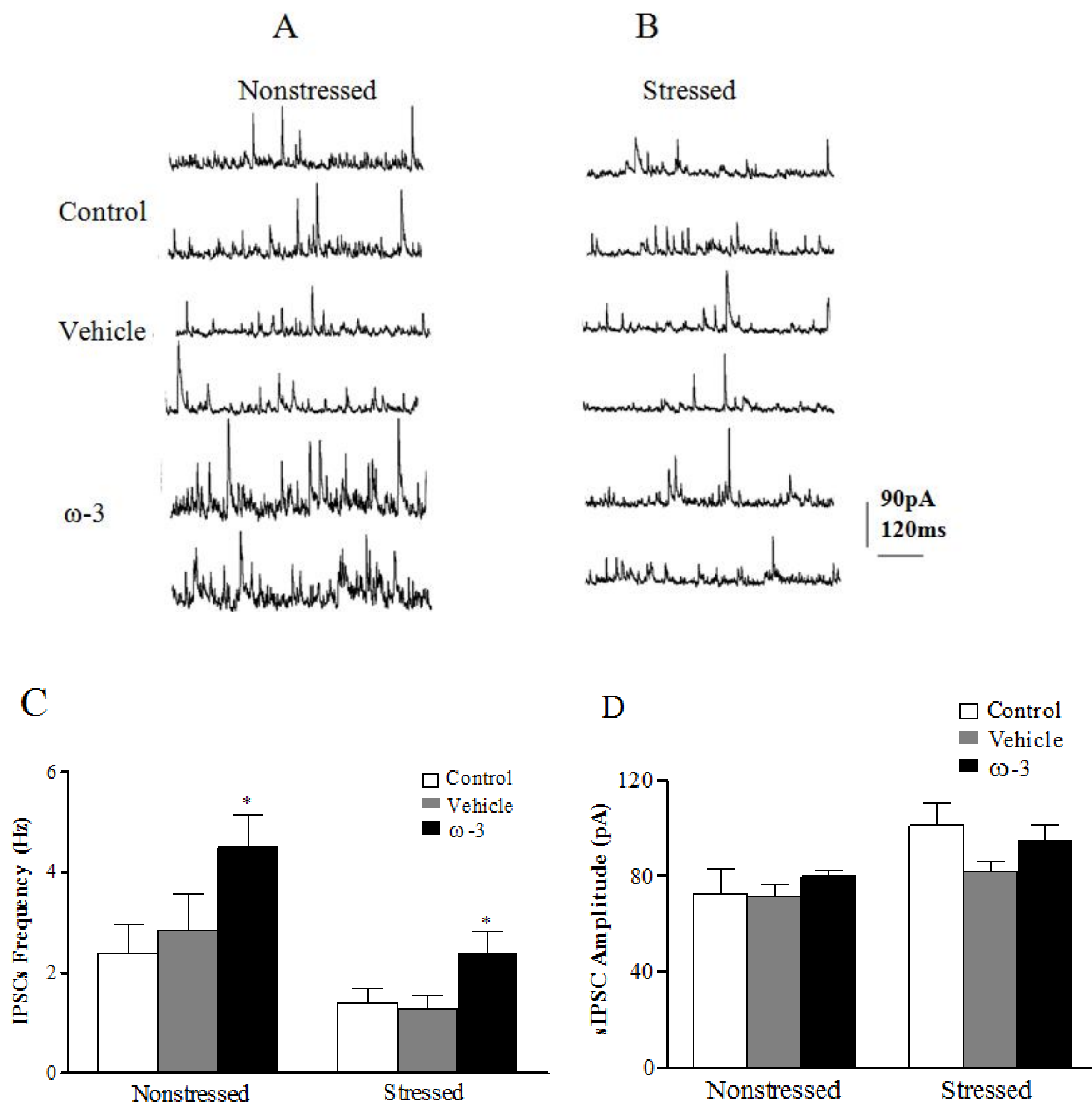


Fig. 5. Frequency of spontaneous inhibitory postsynaptic current (IPSCs) in CA1 hippocampal neurons was reduced in stressed rats and was enhanced in ω-3 supplemented rats *A*, Representative sample traces showing sIPSC recorded in brain slices from nonstressed rats (control, vehicle, ω-3). Traces display a continuous whole-cell voltage clamp recording (0 mV). *B*, sIPSC recorded in a slice from stressed rats (control, vehicle, ω-3).*C*, Summary data showing the sIPSC frequency. D, Summary data showing the sIPSC amplitude in nonstressed and stressed rats.

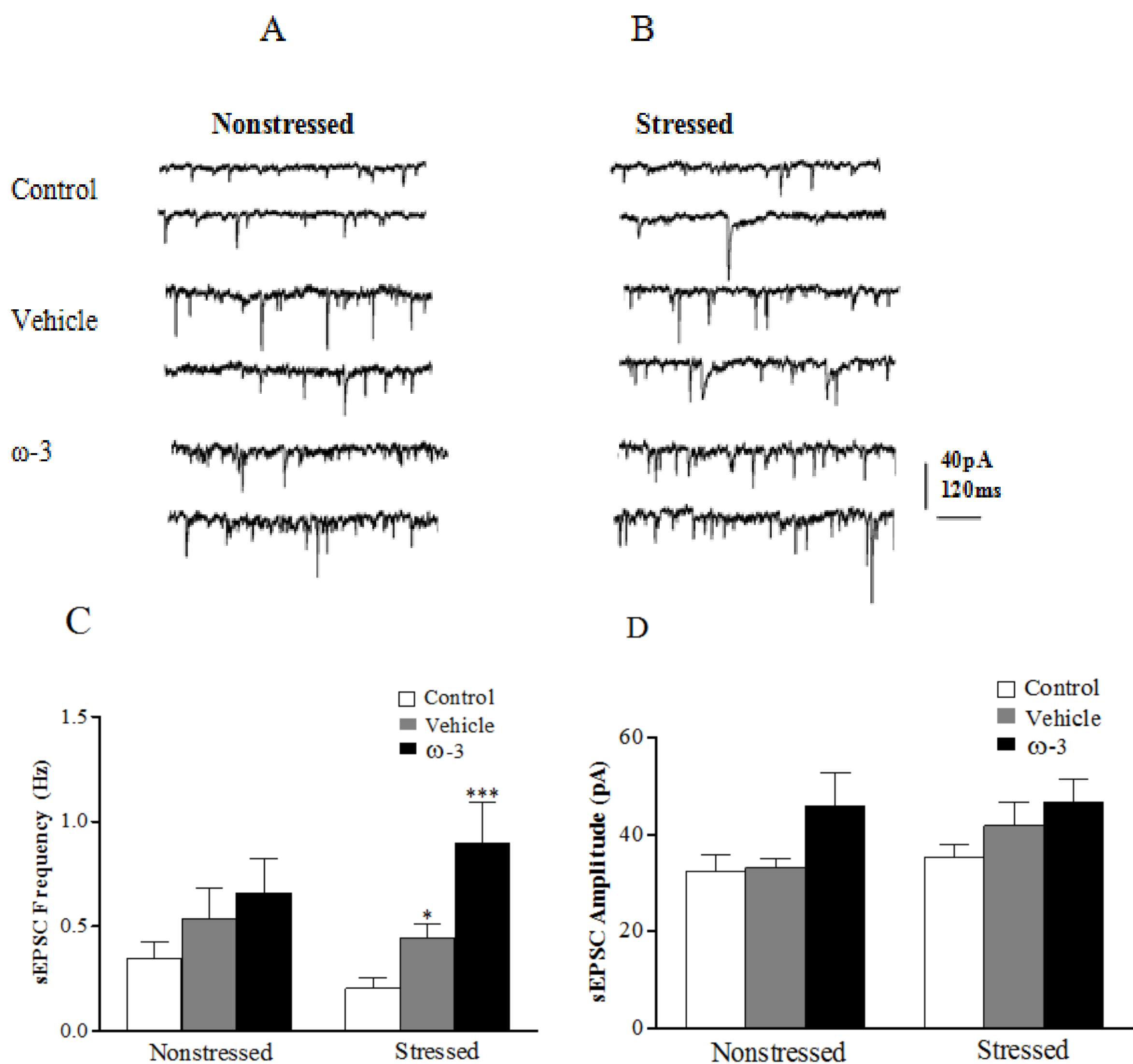


Fig. 6. ω-3 supplementation increased the glutamatergic synaptic efficacy on stressed rats. *A*, Representative sample traces showing spontaneous excitatory postsynaptic current (sEPSC) recorded in brain slices from nonstressed rats (control, vehicle, ω-3). *B*, sEPSC recorded in a slice from stressed rats (control, vehicle, ω-3) .*C*, Summary data showing the sEPSC frequency. D, Summary data showing the sEPSC amplitude in nonstressed and stressed rats.

# ***Capítulo III:*** *Objetivo 3*

*Efecto de la ingesta de AGO sobre la ansiedad, morfología dendrítica y aprendizaje auditivo en ratas sometidas a estrés crónico.*

**Experimental procedures**

***Animals***

Male *Sprague–Dawley* rats (80–100 g, 21 days old at the start of the experiment) were housed in groups of three animals per cage, under a 12/12 light/dark cycle (lights on at 8:00 A.M.). They were maintained in a temperature and humidity controlled room (20 ±1°C, 60%) and weighed every day on a digital scale (Model WLC2/A1, Radwag, Poland). All procedures relating to animal experimentation were in strict accordance with animal care standards outlined in the National Institute of Health (USA) guidelines and approved by the Institutional Animal Ethics Committee of the Universidad de Valparaíso and Universidad Católica del Norte. Efforts were made to minimize the number of animals used and their suffering.

***Preparation of food from scallops gonads* (AGO)**

The gonads of scallops were provided by Dr. Federico Winkler (Universidad Católica del Norte, Coquimbo) and the whole food manufacturing process was at Facultad de Ingeniería en Alimentos (Universidad de la Serena). First, the gonads boil up it for 30 min, thereafter dried at 60°C for 24hrs. Dry gonads were grind up it and mixed with rat food at ratio of 40% scallops gonads and 60% of rats food. Pellet was manually made and dried at 60°C for 24 hrs. AGO was kept at room temperature throughout the experiment. A scallops gonad has 14mg/g of PUFA ω-3, while rat food has 3.4mg/g of PUFA. Therefore AGO mix has an approximate concentration of 10mg/g of PUFA ω-3.

***Experimental Design***

Figure 1 shows a schematic drawing of the experimental design used in this study. Rats were maintained with *ad libitum* access to food (rats chow, Champion®, Santiago, Chile or AGO) and water during all experiments. Each rat ate between 15 -30 g of rat chow or AGO per day; 0.34 % of 1 g of rat chow was ω-3 fatty acids while 1% of 1g of AGO was ω-3 fatty. Rats were randomly assigned into two experimental: Control ($n$ = 27), animals received rat chow during all experiment; AGO ($n$ = 27), rats received a food mix between rat chow and scallops gonads (60 and 40%, respectively). Each experimental group was divided into two subgroups: one that was not subjected to any type of stress [control + unstressed (U), C-U, n= 9; AGO + unstressed, AGO-U, n= 9], and the other one was subjected to a restraint-stress

protocol (control + stress, C-S, n= 9; AGO + stress, AGO-S, n= 9). Stressed and unstressed animals were littermates and after weaning were housed in separate rooms. Unstressed rats were never exposed to stressed rats and the restraint stress was applied in different room.

### *Handling Procedure and Restraint Stress*

Rats were removed every day by hand and transferred to another cage on the pan of a balance to be weighed. Different investigators did this procedure from those applying the restraint stress. All rats in every group were handled with the same procedures. Animals were placed into acrylic restrainers (inner diameter: 6 cm) in their home cages (Fig 2, annex). Restriction was for 6 h every day, beginning at 10 A.M., from the 36 to 57 PND. Restrictors were perforated at their ends to allow ventilation and for avoiding the overheating of the animals. During the stress protocol, animals could breath without problem and urinate and defecate without being in constant contact with their wastes. The following additional parameters were measured to monitor the overall effects of the stress protocol: percentage gain in body weight and anxiety level as determined by performance in the elevated plus maze (see below).

### *Behavioral Procedures*

On day one after the end of the stress protocol rats were analyzed individually in the open field and in Y-maze test. On day two after rats were analyzed EPM.  A separate set of rats was used for this experiment (C-U, n= 9; C-S, n= 9; AGO-U, n= 9; AGO-S, n= 9). Behavioral tests were carried out from 10 am to 2 pm in the test room. The activity of each rat was recorded by Internet Protocol (IP) cameras (VIVOTEK, Sunnyvale CA, USA) fixed above the behavioral apparatus and connected to a computer in another room. Videos were acquired by Nuuo software (Nuuo, Taipei, Taiwan) and analyzed withANY-maze video tracking system (Stoelting Co., IL, USA). Mazes were wiped and cleaned with 5% ethanol solution after each trial. In all experiments, animals from control and stress groups were evaluated at the same time.

### *Open Field Test*

Behavior tests were conducted in a soundproof and temperature-controlled (21 ±1 ºC)

room. Each rat was placed in the center of a black Plexiglass cage (70 x 70 x 40 cm) for 5 min (Fig. 3, annex). The background noise level in the open field was 40 dB (Precision sound level meter, Model # 1100, Quest Technologies, Oconomowoc, WI) and the arena was illuminated by 300 ±20 lux (measured by a digital lux meter, Model # LX-1010B, Weafo Instrument Co., Shanghai, China). The total distance travelled and average speed were determined from the video recordings and analyzed with the ANY-maze video tracking system (Stoelting Co., IL, USA).

***Y-Maze***

Spatial memory was tested on the Y-maze twenty -four hours after completion the analysis of the elevated plus-maze. The Y-maze consisted of three equilaterally intersecting black Plexiglas arms (58 cm long x 19 cm wide x 38 cm high) and several extra-maze cues on the surrounding walls (Fig. 4, annex). The three arms were assigned as Novel, Start and Other, and were counterbalanced among rats. Control and stressed rats were tested at the same time and in separate Y-mazes. Through training, one arm (Novel) was blocked and the animals were placed on the Start to explore for 15 min both the Start and Other arms. After training, the Novel arm was unblocked and rats were returned to their home cages and room. Four hours later, rats were returned to the same start location used during training, and were allowed to freely explore all arms for 5 min. Rats tend to explore novel environments, consequently an intact spatial memory if the rats showed a preference for the Novel arm. Entry into an arm was defined when the animal placed all limbs onto the arm. Behavior was videotaped and entries were converted into percentages. Entries into all arms were counted (total entries) to determine whether locomotor activity levels were similar between experimental groups. To analyze the stress effect on spatial memory ability, a difference score (DS) was measured subtracting the percentage of entries in the alternate arm from the percentage of entries in the novel arm.

***Elevated Plus-Maze***

Twenty-four hours after the open field test, we measured anxiety levels using an EPM test. Each rat was placed individually in an EPM, consisting of two open arms (60 x 15 cm each), two closed arms (60 x 15 x 20 cm each) and a central platform (15 x 15 cm) arranged so

that the two arms of each type were opposite to each other (Fig. 5, annex). The maze was elevated 100 cm above the floor. The illumination was 300 ±10 luxs in the open arms and 210 ±10 luxs in the closed arms. At the beginning of each trial, animals were placed at the center of the maze, facing an open arm. During a 5-min test period we recorded the frequency of entries to the open and closed arms. The percentage of entries and the ratio of open to total arm entries (open/total×100) were used as measures of the anxiety level. Total arm entries were taken as an indicator of general locomotor activity. Entry into an arm was defined as having occurred when the animal placed all four limbs onto the arm.

***Morphometric Study***

A new set of rats (nonstressed, n=9; stressed, n=9) were used to study the effects of AGO intake on dendritic length of CA3 hippocampal neurons. Immediately after completion of the stress protocol each rat was euthanized under deep anesthesia with isoflorane (Lab Baxter, USA). The brain was removed quickly and processed using FD Rapid GolgiStain™ kit (FD Neuro Technologies, Inc., Ellicott City, MD, USA). Both hemispheres were cut in the sagittal plane using a cryostat (Microm®, Walldorf, Model H525, Germany) and 150-µm-thick sections were obtained. Sections were collected serially, dehydrated in absolute alcohol, cleared in xylene, and coverslipped. Slides were coded before quantitative analysis, and the code was broken only after the analysis was completed. For proper Golgi analysis, Golgi-impregnated CA3 pyramidal neurons were chosen based on the following criteria: 1) location in the dorsal portion of the CA3 hippocampal field (Fig. 7, annex); (2) dark and consistent impregnation throughout the extent of all of the dendrites; (3) relative isolation from neighboring impregnated neurons to avoid overlap, and (4) presence of untruncated dendrites. For each brain, 4 CA3 pyramidal cells short-shaft type were selected (Fig. 8, annex). Each selected neuron was drawn at 500X using camera lucida tracings (BH-2, Olympus Co., Tokyo, Japan) and then scanned (eight-bit grayscale TIFF images with 1200 d.p.i. resolution, HP-Deskjet F-380) along with a calibrated scale for subsequent computerized image analysis. Scion image 1.6 software (Scion, Maryland, USA) was used for morphometric analysis of digitized images. In each selected neuron the dendritic length was determined.

***Statistical Analysis***

*Open Field Test and Percentage of Body Weight Gain:* Time, total distance travelled, and average speeds were analyzed with the Student's t-test. Percentage of body weight gain was analyzed using two-ways repeated-measures ANOVA [groups (control, stress) ×days (1, 7, 14, 21)] followed by Bonferroni post-hoc comparisons test. A two-way ANOVA compared groups for anxiety levels in the open field test. The dependent variable for anxiety was the time.

*Spatial memory*: The DS number of entries was analyzed by a two-way ANOVA. The dependent variable for memory was the DS of percentage of number of entries and the independent variables were restraint stress (unstressed and stressed) and diet (control, vehicle and ω-3).

*Anxiety:* A two-way ANOVA compared groups for anxiety levels in the plus-maze. The dependent variable for anxiety was the percentage of open-arm entries and the independent variables were restraint stress (unstressed and stressed) and diet (control, vehicle and ω-3). The corticosterone levels were analyzed by a 2 x 2 factorial ANOVA.

*Morphometric studies:* The total apical length of CA3 hippocampal neurons was analyzed using one-way ANOVA followed by a tukey post hoc comparisons test.

*Verification of assumptions of ANOVA:* The normality of the residuals was assessed through the Kolmogorov-Smirnov test, while the homogeneity of variances was evaluated through to Bartlett'sf or Levene test.

## Results

### *Chronic restraint stress decreased the body weight gain*

Body weight changes were measured during all experimental protocols from weaning to the end of the stress protocol. The two-ways repeated-measures ANOVA showed that chronic restraint stress of rat supplemented with AGO significantly reduced body weight gain beginning on day 4 after stress protocol (C-S: $F(1,16) = 39.58$, $p < 0.0001$). Restraint stress did not affect locomotor activity levels (average speed) (Fig. 2).

### *AGO intake, memory and dendritic length*

Repeated restraint stress and AGO intake of stressed rat did not affect the DS number of entries compared to nonstressed rats on the Y-maze (control group: stressed rats: 1.7 ± 4.30 entries, nonstressed rats: 6.06 ± 4.30; AGO group: stressed rats: 8.1 ± 3.10, unstressed rats: -2.10 ± 4.4, $p > 0.05$) (Fig. 3A). AGO nonstressed rats show lower DS number of entries compared to control group.

A two-way ANOVA analysis showed that chronic restraint stress protocol and AGO intake did not change the total apical length of CA3 hippocampal neurons ($F(1,50) = 1.56$, $p > 0.200$). There were not interactions between stress and diet ($F(1,50) = 3.92.4$, $p > 0.053$) (Fig. 3B).

### *Effects of AGO intake on anxiety*

Stressed rat from control and AGO groups significantly decreased the percentage of entries to open arm (control group: stressed rats: 17.9 ± 3.14 entries, nonstressed rats: 27.3 ± 6.70; AGO group: stressed rats: 16.5 ± 3.3, unstressed rats: 24.4 ± 3.9, $p < 0.05$) (Fig. 4).

### *Effects of AGO intake on Learning*

Figure 5 shows the percentage of conditioned responses (% CR) during the avoidance conditioning to the tone under two dietary regimes (control and AGO intake) in unstressed and stressed conditions. The two-way repeated-measures ANOVA show the main effects of the trials and interactions (trials* diet) on % CR unstressed group rats. There was no main effect of AGO intake on % CR ($F(2,48)= 2.87$, $p =0.17$).

# *Figuras*

## *Capítulo III*

*Objetivo N°3*

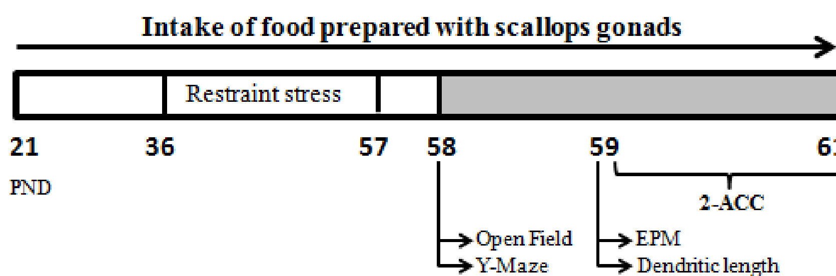


Fig. 1- Schematic drawing of the experimental design. Experimental time line (not to scale) from post natal day (PND) to 61 (intake of food prepared with scallops gonads). Restraint stress was applied from PND 36 to 57, EPM: elevated plus maze test, 2-ACC: Active avoidance conditioning test.

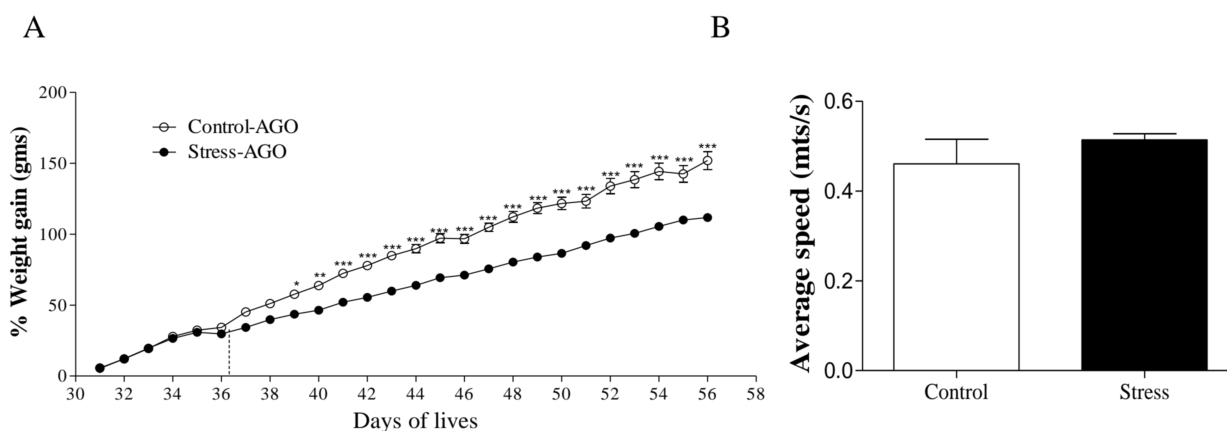


Fig 2. Effects of AGO intake on stress markers anxiety. (A) Comparisons of body weight gain during stress protocol, the weight was registered during all experimental protocol, analyzed using two-ways repeated-measures ANOVA. Stressed rats supplemented with AGO decreasing the percentage of body weight gain. (B) AGO intake does not affect locomotor activities. Data as expressed as mean ± SEM. (*) represent significant differences.

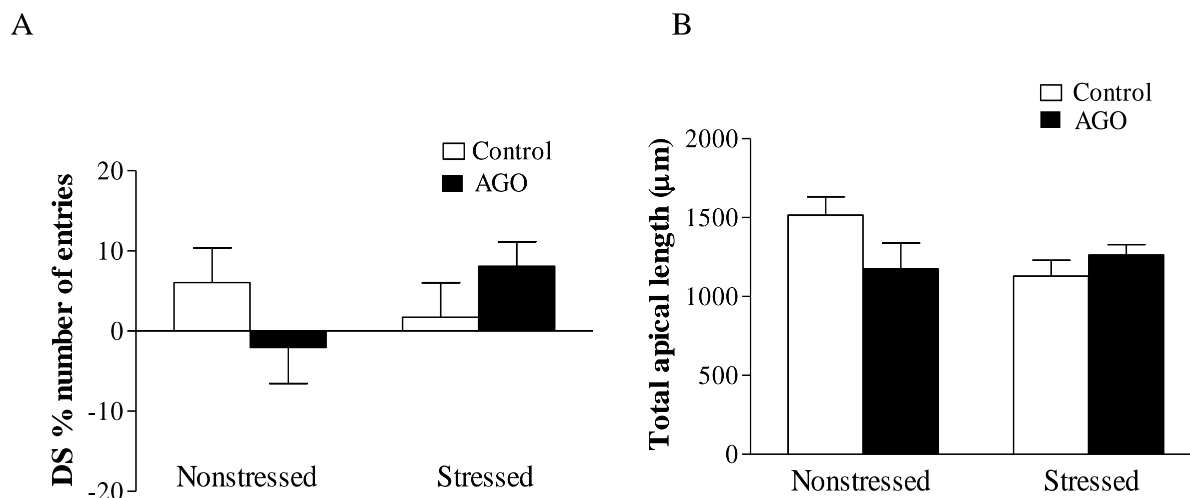


Fig 3. Effect of AGO intake on spatial memory and dendritic length. (A) DS index of two treatment (control and AGO) under two experimental conditions (nonstressed and stressed). Data represent difference between percentage of number of entries to novel arm and number of entries to alternate arm and expressed as mean ± SEM. (B) Morphometric analysis of pyramidal CA3 neurons from nonstressed and stressed rats. Data are expressed as mean ± SEM from 10 to 20 neurons.

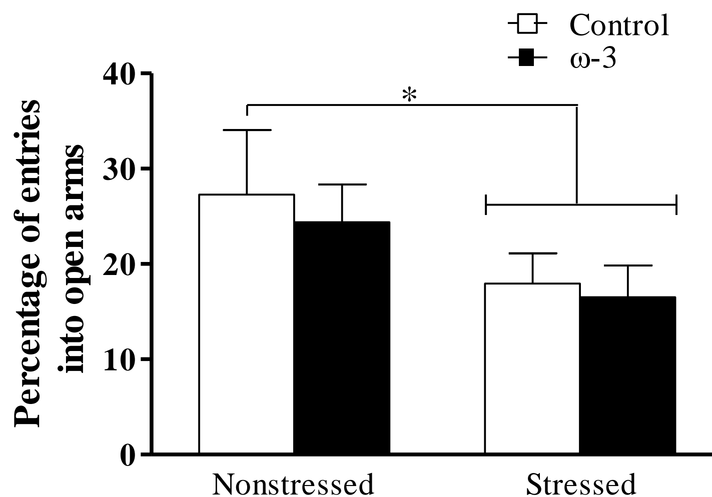


Fig 4. Effects of AGO intake on anxiety. Rats supplemented with AGO subject to restraint stress decreased the % of entries into the open arms compared to control group. (B) AGO intake does not affect locomotor activities. Error bars indicate the means ± SEM. An asterisk (*) indicates significant differences.

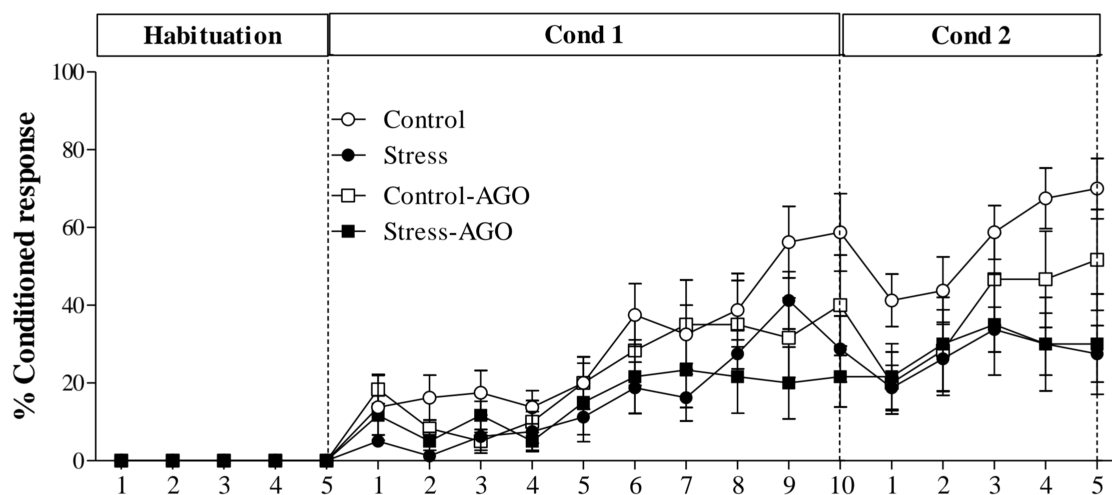


Fig 5. Effects of AGO intake on learning: Restraint stress decreased the percentage of conditioned responses compared to control group of nonstressed rats. The values are the means ± SEM of 9 animals per group. Each point represents the percentages of conditioned avoidance responses (% CR) for the three stages of the test (habituation, conditioning one and two). Data are represented as means ± SEM. An asterisk (*) indicates significant differences.

# *Publicación anexo N•1*

Estado: En prensa

Repeated Restraint Stress Impairs Auditory Attention and GABAergic Synaptic Efficacy in the Rat Auditory Cortex

**Miguel Ángel Pérez**, C. Pérez-Valenzuela, F. Rojas-Thomas, J. Ahumada, M. Fuenzalida y A. Dagnino-Subiabre. Neuroscience (ISI=3.4), 246C:94-107.

# REPEATED RESTRAINT STRESS IMPAIRS AUDITORY ATTENTION AND GABAERGIC SYNAPTIC EFFICACY IN THE RAT AUDITORY CORTEX

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**Abstract—Chronic stress induces dendritic atrophy in the rat primary auditory cortex (A1), a key brain area for auditory attention. The aim of this study was to determine whether repeated restraint stress affects auditory attention and synaptic transmission in A1. Male *Sprague–Dawley* rats were trained in a two-alternative choice task (2-ACT), a behavioral paradigm to study auditory attention in rats. Trained animals that reached a performance over 80% of correct trials in the 2-ACT were randomly assigned to control and restraint stress experimental groups. To analyze the effects of restraint stress on the auditory attention, trained rats of both groups were subjected to 50 2-ACT trials one day before and one day after of the stress period. A difference score was determined by subtracting the number of correct trials after from those before the stress protocol. Another set of rats was used to study the synaptic transmission in A1. Restraint stress decreased the number of correct trials by 28% compared to the performance of control animals ($p < 0.001$). Furthermore, stress reduced the frequency of spontaneous inhibitory postsynaptic currents (sIPSC) and miniature IPSC in A1, whereas glutamatergic efficacy was not affected. Our results demonstrate that restraint stress decreased auditory attention and GABAergic synaptic efficacy in A1.**
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**Key words: stress, attention, learning, auditory system, synaptic transmission.**

## INTRODUCTION

Stress is a complex biological reaction common to all living organisms that allows them to restore homeostasis and adapt to environmental pressure (i.e. stressors) (Selye, 1936; Goldstein and McEwen, 2002). The stress response is mediated strongly by activation of the hypothalamus–pituitary–adrenal (HPA) axis, leading to the secretion of glucocorticoids (corticosterone in rodents and cortisol in humans) from the adrenal gland; which bind to glucocorticoid receptors in the brain and peripheral tissues (Herman et al., 1996, 2003; Smith and Vale, 2006). Stress can be positive (eustress) when the stressors are mild, brief and controllable (Tafet and Bernardini, 2003). Strong and persistent stressors trigger distress or chronic stress (Tafet and Bernardini, 2003). Uncontrollable stressors significantly increase HPA axis activity and plasma corticosterone levels in chronically stressed rats compared to unstressed animals, leading to a maladaptive response (Tafet and Bernardini, 2003; Ferraz et al., 2011).

Limbic structures like the hippocampus, amygdala and medial prefrontal cortex have high concentrations of glucocorticoid receptors (Gray and Bingaman, 1996; Joels, 2001; Wellman, 2001). Chronic glucocorticoid administration and chronic stress alter the dendritic architecture and function of brain areas related to memory, learning and emotional processing (Watanabe et al., 1992; Magariños and McEwen, 1995; Magariños et al., 1998; Wellman, 2001; Vyas et al., 2002; Mitra and Sapolsky, 2008).

### Stress effects on the auditory system and learning

The emotional processing of the acoustic information in the brain depends on the intensity of the acoustic stimuli (McDonald, 1998; Wilensky et al., 2006). Acoustic stimuli are processed at the subcortical level through the neuronal pathway formed by the cochlea nucleus and superior olivary complex–lateral lemniscus. The inferior colliculus (IC) receives all projections from these nuclei, which are then sent to the medial geniculate nucleus (MG, auditory thalamus). Part of the auditory information received in the MG is sent directly to the lateral amygdala (McDonald, 1998; Wilensky et al., 2006). Auditory stimuli $\leqslant$80 dB must be associated with an

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*Abbreviations:* 2-ACT, two-alternative choice task; A1, primary auditory cortex; CE, central amygdaloid nucleus; dB, decibel; DS, difference score; DS-CT, difference score of correct trials; EGTA, ethylene glycol tetraacetic acid; EPSCs, excitatory postsynaptic currents; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPA, hypothalamus–pituitary–adrenal; IC, inferior colliculus; IPSCs, inhibitory postsynaptic currents; ITI, inter-trial interval; kHz, kilohertz; L-CT, latency of correct trials; L-CT/CT, ratio of latency of correct trials/number of correct trials; m, meter; MG, medial geniculate nucleus; mIPSC, miniature inhibitory postsynaptic currents; ms, millisecond; mV, millivolt; PO, posterior thalamic nucleus; PPR, paired-pulse ratio; s, second; sEPSCs, spontaneous excitatory postsynaptic currents; sIPSC, spontaneous inhibitory postsynaptic currents; TTX, tetrodotoxin.

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aversive unconditioned stimulus, such as footshock, to acquire the ability to elicit conditioned fear responses (Monfils et al., 2009). The acquisition of auditory emotional memories in the amygdala is associated with neuronal plasticity in the basolateral amygdala and MG (Maren et al., 2001; Poremba and Gabriel, 2001). Both brain areas exhibit associative plasticity of spike firing during fear conditioning (Maren et al., 2001). On the other hand, acoustic stimuli equal to or higher than 90 dB are sent from the dorsal nucleus of the lateral lemniscus to the posterior thalamic nucleus (PO), located just medially to the posterior intralaminar nucleus (Kudo et al., 1983; Paré et al., 2004). The PO also receives auditory projections from the nucleus of the brachium of the IC (Kudo et al., 1983). The PO efferents are sent directly to the central amygdaloid nucleus (CE) and to the primary somatic sensory cortex, indicating the possibility that the CE receives auditory input from the thalamus (Paré et al., 2004). Through this neuronal pathway, the CE is activated and fear responses such as freezing are performed independent of the pathway formed by the IC–MG–auditory cortex.

Studies in animal models have shown that chronic stress impairs the major nuclei of the auditory system. For instance, a recent study using micro Positron Emission Tomography supports these findings in that chronic mild stress induces significant deactivation in the IC, the main nucleus of the auditory system (Hu et al., 2010a). In this line, we previously demonstrated that restraint stress induces dendritic atrophy in the rat IC, MG, and neurons from layers II/III and V/VI of the primary auditory cortex (A1) (Dagnino-Subiabre et al., 2005, 2009; Bose et al., 2010). Chronic stress significantly impaired auditory learning in rats subjected to a two-way-signaled active avoidance learning procedure, where animals had been trained in a shuttle box to avoid a footshock signaled by acoustic cues (Dagnino-Subiabre et al., 2005).

## Stress effects on synaptic transmission

One factor that has not received sufficient attention yet in this topic is the effect of chronic stress on glutamatergic and GABAergic signaling at the cortical level. The balance of excitation and inhibition in the brain is essential for synaptic plasticity and cognitive functions (Buzsaki and Chrobak, 1995; Cobb et al., 1995). Intracellular recording studies have shown that a synergic effect of excitatory and inhibitory inputs to A1 neurons is key for auditory processing (Wehr and Zador, 2005; Tan and Wehr, 2009). As well interneurons exert a strong control over balance and synchronization of the brain circuit. Thus, modulation of glutamatergic and GABAergic synaptic efficacy is the main regulatory element for auditory learning and complex cognitive processes in both health and disease (Oswald et al., 2006; Levy and Reyes, 2012). Chronic stress and glucocorticoids increase the GABAergic synaptic transmission in the hippocampus (Hu et al., 2010b; Martisova et al., 2012), while in the basolateral amygdala chronic stress decreases GABAergic inhibition (Rodríguez Manzanares et al., 2005; Reznikov et al., 2009; Roozendaal et al., 2009). At the cortical level, stress decreases the inhibition/excitation ratio in the temporal cortex (Garcia-Oscos et al., 2012).

## Attention

All animals live in a world of competition, with multiple stimuli from environment that must be resolved in order so that they behave adaptively. Attention is a complex cognitive function that allows them the ability to select from an overabundance of stimuli, responses, and memories, and in doing so, ignore any that are irrelevant (Raz, 2004).

Attention involves a unitary description of three attentional control systems in the brain: "alerting", "orienting" and "executive" (Posner and Petersen, 1990; Raz and Buhle, 2006). "Alerting," relates to preparedness for an imminent stimulus through maintaining an alert state. Human studies on neuroimaging show that the alert state is associated to activity in the prefrontal and parietal cortices, mainly in the right hemisphere (Coull et al., 1996). "Orienting" is related to the ability to select information from several sensory stimuli; this is associated with activity in both the superior parietal cortex activity and superior colliculus, respectively (Corbetta, 1998; Corbetta et al., 2000). "Executive attention" is the complex monitoring and resolution of conflict between different brain regions.

Attention is studied in rats by the behavioral paradigm two-alternative choice task (2-ACT) (Jaramillo and Zador, 2011). In this paradigm, auditory attention is associated with increases in neural activity in A1, which are required to process attentional information (Hromádka and Zador, 2007; Otazu et al., 2009). Likewise, electrophysiological and behavioral studies have shown a significant positive correlation between the animal's performance in the 2-ACT and increased neuronal activity in A1 (Jaramillo and Zador, 2011).

These findings raise the question of whether complex cognitive functions such as auditory attention are affected by chronic stress, and if this effect is mediated by an alteration of glutamatergic or GABAergic signaling in A1. The objective of this study was to test whether repeated restraint stress impairs auditory attention and decreases synaptic efficacy in A1. We performed four experiments; the first was to measure corticosterone plasma levels of trained rats in a 2-ACT. The second experiment was to analyze whether restraint stress affects the spatial memory of trained rats. In addition, we studied the stress markers of body weight gain and anxiety in all animals. The third experiment was to determine whether restraint stress affects auditory attention in the 2-ACT. Finally, in the fourth experiment we analyzed the efficacy of the glutamatergic and GABAergic systems in A1 of control and chronically stressed rats. The main results of our research were that restraint stress decreased both auditory attention and inhibitory synaptic efficacy in A1 compared to controls.

## EXPERIMENTAL PROCEDURES

### Ethics statement

All procedures related to animal maintenance and experimentation were approved by the Institutional Animal Ethics Committee of the Faculty of Sciences. Universidad de Valparaíso (Chile) and were in strict accordance with animal care standards outlined in National Institute of Health (USA) guidelines. Efforts were made to minimize the number of animals used and their suffering.

### Animals and restraint stress protocol

Male *Sprague–Dawley* rats were housed in groups of three per cage under a 12/12 light/dark cycle (lights on at 08:00 h.), with *ad libitum* access to food (rat chow, Champion®, Santiago, Chile) and water in a temperature and humidity controlled room (20 ± 1 °C, 55% respectively). Animals were weighed every day on a digital scale (Model WLC2/A1, Radom, Radwag, Poland). Beginning on postnatal day 23, the animals were trained for 3 weeks in a 2-ACT, a behavioral paradigm to analyze auditory attention in rats (Fig. 1A). Trained animals were randomly assigned to two experimental groups: control, $n = 48$ and stress, $n = 48$, for all experiments. Control animals, which were littermates of the stress-treated animals, were housed in separate rooms and cages, and not subjected to any type of experimental stress. Restraint-stressed rats were placed in a plastic rat restrainer (6 cm diameter × 12 cm long and then 6 cm diameter × 20 cm long as the rats grew) in their home cages for 6 h daily, beginning at 10.00–16.00 h for 21 consecutive days. Restraint occurred during the light phase the light/night cycle applied. To monitor the overall effects of the stress protocol the percentage gain in body weight of all animals (net change in weight after experiment × 100/ weight at the beginning of experiment) was measured.

### 2-ACT

*Apparatus and stimuli delivery.* To measure auditory attention in rats we used the 2-ACT paradigm. Four modular rat operant chambers and accessories (LE1005, LE10022, LE100575, LE100560, Panlab S.L., Barcelona, Spain) were used in the attention task, each within of a 67 × 67 × 67 $cm^3$ sound-attenuating cubicle lined with 7.5 cm acoustic foam (Vroka S.A., Santiago, Chile). The operant chamber was illuminated to 200 lux (measured by a digital lux meter, Model # LX-1010B, Weafo Instrument Co., Shanghai, China) and the background noise level was 30 dB. During training, the auditory stimuli were delivered through a speaker calibrated with a precision sound level meter (Model # 1100, Quest Technologies, Oconomowoc, WI, USA) to generate 70 dB in the range of 1–15 kHz at the position of the subject. The duration of the auditory stimuli was 0.1 s. The speaker was mounted in front of the three nose-poke, each connected with a liquid dispenser (Fig. 1A). Operant modules were regulated by the Packwin V1.2 software (Panlab S.L., Barcelona, Spain). All experiments were recorded with an IP camera (VIVOTEK, Sunnyvale, CA, USA) fixed above each operant chamber. Videos were acquired by Nuuo software (Nuuo, Taipei, Taiwan).

*Behavioral task.* Three days after weaning, male *Sprague–Dawley* rats (23 days old at the start of the experiment) were trained in the behavioral 2-ACT paradigm. Animals were water deprived over night under a protocol approved by the Institutional Animal Ethics Committee of the Faculty of Sciences, Universidad de Valparaíso, Chile. Afterward, the rat initiated a trial by inserting its nose into the center nose-poke of a three-port operant chamber (Fig. 1A), which triggers the computer to present two types of acoustic stimuli at random one a low-frequency tone of 1 kHz and the other a high-frequency 15 kHz tone. The rats were trained to respond with right pokes for low tones and left pokes for high tones. Correct trials were rewarded with water (Fig. 1A). All operant chambers were thoroughly cleaned with a 5% ethanol solution after each trial. The control and chronically stressed groups were evaluated at the same time.

The 2-ACT training has three steps; in each of which it is possible to independently analyze learning, memory consolidation and auditory attention (Fig. 1B). In the first week of training, rats learned to respond with right pokes for low tones and left pokes for high tones (learning period). In the second week, rats were trained in 50 2-ACT trials until reaching 70% correct trials. At the end of this week the rats' memory related to 2-ACT was consolidated (memory consolidation period). Beginning in the third week, the rats recalled the task and improved their auditory attention increasing correct responses to over 80% of correct trials (auditory attention period).

*Experimental design.* Fig. 1B shows a schematic drawing of the experimental design used in this study. After 3 weeks of training, rats that reached a correct performance of over 80% in 50 trials were included for four experiments, using different sets of animals for each experiment. Experiment 1 analyzed whether repeated restraint stress paradigm affects the stress levels of trained rats (control, $n = 12$, stress, $n = 12$) one day after stress ended. The most conventional method to determine if the animals are stressed is to measure the plasma levels of the stress hormone corticosterone. Animals were subjected to a new stressor (swimming in a water maze) and corticosterone plasma levels were quantified before and after water maze exposure. Experiment 2 consisted of analyzing both 2-ACT training and restraint stress effects on the locomotor activity (open field), anxiety (elevated plus-maze), and memory (Y-maze) (control, $n = 9$, stress, $n = 9$). In experiment 3 we studied the effect of restraint stress on auditory attention. The animals were randomly assigned to two groups: control, $n = 15$, and stress, $n = 15$, and one day before and after the stress protocol, the rats were subjected to 50 trials of 2-ACT

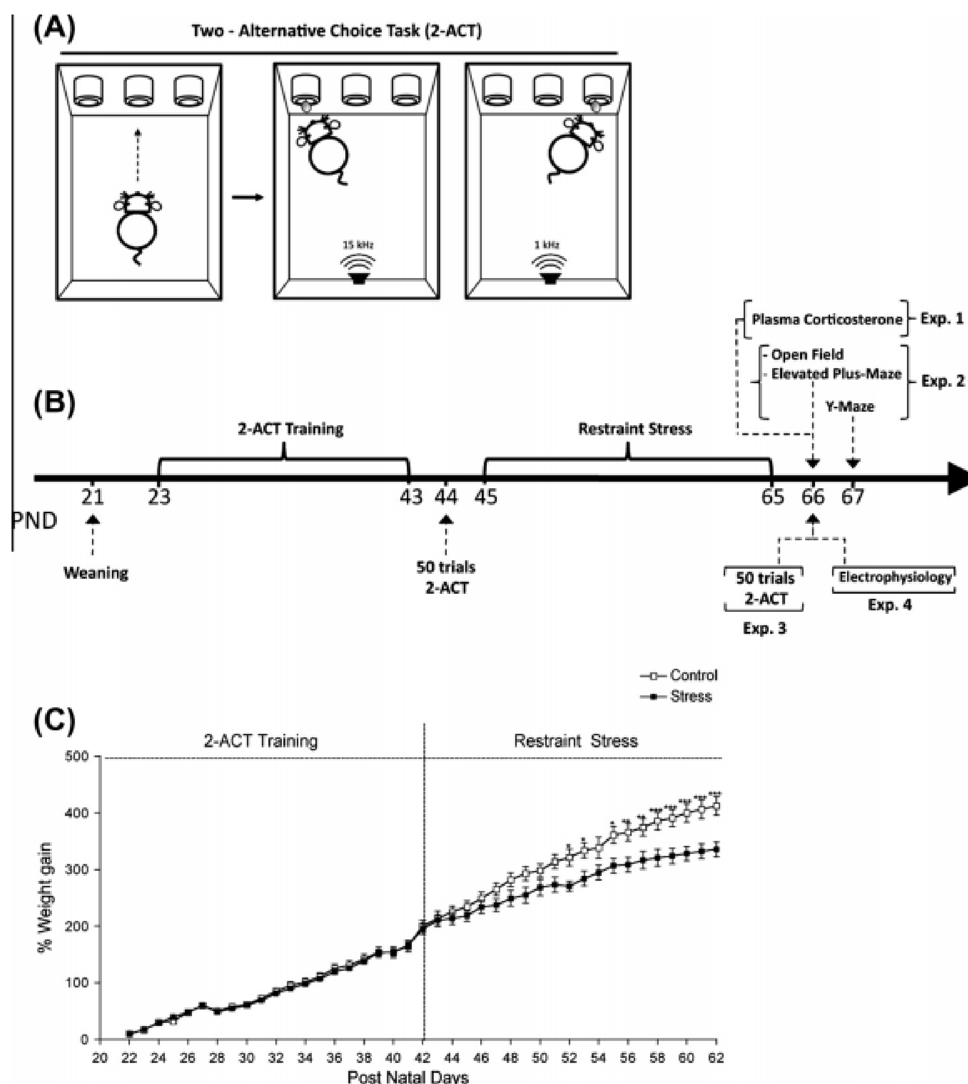


**Fig. 1.** Structure of the basic two-alternative choice task (2-ACT), schematic drawing of the experimental design, and the influence of restraint stress on the body weight. (A) 2-ACT is an auditory attentional task; the rat initiates a trial when decides to introduce its nose into the center port (*left*). This elicits the computer to randomly present two types of auditory stimuli, a high (15 kHz) or low (1 kHz) frequency tone (*right*). Rats were trained to respond with a left poke for high tones and a right poke for low tones; correct trials were rewarded with water. (B) The arrow represents the postnatal days of the animals (PND). After weaning, rats were trained in the 2-ACT for 20 days. Afterward, animals were randomly assigned to two experimental groups: control, $n$ = 48, and stress, $n$ = 48. Twenty-four trained rats (control, $n$ = 12, stress, $n$ = 12) were used in the experiment 1 (Exp. 1) to measure plasma corticosterone levels one day after stress ended. Eighteen trained rats (control, $n$ = 9, stress, $n$ = 9) were used in the experiment 2 (Exp. 2) to analyze the stress effects on locomotor activity (open field), anxiety (elevated plus-maze), and memory (Y-maze) on days 66 and 67, respectively. In experiment 3 (Exp. 3), to analyze the stress effects on auditory attention, thirty trained rats, (control, $n$ = 15, stress, $n$ = 15) were subjected to 50 2-ACT trials one day before and after the restraint stress protocol. A difference score was then determined by subtracting the correct trials after from those before chronic stress (DS-CT). Experiment 4 (Exp. 4) analyzed synaptic transmission in A1 (control, $n$ = 12, stress, $n$ = 12). (C) Training in the auditory task (2-ACT) did not affect the percentage of weight gain. At the beginning of the restraint stress, control animals gained weight gradually throughout the study, however chronically stressed rats failed to gain weight. Data are represented by mean ± SEM.

(Fig. 1B). Experiment 4 analyzed stress effects on synaptic transmission in A1 (control, $n$ = 12, stress, $n$ = 12).

### Experiment 1

*Plasma corticosterone measurement.* A separate set of animals was used to measure the concentration of corticosterone in plasma, in order to avoid the stressfulness of blood collection on behavioral or electrophysiological experiments. One set of rats (control, $n$ = 6, stress, $n$ = 6) were given a 60-s probe trial in a water maze at 11:00 h after which the animals were transferred to a heated holding cage for 10 min. Afterward, the animals were transported to a separate room (time used approximately 10 s) and were quickly anesthetized with isoflurane (time used approximately 5 s) and immediately sacrificed via decapitation under deep anesthesia to blood collection. Animals were not

exposed to other decapitated animals before deep anesthesia. Another set of rats (control, $n = 6$, stress, $n = 6$) were not disturbed and sacrificed at 11:11 h under deep anesthesia. The Morris water maze consisted of a blue circular tank (183 cm diameter) in a room that was rich with spatial cues. The tank contained non toxic colored water at 19 °C (black nontoxic tempura paint).

Blood (1 ml) was collected in heparinized microcapillary tubes and centrifuged (Model # MiniSpin Plus; Eppendorf AG, Hamburg, Germany) at 10,000 rpm for 10 min to obtain plasma and then stored at −70 °C. Total corticosterone was determined by an Enzyme Immunoassay kit (Corticosterone BioAssay, Catalog. # C7903-30) purchased from US Biological (Swampscott, MA, USA). Optical density values were measured at 450 nm using a micro-plate reader (Tecan GENios, Tecan Group Ltd., Switzerland). Samples were diluted 1:10 and then processed in duplicate and averaged final values were represented as ng/ml.

## Experiment 2

*Behavioral testing.* The open field and elevated plus-maze tests were conducted 24 h after final completion of the stress protocol, and a day after the Y-maze test was applied (Fig. 1B). All animals were naive to the test situations. Behavioral tests were carried out from 10.00 to 14.00 h. in the test room. The activity of each rat was recorded by IP cameras fixed above the behavioral apparatus and connected to a computer in another room. Videos were acquired by Nuuo software (Nuuo, Taipei, Taiwan) and analyzed using ANY-maze video-tracking system (Stoelting Co., Wood Dale, IL, USA). All mazes were wiped clean thoroughly with 5% ethanol solution after each trial. In all experiments, animals from control and stress experimental groups were evaluated at the same time.

*Open field test.* Behavioral tests were conducted in a sound-proof and temperature-controlled (21 ± 1 °C) room. Each rat was placed in the center of a black Plexiglass cage (70 × 70 × 40 cm) for 5 min. The background noise level in the open field was 40 dB (Precision sound level meter, Model # 1100, Quest Technologies, Oconomowoc, WI, USA) and the arena was illuminated to 300 lux (measured by digital lux meter, Model # LX-1010B, Weafo Instrument Co., Shanghai, China). Total distance traveled and average speed was analyzed from video recordings and analyzed using ANY-maze video-tracking system (Stoelting Co.).

*Elevated plus-maze.* Immediately after the analysis of the open field (approximately 10 s) we measured anxiety levels using an elevated plus-maze test. Each rat was individually placed in an elevated plus-maze, consisting of two open arms (60 × 15 cm each), two closed arms (60 × 15 × 20 cm each) and a central platform (15 × 15 cm), arranged so that two arms of each type were opposite to each other. The maze was elevated 100 cm above the floor. The illumination was 300 lux in the open arms and 210 lux in the closed arms. At the beginning of each trial animals were placed at the center of the maze, facing an open arm. During a 5-min test period we recorded the frequency of entries to the open and closed arms, the total number of arm entries, and the amount of time spent in each section of the maze. The number of entries and time spent in the open arms, and the ratio of open to total arm entries (open/total × 100) were used as measures of the anxiety level (Dagnino-Subiabre et al., 2006). Total arm entries were taken as an indicator of general locomotor activity. Entry into an arm was defined as having occurred when the animal placed all four limbs onto the arm.

*Y-maze.* Spatial memory was tested on the Y-maze 24 h after completing the analysis of the elevated plus-maze. The Y-maze consisted of three equilaterally intersecting black Plexiglas arms (58 cm long × 19 cm wide × 38 cm high) and several extra-maze cues on the surrounding walls. The three arms were assigned as Novel, Start and Other, and were counterbalanced among rats. Control and stressed rats were tested at the same time and in separate Y-mazes. Through training, one arm (Novel) was blocked and the animals were placed on the Start to explore for 15 min both the Start and Other arms. After training, the Novel arm was unblocked and rats were returned to their home cages and room. Four hours later, rats were returned to the same start location used during training, and were allowed to freely explore all arms for 5 min. Rats tend to explore novel environments, consequently an intact spatial memory if the rats showed a preference for the Novel arm. Entry into an arm was defined when the animal placed all limbs onto the arm. Behavior was videotaped and entries were converted into percentages. Entries into all arms were counted (total entries) to determine whether locomotor activity levels were similar between experimental groups. To analyze the stress effect on spatial memory ability, a difference score (DS) was measured subtracting the percentage of entries in the alternate arm from the percentage of entries in the novel arm.

## Experiment 3

Trained rats were subjected to 50 trials of 2-ACT one day before and after the stress protocol (Fig. 1B). The following parameters were measured using the Packwin software (Panlab, Barcelona, Spain) in each trial: number of correct trials, ratio of latency of correct trials (L-CT)/number of correct trials (L-CT/CT), and inter-trial interval (ITI). The ratio of L-CT/CT indicates the time used in each correct trial. To analyze the stress effects on the auditory attention, a DS was measured by subtracting the number of correct trials determined before stress (DS-CT) from the number of correct trials obtained on the 50 trials after the stress period. A DS was also calculated for the ratio of L-CT/CT [(DS-(L-CT/CT)] and ITI (DS-ITI).

**Experiment 4**

*Electrophysiology.* A new set of trained rats (control, $n = 12$, stress, $n = 12$) was used to study the chronic stress effects on the efficacy of the glutamatergic and GABAergic systems in A1. After completion of the stress protocol each rat was decapitated under deep anesthesia with isoflurane. The brain was removed quickly and submerged in cold (~4 °C) artificial cerebrospinal fluid (in mM: 124.00 NaCl, 2.69 KCl, 1.25 $KH_2PO_4$, 2.00 $MgSO_4$, 26.00 $NaHCO_3$, 2.00 $CaCl_2$, 10.00 glucose). The pH of the artificial cerebrospinal fluid was stabilized at 7.4 by bubbling carbogen (95% $O_2$ and 5% $CO_2$). Coronal brain slices (300–350 µm) were cut with a Vibratome (Campden Instruments, model MA752, England) and incubated in the artificial cerebrospinal fluid (> 1 h, at room temperature; 20–22 °C). Slices were transferred to a 2-ml chamber fixed to an upright microscope stage (NIKON, model Eclipse FN1, Tokyo, Japan) equipped with infrared differential interference contrast (DIC) video microscopy and 40× water immersion objectives. Slices were superfused with carbogen-bubbled artificial cerebrospinal fluid (2 ml/min) and maintained at room temperature (22–24 °C). 2-Amino-5-phosphonopentanoic acid (D-AP5; 50 µM) and 7-nitro-2,3-dioxo-1,4-dihydroquinoxaline-6-carbonitrile (CNQX; 20 µM) were added to the artificial cerebrospinal fluid as needed.

Recordings were made in neurons located 300–450 mm from the pia surface corresponding to layers II–III of rat auditory neocortex. Whole-cell recordings were made with patch pipettes (4–6 MΩ) filled with an internal solution that contained in mM: 100 Cs-Gluconate, 10 HEPES, 10 EGTA, 4 $Na_2$-ATP, 10 TEA-Cl and 1 $MgCl_2$–$6H_2O$, buffered to pH 7.2–7.3 with CsOH. Recordings were performed in voltage-clamp modes using an EPC-7 patch-clamp amplifier (HEKA, Instruments). In voltage-clamp experiments the Vh was adjusted to −65 or 0 mV as needed. In the voltage-clamp configuration the series resistance was compensated to ~70% and neurons were accepted only when the seal resistance was > 1 GΩ and the series resistance (7–14 MΩ) did not change > 10% during the experiment. The liquid junction potential was measured (~6 mV) but was not corrected. Voltage-clamp data were low-pass filtered at 3.0 kHz and sampled at rates between 6.0 and 10.0 kHz using an A/D converter (ITC-16, InstruTech) and stored with Pulse FIT software (Heka Instruments). The Pulse Fit program was used to generate stimulus timing signals and transmembrane current pulses. The recording analysis was made off-line with pClamp software (Clamp-fit, Molecular Devices). Inhibitory postsynaptic currents (IPSCs) were evoked with a concentric bipolar electrode (60 µm diameter, tip separation ~100 µm (FHC Inc., ME, Bowdoin), placed at about 100–200 µm lateral from pyramidal neuron somata to stimulate the GABA interneurons or at the base of layer III/IV, in line with the recording electrode to stimulate the thalamocortical axons.

An average of IPSC ($n = 10$) was obtained under voltage clamp by repeated stimulation at 0.3 Hz. Chemicals were purchased from Sigma–Aldrich Chemistry (Santiago, Chile), and Tocris (Bioscience, USA). The paired pulse ratio (PPR) was calculated as 1-(R2/R1) ∗ 100, where R1 and R2 are the peak amplitudes of the first and second IPSCs, respectively.

To determine whether chronic stress could simultaneously affect the glutamatergic and GABAergic pyramidal neuron synapses; we voltage-clamped CA1 PNs at the reversal potential for evoked excitatory or inhibitory synaptic currents (EPSCs or IPSCs, respectively). Values of the reversal potential of EPSCs and IPSCs were estimated from current–voltage relationships of EPSCs (0.3 ± 0.5 mV; $n = 10$) and IPSCs (−64.2 ± 2.3 mV; $n = 10$), respectively. Moreover, in some experiment the excitatory or inhibitory synaptic transmission were isolated after blocking GABAA with picrotoxin (10 µM) or NMDA and AMPA receptors with D-AP5 (50 µM) and CNQX (20 µM).

The spontaneous inhibitory and excitatory postsynaptic currents (sIPSCs or sEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) were analyzed off-line using the Minianalysis software (Minianalysis; Synaptosoft, Decatur, GA, USA), which allowed visual detection of events and selection for analysis of those that exceeded an arbitrary threshold.

*Calculation of the multiplicity factor.* The multiplicity index was calculated in order to estimate the degree of connectivity between interneurons and pyramidal neurons in A1 (Hsia et al., 1998; Groc et al., 2003). The spontaneous and miniature (action potential-independent) GABAergic postsynaptic currents, sIPSC and mIPSC respectively, were compared in A1 neurons of rats from control and stress groups. To determine the index of multiplicity, first the amplitude and frequency mean values of sIPSC and mIPSC alone were obtained and recorded respectively before and after adding tetrodotoxin (TTX). Multiplicity was calculated as the mean amplitude of action potential-driven events ("*a*") divided by mean quantal size ("*q*": mean amplitude of mIPSC recorded in TTX). The "*a*" value was determined for each cell, subtracting the contribution of mIPSC to the pool of events collected in the absence of TTX, using the expression for "*a*":

$$a = \frac{f_b b - f_q q}{f_b - f_q}$$

where "$f_b$" and "$f_q$" denote the mean frequency values from events recorded before and after the addition of TTX to the perfusion media, respectively, and "*b*" is the mean amplitude of both sIPSC and mIPSC.

**Statistical analysis**

*Behavioral studies.* Locomotor activity, anxiety, and memory studies were analyzed by a Student's unpaired *t*-test. Body weight gain and DS for correct trials, ratio (L-CT/CT), and ITI were analyzed using a two-way repeated-measure ANOVA [Body weight [groups (control, stress) × post-natal days (22–62)]; DS [groups

(control, stress) × trials (1–50)] followed by a Bonferroni post hoc comparison test.

*Plasma corticosterone levels.* Results were analyzed by one-way ANOVA to compare groups (control and stress) and swimming conditions (no swimming or swimming on the day of plasma collection) followed by Bonferroni's multiple comparison test.

*Electrophysiological studies.* Data analysis and statistical evaluations were made with both the pClamp program (Molecular Devices Corporation, Chicago, USA) and Origin 7.0 (Originlab Corporation, Northampton, MA, USA). Results are presented as percentages of control. Statistical analysis was performed using Student's two-tailed *t*-test.

Results are presented as the mean ± SEM, for the electrophysiological studies $n$ = number of cells. A probability level of 0.05 or less was accepted as significant.

## RESULTS

### Effects of restraint stress on body weight gain

2-ACT training did not affect body weight gain (Fig. 1C). Restraint stress attenuated the percentage of body weight gain compared to controls (Fig. 1C). A 2 × 41 mixed factor ANOVA, with treatment (control, $n = 21$, stress, $n = 21$) as the between-subjects factor and the day (22–62) as the repeated measure showed a significant treatment-day interaction, ($F_{(40,1600)} = 55.65$, $p < 0.0001$), a significant main effect of treatment, ($F_{(1,40)} = 18.51$, $p < 0.0001$), and a significant main effect of day, ($F_{(40,1600)} = 4556$, $p < 0.0001$). At the start of the stress protocol, there were no differences between the body weights of the animals from control and stress groups (Fig. 1C). However, rats that received restraint stress showed less weight gain during the 21 days of the stress protocol (postnatal day 42–62) than to control rats ($p < 0.0001$) (Fig. 1C).

### Experiment 1

*Effects of restraint stress on plasma corticosterone levels.* Stressed animals showed an increase in HPA axis activity and plasma corticosterone levels compared to controls after exposure to an uncontrollable stressor, leading to maladaptive response (Tafet and Bernardini, 2003). In this way, acute swim stress in a water maze increases plasma corticosterone levels of *Sprague–Dawley* rats (McFadden et al., 2011). Therefore, we measured the plasma corticosterone levels of trained animals one day after of the last restraint session, when behavioral and electrophysiological experiments were initially conducted. Fig. 2 shows the level of circulating corticosterone in rats subjected to a 60-s probe trial in the water maze and in animals that were not disturbed. Controls and the rats that were subjected to restraint stress swimming for 60 s in the water maze had higher corticosterone levels than those that were left

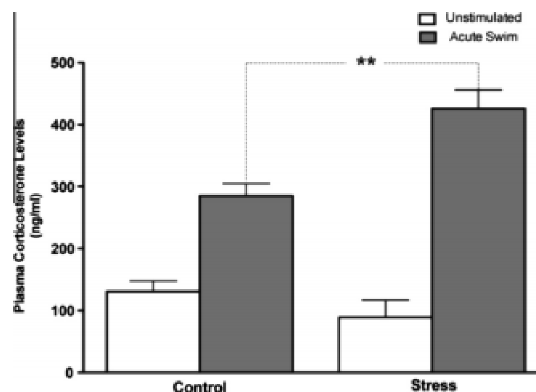


**Fig. 2.** Stress levels after 21 days of restraint stress paradigm. Acute 60 s swim in a water maze at 19 °C given 10 min before plasma collection caused a significant increases in plasma corticosterone levels in the rats of all experimental groups (gray bars); whereas after acute swim chronically stressed rats had significantly increased serum corticosterone levels compared to control animals. The asterisk (*) indicates a significant difference relative to control animals. Data are represented by mean ± SEM.

undisturbed (control group: undisturbed = 130.60 ± 17.06 ng/ml, $n = 6$, acute swimming = 285.10 ± 19.88 ng/ml, $n = 6$, $p < 0.01$; stress group: undisturbed = 89.12 ± 27.78 ng/ml, $n = 6$, acute swimming = 426.00 ± 29.97 ng/ml, $n = 6$, $p < 0.001$). Following acute swimming, animals from the stress group had significantly higher corticosterone levels than control rats (stress group = 426.00 ± 29.97 ng/ml, $n = 6$; control group: 285.10 ± 19.88 ng/ml, $n = 6$; $p < 0.01$).

### Experiment 2

*Effects of restraint stress on locomotor activity, anxiety and memory.* Table 1 shows the effects of restraint stress on locomotor activity, anxiety, and memory. Statistical analysis revealed that the restraint stress protocol did not affect locomotor activity in the open field [(total distance traveled, stress: 16.25 ± 1.60 m, $n = 9$;

**Table 1.** Influence of restraint stress on the locomotor activity, anxiety, and memory of trained rats

| | Control | Stress |
|---|---|---|
| *Locomotor activity (Open Fiel)* | | |
| Total distance traveled (m) | 14.9 ± 3.2 | 16.3 ± 1.6 |
| Average speed (m/s) | 0.05 ± 0.01 | 0.05 ± 0.01 |
| *Anxiety (EPM)* | | |
| Number of open arm entries | 4.8 ± 1.3 | 2.2 ± 0.4* |
| Number of closed arm entries | 12.4 ± 1.2 | 10.2 ± 0.8 |
| Number of total entries to the arms | 8.6 ± 1.5 | 6.2 ± 1.4 |
| *Memory (Y-maze)* | | |
| Difference score (% Novel–% Other) | 6.7 ± 4.4 | 0.0 ± 2.9* |
| Number of total entries to the arms | 15.2 ± 2.9 | 12.8 ± 1.4 |

The asterisk (*) indicates significant difference relative to control animals. Data are represented by mean ± SEM.

control: 14.89 ± 3.24 m, $n = 9$; $p = 0.7177$) (average speed, stress: 0.0540 ± 0.0053 m/s, $n = 9$; control: 0.0494 ± 0.011 m/s, $n = 9$; $p = 0.7148$)]. Restraint stress induced a significant reduction in the frequency of the open-arm entries (stress: 2.2 ± 0.4, $n = 9$; control: 4.8 ± 1.2, $n = 9$; $p < 0.05$) on the elevated plus maze (Table 1). There were no treatment differences in the total arm entries, indicating that the stress protocol did not affect locomotor activity (stress: 6.2 ± 1.4, $n = 9$; control: 8.6 ± 1.5, $n = 9$; $p = 0.3055$) (Table 1). These results are indicative of an enhanced anxiety response in the trained stressed animals. Restraint stress impaired the trained rats' performance on the Y-maze. This finding was supported by the 4-h delay version of the Y-maze, restraint stress significantly decreased the %DS between Novel and Other arm entries (stress: 0.0 ± 2.9, $n = 9$; control: 6.7 ± 4.4, $n = 9$; $p < 0.05$). Statistical analysis revealed that restraint stress did not affect the total number of entries made into the arms of the Y-maze (stress: 4.3 ± 0.3, $n = 9$; control: 5.1 ± 0.6, $n = 9$; $p = 0.1515$).

**Experiment 3**

*Auditory attention task.* The purpose of this experiment was to analyze the effect of the restraint stress protocol on the auditory attention of rats that were trained in the 2-ACT, a behavioral paradigm to study attention in rats (Fig. 1A, B). Restraint stress did not affect the DS-CT through the first 10 trials of the 2-ACT (Fig. 3A). After of the 10th trial, restraint stress decreased the DS-CT compared to that of control animals (Fig. 3A). Restraint stress did not affect interaction between treatment and trial ($F_{(9,252)} = 1.57$, $p = 0.1250$), while treatment effect was significantly altered ($F_{(1,28)} = 139.3$, $p < 0.0001$). Stress and control group rats had DS-CTs of −9.4 ± 0.6 and 4.6 ± 0.6 correct trials, respectively (Fig. 3B). The majority of control animals had a significantly higher number of correct trials on postnatal day 66 (Fig. 3C). In terms of percentages, the percentages of DS-CT for animals of stress and control groups were −18.8 ± 1.2% and 9.2 ± 1.2% respectively. This is a 28% reduction in the 2-ACT performance of stressed rats compared to that of controls on postnatal day 66 (Fig. 3B). Stressed rats had over 80% of correct trials before restraint stress and 61.2% after the stress period, indicating that restraint stress impaired auditory attention and did not significantly affect memory consolidation related to the 2-ACT after the stress period (Fig. 3C).

Restraint stress decreased the DS-(L-CT/CT) compared to controls (Fig. 3D). Interactions between treatment and trial, and treatment effect decreased significantly [(Interaction: $F_{(9,252)} = 2.51$, $p < 0.001$); (Treatment: $F_{(1,28)} = 5.86$, $p < 0.05$)] (Fig. 3D). Restraint stress significantly decreased the average of total DS-(L-CT/CT) (Fig. 3E). This result demonstrates that control rats take more time for each correct trial than rats from the stress group.

Rats that were subjected to restraint stress showed a significant increase of the DS-ITI relative to control animals (Fig. 3F). There was no effect in the interaction between treatment and trial (Interaction: $F_{(9,252)} = 0.45$, $p = 0.91$), but the treatment effect was significantly affected ($F_{(1,28)} = 16.35$, $p < 0.0001$) (Fig. 3F). The restraint stress protocol significantly increased the average of total DS-(L-CT/CT) (Fig. 3G). This result shows that rats from the stress group used more time compared to controls for decision-making to perform each correct trial in the 2-ACT paradigm.

**Experiment 4**

*Effects of restraint stress on glutamatergic and GABAergic synaptic transmission in A1.* To determine whether the restraint stress protocol affects the efficacy of GABAergic synapses on pyramidal neurons of A1, we evoked isolated IPSC by the paired-pulse protocol. Afterward, modifications of the paired-pulse depression (PPR) were quantified by an index (1-(R2/R1) ∗ 100). We observed that in rats from both experimental groups, the PPR was characterized by depression, with the latter (R2) IPSC lower than the former (R1), indicating that the group with stimulated inhibitory synapses had a high release probability. We observed that restraint stress decreased the PPR (24.9 ± 4.9%) compared to controls (42.6 ± 12.2%). These effects suggest that restraint stress protocol induced a depression of GABA release (Fig. 4A, B), which is illustrated by representative neurons in Fig. 4A, top recordings.

According to the presynaptic locus, we observed that restraint stress decreased the frequency of sIPSC and mIPSC. The sIPSC frequency of rats from the stress group was 1.9 ± 0.4 Hz, whereas the frequency reached 2.8 ± 0.4 Hz in control animals ($p < 0.05$; $n = 8$; respectively; Fig. 5A, B). In addition, the mIPSC frequency of rats after restraint stress was 0.4 ± 0.04 Hz and 1.93 ± 0.3 Hz for control rats. The reductions in the sIPSC and mIPSC frequencies in the rats of the stress group were also observed after comparing the cumulative probability plots of the sIPSC frequency relative to controls (Fig. 5B), suggesting that stress-induced reduction of GABA release occurs presynaptically as a result of a decreased probability of release. Moreover, we observed that restraint stress had no effect on postsynaptic GABA efficacy. Thus, the sIPSC and mIPSC amplitudes were 98.1 ± 11.2 and 50.8 ± 4.2 pA in the rats subjected to restraint stress, while in control animals they were 124.3 ± 13.9 and 55.3 ± 1.0 pA ($n = 6$; $p = 0.08$; Fig. 5B). Restraint stress significantly decreased the sIPSC and mIPSC frequencies, whereas the amplitude was not affected than controls (Fig. 5C). In addition, the multiplicity index was examined to estimate synaptic network connectivity (Hsia et al., 1998; Groc et al., 2003). The sIPSC was recorded first and after addition of TTX (500 nM), the mIPSC was recorded ($n = 6$). Afterward, the multiplicity index was calculated for each cell (Groc et al., 2003; Riebe and Hanse, 2012). The multiplicity index was on average 1.8 ± 0.1 for stressed rats and 2.3 ± 0.3 for controls ($n = 4$, respectively; data not shown), indicating

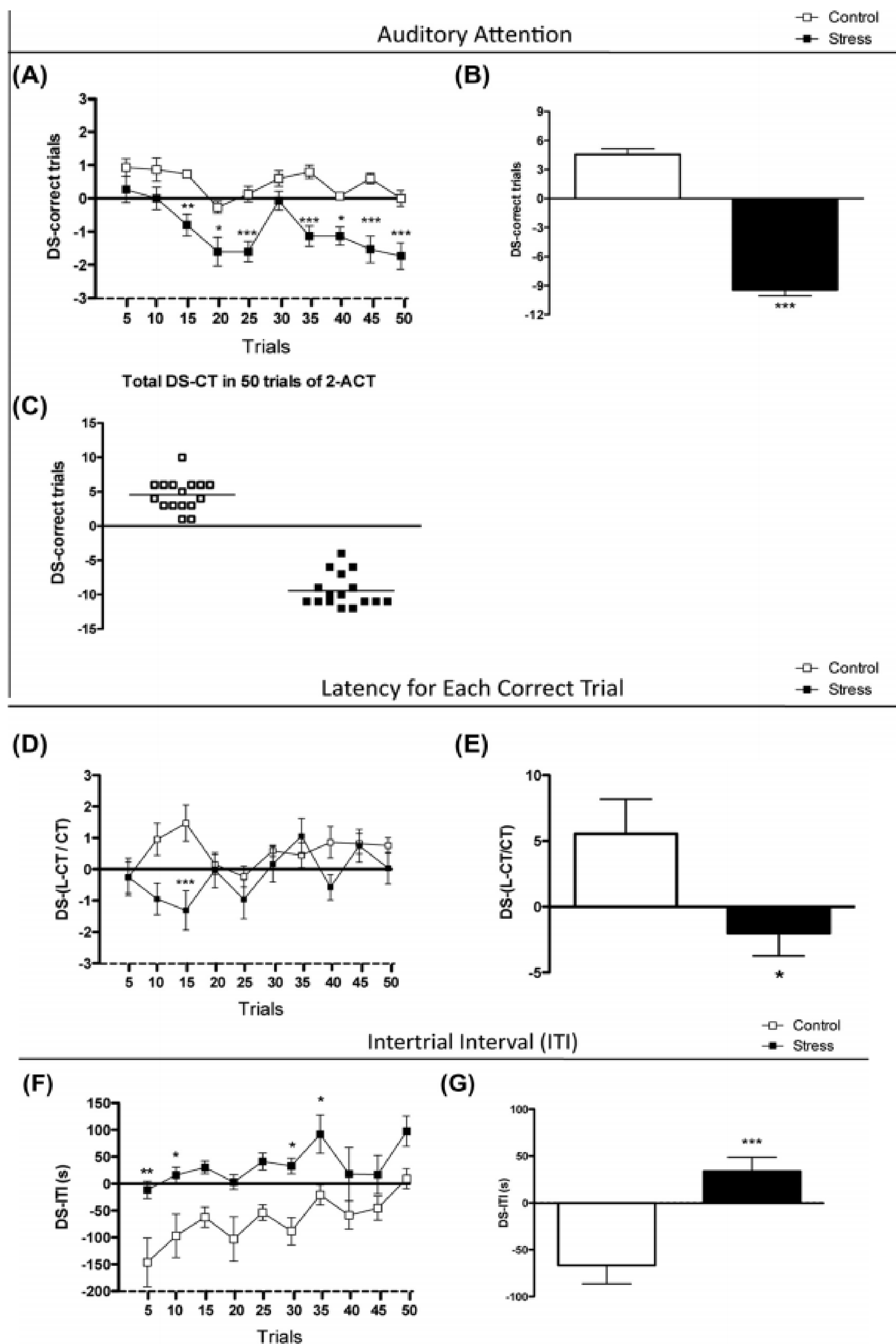


**Fig. 3.** Influence of restraint stress on the auditory attention, latency of correct trials, and inter-trial interval (ITI). (A) The DS-CT significantly decreased in the stressed rats compared to controls. (B, C) Restraint stress significantly decreased the average of total correct trials in 50 trials of 2-ACT. (D) Ration of DS-(L-CT/CT) was increased in the control rats compared to the stressed rats. (E) Restraint stress significantly decreased the average of total DS-(L-CT/ CT). (F) Restraint stress decreased the DS-ITI. (G) Restraint stress significantly increased the average of total ITI. The asterisk (*) indicates significant difference relative to control animals. Data are represented by mean ± SEM.

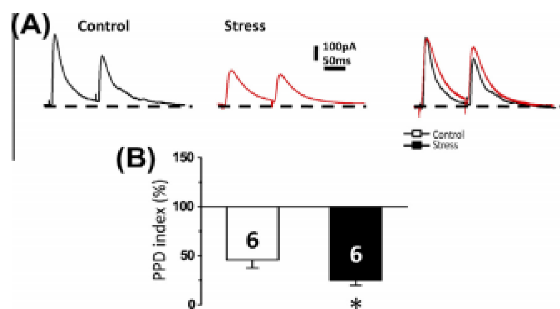


**Fig. 4.** Restraint stress impaired the probability of GABA release. (A) representative averaged IPSC recorded under control and stress conditions respectively. At the right, superimposed representative averaged IPSC recorded under control (black trace) and stress conditions (red trace). (B) Summary data showing the changes in PPR index between stressed and control rats. The numbers in the bars represent sample size (*n*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that restraint stress did not affect the degree of connectivity between an interneuron and A1 pyramidal neurons.

In a parallel experiment, we analyzed the effect of restraint stress on glutamatergic synaptic transmission. Restraint stress did not affect the sEPSC frequency and amplitude. Fig. 6A shows a representative sEPSC recording from A1 pyramidal neurons in both restraint stress and control condition. The sEPSC frequencies were 0.9 ± 0.3 Hz in the rats of the stress group and 1.1 ± 0.3 Hz in control rats ($p > 0.05$; $n = 6$, respectively), whereas the amplitude reached to 46.6 ± 6.9 pA in the animals that were subjected to restraint stress and 54.8 ± 7.2 pA in controls (Fig. 6B). Furthermore, the cumulative probability plots of the sEPSC frequency and amplitude confirm that restraint stress did not affect the efficacy of A1 excitatory synaptic transmission (Fig. 6C).

## DISCUSSION

The present study shows that repeated restraint stress impaired the auditory attention and GABAergic synaptic efficacy in the rat A1. The first step of our investigation was to analyze the stress levels of trained rats one day after the stress protocol ended. Fig. 2 shows that non-stimulated rats of the stress and control groups had similar corticosterone levels suggesting that trained rats would adapt to 21 days of restraint stress. Previous studies have shown that 3 or 6 h per day of restraint stress significantly increases corticosterone plasma levels during the first week, while in the second and third weeks of restraint stress the increases of corticosterone levels were less pronounced (Galea et al., 1997; Cook and Wellman, 2004). Therefore, if the

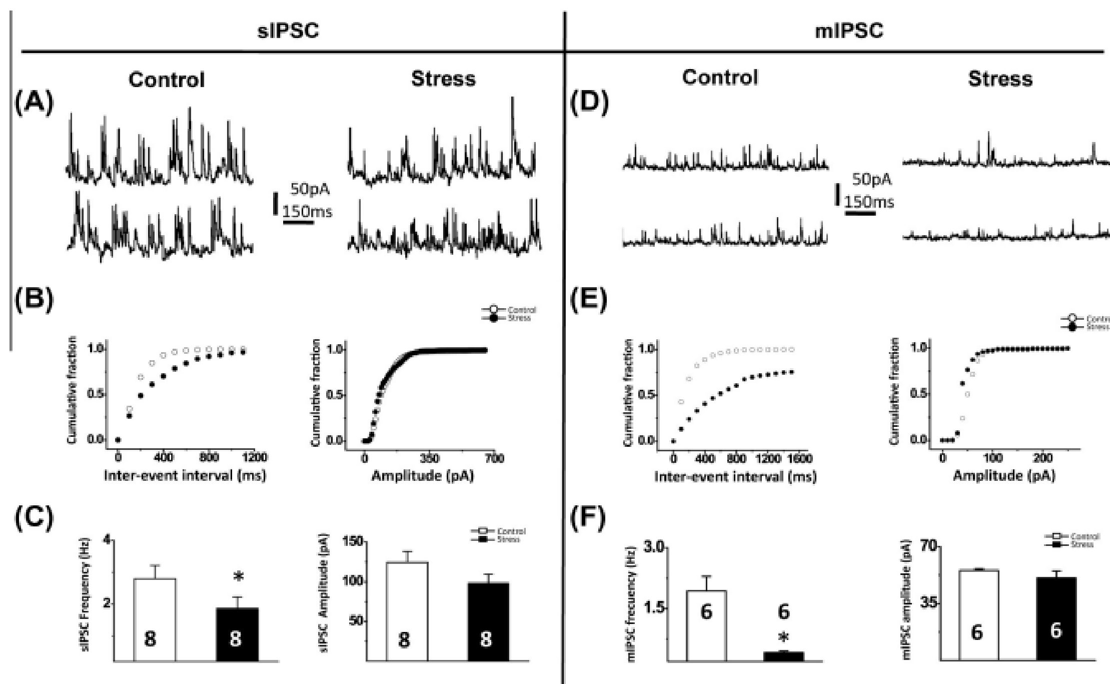


**Fig. 5.** Restraint stress reduced spontaneous GABAergic activity. (A) Representative sample traces showing sIPSC recorded under control and restraint stress conditions. (B) Cumulative probability histogram of sIPSC frequency and amplitude of control (open circles) and stress (filled circles) conditions. (C) Summary data showing the sIPSC frequency (left) and amplitude (right) under control and stress conditions. The numbers in the bars represent sample size (*n*). (D) Representative traces showing mIPSC recorded for control and stressed rats. (E) Cumulative probability histogram of mIPSC frequency and amplitude of control (open circles) and stress (filled circles) conditions. (F) Summary data showing the mIPSC frequency (left) and amplitude (right) for control and stressed rats. The numbers in the bars represent sample size (*n*).

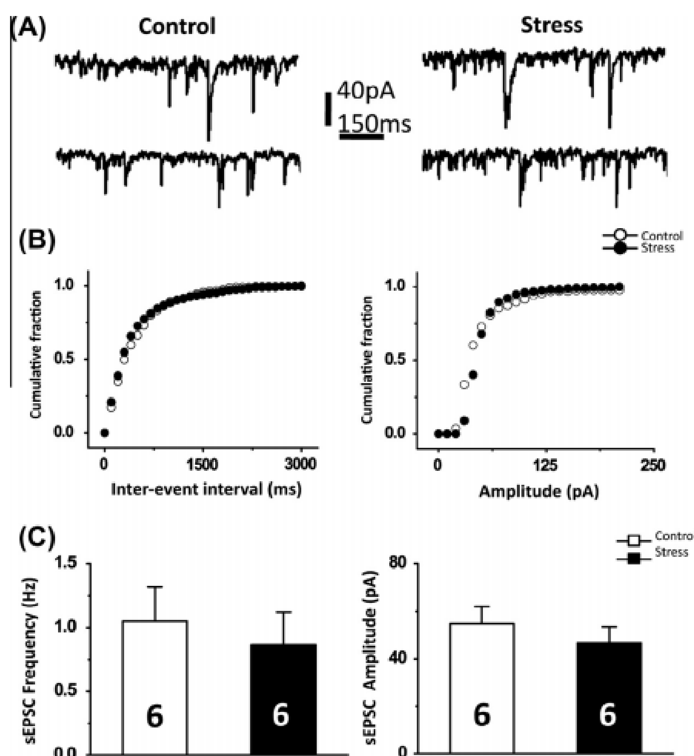


**Fig. 6.** Restraint stress did not affect Glutamatergic synaptic efficacy. (A) Representative sample traces showing sEPSC recorded under control and stress conditions. (B) Cumulative probability histogram of sEPSC frequency and amplitude of control (open circles) and stress (filled circles) conditions. (C) Summary data showing the sIPSC frequency and amplitude under control and stress conditions. The numbers in the bars represent sample size (*n*).

effects of restraint stress on the HPA axis activity and corticosterone response had been lost during the 21 days of restraint stress, the rats of control and stress groups would have shown comparable plasma corticosterone levels after exposure to a new uncontrollable stressor (acute swimming). However, rats that were subjected to restraint stress had significantly higher plasma corticosterone levels than control rats following one minute of swimming (Fig. 2). The conclusion of this experiment is that one day after restraint stress ended, trained rats of the control and stress groups showed similar HPA axis activity in an environment without stressors. On the other hand, rats of the stress group still showed higher levels of the HPA axis activity than controls animals when were exposed to a new uncontrollable stressor. This neuroendocrine alteration, which induces maladaptive responses to stressors, is characteristic of stressed animals (Tafet and Bernardini, 2003; Ferraz et al., 2011).

After 2-ACT training, chronic stress reduced the percentage of body weight gain compared to that of control animals (Fig. 1C). In addition, restraint stress enhanced anxiety in the elevated plus-maze (Table 1), as reported previously for physiological and behavioral stress markers (Dagnino-Subiabre et al., 2009).

Experiment 2 analyzed whether restraint stress affects the locomotor activity and spatial memory of trained rats. Restraint stress did not affect the distance traveled and average speed in the open field (Table 1). As well stressed- and control rats did not show differences in the number of total arm entries in both the elevated plus-maze and Y-maze (Table 1), indicating that restraint stress did not affect the locomotor activity. Moreover, restraint stress significantly decreased the percentage of DS in the Y-maze. This result demonstrates that restraint stress impaired the spatial memory of trained rats (Table 1). Comparable stress paradigms, such as immobilization and chronic unpredictable stress, show that stress-induced impairment on the spatial recognition memory is correlated with dendritic atrophy of the CA3c pyramidal neurons and decreases of the neurogenesis in the *dentate gyrus* of the hippocampus, a main brain area for the spatial memory (Vyas et al., 2002; McLaughlin et al., 2007).

#### Effects of restraint stress on the auditory attention

The stress-induced enhancement of anxiety and spatial memory impairment did not affect rat performances in

the 2-ACT during the first trials (Fig. 3A). Positive values for the DS-CT were associated with an increase in correct trials after the stress period compared to correct trials before restraint stress. Stressed rats had a DS-CT of −9.4 ± 0.6 correct trials (Fig. 3B), indicating that restraint stress decreased 2-ACT performance by 18.8%. Therefore, stressed rats had over 80% correct trails before restraint stress and 61.2% after the stress period. This result demonstrates that stressed rats recalled the task because 2-ACT performance over 60% indicates the animals know to respond with right pokes for low tones and left pokes for high tones. In support of this idea, animals of control and stress groups showed similar DS-CT during the first 10 trials of the 2-ACT. If restraint stress impaired the memory related to 2-ACT, which was consolidated before stress period, the stressed rats should have had negative values for the DS-CT during all the first 10 trials of the 2-ACT and significantly fewer than 50% correct trials after the stress period, similar to the performances at the beginning of the second week of training. Interesting, most of stressed animals significantly decreased their 2-ACT performances after the first ten trials (Fig. 3A). This result suggests that auditory attention was significantly impaired in the stressed rats compared to that in control animals (Fig. 3A–C).

After the stress period, the DS-(L-CT/CT) significantly decreased in the rats of the stress group (Fig. 3D, E). Thus, control rats took more time in each 2-ACT trial to achieve a correct response. It was previously reported that neuronal activity in A1 regulated the rat performance in the 2-ACT (Jaramillo and Zador, 2011). In addition, the balance between the excitatory and inhibitory systems is essential for cortical functions such as auditory attention (Buzsaki and Chrobak, 1995; Cobb et al., 1995; Isaacson and Scanziani, 2011). Therefore, we speculate that the stress-induced dendritic atrophy in A1 (Bose et al., 2010) and decreasing of the probability of GABA release (Figs. 4 and 5) may affect the balance between excitation and inhibition in A1; which in turn impairs the sensory representation and perception of the stressed rats through the 2-ACT. Under these conditions, tone frequency discrimination and response accuracy, and auditory attention decrease in rats subjected to restraint stress (Fig. 3A–C).

Rats from the stress group used significantly more time during ITI than the control animals (Fig. 3F, G). It is possible that an increase in DS-CT in controls rats improves the motivation to perform the auditory task. In contrast, the rats of the stress group did not change their motivation to perform the auditory task after restraint stress (Fig. 3F, G).

#### Effects of restraint stress on synaptic transmission in A1

It has been in this study that repeated restraint stress decreased GABAergic transmission in A1 without affecting glutamatergic transmission (Figs. 4–6). According to the presynaptic locus of expression, we observed that restraint stress decreased the frequency of sIPSC and mIPSC in A1, while the amplitude of sIPSC and mIPSC did not change after restraint stress. However, the cellular mechanisms that underlie this form of stress-dependent GABAergic depression in A1 are currently unknown.

It has been shown that chronic stress affects GABAergic synaptic transmission at the subcortical level (Hu et al., 2010a; Rodríguez Manzanares et al., 2005). Recent evidence suggests that both stress and the glucocorticoids affect GABAergic synaptic efficacy (Hu et al., 2010a), which could be due to direct actions via the neuronal glucocorticoid receptors. Glucocorticoid receptors are expressed widely in the brain; including in layers II–III of the neocortical regions (Sah et al., 2005). Corticosterone binds to cytosolic glucocorticoid receptor, thereby increasing the gene expression of proplastic genes, such as neuronal cell adhesion molecules, NCAM and L1 (De Kloet et al., 1998; Sandi, 2004; Meltser and Canlon, 2011). These molecules are implicated in neurite extension, cell survival and synaptic plasticity (Kiss et al., 2001). Thus, restraint stress may down-regulate the expression of glucocorticoid receptors and proplastic genes in A1 and affect inhibitory parvalbumin-positive neurons and pyramidal neurons, both types of neurons present in A1 (Letzkus et al., 2011). In support of this idea, chronic stress reduces the number of inhibitory parvalbumin-positive neurons in the hippocampus (Hu et al., 2010b) and causes atrophy of the basilar dendrites of pyramidal neurons in layers II and III of A1 (Bose et al., 2010).

Both the stress-dependent GABAergic depression in A1 demonstrated in this study (Fig. 5) and the dendritic atrophy induced by chronic stress in A1 (Bose et al., 2010) can modify the threshold of synaptic plasticity and induce an imbalance between the excitatory and inhibitory systems in A1, which in turn, could have a long-term effect on rat performance on the 2-ACT, specifically by decreasing the auditory attention of the rats that were subjected to restraint stress with respect to control animals in the last forty trials of the 2-ACT, as shown in Fig. 3.

The acoustic environment has a key role in the development of cortical circuits and auditory synaptic plasticity. This process is strongly modulated by the balance between excitation and inhibition that determines the development of the synaptic receptive field in A1 (Sun et al., 2010). In this context, sensory experience induces the refinement of intracortical inhibition, which regulates the organization and functioning of A1 (Dorrn et al. 2010; Sun et al., 2010). Therefore, if balanced excitation and inhibition is necessary to the temporal precision of neuronal activity in A1, restraint stress may disrupt the excitatory-inhibitory balance and change the auditory plasticity affecting complex cognitive functions associated with A1 such as auditory attention.

Agonist for alpha 7 nicotinic receptors increases the probability of GABA releases (Arnaiz-Cot et al., 2008). These receptors are expressed in A1 (Broide et al., 1995) and could increase GABA release in A1 of the stressed rats. Thus, agonist for alpha 7 nicotinic

receptors may improve auditory attention in the stressed rats.

The animal model and the behavioral paradigm to analyze auditory attention used in our research may be useful to study the cellular and behavioral mechanisms associated with attentional deficit in humans with psychosocial stress (Simoens et al., 2007) and patients suffering stress-related disorders. For example, patients with Post-Traumatic Stress Disorders show attentional disturbances (Kimble et al., 2010) and reduction in pre-attentive auditory sensory memory (Kimble et al., 2010). In addition, patients with major depressive disorders show impairment in early auditory processing (Kähkönen et al., 2007).

## CONCLUSION

The data presented here demonstrates that repeated restraint stress impairs auditory attention in rats. This result correlates with decreased GABAergic synaptic efficacy in the A1. We propose that stress-induced inhibition of GABA release in A1 could be triggered by the effect of glucocorticoids on synaptic plasticity in A1 during the stress period. This novel molecular mechanism may underlie the effects of restraint stress on auditory attention in rats.

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Corticosterone Treatment Impairs Auditory Fear Learning and the Dendritic Morphology of the Rat Inferior Colliculus

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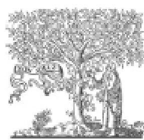

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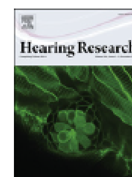

Research paper

# Corticosterone treatment impairs auditory fear learning and the dendritic morphology of the rat inferior colliculus

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ABSTRACT

Stress leads to secretion of the adrenal steroid hormone corticosterone (CORT). The aim of this study was to determine the effects of chronic CORT administration on auditory and visual fear conditioning. Male Sprague–Dawley rats received CORT (400 mg/ml) in their drinking water for 10 consecutive days; this treatment induces stress levels of serum CORT. CORT impaired fear conditioning ($F_{(1,28)} = 11.52$, $p < 0.01$) and extinction ($F_{(1,28)} = 4.86$, $p < 0.05$) of auditory fear learning, but did not affect visual fear conditioning. In addition, we analyzed the CORT effects on the neuronal morphology of the inferior colliculus (flat neurons, auditory mesencephalon, a key brain area for auditory processing) and superior colliculus (wide-field neurons, related to visual processing) by Golgi stain. CORT decreased dendritic arborization of inferior colliculus neurons by approximately 50%, but did not affect superior colliculus neurons. Thus, CORT had more deleterious effects on the auditory fear processing than the visual system in the brain.

## 1. Introduction

Stress is a complex biological reaction that restores homeostasis, allowing organisms to adapt to environmental pressure (i.e., stressor) (Selye, 1936; McEwen, 2007). The stress response is mediated heavily by activation of the hypothalamic–pituitary–adrenal (HPA) axis, leading to secretion of glucocorticoids (GCs) from the adrenal gland; GCs are bound to glucocorticoid receptors (GRs) in the peripheral tissues and the brain (Herman et al., 1996, 2003; Smith and Vale, 2006; McEwen, 2007). Limbic structures like the hippocampus, amygdala and medial prefrontal cortex have high concentrations of GRs (Gray and Bingaman, 1996; Joels, 2001; Wellman, 2001). Chronic glucocorticoid (GC) treatment produces dendritic atrophy in the hippocampus (McEwen, 1992; Watanabe et al., 1992; Magariños et al., 1998) and medial prefrontal cortex (Magariños et al., 1998). Conversely, acute GC treatment induces dendritic hypertrophy in the basolateral amygdaloid nucleus and enhances anxiety and conditioned fear responses (Cordero et al., 1998; Conrad et al., 2004; Mitra and Sapolsky, 2008). The acquisition of auditory emotional memories in the amygdala is associated with neuronal plasticity in the basolateral amygdala and medial geniculate nucleus (MG, auditory thalamus) (Maren et al., 2001; Poremba and Gabriel, 2001) (Fig. 1). Both brain areas exhibit associative plasticity of spike firing during fear conditioning (Maren et al., 2001). In contrast to the MG, the lateral amygdala receives only indirect projections from the lateral geniculate nucleus (LG) of the visual thalamus (LeDoux et al., 1984; McDonald, 1998; Aboitiz et al., 2003) (Fig. 1).

Chronic stress also alters dendritic architecture and function of brain areas related to memory and emotional processing, such as the hippocampus, the amygdala and medial prefrontal cortex (Magariños and McEwen, 1995; McEwen and Chattarji, 2004). Similarly, the auditory system is sensitive to stress-induced damage. For example, in rats, chronic stress causes dendritic atrophy in the

*Abbreviations:* ACx, primary auditory cortex; BNST, bed nucleus of stria terminalis; CORT, corticosterone; CS, conditioned stimulus; dB, decibel; ELISA, enzyme-linked immunoassay; GCs, glucocorticoids; GR, glucocorticoid receptor; HPA, hypothalamus–pituitary–adrenal axis; IC, inferior colliculus; LG, lateral geniculate nucleus; MG, medial geniculate nucleus; ms, millisecond; MR, mineralocorticoid receptor; s, second; SC, superior colliculus; US, unconditioned stimulus.

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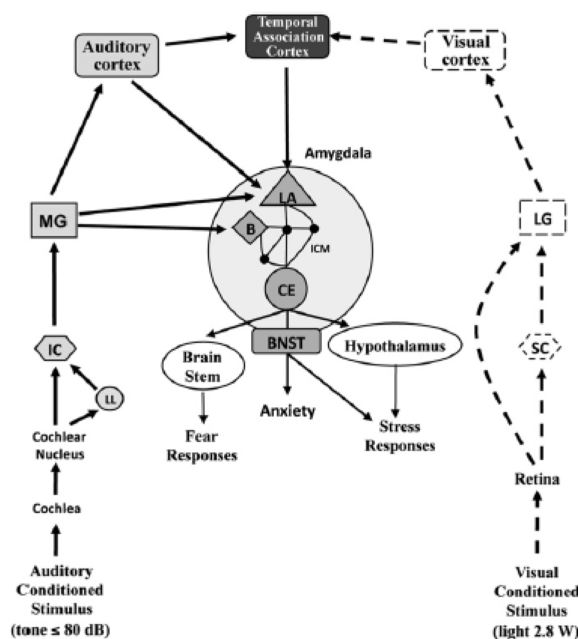


**Fig. 1.** The main ascending neuronal pathway involved in auditory and visual emotional processing in rats. The auditory and visual systems have direct and indirect connections to the amygdala. This scheme illustrates only the direct connections between the major nuclei of the auditory and visual pathway to the amygdala. Auditory conditioned stimuli (tone, CS, continuous line) are received in the cochlear nucleus and send projections to the lateral lemniscus (LL) and inferior colliculus (IC). From the IC, efferents are sent to the medial geniculate nucleus (MG) and the auditory cortex, which in turn projects glutamatergic inputs to the lateral and basal amygdaloid nuclei. Visual conditioned stimuli (light, dotted line) are received in the retina and are then sent to the lateral geniculate nucleus (LG) and the superior colliculus (SC). From the LG, projections are sent to the primary visual cortex. Information received in the lateral and basal amygdaloid nuclei is sent to the central amygdaloid nucleus through the intercalated cell masses. The central amygdaloid nucleus projects to hypothalamic sites and several brain stem nuclei that participate in the stress and fear responses (such as freezing). From the central amygdaloid nucleus, projections are directed to the bed nucleus of stria terminalis (BNST), inducing anxiety.

inferior colliculus (IC), a main component of the auditory nervous system which has a critical role in auditory fear learning (LeDoux et al., 1984). In contrast, stress does not affect superior colliculus (SC) neurons related to visual processing (Dagnino-Subiabre et al., 2005). The IC and SC are counterparts and their principal neuron types are flat neurons and wide-field neurons respectively. These neurons project to collothalamic nuclei, for example, flat neurons in the IC project to MG and wide-field neurons in the SC project to the LG (visual thalamus), thus both types of neurons are comparable (Peruzzi et al., 2000; Hilbig et al., 2000).

The stress-induced IC dendritic atrophy is correlated with auditory learning impairment. Using a two-way signaled active avoidance (2-AA) learning procedure, where rats are trained in a shuttle box to avoid a foot shock signaled by an auditory or visual stimulus, chronic stress strongly impairs the conditioned avoidance response to auditory stimuli, but does not affect visual avoidance conditioning (Dagnino-Subiabre et al., 2005). By fifteen days after stress, the IC neurons recover their structure completely, and this neural plasticity is correlated with improved auditory learning (Dagnino-Subiabre et al., 2005). A recent study using micro Positron Emission Tomography supports these findings, in that chronic mild stress induces significant decrease of glucose metabolism in the IC, but not in the SC (Hu et al., 2010).

Stress impairs other brain nuclei of the rat auditory system. Magnocellular neurons of the MG and pyramidal neurons of the primary auditory cortex (ACx) are atrophied after chronic stress (Bose et al., 2010; Dagnino-Subiabre et al., 2009). These findings raise the question of whether auditory emotional processing is affected by GCs. The objective of this study was to test whether chronic treatment with stress levels of corticosterone (CORT; the main GC of rats) affects the dendritic morphology of IC and SC neurons, and alters auditory fear learning and visual fear conditioning in rats.

## 2. Materials and methods

### 2.1. Experimental animals

Adult male Sprague–Dawley rats (180–200 g, ~50 days old at the start of the experiment) were housed in groups of three under a 12/12 light/dark cycle (lights on at 7:00 A.M.), with ad libitum access to food and water in a temperature–humidity-controlled room (21 °C, 55%). Rats were randomly assigned to two groups: vehicle-treated, $n = 60$ and CORT-treated, $n = 60$, *for* behavioral and *morphologic* studies. Vehicle animals, which were littermates of the CORT-treated animals, were housed in separate rooms and separate cages, and not subjected to any type of experimental stress. *All procedures related to animal maintenance and experiments were* approved by the Stanford University Administrative Panel on Laboratory Animal Care (APLAC), and the Institutional Animal Ethics Committee of the Faculty of Sciences-Universidad de Valparaíso (Chile). The experimental protocols were in accordance with the animal care standards in National Institutes of Health (NIH) guidelines. Efforts were made to minimize the number of animals used and their suffering. The following additional parameters were measured to monitor the overall effects of the CORT and vehicle administration: Body weight gain and anxiety level as determined by performance in the elevated plus-maze.

### 2.2. CORT treatment

Corticosterone (CORT) (Sigma–Aldrich, St. Louis, MO) was dissolved in 2.4% ethanol. Both CORT and vehicle experimental groups received the same amount of ethanol in their drinking water (2.4%). Vehicle or CORT was administered through the drinking water (400 µg CORT/ml) over a 10-day period. CORT drinking solution was made using a stock solution of CORT in 100% ethanol (16.6 mg/ml). This concentration and route of administration results in stress levels of serum CORT (Magariños et al., 1998; Conrad et al., 2004). CORT and vehicle were administered for 10 days, then removed and replaced with tap water. Behavioral and morphological endpoints were measured between 24 h and 48 h after the chronic CORT treatment. Separate sets of animals were used for behavioral, morphological and enzyme-linked immunoassay (ELISA) studies.

### 2.3. Plasma CORT measurement

We first analyzed the effects of CORT treatment on CORT plasma level. A separate set of animals was used to measure the concentration of CORT in plasma, in order to avoid the stressfulness of blood collection on morphological or behavioral experiments. One set of rats was sacrificed via decapitation at 09:00 h (vehicle, $n = 6$, CORT, $n = 6$) and other set prior to lights off at 19.00 h (vehicle, $n = 6$, CORT, $n = 6$) on day when behavior and morphological experiments were initially conducted. Blood (1 ml) was collected in heparinized microcapillary tubes and centrifuged (Model # MiniSpin Plus; Eppendorf AG, Hamburg, Germany) at 10,000 rpm for 10 min to obtain plasma and then stored at −70 °C. Total CORT was

determined by an Enzyme Immunoassay kit (Corticosterone Bio-Assay™, Catalog. # C7903-30) purchased from US Biological (Swampscott, MA). Optical density values were measured at 450 nm using a microplate reader (Model # Anthos 2010 Micro-plate Reader, Biochrom Ltd, UK). Samples were diluted 1:10 and then processed in duplicates and averaged final values were represented as μg/dL.

### 2.4. Behavioral testing

To rule out the possibility of CORT causing unspecific motor changes in the fear conditioning, morphologic and ELISA studies, an independent group of rats (vehicle, $n = 9$, CORT, $n = 9$) were tested for their locomotor activity and anxiety using the open field and elevated plus-maze tests respectively. These behavior tests were conducted 24 h after completion of the CORT treatment. All animals were naive to the test situations. Behavioral tests were carried out from 10.00 to 14.00 h in the test room. The activity of each rat was recorded by IP cameras (VIVOTEK, Sunnyvale CA, USA) fixed above the behavioral apparatus and connected to computer in another room outside of the vivarium. Videos were acquired by Nuuo software (Nuuo, Taipei, Taiwan) and analyzed using ANY-maze video tracking system (Stoelting Co., Illinois, USA). The maze was wiped clean thoroughly with 5% ethanol solution after each trial. In all experiments, animals from vehicle and CORT were evaluated at the same time.

#### 2.4.1. Open field test

The behavior tests were conducted in a sound-proof and temperature-controlled ($21 \pm 1$ °C) room. Each rat was placed in the center of a black Plexiglass cage ($70 \times 70 \times 40$ cm) for 5 min. The noise into the open field was 40 dB (Precision sound level meter, Model # 1100, Quest Technologies, Oconomowoc, WI) and the arena was illuminated to 300 lux (measured by digital lux meter, Model # LX-1010B, Weafo Instrument Co., Shanghai, China). Time spent in the center and border zone of the arena, total distance travelled and average speed were analyzed from video recordings.

#### 2.4.2. Elevated plus-maze

Immediately after the analysis of the open field (approximately 10 s) we measured anxiety levels by using the elevated plus-maze test. Each rat was individually placed in an elevated plus-maze, consisting of two open arms ($60 \times 15$ cm each), two closed arms ($60 \times 15 \times 20$ cm each) and a central platform ($15 \times 15$ cm), arranged in a way so that the two arms of each type were opposite to each other. The maze was elevated 100 cm above the floor. The illumination was 300 lux in the open arms and 210 lux in the closed arms. At the beginning of each trial, animals were placed at the center of the maze, facing an open arm. During a 5-min test period, we recorded the frequency of open and closed arm entries, total arm entries, the amount of time spent in each section of the maze. The number of entries and time spent in the open arms, and the ratio of open to total arm entries (open/total $\times$ 100) were used as measures of the anxiety level (Dagnino-Subiabre et al., 2006a,b). Total arm entries were taken as an indicator of general locomotor activity. Entry into an arm was defined as the animal placing all four limbs onto the arm.

#### 2.4.3. Fear conditioning

*2.4.3.1. Apparatus and stimuli.* To measure fear conditioning, we used two modified observation chambers ($30 \times 24 \times 40$ cm; Med Associates, St. Albans, VT) contained in sound-proof cubicles (Med Associates). Two types of conditioned stimuli (CS) were applied: a 5 kHz tone amplified to 80 dB, with the speaker mounted in front of the pellet receptacles, or a light pulse (2.8 W), with the LED stimulus light accessory mounted above the pellet receptacles. The unconditioned stimulus (US) was a brief (500 ms) delivery of direct current (0.5 mA) produced by a grid floor shocker (Med Associates). Both CS and US delivery were regulated by computer-based operant software (MedPC-IV; Med Associates). Behavior was videotaped for analysis using a webcam (Logitech, C905, Fremont, CA) mounted to the ceiling. The fear conditioning chambers were cleaned with 5% ethanol each time a rat was removed from the chamber.

*2.4.3.2. Auditory and visual fear conditioning procedure.* We used one set of rats for auditory fear learning (vehicle, $n = 15$, CORT, $n = 15$) and another set for visual fear conditioning experiments (vehicle, $n = 15$, CORT, $n = 15$).

Fear conditioning was conducted over three days, beginning one day after the end of chronic CORT treatment. Rats were placed in the conditioning chamber for a 10-min acclimation period, without CS presentation (day 0). Rats were then returned to their home cages and colony room. On day 1, all rats were first exposed to a 3-min acclimation period, followed by five habituation trials. For auditory fear learning, we used a 20-s tone (5 kHz, 80 dB), and for visual fear conditioning, we used a 20-s light pulse (2.8 W) in one habituation trial. Rats then underwent fear conditioning, consisting of seven conditioning trials. During each, the presentation of CS coterminated with the foot shock US (500-ms, 0.5 mA). Rats were returned to their home cages for 1 h; afterwards, they were returned to the conditioning chamber and received extinction trials consisting of CS alone. To ensure comparable levels of extinction learning between vehicle and CORT groups, on day 1, extinction trials continued until the rats exhibited less than 10% (3 s) freezing on four consecutive trials. The number of trials to criterion was similar across experimental groups. After extinctions trials, rats were returned to their home cages and to the housing room.

On day 2, rats were placed in the conditioning chamber for a 3-min acclimation period, followed by extinction trials consisting of fifteen CS alone to analyze the recall of fear learned during the conditioning trials. Freezing was continuously recorded during and later scored to determine the degree to which rats acquired the conditioned association (see Measurement of freezing behavior). Mean inter-trial interval was 4 min throughout habituation, conditioning and extinction trials.

*2.4.3.3. Measurement of Freezing Behavior.* Freezing was used to measure the conditioned emotional fear response and was defined as the absence of any visible movements with the exception of respiration-related movement and non-awake or resting body posture (Monfils et al., 2009; Paré et al., 2004). For all trials, the duration of freezing during the 20-s CS was measured with a digital stopwatch by an observer blind to experimental conditions. Percent freezing (seconds spent freezing/20-s CS) during habituation, fear conditioning, and extinction were calculated.

*2.4.3.4. Sensitivity to foot shock.* One day after the completion of all extinction trials, animals were tested for sensitivity to foot shock. Rats were placed into the conditioning chamber and given unsignaled foot shocks of increasing amplitudes beginning with 0.005 mA. Foot shock was increased in 0.05 mA increments until a jumping response was induced. An observer blind with respect to experimental group assignment measured thresholds.

### 2.5. Morphological data analysis

A new set of rats (vehicle, $n = 9$, CORT, $n = 9$) was used for morphometric studies. One day after the end of vehicle and CORT administration, animals were killed under deep anesthesia with sodium pentobarbital. The brain was removed quickly and

processed using FD Rapid Golgi Stain™ kit (FD Neuro Technologies, Inc., Ellicott City, MD, USA). Both hemispheres were cut in the sagittal plane using a cryostat (Microm International) and 100-μm-thick sections were collected onto super-frost plus slides. Sections were collected serially, dehydrated in absolute alcohol, cleared in xylene, and cover-slipped. Slides were coded before quantitative analysis, and the code was broken only after the analysis was completed. To compare the present study with our previous results (Dagnino-Subiabre et al., 2005), we analyzed the effects of vehicle and CORT administration on the flat IC neurons and the wide-field neurons of the SC. The morphometric analysis of both types of neurons was restricted to those located between bregma −1.2 mm and 6.1 mm in the IC, and between bregma −0.1 mm and 6.8 mm in the SC. Random selection was made of 10 flat neurons and 10 wide-field neurons, in the center of the IC and SC respectively, which fulfilled the following selection criteria: (1) presence of untruncated dendrites, (2) consistent and dark impregnation along the entire dendritic field, and (3) relative isolation from neighboring impregnated neurons to avoid overlap. In order to reduce error in data acquisition and subjectivity of the experimenter, the latter was blinded to treatment (but knew whether the sample was from the IC or SC). Camera lucida tracings (BX31-U-DAL 10X, Olympus Co., Tokyo, Japan) were obtained from selected neurons and then scanned (eight-bit grayscale TIFF images with 1200 d.p.i. resolution; EPSON ES-1000C) along with a calibrated scale for subsequent computerized image analysis. Custom designed macros embedded in NIH Image 1.6 software were used for morphometric analysis of digitized images. Dendritic length and the number of branch (bifurcation) points were determined in each neuron.

### 2.6. Statistical analysis

Locomotor activity, anxiety, foot shock sensitivity, and morphological studies were analyzed by a Student's unpaired *t*-test. Body weight, CORT plasma levels and percent freezing during fear conditioning were analyzed using two-way repeated-measures ANOVA [Body weight [groups (vehicle, CORT) × Days (1, 4, 7, and 10)]; CORT plasma levels [groups (vehicle, CORT) × Hours (09.00 h, 19.00 h)]; Percent freezing [groups (vehicle, CORT) × trials (habituation, conditioning, extinction, recall)] followed by a Bonferroni post hoc comparisons test. Results are presented as the mean ± SEM. A probability level of 0.05 or less was accepted as significant.

## 3. Results

### 3.1. Effects of CORT on physiological parameters

Fig. 2A shows level of circulating CORT on morning (09:00 h) and evening (19.00 h) after 10 days of vehicle or CORT administration. A 2 × 2 mixed factor ANOVA with treatment (vehicle, $n = 6$, CORT, $n = 6$) as the between-subjects factor and time (09.00 h and 19.00 h) as the repeated measure showed no significant differences in CORT levels between experimental groups obtained at 19.00 h (CORT: 17.57 ± 2.06, $n = 6$; vehicle: 23.73 ± 3.13, $n = 6$; $p > 0.05$). However, CORT-treated animals had higher CORT levels than vehicle controls at the morning, a significant treatment by hour interaction, ($F_{(1,10)} = 7.14$, $p < 0.05$), and a significant main effect of treatment at 09:00 h (CORT = 18.33 ± 3.73, Vehicle = 5.67 ± 1.73, $p < 0.001$).

Body weight was measured daily to validate that 10 days of CORT administration reduced weight gain, as occurs with chronic stress; this was the case (Fig. 2B). A 2 × 4 mixed factor ANOVA with treatment (vehicle, $n = 9$, CORT, $n = 9$) as the between-subjects factor and day (1, 4, 7, and 10) as the repeated measure showed

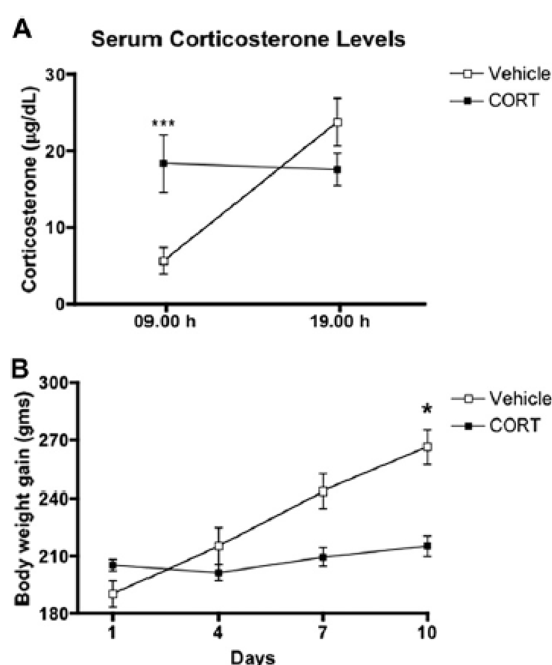


**Fig. 2.** The influence of chronic CORT treatment on CORT plasma level and body weight. (A) Rats treated with CORT in their drinking water had significantly increased serum CORT levels in the morning (09:00 h). (B) Although all rats had similar body weights at the start of the study, rats given CORT in their drinking water (CORT) failed to gain weight. In contrast, vehicle-treated rats gained weight gradually throughout the study (Vehicle). Significant treatment by day interaction, $F_{(3,48)} = 105.2$, $p < 0.0001$. Data are represented by mean ± SEM.

a significant treatment by day interaction, ($F_{(3,48)} = 105.2$, $p < 0.0001$), a significant main effect of treatment, ($F_{(1,16)} = 4.9$, $p < 0.05$), and a significant main effect of day, ($F_{(3,48)} = 189.4$, $p < 0.0001$). However, rats that received CORT showed decreased weight gain during 10 days of treatment, relative to vehicle control rats ($p < 0.05$).

### 3.2. Effects of CORT on locomotor activity and anxiety

CORT administration did not affect locomotor activity, including the total distance travelled, average speed, or time spent in central and border zone of the arena (Fig. 3). Moreover, CORT treatment did not affect measures of anxiety (i.e., the frequency of open arm entries or time spent in open arms in the elevated plus-maze, as well as the ratio of open to total arm entries) (Fig. 4). There were no treatment differences in the number of total arm entries.

### 3.3. Auditory and visual fear conditioning

CORT treatment did not significantly affect unconditioned responses to tone alone (Fig. 5A). During the habituation phase, there was no main effect of treatment on freezing ($F_{(1,16)} = 1.12$, $p > 0.05$) and no interaction of group and trial ($F_{(4,16)} = 0.36$, $p > 0.05$). In the conditioning phase, CORT treatment significantly decreased the auditory conditioned responses compared with vehicles; there was a significant difference between the CORT and vehicle groups ($F_{(1,28)} = 11.52$, $p < 0.01$), and a significant interaction of group and trial ($F_{(6,168)} = 3.64$, $p < 0.01$) (Fig. 5A). Freezing percentage varied significantly across trials ($F_{(6,168)} = 15.87$,

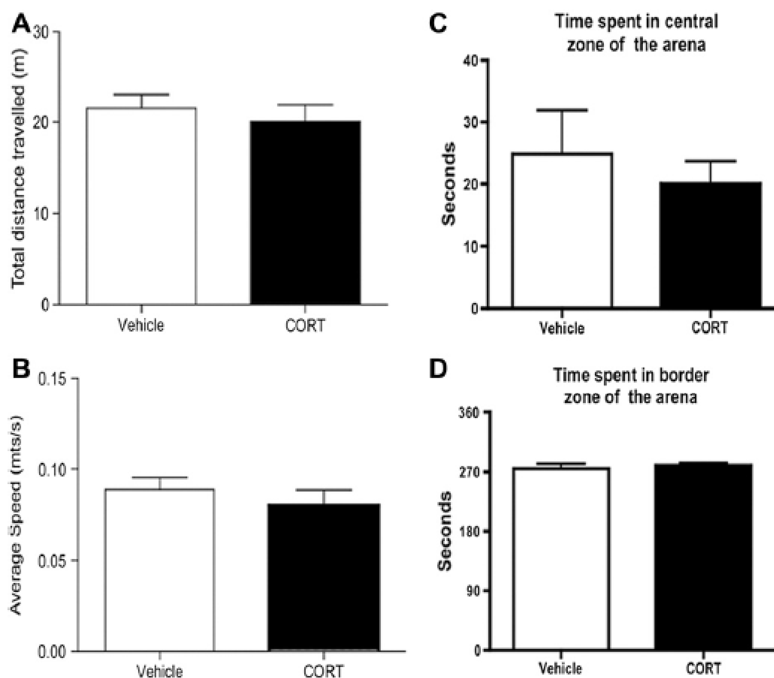


**Fig. 3.** Effect of CORT on locomotor activity in rats. CORT administration did not affect the total distance travelled (A), total average speed (B), time spent in center (C), and time spent in perimeter (D) in a 5 min observation period. Values are the mean ± SEM.

$p < 0.0001$), with both CORT and vehicle groups acquiring the auditory conditioned fear response. In the extinction phase, the interaction between the experimental groups with trials was altered by CORT administration ($F_{(14,392)} = 7.31$, $p < 0.001$) (Fig. 5A). In both groups, the conditioned fear responses were diminished with repeated presentation of tone alone ($F_{(14,392)} = 45.81$, $p < 0.0001$) (Fig. 5A), and CORT significantly reduced the extinction ($F_{(1,28)} = 4.86$, $p < 0.05$).

In the recall phase, vehicle- and CORT-treated rats showed equivalent recall of fear conditioning (group effect: $F_{(1,16)} = 0.46$, $p > 0.05$) (Fig. 5A), with such recall decreasing in both groups ($F_{(14,224)} = 6.11$, $p < 0.0001$).

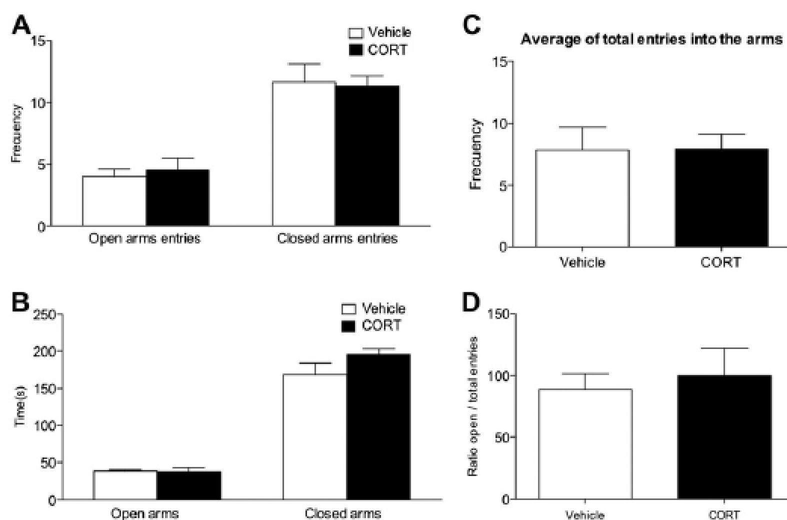


**Fig. 4.** Effect of CORT administration on anxiety. CORT did not affect the frequency of entries (A) and the time (B) spent on open arms of the elevated maze, total arm entries (C), and the ratio of open/total arm entries (D). These results indicate that CORT did not affect anxiety. Values are the mean ± SEM.

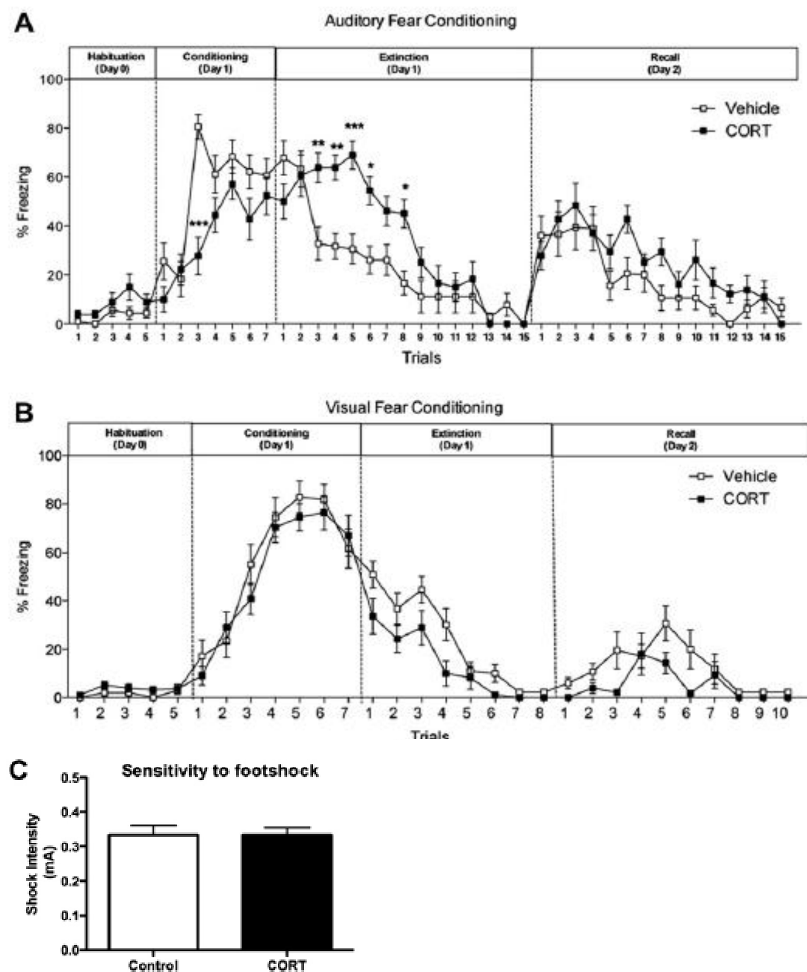


**Fig. 5.** Effect of CORT administration on fear conditioning. Mean and average percent freezing to tone (A) and light (B) in control (open squares, $n = 9$) versus CORT-treated rats (filled squares) across habituation, conditioning, extinction, recall, and trials. CORT impaired the extinction of auditory fear learning [interaction ($F_{(14,224)} = 2.51$), $p < 0.01$], but did not affect visual fear conditioning. (C) Influence of CORT on foot shock response thresholds for the experimental groups. CORT did not alter sensitivity to foot shock. Vertical bars represent SEMs.

Since CORT decreased auditory fear conditioning and extinction, we analyzed the CORT effects on visual fear conditioning and extinction for comparison. CORT did not affect the freezing percentage during the habituation phase (Fig. 5B). There was no main effect of treatment on freezing ($F_{(1,28)} = 3.88$, $p > 0.05$) and no interaction of group and trial ($F_{(4,112)} = 0.48$, $p > 0.05$). During conditioning trials, the percentage freezing varied significantly across trials ($F_{(6,168)} = 36.88$, $p < 0.0001$), with both groups acquiring a visual conditioned fear response. There were no group ($F_{(1,28)} = 0.57$, $p > 0.05$) or interaction effects ($F_{(6,168)} = 0.71$, $p > 0.05$). Both vehicle- and CORT-treated rats showed a diminished visual conditioned fear response with repeated presentation of light alone through extinction and recall phases [Extinction: ($F_{(7,196)} = 19.77$, $p < 0.001$); Recall: ($F_{(9,252)} = 5.88$, $p < 0.001$)]. For visual fear conditioning, CORT did not affect the rate of extinction and recall compared to vehicles (Fig. 5B) [Extinction: effect of treatment, ($F_{(1,28)} = 3.90$, $p > 0.05$); for interaction of group and trial, ($F_{(7,196)} = 0.21$, $p > 0.05$)] [Recall: effect of treatment, ($F_{(1,28)} = 3.82$, $p > 0.05$); for interaction of group and trial, ($F_{(9,252)} = 0.49$, $p > 0.05$)] compared to vehicles (Fig. 5B).

#### 3.3.1. *Foot shock sensitivity*

Vehicle- and CORT-treated rats showed comparable sensitivity to lower shock intensity (CORT $= 0.33 \pm 0.02$, Vehicle $= 0.33 \pm 0.03$, $p > 0.05$) (Fig. 5C), ruling out increased sensitivity as an explanation for the CORT effect on auditory fear learning.

### 3.4. *Effects of CORT treatment on dendritic morphology of the inferior and superior colliculus*

Photomicrographs of representative Golgi-impregnated flat neurons of the IC from vehicle- and CORT-treated animals, and their respective camera lucida drawings are shown in Fig. 6A. CORT decreased the number of branch points in flat neurons in the IC (CORT: $2.8 \pm 0.3$, $n = 9$; Vehicle: $5.1 \pm 1.1$, $n = 9$; $p = 0.023$), but did not change total dendritic length (Fig. 6B).

Photomicrographs of representative Golgi-impregnated neurons of the SC from vehicle- and CORT-treated animals, and their respective camera lucida drawings are shown in Fig. 6A. CORT did not affect either dendritic length neurons or branch points of the SC neurons (Fig. 6B).

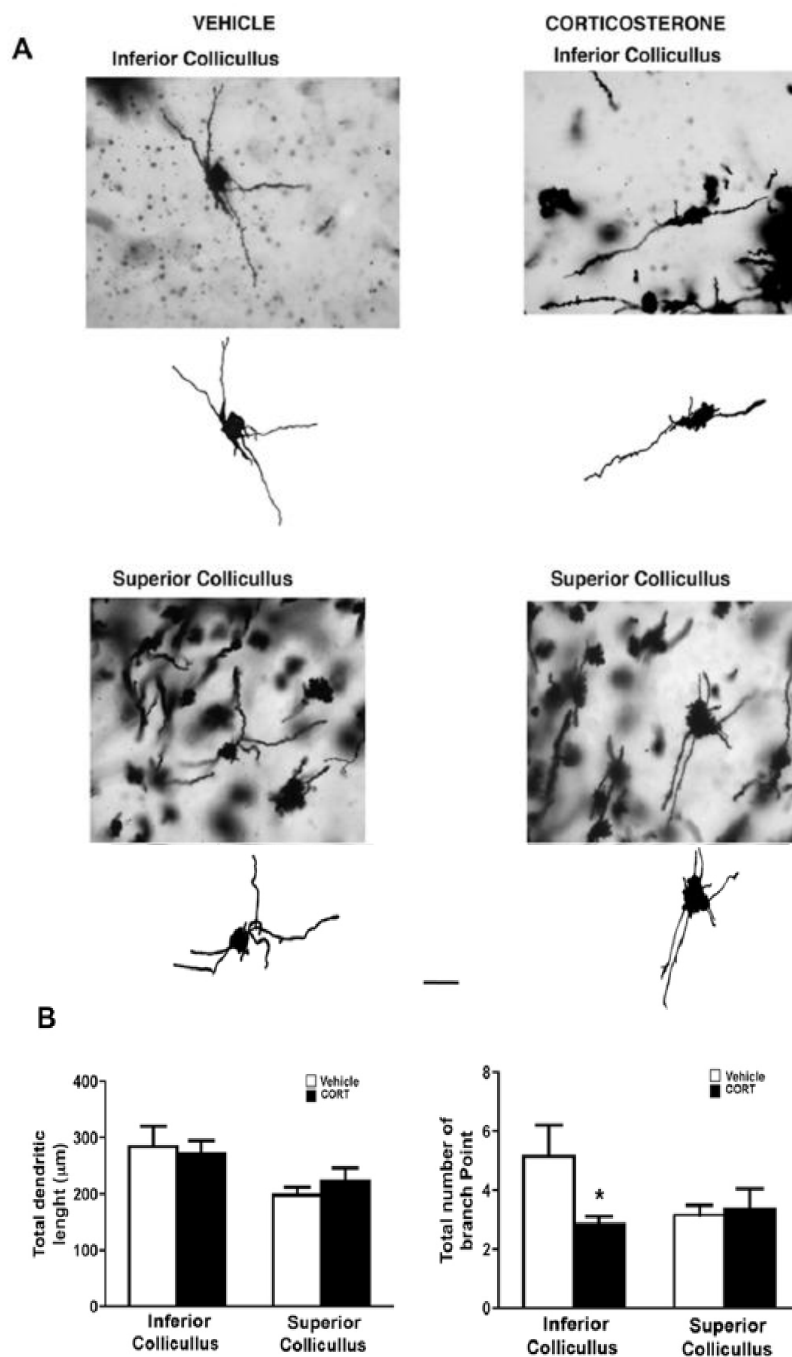


**Fig. 6.** Morphometric analyses of the IC and SC neurons. (A) Photomicrographs and camera lucida tracings of representative golgi-impregnated flat neurons of the IC and wide-field neurons of the SC, in vehicle- and CORT-treated rats. Scale bar, 20 μm. (B) Morphometric analysis of neurons from vehicle- and CORT-treated rats. After 10 days of CORT administration ($n = 9$ animals), the total branch number of the IC neurons was significantly reduced compared with vehicle-treated rats ($n = 9$ animals) (*$p < 0.05$). There were no CORT-induced changes observed in the total dendritic length of SC neurons (CORT, $n = 9$ animals; vehicle, $n = 9$ animals). The values are the mean ± SEM. Asterisk (*) indicates significant differences relative to vehicle-treated rats.

## 4. Discussion

The present study shows that CORT administration impairs both auditory fear learning and extinction (Fig. 5), and decreases the dendritic arborization of the IC neurons (Fig. 6). On the other hand, CORT did not affect visual fear conditioning or dendritic morphology of SC neurons (Figs. 5 and 6).

### 4.1. *Effects of chronic CORT administration on fear conditioning and neuronal morphology*

CORT impaired the acquisition of auditory conditioned fear responses and attenuated conditioned fear extinction (Fig. 5A). In contrast, CORT administration did not affect visual fear conditioning (Fig. 5B). These results were unlikely to be caused by changes in

sensitivity to foot shock between vehicle- and CORT-treated animals, as both groups exhibited similar foot shock threshold (Fig. 5C).

CORT-induced alterations of auditory fear conditioning and extinction could be related to decreased dendritic arborization in IC induced by CORT (Fig. 6A and B). In addition, it is possible that neuronal morphologic changes in other regions, such as in the MG or auditory cortex, may affect auditory fear learning. Dendritic atrophy in neurons of the IC, MG or auditory cortex might impair the ability to receive and deliver the auditory CS to the amygdala and decrease freezing responses through the conditioning trials (Fig. 5A). Lesion studies on the IC and MG demonstrate that an association between the auditory CS and foot shock US is necessary to acquire aversive memories (LeDoux et al., 1984). During the extinction trials, there is acquisition of a new memory concerning the failure to associate tone with foot shock, and freezing then is decreased in the extinction phase. Regardless of whether CORT treatment decreased the dendritic arborization of IC neurons, the deliver of the auditory CS to the amygdala could be decreased in the extinction trials, resulting in CORT-treated rats being slower to extinguish learned fear (Fig. 5A). These results are supported by our previous finding regarding the effects of chronic stress on auditory learning during avoidance conditioning. Fear conditioning and 2-AA are associated with auditory learning (Dagnino-Subiabre et al., 2005, 2009). Stress-induced IC dendritic atrophy correlated with auditory learning impairment in the 2-AA (Dagnino-Subiabre et al., 2005). On the other hand, CORT-induced IC dendritic atrophy also correlated with auditory learning impairment on fear conditioning (Fig. 5A).

CORT-induced impairment of fear extinction (Fig. 5A) appears to contradict previous findings related with the effects of chronic stress on fear-conditioned extinction. Stress-induced IC dendritic atrophy was not associated with extinction impairment of fear conditioned after one week (Miracle et al., 2006), 15 days (Dagnino-Subiabre et al., 2009) or 21 days (Baran et al., 2009) of restraint stress. In this respect, anxiety may affect freezing during extinction trials in fear conditioning; for example, the anxiogenic drugs Amphetamine (Vuong et al., 2010) and Yohimbine (Braun et al., 2011) blunt freezing throughout extinction (Mueller et al., 2009). Conversely, the anxiolytic effect of Fluoxetine (Ampuero et al., 2010) and Citalopram (Sun et al., 2010) correlate with increased freezing during extinction (Burghardt et al., 2007). Anxiety is regulated by the basolateral amygdala and chronic stress induces dendritic hypertrophy in this region (Vyas et al., 2002); we speculate that this morphological alteration can increase the neuronal activity in the amygdala. Stress-induced IC dendritic atrophy could decrease the deliver of auditory CS to the amygdala, thus decreasing freezing on extinction trials. This alteration could be prevented by increases of neuronal activity in the amygdala of stressed rats. In our experiments, CORT administration did not affect anxiety (Fig. 4); consequently CORT-treated rats were slower to extinguish learned fear (Fig. 5). Effects of IC dendritic atrophy on auditory fear learning can be measured in behavioral paradigms that induce low levels of freezing in the animals. In support of this idea, control rats subjected to 2-AA have less freezing, as compared to fear conditioning (Bose et al., 2010; Choi et al., 2010), and thus stressed rats show decreased auditory fear learning when subjected to 2-AA (Dagnino-Subiabre et al., 2005). In contrast, stressed rats subjected to fear conditioning did not differ in freezing responses on conditioning trials (Dagnino-Subiabre et al., 2009). In conclusion, effects of IC dendritic atrophy on auditory fear learning could be measured in less aversive behavioral paradigms due to the low levels of freezing that these induces on rats.

Neither CORT administration nor restraint stress affected SC morphology (Dagnino-Subiabre et al., 2005, 2009); as would thus be expected because CORT treatment did not affect visual fear learning (Fig. 5B). It is difficult to determine the impact of CORT on visual extinction and recall trials because the animals showed less freezing on the last conditioning trial with respect to the preceding trial (Fig. 5B). Apparently the rats in the last visual conditioning trial began to adapt to the unconditioned stimulus, as has been reported previously (Bouton et al., 2008).

The dendritic lengths of IC flat neurons measured in this study were smaller than other Golgi analyses of dendritic morphology in IC neurons (Malmierca et al., 1993, 1995, 2011). These studies used female *Rattus novegicus* rats (Malmierca et al., 2011), in contrast to our *Sprague–Dawley* males. Ovarian hormones and strain type might affects neuronal morphology; as precedent, 17 β-estradiol increases the apical dendritic length of hippocampal CA1 pyramidal neurons in female rats (McLaughlin et al., 2010), and the total dendritic length of CA3 neurons from *Wistar* rats is greater than in *Sprague–Dawley* rats (Magariños and McEwen, 1995). Therefore, it is possible that the IC flat neurons from female *R. novegicus* are bigger than comparable neurons in male *Sprague–Dawley* rats.

### 4.2. *Possible cellular mechanisms underlying dendritic changes in the inferior colliculus*

The CORT effects in the IC may be due to direct actions via the GR in such neurons. GR are expressed in the IC (Mazurek et al., 2010) and the ultradian release of CORT from the adrenal glands regulates the activation of GR in neurons (Stavreva et al., 2009). CORT binds to cytosolic GR, inducing GR dimerization and translocation to the nucleus, thereby increasing the gene expression of pro-plasticity genes, such as neuronal cell adhesion molecules, NCAM and L1 (De Kloet et al., 1998; Sandi, 2004; Meltser and Canlon, 2011). Moreover, the CORT–GR complex increases the nuclear translocation of NF-kB, increasing expression of neurotrophins such as BDNF and NT-3 in IC neurons (Reichardt, 2006). These molecules are implicated in neurite extension, cell survival and synaptic plasticity (Kiss et al., 2001). In contrast, both chronic CORT administration (Fig. 2A) and chronic mild stress affects the ultradian CORT release and increases the plasma CORT levels (Grippo et al., 2005; Ushijima et al., 2006). These alterations are correlated with a significant decrease of glucose metabolism in the IC after chronic mild stress, but not in the SC, suggesting that the IC could be more sensitive to stress and higher level of CORT compared to the SC (Hu et al., 2010). Chronic stress down regulates GR expression in the IC at the time that plasma CORT levels have returned to baseline (Mazurek et al., 2010). Therefore, chronic CORT administration could down regulate the GR expression in the IC and decrease the levels of pro-plasticity proteins; this may lead to decreased dendritic arborization of the IC neurons. We propose that chronic CORT treatment may have either none or an opposite effect in the SC.

Dexametasone and RU 486 are agonist and antagonist of GR respectively (Jadavji et al., 2011). In this context, we speculate that the circadian level of GR agonists, might increase the neuronal activity in the IC, conversely, GR antagonist, may inhibit the GR and NF-kB nuclear translocation and decrease the expression of neurotrophic factors in the IC neurons, which in turn may affect the neuronal morphology of the IC (as shown in Fig. 6). Regardless of whether chronic CORT treatment down regulates GR expression in the IC, we speculate that the mineralocorticoid receptor (MR) antagonist Spironolactone (Kumar et al., 2007) could have a possible neuroprotective effects on IC neurons. Spironolactone might decrease the availability of MR in the cytoplasm of IC neurons, increasing the probability that CORT binds to GR. As a result, the GR–CORT complex and NF-kB nuclear translocation up regulate the expression of protective neurotrophic factors and pro-plasticity proteins in IC neurons.

*4.3. Auditory and visual pathways, and their connectivity with the amygdala: the possible role in the CORT effects on fear conditioning*

Other possible explanations for our finding is that CORT-induced IC plasticity is indirectly produced by morphologic changes propagated from upper or lower levels of the amygdala–auditory pathway (for example, from the basolateral amygdala or cochlear nucleus, respectively; Fig. 1). There exists evidence indicating that an intact basolateral amygdaloid nucleus is essential for developing the associative neuronal plasticity in the MG throughout aversive learning (Maren et al., 2001). In addition, CORT produces dendritic hypertrophy of the spiny pyramidal-like neurons of basolateral amygdaloid nucleus (Mitra and Sapolsky, 2008). It is possible that the chronic CORT administration in the present study produces plasticity in the basolateral amygdaloid nucleus; while not sufficient to enhance anxiety, this is sufficient to produce morphologic changes in the MG. This process may be propagated to even lower levels in the auditory pathway and influence plasticity at the mesencephalic level in the IC. In contrast to the MG, the neighboring LG of the visual thalamus does not directly project to the lateral amygdala (McDonald, 1998). During classical visual fear conditioning, the expression of conditioned fear is produced directly from both the SC by the lateral posterior nucleus–lateral amygdala pathway and the retina by the LG-primary visual cortex–temporal association cortex–lateral amygdala pathway (Doron and Ledoux, 1999; Shi and Davis, 2001). It is probably the case that such projections are not as robust as the auditory projections from the MG to the lateral amygdala (LeDoux et al., 1990). In this context, the CORT-induced structural changes in lateral amygdala may not be propagated to the LG of the visual thalamus. On the other hand, there are direct projections from the basal amygdala to IC (Marsh et al., 2002). Therefore, CORT administration can increase the NMDA-receptor-mediated synaptic currents in the basolateral amygdala, which in turn, could directly reduce NMDA-receptor-mediated synaptic currents in the IC. NMDA-induced increases in intracellular calcium concentrations regulate BDNF expression by the aryl hydrocarbon receptor and BDNF is known to be a key regulator of dendritic morphology (Lin et al., 2009; Lakshminarasimhan and Chattarji, 2012); thus, CORT administration can increase both BDNF expression and growth of dendrites in the basolateral amygdala, while down regulating BDNF and inducing dendritic atrophy in the IC (Lakshminarasimhan and Chattarji, 2012).

The IC is strongly innervated by afferents from the cochlear nucleus (Fig. 1); thus, neuronal plasticity in the cochlea might be propagated to upper levels of the auditory pathway. GRs are expressed in the hair cells, spiral ganglion neurons and the spiral ligament of the cochlea (Meltser et al., 2009; Tahera et al., 2006; ten Cate et al., 1992, 1993; Zuo et al., 1995). Also GR have a protective role after acute stress and acoustic trauma (Meltser and Canlon, 2011; Kraus and Canlon, 2012). Thus, we propose that both chronic CORT administration and chronic restraint stress have negative effects on the cochlea; this may be due to down regulation of GR mRNA expression and to decreased expression of neurotrophic factors in the cochlea. Moreover, it is possible that a mixture of direct and indirect effects of CORT on the IC could be necessary to produce plasticity in the IC neurons.

*4.4. Conclusions*

The data presented here demonstrate that CORT treatment impaired auditory fear learning and the extinction of auditory conditioned fear, without affecting the visual fear conditioning. Additionally, CORT treatment decreased dendritic arborization of the IC neurons, a major auditory nucleus, but did not affect the SC. Overall, these results show a sensory modality-specific CORT effect on auditory fear processing in the rat brain. Potentially, similar behavioral and morphological changes could be induced by psychosocial stress on sound processing in humans (Simoens et al., 2007).

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## Discusión

El presente estudio demuestra que la suplementación con PUFAs ω-3 mejora las alteraciones inducidas por el estrés crónico sobre funciones cognitivas tales como el aprendizaje auditivo y la memoria espacial. Además, la suplementación con PUFAs ω-3 posee un efecto ansiolítico y regulador sobre la arquitectura dendrítica y sobre la transmisión GABAérgica, resultados que han permitido relacionar el deterioro del balance de la actividad sináptica GABAérgica en la región CA1 del hipocampo con el déficit en la memoria espacial, funciones que han sido restauradas mediante la suplementación con PUFAs ω-3. Durante el desarrollo de este estudio, en primer lugar investigamos si nuestro protocolo de estrés fue efectivo en inducir la respuesta al estrés. Las ratas sometidas a estrés crónico por restricción de movimiento en todos los grupos experimentales disminuyó el porcentaje de ganancia en peso en comparación a los grupos controles. Este marcador de estrés, demuestra que nuestro protocolo de estrés utilizado fue efectivo. Resultados comparables han sido publicados utilizando protocolos de estrés similares y en estudios con administración de glucocorticoides (Bourre et al., 1989; Watanabe et al., 1992; Kleen et al., 2006).

**Capítulo I:** *Publicación N° 1, en prensa*

### *1.1 Efecto de la suplementación con PUFAs ω-3 sobre la ansiedad, nivel de CORT en ratas sometidas a estrés crónico.*

Las ratas estresadas del grupo control y vehículo disminuyen el número de entradas al brazo abierto en la prueba de ansiedad y se mantienen durante una mayor cantidad de tiempo en el centro en la prueba de campo abierto. Estas conductas están relacionadas con un efecto ansiogénico inducido por el estrés crónico y por el tratamiento vehículo. Por el contrario, la suplementación con PUFAs ω-3 en ratas estresadas aumenta el número de entradas al brazo abierto, lo cual está relacionado con un efecto ansiolítico. La ansiedad es regulada principalmente por la BLA y por BNST (Davis, 1998; Pêgo et al., 2008; Davis et al., 2010). En algunos protocolos de estrés, tal como el estrés crónico impredecible, el aumento de la ansiedad ha sido correlacionado con la hipertrofia de BLA y BNST (Vyas et al., 2002, 2003, 2004). Es posible que nuestro protocolo de estrés y tratamiento vehículo indujeran la hiperactividad de BLA y/o BNST, lo que conlleva a un aumento de la ansiedad, actividad del eje HPA y aumento del nivel de CORT. El núcleo dorsal del rafe (DRN) modula la

excitabilidad neuronal de BNST (Guo y Rainnie, 2010), proyecciones serotoninérgicas son enviadas de DRN al BNST, activando los receptores de serotonina 5-$HT_{1A}$ y 5-$HT_{1B}$, produciendo la inhibición de la excitabilidad de BNST (Levita et al., 2004; Guo y Rainnie, 2010). El nivel de 5-HT se ha encontrado reducido en las ratas sometidas a estrés crónico (Sunanda et al., 2000; Torres et al., 2002), por lo que la activación de los receptores 5-$HT_{1A}$ y 5-$HT_{1B}$ es mínima. Esta alteración puede contribuir al incremento en la excitabilidad en el BNST y al aumento de la ansiedad en las ratas estresadas (Fig. 3A).

La suplementación con PUFAs ω-3 reduce el nivel de CORT en las ratas estresadas, sugiriendo que la suplementación previene la hiper-activación del eje HPA, disminuyendo el efecto de CORT sobre la morfología dendrítica y sobre la actividad neuronal de BLA y BNST. Se ha encontrado que la suplementación con PUFAs ω-3 incrementa el nivel de 5-HT en el cerebro de ratas estresadas (Vancassel et al., 2008), lo cual podría reducir la excitabilidad neuronal de BNST mediante la activación de los receptores 5-$HT_{1A}$ y 5-$HT_{1B}$, disminuyendo la ansiedad en las ratas estresadas suplementadas.

La suplementación con PUFAs ω-3 tuvo un efecto ansiogénico en las ratas no sometidas a estrés. Sin embargo redujo la concentración de CORT comparado con las ratas no estresadas tratadas con vehículo al exponerlas a un nuevo estresor (Fig. 3B). Es posible que la suplementación previniese la hipertrofia en BLA y/o BNST dando como resultado una disminución en el nivel de CORT. Se ha observado que la suplementación con PUFAs ω-3 incrementa el nivel de 5-HT (Vancassel et al., 2008), por lo tanto sugerimos que como mecanismo compensatorio al aumento de 5-HT, ocurre una disminución en la expresión de los receptores 5-$HT_{1A}$ y 5-$HT_{1B}$ en las ratas no estresadas suplementadas, provocando un aumento en la excitabilidad de BNST por pérdida del control inhibitorio de 5-HT sobre BNST, dando como resultado un aumento de la ansiedad en las ratas controles suplementadas con PUFAs ω-3 (Fig 3A).

El nivel de CORT ha sido similar en los grupos no estresados y en los sometidos a estrés crónico, estos resultados sugieren que las ratas estresadas se adaptan al protocolo de estrés por restricción de 21 días. Varios estudios han mostrado que el estrés por restricción durante 3 o 6 horas por día aumenta el nivel plasmático de CORT durante la primera semana, mientras que en la segunda y tercera semana de estrés por restricción el incremento de CORT es menos pronunciado (Galea et al., 1997; Cook and Wellman, 2004). Por lo tanto, si el efecto

de 21 días de estrés crónico sobre la actividad del eje HPA y nivel CORT se perdiera, el nivel de CORT entre las ratas no estresadas y estresadas podría ser similar después de someterlas a un nuevo estresor. Sin embargo, las ratas sometidas a estrés por restricción tienen un nivel de CORT significativamente superior comparado con las ratas no estresadas después de exponer a las ratas durante 1 minuto al nado forzado (Fig. 3B). Estos resultados sugieren que después del protocolo de estrés, las ratas no estresadas y estresadas poseen una actividad similar del eje HPA en un ambiente libre de estresores. Sin embargo, al someterlas a un nuevo estresor, el grupo de ratas sometidas a estrés crónico, incrementa la actividad de eje HPA por sobre la actividad de las ratas no estresadas. Esta alteración en la actividad del eje HPA, es la responsable de una mala respuesta adaptativa frente a los estresores, alteración que está asociada con el desarrollo de enfermedades neuropsiquiátricas.

***1.2 Efecto de la suplementación sobre el aprendizaje auditivo en ratas sometidas a estrés***

El estrés crónico y el tratamiento con vehículo son situaciones estresantes, debido a que ambos tratamientos incrementan la concentración plasmática de CORT. Ambos tratamientos producen un deterioro en el aprendizaje auditivo durante el condicionamiento (Fig. 4A, B). Estudios de lesiones de los principales núcleos del sistema auditivo que regulan el aprendizaje, el CI y MG han demostrado que estas dos estructuras cerebrales son factores claves para la adquisición de memorias aversivas en respuesta a estímulos auditivos durante el condicionamiento al miedo en ratas (LeDoux et al., 1984). Además, el estrés por restricción induce atrofia dendrítica del CI, MG y corteza auditiva primaria (Dagnino-Subiabre et al., 2005, 2009; Bose et al., 2010), afectando el procesamiento auditivo. En nuestro estudio, el deterioro en el aprendizaje auditivo podría haber sido causado por la atrofia del CI y MG inducida por el estrés crónico. El tratamiento vehículo tanto en ratas no estresadas como en los animales estresados, disminuye el aprendizaje en comparación con el control no estresado. Contrariamente, la suplementación con PUFAs ω-3 revierte este efecto, posiblemente mediante la prevención de la atrofia del CI y MG. De hecho, se ha demostrado que la deficiencia de PUFAs ω-3 deteriora el condicionamiento de evitación activa (Bourre et al., 1989). Además, alteraciones en el aprendizaje se han asociado con una deficiencia materna de LNA, la cual se previene por la suplementación de LNA después del destete (Ikemoto et al., 2001). Probablemente, la suplementación previene el daño en el aprendizaje en ratas inducido

por la deficiencia de PUFAs ω-3.

Otros núcleos claves para la adquisición de respuestas condicionadas evocadas de evitación activa son la amígdala lateral y basal (Choi et al., 2010). Por lo tanto, otra posible explicación para nuestros resultados durante la prueba de aprendizaje es que el estrés crónico y la suplementación con PUFAs ω-3 tengan efectos opuestos sobres estos núcleos, induciendo el estrés crónico la hipertrofia de la LA, y que esta remodelación dendrítica aumente la ansiedad a través del BNST (Vyas et al., 2003). Por otro lado, la suplementación con PUFAs ω-3 puede prevenir estas alteraciones morfológicas y producir efectos ansiolíticos en las ratas estresadas, lo cual podría resultar en una mejora del aprendizaje, como ha sido observado durante la administración del ansiolítico midazolam, que facilita la recuperación de la evitación condicionada en ratas (Obradovic et al., 2004).

**Capítulo II:**

***Efecto de la suplementación con PUFAs ω-3 sobre el deterioro que induce el estrés crónico en la memoria espacial, morfología dendrítica y en la plasticidad sináptica.***

El déficit en la memoria espacial inducido por el estrés crónico, fue restaurado mediante la suplementación con PUFAs ω-3, alcanzando un nivel de memoria comparable al de ratas controles no sometidas a estrés (Fig. 1 parte superior). Se ha sugerido que el estrés crónico podría producir un déficit en la memoria espacial por cambios en la morfología dendrítica hippocampal (Conrad et al., 1996). El estrés crónico produce atrofia de las dendritas apicales de las neuronas piramidales de la región CA3 del hipocampo (Magariños and McEwen 1995; Conrad et al., 1996; Kleen et al., 2006). Sin embargo, cuando el estresor es expuesto por una duración que no induce retracción dendrítica, tal como en protocolos de 7 o 14 días (Magariños and McEwen 1995), o cuando la retracción dendrítica inducida por estrés crónico presenta un período post-estrés carente de estresores, por un tiempo superior a 10 días (Conrad et al., 1999), la memoria espacial dependiente del hipocampo se recupera (Luini et al., 1994, 1996). Nuestros resultados indican que el estrés crónico induce retracción dendrítica, mientras que a nivel conductual se correlaciona con una disminución en la memoria espacial. El déficit en la memoria espacial en ratas estresadas, fue revertido mediante la suplementación con PUFAs ω-3. El efecto positivo del tratamiento con PUFAs ω-3 sobre la memoria pudo haber estado relacionado con una mejora de la arborización dendrítica de las neuronas

piramidales (Fig. 2A, B). Se ha demostrado que en las neuronas, el DHA se encuentra en las dendritas, donde puede participar en la extensión y generación de la arborización dendrítica, proceso que ocurre durante la formación de la memoria (Marszalek y Lodish, 2005). Contrariamente, hemos encontrado que la suplementación con PUFAs ω-3 en ratas no estresadas deterioró la memoria espacial, lo cual no se correlacionó con cambios en la morfología dendrítica. Este resultado podría indicar que existen otros mecanismos involucrados en el deterioro cognitivo, independientes de la arquitectura dendrítica y que en nuestro estudio hemos encontrado que estar relacionado con el control de la transmisión GABAérgica, debido principalmente a que no encontramos diferencias en la transmisión Glutamatérgica, estos resultados coinciden en parte a los mecanismos propuestos en los cambios en la arquitectura dendrítica, que son considerados como un mecanismo compensatorio en respuesta al aumento de la estimulación y liberación de aminoácidos excitatorios, que podrían matar a las neuronas (Conrad, 2006).

Diferentes moléculas han sido estudiadas para entender las bases moleculares de los cambios estructurales de las neuronas y de la fisiología de los circuitos excitatorios e inhibitorios. La molécula de adhesión celular neuronal (NCAM) (De Kloet et al., 1998; Meltser y Canlon, 2011) es un candidato importante que media estos cambios. NCAM se expresa en la superficie de las neuronas y glías y tiene un rol importante en la extensión de neuritas, supervivencia celular, plasticidad sináptica, la memoria y el aprendizaje (Kiss et al., 2001). Por lo tanto, otra posible explicación para el déficit en la memoria espacial inducido por el estrés crónico y en ratas controles suplementadas con PUFAs ω-3 es que se produzca una disminución en la expresión de NCAM en las neuronas del hipocampo, afectando la estructura tanto de neuronas excitatorias como inhibitorias. En este sentido, se ha encontrado que el estrés crónico causa atrofia de las neuronas piramidales de la región CA3 del hipocampo (Hu et al., 2010a) y reduce el número de neuronas inhibitorias palvalbumina-positiva en el hipocampo (Hu et al., 2010a).

Hemos encontrado que el tratamiento vehículo produce un bajo rendimiento en la prueba de memoria tanto en ratas no estresadas como en las sometidas a estrés, obteniendo prácticamente el mismo nivel. Tal rendimiento cognitivo se correlacionó con una disminución en el largo dendrítico comparado a la situación control. En ambos grupos del tratamiento vehículo (estresados y no estresados), el largo dendrítico es similar, demostrando que tal

tratamiento por sí mismo, produce un cierto grado de estrés, lo cual es apoyado por los niveles de CORT obtenidos en ambos grupos (Fig. 3B, publicación N°1). Por lo tanto, el déficit en la memoria espacial observada en las ratas sometidas a estrés y en el tratamiento vehículo y su incremento mediante la suplementación con PUFAs ω-3 podrían ser el resultado de cambios en la morfología hipocampal.

***2.1 Efecto del estrés crónico y suplementación en la transmisión sináptica***

Además de las alteraciones en la arquitectura neuronal, diferentes experimentos de electrofisiología indican que el estrés y niveles altos de CORT inducen la activación de estructuras que cumplen un rol fundamental en la memoria y aprendizaje, produciendo un incremento en la excitabilidad de las neuronas del hipocampo (Karst y Joëls, 2002) y amígdala (Duvarci y Pare, 2007; Roozendaal et al., 2009). El estrés crónico y la administración de CORT durante tres semanas, provoca atrofia de las dendritas apicales de las neuronas CA3 del hipocampo (Magariños and McEwen 1995; Conrad et al., 1996; Kleen et al., 2006). La retracción dendrítica inducida por el estrés o CORT es bloqueada mediante la administración de drogas que reducen la neurotransmisión de aminoácidos excitatorios, tal como Fenitoína (Watanabe et al., 1992) o a través de drogas que reducen la excitabilidad general mediante la estimulación del sistema GABAérgico con benzodiazepinas (adinazolam) (Magariños et al., 1999), indicando que múltiples neurotransmisores y hormonas se encuentran involucrados en la atrofia del hipocampo inducida por el estrés crónico (Watanabe et al., 1992; Magariños et al., 1999). El incremento en la excitabilidad puede ser el resultado de una reducción en la transmisión inhibitoria inducida por el estrés crónico, la cual ha sido observada en el hipocampo (Holm et al., 2011) y la amígdala (Davis, et al., 1994).

En nuestro estudio hemos encontrado que el estrés crónico disminuye la transmisión GABAérgica en la región CA1 del hipocampo sin provocar cambios significativos en la transmisión glutamatérgica (Fig. 3-6). Las ratas sometidas a estrés por restricción del grupo control y vehículo presentan una disminución en la frecuencia de las corrientes postsinápticas inhibitorias espontáneas (sIPSC) y en la probabilidad de liberación de GABA. Tales alteraciones en la transmisión GABAérgica fueron revertidas mediante la suplementación con ω-3 (Fig. 3-6). Probablemente, esta rectificación en la transmisión sináptica GABAérgica mediante la suplementación puede estar relacionada con la disminución en los niveles de

CORT, lo cual disminuiría la activación de los GRs en el hipocampo, reduciendo la excitabilidad neuronal. Resultados similares han sido encontrados en ratas sometidas a estrés impredecible, en las cuales las sIPSC están reducidas, lo cual está asociado con una reducida probabilidad de liberación de GABA (Holm et al., 2011). Estudios de microdiálisis apoyan estos resultados, indicando que los niveles de GABA se encuentran disminuidos en ratas sometidas a estrés crónico impredecible (Gronli et al., 2007). Por el contrario, varios antidepresivos y estabilizadores el ánimo incrementan el tono GABAérgico (Brambilla et al., 2003), mientras que en tratamientos con antidepresivos disminuyen los niveles de CORT (Zhang et al., 2010).

Otra posible explicación a nuestros resultados puede estar vinculada con el control de la movilización "*turnover*" de ácidos grasos en las membranas plasmáticas. Se ha demostrado que el estrés crónico disminuye el contenido de PUFAs ω-3 en las membranas (Farooqui et al., 1997), posibilitando el aumento de AA y la síntesis de eicosanoides, que ha sido asociado con neuropatologías inflamatorias (Farooqui et al., 1997). Varios estudios han demostrado que el aumento en la dieta de PUFAs ω-6, tal como AA o su precursor LA, aumentan la concentración de 2-araquidonilglicerol (2-AG) y araquidonoil-etanolamida (AEA) (Matias et al., 2008), que corresponden a lípidos sintetizados parte del sistema endocanabinoide (eCB) que son liberados de manera retrograda desde la neurona post-sináptica hacia los terminales pre-sinápticos, activando a los receptores de canabinoides tipo 1 (CB1), cuya activación puede inducir depresión a corto o a largo plazo en sinapsis inhibitorias y excitatorias en distintas regiones del cerebro incluido el hipocampo (Chevaleyre and Castillo, 2003; Rapoport, 2008; Hill y McEwen, 2009; Häring et al., 2012). Estudios de inmunohistoquímica han mostrado que CB1 se encuentran localizados primariamente en las neuronas GABAérgicas (Katona et al., 1999; Tsou et al., 1999; Irving et al., 2000). Se ha determinado que la activación de los receptores CB1 se puede realizar mediante dos vías de señalización e inducir una disminución de la probabilidad de liberación de neurotransmisor (Freund, 2003; Chevaleyre et al., 2007; Lovinger, 2008). El dímero βγ de la proteína G asociada al receptores CB1 bloquea canales de $Ca^{+2}$ dependientes de voltaje (VGCC) del tipo N (Alger, 2002; Chevaleyre et al., 2007; Lovinger, 2008), mientras que la subunidad α de esta proteína G inhibe a la adenilato ciclasa (AC), disminuyendo el nivel de cAMP y terminando con la disminución de la actividad de la proteína kinasa A (PKA) (Chevaleyre et al., 2007). Nuestros resultados muestran que el estrés

crónico disminuye la eficacia sináptica GABAérgica (Fig. 3-6), mientras que la suplementación con PUFAs ω-3 la restaura. Se ha encontrado que el estrés crónico produce un aumento de la razón ω-6/ω-3 (Parker et al., 2006; Ross et al., 2007). Por lo tanto, el posible mecanismo podría involucrar la disminución del contenido de AA, mediante la suplementación con PUFAs ω-3, inhibiendo la síntesis de eicosanoides (Farooqui et al., 1997), formación de eCB e inhibición de transmisión sináptica GABAérgica, lo cual es apoyado por los resultados de Betetta y colaboradores (2009) quienes encontraron que la ingesta de PUFAs ω-3 durante 4 semanas disminuye la concentración de AEA y 2-AG. Como resultado, se restablece el normal funcionamiento de los sistemas neuronales para el desarrollo de pruebas cognitivas tales como la memoria espacial y aprendizaje auditivo.

**Capítulo III**:

***3.1 Efecto de la ingesta de AGO sobre los principales marcadores del estrés, ansiedad, memoria espacial y aprendizaje auditivo en ratas sometidas a estrés crónico.***

Los alimentos de origen marino son considerados como la principal fuente de PUFAs ω-3. Su consumo ha sido asociado con efectos beneficiosos sobre la salud humana. Sin embargo la ingesta en países desarrollados o en vías de desarrollo se encuentra muy por debajo de los niveles recomendados (600 mg al día), produciendo un aumento de la razón omega-6/omega-3, que ha sido catalogada como un factor de riesgo para el desarrollo de enfermedades de gran impacto, tal como las enfermedades cardiovasculares, obesidad, esquizofrenia y Alzheimer (Soderberg et al., 1999; Peet et al., 2001; Artemis y Simopoulos, 2002; Sethom et al., 2010). La ingesta de un alimento elaborado con gónadas de ostión a una concentración de 100-300 mg por día, no produjo un aumento significativo sobre la memoria espacial y aprendizaje auditivo en ratas sometidas a estrés crónico (Fig 3A y 5), como ha sido observado mediante la suplementación con PUFAs ω-3. Si bien existen diferencias en la forma en que se ingieren estos PUFAs ω-3, a través de un alimento enriquecido y mediante capsulas que contienen aceite de pescado, se ha determinado que mediante ambas formas de incorporación las concentraciones en el plasma y tejidos de éstos PUFAs ω-3 no varían (Higgins et al., 1999; Wallace et al., 2000).

Por otra parte, se ha encontrado que a los individuos controles alimentado con AGO, experimentan una disminución no significativa de la memoria espacial y un aumento de la

ansiedad, al igual que los resultados obtenidos con la suplementación PUFAs ω-3. Estos resultados nos sugieren que probablemente, en ratas controles se produce un aumento en el nivel de PUFAs ω-3 generando un desbalance en membrana plasmática, alteración que podría afectar la actividad del BNST, aumentando la ansiedad. Estos resultados difieren con los resultados encontrados en otros estudios en los cuales se ha demostrado que la ingesta de PUFAs ω-3 reduce la concentración de PUFAs ω-6, aumenta la concentración en el plasma de PUFAs ω-3, reduciendo la ansiedad (Buydens-Branchey y Branchey, 2006). Probablemente, en ratas sometidas a estrés, la ingesta de AGO no alcanza a suplir los requerimientos diarios de PUFAs ω-3. Se ha estimado que los requerimientos diarios para una persona normal oscilan entre 100-300 mg (Guesnet y Alessandri, 2011) hasta 600 mg (Kris-Etherton et al., 2000). Además, se ha demostrado que el estrés crónico disminuye el contenido de PUFAs ω-3 y bloquea la incorporación de DHA libre en las membranas neuronales (Vancassel et al., 2008; Hennebelle et al., 2012). Sin embargo, aún se desconoce cuáles deben ser los requerimientos diarios en condiciones de estrés, considerando su disminución por medio de la movilización de ácidos grasos por acción de las fosfolipasas. Varios estudios nutricionales muestran que la ingesta de PUFAs ω-3 no satisface las necesidades fisiológicas ya que los PUFAs (EPA, DHA) se encuentran sólo en los organismos de origen marino y la conversión a partir de sus precursores LNA no es eficiente (Horrocks y Yeo, 1999). Sin embargo, la suplementación con PUFAs ω-3 también puede ser perjudicial, porque puede promover la peroxidación lipídica y la posterior propagación de radicales libres (Reed, 2011) que han sido involucrados en una amplia variedad de trastornos neurodegenerativos (Grisham y Granger, 1988) y cáncer (Brasky et al., 2011).

Actualmente existe una gran diversidad de productos alimenticios con alto contenido de PUFAs ω-3, existen los alimentos enriquecidos con PUFAs ω-3 y las capsulas con aceites de pescado, que en el comercio son a menudos llamados alimentos funcionales. El desarrollo de productos enriquecidos con aceites de pescado, así como el de otros tipos de alimentos, debe estar basado en el conocimiento científico de sus efectos sobre la salud humana. Si bien, este trabajo colabora al conocimiento con respecto a los efectos de la suplementación sobre funciones cognitivas que son afectadas en condiciones de estrés crónico, la incorporación de estos y las formaciones de derivados a partir de PUFAs ω-3 aún no se han evaluado.

***4. Conclusiones***

***Impacto clínico de la suplementación con PUFAs omega-3 sobre trastornos relacionados al estrés.***

Estudios preclínicos y clínicos apoyan el uso de la suplementación con PUFAs ω-3 en desórdenes relativos al estrés tal como los desórdenes depresivos y de ansiedad. Por ejemplo, dietas ricas en PUFAs ω-3 mejoran el efecto de antidepresivos en modelos animales que presentan conductas similares a la depresión (Venna et al., 2009; Laino et al., 2010), así como en pacientes con depresión mayor (Sánchez-Villegas et al., 2009; Rienks et al., 2013) y con desórdenes de ansiedad (Ross, 2009). En este contexto, nosotros proponemos que debido al efecto ansiolítico y anti-estrés, la suplementación de PUFAs ω-3 puede mejorar los síntomas de pacientes con desórdenes depresivos y de ansiedad. Por el contrario, dietas pobres en PUFAs ω-3 podrían ser un factor de riesgo para el desarrollo de desórdenes depresivos y de ansiedad.

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# ANEXO

**Tabla I**. contenido de ácidos grasos ω-3 y ω-6 en aceites vegetales, carne y productos del mar.

| | Ácidos grasos ω-3 | | | Ácidos grasos ω-6 | |
|---|---|---|---|---|---|
| | LNA (18:3) | EPA (20:5) | DHA (22:6) | LA (18:2) | AA (20:4) |
| **Aceites** | | | | | |
| **Maíz** | 0,8 | --- | --- | 52 | --- |
| **Olivo** | 0,5 | --- | --- | 10 | --- |
| **Soya** | 8 | --- | --- | 54 | --- |
| **Productos del mar** | | | | | |
| **Ostión del norte** | 1.5 | 16 | 14.2 | 1.1 | 0,6 |
| **Salmón** | 1.1 | 13.5 | 18.9 | 1.6 | 0,7 |
| **Trucha** | 1.7 | 7 | 20.4 | 4.8 | 0,8 |
| **Carnes** | | | | | |
| **Ave** | 0,9 | 3 | 0,6 | 12.2 | 0,5 |
| **Vacuno** | 0,3 | traza | traza | 2.1 | 0,4 |
| **Cerdo** | 0,5 | traza | 0,4 | 8.1 | 0,4 |

Valores en g/100g de ácidos grasos totales. Los niveles de ácidos grasos basan en los reportes de Innis, 2004; Larsson et al., 2004 y Caers et al., 1999

Tabla II. Distribución de los ácidos grasos en cada uno de las especies de fosfolípidos

| | | PC | PS | PI | PE |
|---|---|---|---|---|---|
| **Ácido palmítico** | 16:0 | 38.8 | 6.2 | 2.8 | 9.8 |
| **Ácido esteárico** | 18:0 | 6.2 | 48.1 | 55.7 | 24.9 |
| **Acido oleico** | 18:1 (ω-9) | 54 | 41.6 | 12.3 | 37 |
| **Acido araquidónico** | 20:4 (ω-6) | 0,6 | 1.1 | 28.2 | 14.9 |
| **Acido docosahexaenoico** | 22:6 (ω-3) | 0,4 | 5.1 | 1 | 13.4 |

Los porcentajes están basados en los valores reportados por Knapp y Wurtman, 1999

# Figuras

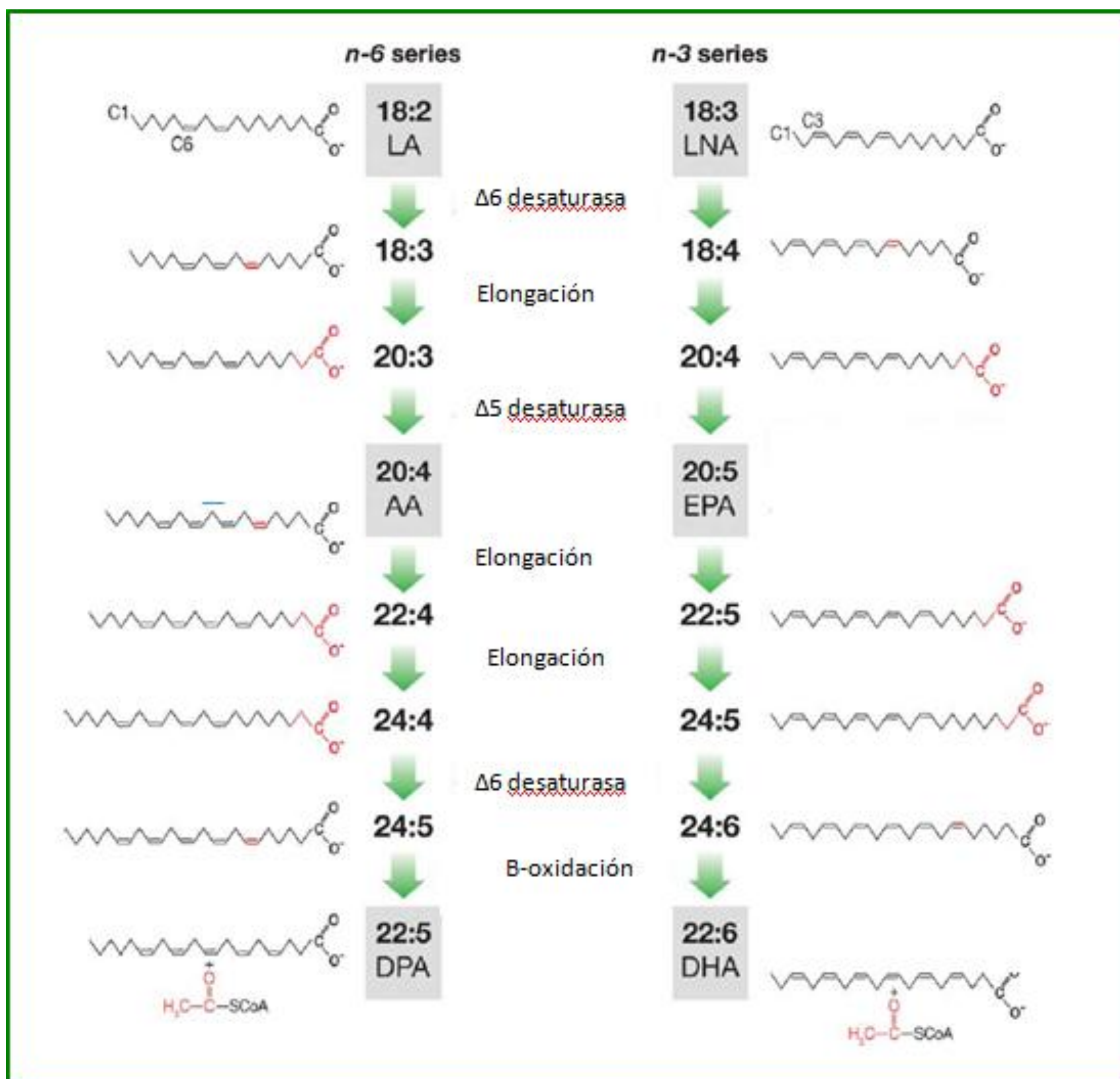


Figura 1. Biosíntesis de los ácidos grasos poli-insaturados ω-3 y ω-6 a partir de sus precursores de 18 carbonos. El grupo metilo terminal corresponde al carbono 1. Los ácidos grasos ω-3 y ω-6 son llamados se acuerdo a la posición del primer doble enlace. LA, ácido linoleico; LNA, ácido linolénico; AA, ácido araquidónico; EPA, ácido eicosapentanoico; DPA, ácido docosapentaenoico; DHA, ácido docosahexaenoico.

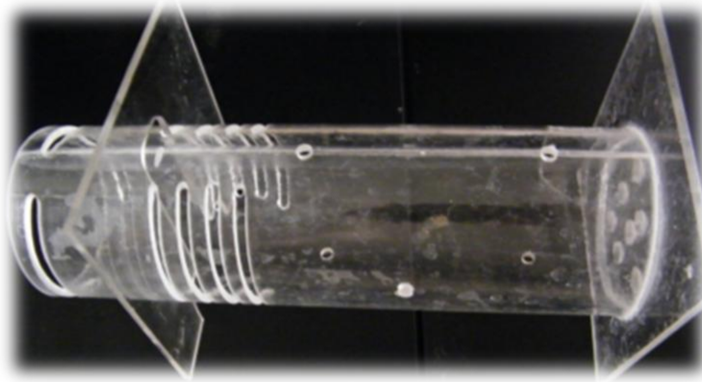

Figura 2. Fotografía de la cámara de restricción utilizada para desarrollar el protocolo de estrés. Tubo circular de acrílico de 30 cms de largo que presenta perforación para la entrada de oxígeno.

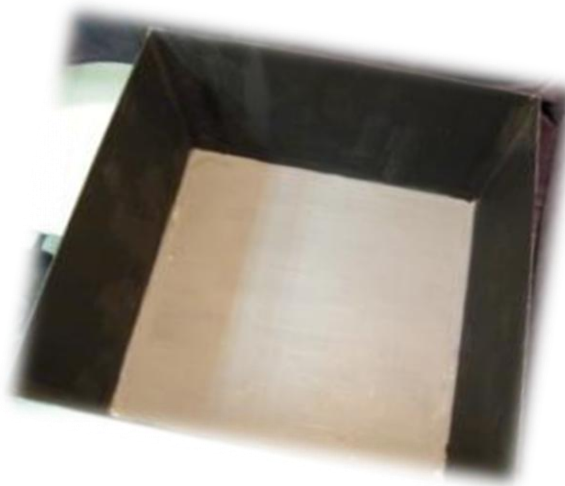

Figura 3. Fotografía del aparato utilizado para cuantificar la actividad locomotora en ratas. Caja de acrílico de 70 cms de ancho y largo, y de 40 cms de altura. La rata se deja en el centro y se filma su exploración durante 5 minutos.

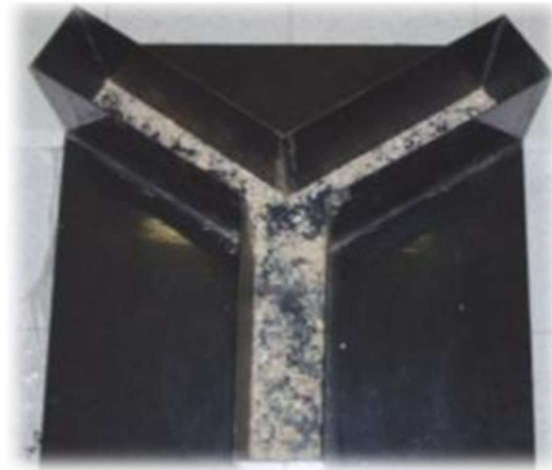

Figura 4. Fotografía del aparato utilizado para cuantificar la memoria espacial en ratas. Laberinto en forma de Y, formado por 3 brazos iguales de 40 cms. El experimento se realiza en dos etapas, en la primera etapa se deja inaccesible uno de los brazos (brazo nuevo) y se deja explorar durante 15 minutos. Y luego de 4 horas se vuelve a someter a la rata durante 5 minutos al laberinto, pero en esta ocasión se dejan accesibles los tres brazos.

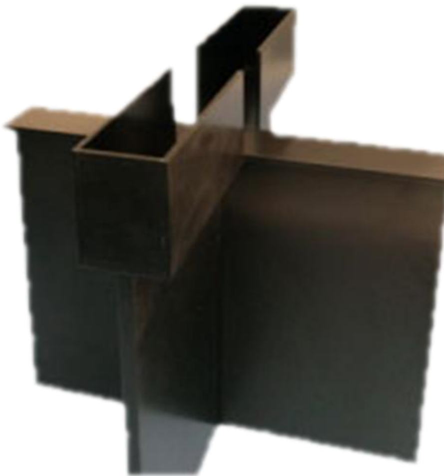

Figura 5. Fotografía del aparato utilizado para cuantificar la ansiedad en ratas. Laberinto elevado a 50 cms en forma de cruz, formado por 4 brazos de 60 cms, dos de los cuales se encuentran abiertos (brazos abiertos) y dos poseen una placa lateral de 10 cms de altura (brazos cerrados). La rata se coloca en el centro del laberinto y se permite la exploración por 5 minutos.

Figura 6. Fotografía del aparato utilizado para cuantificar el aprendizaje acústico. Cámara de condicionamiento, formada por cámaras que tienen comunicación, cada una capaz de producir el estímulo condicionante y por una rejilla sensible al movimiento que genera el estimulo no condicionante (corriente 0,5 mA)

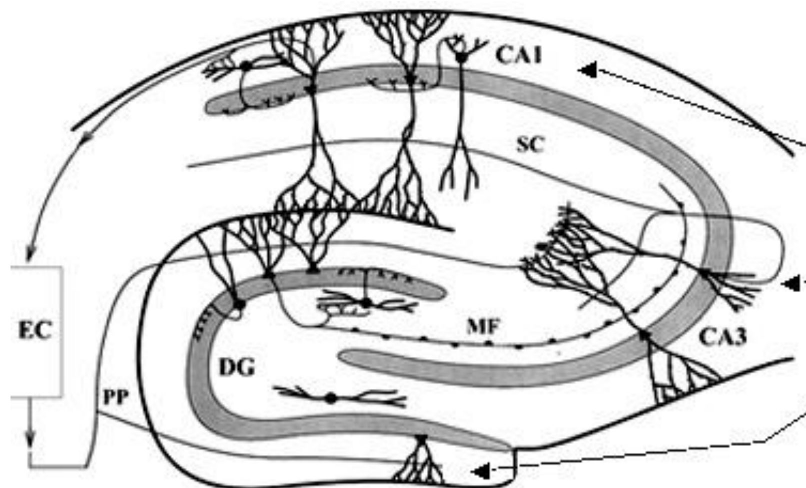


Figura 7. Corte coronal del cerebro de la rata, sección del hipocampo dorsal. Las neuronas estudiadas corresponden a las del área $CA_3$.

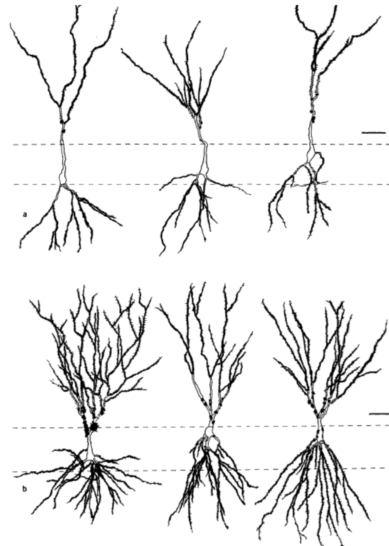

Figura 8. Tipos de neuronas encontradas en el hipocampo en la región $CA_3$. En la parte superior se encuentran las neuronas piramidales del tipo Long-Shaft y en la parte inferior se encuentran las neuronas piramidales del tipo Short-Shaft.

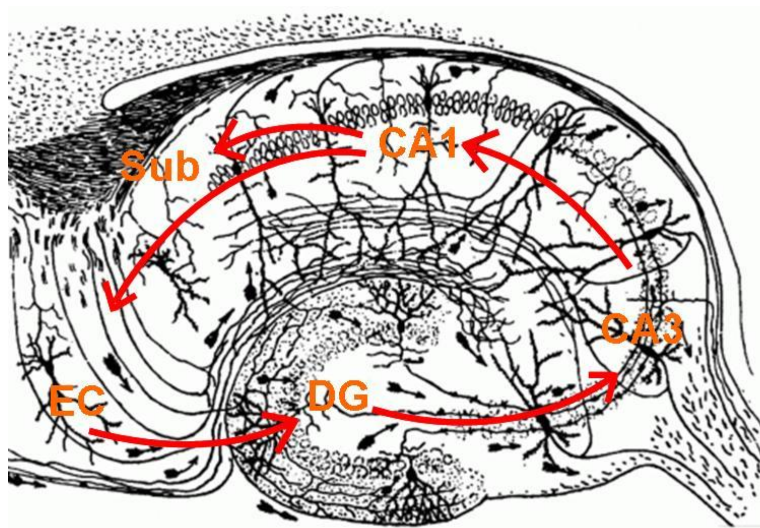


Figura 9. Esquema de la formación hipocampal dibujado por Santiago Ramón y Cajal. Se señala el circuito excitatatorio intrínseco entre las distintas áreas de la formación del hipocampo (flechas rojas). EC, corteza entorrinal, DG, giro dentado, CA3, cornu ammonis 3, CA1, cornu ammonis 1 y Sub, subiculum.