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EFECTOS COMPORTAMENTALES Y MOLECULARES DEL ESTRÉS AGUDO, ETANOL Y LA CAFEÍNA EN EL PEZ CEBRA

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Prefacio

El presente trabajo de tesis surge de mi interés por comprender los mecanismos moleculares en el cerebro cuando esta expuesto a determinadas sustancias o tratamientos. El conflicto armado en Colombia genera una variedad de trastornos mentales y problemas sociales a los afectados. Hay una limitación importante al realizar análisis de tejido cerebral de humanos y son las múltiples interacciones con el ambiente, limitaciones en obtener una muestra, el gran volumen cerebral, las diferentes áreas que lo componen y la variedad de patologías que hayan sido diagnosticadas. Los modelos animales son una alternativa para analizar los cambios del comportamiento y cambios moleculares que pueda generar un tratamiento. Esta tesis explora los cambios comportamentales y moleculares bajo condiciones de estrés ambiental y exposición a sustancias como el alcohol y la cafeína en el pez cebra.

Durante el desarrollo del presente trabajo se revisaron los software para análisis del comportamiento y se encontraron algunas limitaciones como costos en el acceso, largos procesos y variabilidad de resultados. Posteriormente, tuve la oportunidad de trabajar con la Universidad Industrial de Santander con el Dr. Fabio Martínez y el estudiante Edgar Montenegro y desarrollar un algoritmo de libre acceso que permite realizar el análisis automático del comportamiento. Se espera que este trabajo de tesis aporte con el entendimiento de los mecanismos de los trastornos mentales y, que la herramienta de análisis del comportamiento ayude a los investigadores a entender mejor sus datos.

Resumen

El estrés es un factor de riesgo para diversas enfermedades orgánicas incluyendo trastornos psiquiátricos como la ansiedad, depresión, consumo de sustancias adictivas entre otros. El alcohol es una sustancia ampliamente usada en el mundo y sus efectos agudos a nivel comportamental son conocidos. El efecto del estrés ambiental y sustancias sobre el comportamiento se ha estudiado por separado. El estrés a corto y largo plazo en el pez cebra puede generar alteraciones en el SNC afectando los niveles de ansiedad en diferentes pruebas del comportamiento tales como la conducta motora, social, aprendizaje y memoria. Sin embargo, a nivel molecular en el cerebro son poco conocidos los efectos del estrés ambiental en conjunto con el alcohol y otras sustancias como la cafeína, consumidas en todo el mundo.

La presente tesis de doctorado demuestra que el estrés ambiental agudo por sí solo y en conjunto con el alcohol o la cafeína disminuye la actividad locomotora del pez cebra y altera los patrones de comportamiento de ansiedad, agresividad y social, determinado por tiempos de permanencia en zonas de interés. Se realizó una revisión de software de uso libre que describe las ventajas y limitaciones de cada uno. Con la finalidad de mejorar el análisis del comportamiento, se desarrolló ZebraMov, un algoritmo que permite el análisis de los videos más detallado y preciso, aplicable a otras especies. El estrés asociado a la exposición de cafeína mostró un aumento de la cohesión social (efecto ansiogénico) con disminución de la locomoción.

El análisis del comportamiento de la prueba de agresividad con el espejo evidenció la disminución significativa de la locomoción en peces sometidos a estrés más 0.75% de etanol comparado con el control. En un estudio piloto con muestras de cerebro completo, se analizó la expresión de mRNA en los grupos sometidos a estrés y estrés más 0.75% de alcohol. Se evidenció una disminución de la expresión de 5 veces en los genes comta, slc6a3 (dat) y bdnf3; relacionados con la enzima que degrada las catecolaminas, transportador de dopamina y el factor neurotrófico derivado del cerebro, respectivamente. De forma aguda, el estrés puede generar cambios comportamentales y moleculares y conducir a la neurodegeneración, alterando la biodisponibilidad de moléculas esenciales para la transmisión sináptica que pueden llevar al desarrollo de trastornos psiquiátricos. Debido a los diversos mecanismos involucrados en el estrés y exposición a sustancias, persiste la necesidad de analizar otros tipos de comportamiento y efectos moleculares que sean útiles como estudios preclínicos para el desarrollo de tratamientos terapéuticos más efectivos.

Abstract

Stress is a risk factor for several organic diseases including psychiatric disorders such as anxiety, depression, and substances use disorders, among others. Alcohol is a widely used substance in the world and its acute effects on a behavioral level are known. The effect of environmental stress and substances on behavior has been studied separately. Short and long-term stress in zebrafish can generate alterations in the CNS affecting anxiety levels in different behavioral tests such as locomotion, social, learning and memory behavior. However, in the brain, the molecular effects of environmental stress are poorly understood in association with alcohol and other substances such as caffeine, consumed throughout the world.

The present doctoral thesis demonstrates that acute environmental stress by itself and associated with alcohol or caffeine, decreases the locomotor activity of zebrafish and alters the patterns of anxiety, aggressiveness and social behavior, determined by times of permanence in areas of interest. A freely software review was performed and describes the advantages and limitations of each one. In order to improve behavior analysis, ZebraMov was developed, an algorithm that allows more detailed and accurate video analysis, applicable to other species. The stress associated with caffeine exposure showed an increase in social cohesion (anxiogenic effect) with a decrease in locomotion.

The behavior analysis of the aggressiveness test with a mirror showed a significant decrease in locomotion in fish subjected to stress plus 0.75% ethanol compared to the control. In a pilot study with whole brain samples, mRNA expression was analyzed in the groups subjected to stress and stress plus 0.75% alcohol. A significant 5-fold down-regulation were observed for the genes comta, slc6a3 (dat) and bdnf3; related to the catecholamine-degrading enzyme, dopamine transporter, and brain-derived neurotrophic factor, respectively. Acute stress can generate behavioral and molecular changes and lead to neurodegeneration, altering the bioavailability of essential molecules for synaptic transmission that can lead to the development of psychiatric disorders. Due to the several mechanisms involved in stress and exposure to substances, other types of behavior and molecular effects need to be analyzed. Preclinical studies are useful for the development of more effective therapeutic treatments and understand diseases mechanism.

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Lista de Abreviaturas y acrónimos

°C: grados centígrados
µM: micro Molar
µL: microlitro
µg/L: microgramos por litro
bdnf3: factor neurotrófico derivado del cerebro 3
BS: Background Subtraction
cDNA: ácido desoxirribonucleico complementario
cm: centimetros
comta: catecol -o- metiltransferasa a
C_T: Cycle threshold, Umbral de ciclo
dH2O: agua destilada estéril
elf1a: factor de elongación a1
L: litros
mL: mililitro
mg/L: miligramos por litro
mRNA: RNA mensajero
PCR: Reacción en cadena de la polimerasa
qRT-PCR: PCR cuantitativa en tiempo real
ROI: region of interest
RT-PCR: PCR en tiempo Real
slc6a3: transportador de dopamina
SNC: Sistema nervioso central

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Capítulo 1. Introducción

Los trastornos psiquiátricos a nivel mundial y en Colombia representan una de las principales cargas de la enfermedad, años de vida saludable perdidos por discapacidad y vividos con discapacidad (Steel et al., 2014; GBD, 2019). Se han identificado factores de riesgo como el estrés psicosocial y el consumo de alcohol para desarrollar trastornos mentales y enfermedades crónicas cardiovasculares, digestivas, metabólicas, entre otras (Daré et al., 2019). Un estudio realizado a 1026 victimas del conflicto armado en Colombia de 13 a 65 años de edad, evidenció a través de encuestas y escalas (CIDI, SIDUC, CICAD/OEA y el DSM IV) que el 16,4 % presentaban depresión mayor, el 9,9 % estrés postraumático, el 7,2 % ansiedad por separación, el 5,8 % trastornos de conducta, y un 5,6 % déficit de atención; además, se exploró el consumo de sustancias con una prevalencia de vida de consumo de alcohol de 68,7 %; de tabaco 31,3 %, de marihuana 11,2 %, de cocaína 3,5 %, de bazuco 2,0 % y de inhalables 2,3 % (Castaño et al, 2017).

En pacientes diagnosticados con trastorno depresivo mayor y trastorno afectivo bipolar, se ha evidenciado la alteración de las concentraciones de neurotransmisores en plasma, incluyendo el sistema GABAérgico, catecolaminérgico y serotoninérgico, lo cual explica los síntomas presentes en los pacientes y la alteración del comportamiento (Pan et al., 2018). Se ha analizado en muestras de tejido cerebral de suicidas con trastorno depresivo previamente diagnosticado y se han reportado variaciones en la expresión de genes relacionados con la regulación del estado de animo tales como receptores, transportadores y enzimas de serotonina, dopamina, GABA y otras moléculas que intervienen para el correcto funcionamiento neuronal (Yin et al., 2016). El factor genético desempeña un papel significativo en la posible instauración de múltiples enfermedades, por ejemplo, el gen APOE (que codifica para la apolipoproteina E) y la presencia de un alelo E4, es un factor de riesgo para desarrollar la enfermedad de Alzheimer, pero no en todos los portadores se presentaran los síntomas (Forero et al., 2016. Producto # 1). Como es evidente, el ambiente y los sucesos que rodean la vida de un individuo puede influir en la aparición de enfermedades y generar cambios a escala molecular.

Reconocer los mecanismos de la enfermedad es fundamental para el uso y desarrollo de nuevas moléculas que mejoren los síntomas existentes, por lo tanto, se necesitan más estudios que permitan establecer las consecuencias de los factores de riesgo, pero dada las limitaciones en los humanos la experimentación en modelos animales es una alternativa. En ese sentido, el pez cebra o *Danio rerio* ha demostrado ser un modelo animal extremadamente útil en la investigación en ciencias básicas y en el campo de las neurociencias, facilitando al investigador la manipulación del entorno, extracción y correlación de datos del comportamiento con muestras biológicas (Kalueff, Stewart & Gerlai, 2014). El pez cebra cuenta con múltiples ventajas tales como su naturaleza prolífica, las hembras tienen la capacidad de desovar entre 100-300 huevos, rápido desarrollo, comportamiento tipificado, secuencia completa del genoma, mantenimiento fácil y económico que lo hacen idóneo para la investigación (Howe et al., 2013; Kalueff et al., 2013).

Mas recientemente, el uso de herramientas computacionales para el análisis de imágenes como los softwares y el diseño de algoritmos se han vuelto parte fundamental para entender diferentes fenómenos del comportamiento. Se realizó una búsqueda de los software de uso libre y sus características y capacidades disponibles tienen en común el análisis del comportamiento locomotor, generando datos básicos de distancia, velocidad y aceleración de forma automática, semiautomática o manual; en su mayoría cuentan con capacidad de analizar la locomoción por regiones y tiempos de permanencia; otros software mediante el aprendizaje de maquina se han especializado en el reconocimiento de múltiples individuos y de patrones característicos de una especie (Franco-Restrepo et al., 2017. Producto # 2).

En consecuencia, el problema principal radica en la necesidad del investigador de usar dos o más programas para analizar más variables cinemáticas; además sus algoritmos no son uniformes por lo que pueden generarse diferencias en el análisis cinemático y finalmente, obtener datos específicos del comportamiento limita su aplicación en otras especies. En ese sentido, se desarrollo un algoritmo usando el método de binarización (background subtraction) y de trayectorias densas (flujo óptico); con la capacidad de analizar de forma automática los patrones cinemáticos (posición, distancia, velocidad y aceleración); generar mapas de ocurrencia por individuo; promedio de mapas con desviación estándar por grupo; y la aplicación de correlación estadística (Franco-Restrepo et al., 2021. Producto # 4). La valoración de los mapas de ocurrencia es una tarea subjetiva, a tal punto que un investigador podría clasificar dentro de un grupo dos mapas que pertenecen a grupos diferentes. Por lo tanto, con la finalidad de hacer esta tarea mas objetiva, se empleó aprendizaje de máquina y una red neuronal convolucional para clasificar los mapas de ocurrencia generados previamente, diferenciando entre los peces control, los sometidos a estrés y los sometidos a estrés más la exposición a alcohol o cafeína (Montenegro et al., 2021. Producto # 5). Esta herramienta permite verificar el efecto de un tratamiento particular con cada uno de los animales experimentales con una precisión del 84%.

Por otra parte, varios autores han documentado los efectos del estrés ambiental y exposición a sustancias en el pez cebra en términos del análisis del comportamiento y pruebas de genética molecular (Abreu et al., 2014; Zhou et al., 2019). Sin embargo, no se realiza el análisis del comportamiento locomotor de forma completa, enfocándose en un dato específico de comportamiento, por ejemplo, la latencia en segundos en llegar a una región específica del acuario. El comportamiento del pez cebra sometido a estrés agudo y crónico presenta alteración en su patrón locomotor con disminución de la distancia recorrida; en pruebas de medición de estrés y ansiedad como la búsqueda de refugio, los peces estresados buscan en menos tiempo el refugio en comparación con los controles (Piato et al., 2011; Manuel et al., 2014). En pruebas de expresión de mRNA el estrés y el alcohol alteran la expresión de receptores, factores de crecimiento y enzimas en muestras de tejido cerebral (Pan et al., 2011; Tran et al., 2016). La combinación de ambos factores (estrés y alcohol), producen una disminución de la expresión de genes candidato tales como el transportador de dopamina (slc6a3), el factor neurotrófico derivado del cerebro (bdnf3), la comta- catecol -o-metiltransferasa a (comta) y aumento no significativo en la expresión del transportador de

serotonina (slc6a4a); relacionados con la homeostasis neuronal y que influyen en la posible instauración de trastornos mentales (Franco-Restrepo et al., 2021. Producto # 3).

Con la finalidad de caracterizar y analizar el efecto del estrés ambiental asociado a la exposición a sustancias en términos del comportamiento y efecto genético, en el presente trabajo de tesis se adapto un protocolo de estrés ambiental agudo de tres días el cual consiste en cambio de temperatura del agua, persecución e inmovilización. Posteriormente, los peces fueron expuestos a alcohol o cafeína (sustancia depresora y estimulante del SNC, respectivamente) a diferentes concentraciones para luego ser trasladados a un tanque y grabar su comportamiento en una prueba determinada (exploración/ansiedad, agresividad y social). Los videos se analizaron con software de libre acceso y mediante nuevas estrategias computacionales. En un estudio piloto, con muestras de tejido cerebral de peces bajo estrés y alcohol, se analizaron los niveles de expresión de mRNA mediante PCR en tiempo real (RT-PCR) y el método comparativo C_T.

1.1 Objetivo general y específicos

Explorar y describir los cambios del comportamiento y cambios moleculares del pez cebra bajo condiciones de estrés ambiental repetitivo y administración de alcohol y cafeína.

Específicos:

1. Analizar software de uso abierto disponibles, sus capacidades y limitaciones en el análisis de videos del comportamiento.

2. Analizar y caracterizar el comportamiento del pez cebra bajo condiciones de estrés ambiental en pruebas de exploración/ansiedad, agresividad y social.

3. Analizar y caracterizar el comportamiento del pez cebra bajo condiciones de estrés ambiental y exposición a alcohol y cafeína en pruebas de agresividad, exploración/ansiedad y social.

4. Analizar la expresión de genes de interés en el pez cebra sometido a estrés ambiental y exposición a alcohol.

5. Explorar la posibilidad de desarrollar herramientas que nos ayuden a obtener datos para entender el comportamiento del pez cebra con el uso de herramientas computacionales como la automatización de procesos, aprendizaje de maquina y redes neuronales convolucionales.

Esta tesis analiza y caracteriza la alteración del comportamiento en pruebas de ansiedad, agresividad y conducta social y adicionalmente explora desde el punto de vista de la genética molecular en el pez cebra las potenciales relaciones entre condiciones de estrés ambiental agudo y exposición a alcohol y cafeína. Su aplicación potencial en el campo de las neurociencias es facilitar la comprensión de los mecanismos neuronales generado por el estrés y el uso de sustancias. Por otra parte, la aplicación de los algoritmos ahorrará tiempo en la extracción de datos, aumentando la precisión de seguimiento, generando datos adicionales como promedios, desviación estándar y correlación estadística a partir de mapas de ocurrencia para posición y locomoción. La herramienta empleada usó aprendizaje de maquina y redes neuronales que pueden catalogar y deducir el comportamiento de un

individuo por medio de las secuencias de imágenes analizadas. Los análisis realizados facilitarán a los investigadores en campos del comportamiento a entender los fenómenos subyacentes y la correlación con pruebas moleculares; en campos como la farmacología y toxicología ayudará a la comprensión de los efectos de una sustancia y a la toma de decisiones según las dosis empleadas.

Capítulo 2. Estado del Arte

La metodología empleada se describe brevemente a continuación y se profundiza en cada sección más adelante. Inicialmente se sometió a los peces cebra adultos a estrés ambiental durante tres días y posteriormente a la exposición aguda a sustancias, pruebas de comportamiento, eutanasia, extracción de cerebro completo y finalmente pruebas de genética molecular. El análisis de los videos del comportamiento es parte fundamental para la correlación de resultados moleculares, por lo tanto, a través de estrategias computacionales se desarrolló un algoritmo capaz de medir patrones cinemáticos de forma precisa y con capacidad de generar datos adicionales para facilitar la comprensión del fenómeno comportamental.

2.1 Animales y mantenimiento

El pez cebra o *Danio rerio* es un organismo vertebrado originario de la India, Nepal y Bangladesh que se describió por primera vez en el año 1822, perteneciente a la clase de los teleósteos (que poseen esqueleto, escamas y vejiga natatoria) y habita es estado natural en los ríos de poca profundidad (Hamilton, 1822). En el año 1920 fue identificado como un organismo genéticamente tratable, principalmente los de patrón de pigmentación con líneas (Goodrich, 1929; Holtzman et al., 2016). Para el año 1981 se realizaron los primeros estudios para evaluar la segregación de alelos y mutaciones (Streisinger et al., 1981). Mas recientemente, el comportamiento del pez cebra se ha caracterizado, con aproximadamente 190 tipos de comportamientos que están incluidos dentro de los principales dominios del comportamiento como lo son el locomotor, ansiedad/miedo, social, cognitivo y sensorial (dolor y sentidos) (Kalueff et al., 2013). En el año 2013, en su versión número tres, se secuenció el genoma completo del pez cebra, siendo una de las especies con duplicación génica, contando con 25 pares de cromosomas (1.412 Gb), 26.206 genes que codifican proteínas y una homología con los humanos de 71,4% (Howe et al., 2013). Su cerebro ha sido mapeado y cuenta con sistemas y neurotransmisores de serotonina, GABA, catecolaminas, glutamato, entre otros (Asakawa et al., 2008; Randlett et al., 2015). Comparado con otros modelos animales, cuenta con ventajas como la transparencia de los embriones y cepas sin pigmentación en etapa adulta, alta tasa de reproducción, rápido crecimiento y costos de mantenimiento económico (Stewart et al., 2014). Estas características hacen que sea el modelo adecuado para investigación en ciencias básicas de la salud y un modelo animal de utilidad en medicina traslacional (Stewart et al., 2014; Vargas, 2017).

En el presente trabajo se emplearon peces cebra adultos tipo silvestre que fueron obtenidos en una tienda local de animales. Cada acuario contenía un volumen de 7 L, 400 mL por

espécimen, permitiendo contener alrededor de 18 peces por tanque. La temperatura de los acuarios se mantuvo a 27 ± 1°C, con filtros que permitieron la aireación y distribución del agua y garantizaban una adecuada temperatura y calidad del agua. De igual forma el sistema de iluminación garantiza ciclos de luz oscuridad de 12:12 horas respectivamente (Kim et al, 2009). La alimentación se realizó diariamente con dieta estandarizada para peces tropicales (Tetramin®) disponible comercialmente. Diariamente se hacían mediciones de temperatura y pH para garantizar niveles óptimos entre 6.8 y 7.4 (Trevarrow, 2004); además se observaban los peces para identificar anormalidades anatómicas, patologías y condiciones de estrés que pudieran generar cambios de conducta. De ser necesario, los animales con algún tipo de deterioro se les practicaba eutanasia con crioanestesia a -4°C.

2.2 Población y aspectos éticos

Se emplearon un total de 130 ejemplares de peces cebra adultos tipo silvestre, sanos, sin evidencia de enfermedades, los cuales fueron asignados a grupos control o experimental de forma aleatoria. Los grupos experimentales fueron sometidos a estrés y exposición a alcohol o cafeína con dosis según lo reportado en estudios previos (ver adelante). Cada grupo incluyó una muestra de seis peces lo cual está en concordancia con las muestras reportadas en publicaciones de este tipo y se ajusta al principio bioético de las 3 R´s (Russell y Burch, 1959) y la Guía para el Cuidado y Uso de Animales en Neurociencia y en Investigación del Comportamiento (National Academies Press, 2003). Los grupos experimentales resultantes fueron grupo control, solo estrés y estrés más alguna dosis de alcohol o cafeína. La experimentación fue aprobada por el comité de ética para investigación con animales de la Universidad Antonio Nariño (Junio/2017).

2.3 Diseño experimental: Exposición a estrés ambiental y sustancias

Con el fin de evaluar el comportamiento en el pez cebra, se sometieron ejemplares a un protocolo de estrés ambiental agudo (adaptado de Piato et al, 2011) durante 3 días el cual incluyó:

Día 1: cambio de tanque con temperatura del agua a 33°C por 30 minutos, seguido de cambio del tanque con temperatura del agua a 23°C por 30 minutos. Día 2: persecución con red durante 10 minutos en tanque de almacenamiento. Día 3: inmovilización en tubos Eppendorf de 2 mL (abiertos por ambos polos para permitir la circulación de agua oxigenada) por 60 minutos (Figura 1). Durante el día 4 se le realizó la exposición a sustancias y evaluación del comportamiento en diferentes pruebas para lo cual se grabaron videos de 5 minutos de duración para su posterior análisis.

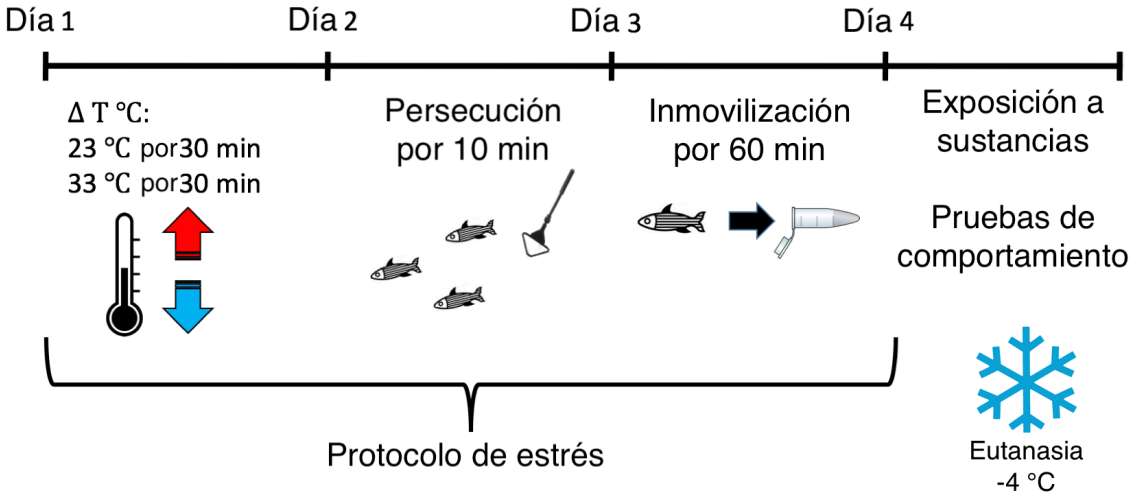

Figura 1. Protocolo de estrés ambiental

En el día 1 los peces son cambiados a tanques con temperaturas de 23 y 33°C durante 30 minutos en cada temperatura. En el día 2 se introduce una red y se persiguen durante 10 minutos. En el día 3 se inmovilizan los peces dentro de un tubo de Eppendorf (abierto en ambos extremos para la oxigenación) durante una hora. En el día 4 los peces son expuestos a etanol o cafeína y posteriormente se realiza la prueba del comportamiento.

El efecto del estrés agudo como el traslado de un pez a otro tanque y ser transportados por 30 minutos en un vehículo, produce una disminución del comportamiento locomotor y exploración en pruebas tales como el tanque trapezoidal y prueba de la caja con luz/oscuridad (Song et al., 2016). Con enfoques similares se ha estudiado en el pez cebra las diferencias de género en el comportamiento agresivo y cohesión social, mostrando una exploración reducida, más episodios de congelación y tendencia al agrupamiento social (Piato et al., 2011; Rambo et al., 2017).

Las dosis administradas están relacionadas con las reportadas en la literatura para este modelo y se diluyeron en un tanque de 1 L para alcanzar la concentración deseada de cada compuesto. La exposición a etanol se realizó a concentraciones de 0 (solo estrés), 0.25, 0.5, 0.75 y 1% con una duración de 40 minutos, tiempo mínimo necesario para alcanzar concentraciones de alcohol en la sangre y cerebro similares a la concentración del tanque (Gerlai, 2009). El alcohol a nivel neuronal tiene un efecto principalmente inhibitorio, bloqueando canales iónicos de sodio dependientes de voltaje y de potasio activados por calcio, aumentando la neurotransmisión de GABA en la médula espinal y afectando a largo plazo las vías de recompensa de la dopamina (Sullivan et al., 2010). La exposición aguda a dosis bajas a moderadas en el pez cebra adulto induce cambios en el comportamiento que incluye disminución de la ansiedad; aumento en la locomoción y agresividad; cambios en la preferencia de lugar y en el comportamiento social (Echevarria et al., 2010; Mathur et al., 2011). En contraste, la exposición crónica al alcohol produce tolerancia y abstinencia incrementando la ansiedad y disminuyendo la tendencia al agrupamiento social (Cachat et al., 2010).

Para la exposición a cafeína se usaron concentraciones de 0 (solo estrés), 10 y 100 µM, equivalentes a 1.94 y 19.41 mg/L, con exposición aguda durante 20 minutos. Las dosis y

tiempos de exposición han sido reportadas previamente en diversos estudios, con variaciones de exposición de 5 a 30 minutos y rango de dosis de 0.01 µg/L a 200 mg/L (Rosa et al., 2018; Zhou et al., 2019). Posteriormente cada pez era transferido a un tanque con agua fresca por 5 minutos y luego transferido al tanque del comportamiento para grabar la prueba. La cafeína es un agonista competitivo, no selectivo que bloquea principalmente los receptores A_1, A_{2A}, A_{2B} y D_2; con efectos principalmente en el SNC y en el comportamiento del pez cebra, incrementando la ansiedad, agresividad y estado de alerta (Ferré, 2016; Rosa et al., 2018). Las altas dosis de cafeína y el efecto causado principalmente por la unión a los receptores A_1, se han relacionado con reducción de la exploración y aumento en los niveles de cortisol, lo que puede ser traducido en un efecto ansiogénico (Ladu et al., 2015; Ferré, 2016). Los efectos del comportamiento social bajo condiciones de estrés y exposición a cafeína combinados no se han estudiado previamente en el pez cebra.

2.4 Pruebas de comportamiento

En el pez cebra se han caracterizado diversos tipos de comportamiento. Con base en el catálogo de comportamiento del pez cebra, se tuvo en cuenta el comportamiento locomotor; geotaxis que hace referencia a la preferencia por las zonas profundas del tanque; tigmotaxis que es la tendencia a permanecer cerca de las paredes, comportamiento social y de ansiedad (Kalueff et al, 2013). Se realizaron grabaciones y se analizó el comportamiento de los peces cebra por 5 minutos en los siguientes tanques (Figura 2):

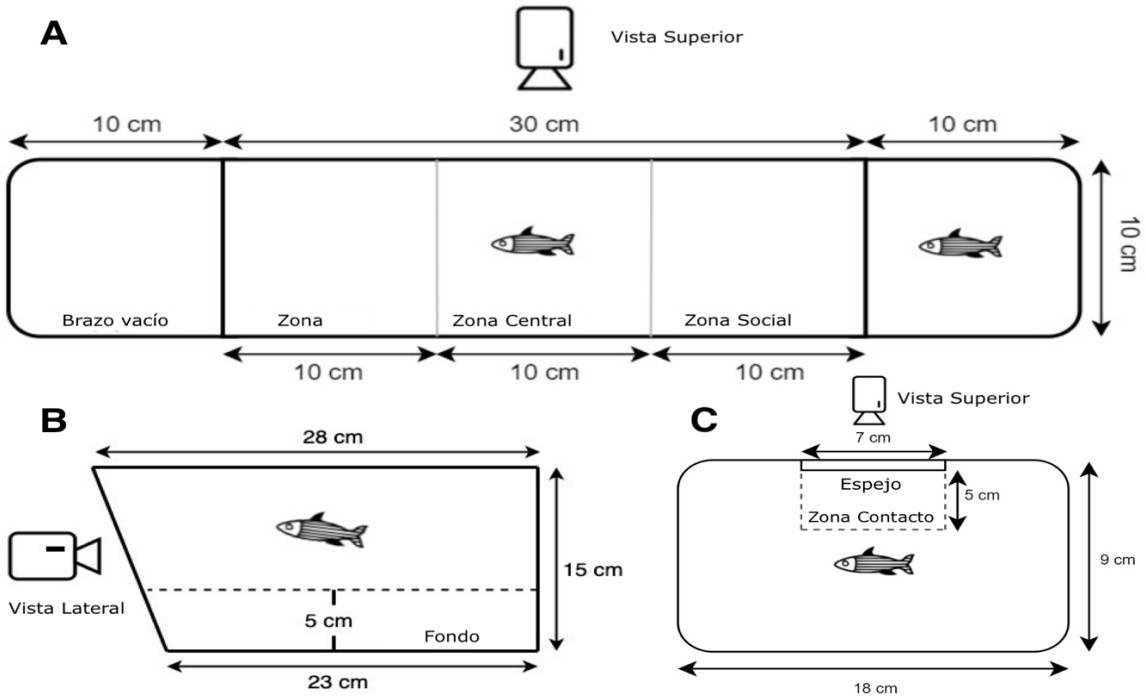

Figura 2. Tanques del comportamiento.

A. Tanque para la prueba del comportamiento social, la región de 30 cm se divide en tres zonas virtuales de 10 cm cada una (social, media y lateral). **B**. Tanque trapezoidal para prueba de ansiedad/exploración, el fondo se divide en dos regiones, tercio inferior (fondo) de 5 cm y tercio superior de 10 cm. **C**. Tanque para prueba del comportamiento de agresividad con prueba del espejo con una zona de contacto (líneas negras punteadas).

En la prueba de exploración/ansiedad en ambiente novedoso estudios previos han empleado el tanque trapezoidal, dividido virtualmente en dos secciones, tercio inferior y dos tercios superiores del tanque. Generalmente el pez presenta ansiedad manifestándose con mayor tiempo en el fondo. Se evaluaron los tiempos en la exploración en un tanque con medidas de longitud en la base de 23 cm, longitud superior de 28 cm, altura 15 cm y 7 cm de ancho (Grossman et al, 2010).

La prueba de agresividad con prueba de espejo permite valoración de agresividad mediante el tiempo de permanencia en la zona cercana al espejo, previamente se ha estudiado en tanque rectangular con un espejo en su interior, los peces cebra exhiben comportamientos como mordidas, aumento del nado en dirección hacia el otro pez y despliegue de aletas dorsales y pectorales del pez dominante hacia el subordinado, esto a través de análisis manual y semiautomático del comportamiento (Filby et al., 2010; Way et al, 2015).

En la prueba de preferencia social con un tanque de 50 cm de longitud con divisiones transparentes en sus extremos de 10 cm, el pez valorado se encuentra en el centro (30 cm) con división virtual de 3 zonas de 10 cm (social, media y lateral). Permite la valoración de la preferencia social al introducir un pez en uno de los extremos de 10 cm del tanque, el pez sujeto a prueba se moverá libremente (zona de 30 cm) cruzando las zonas. Los peces cebra tienden a permanecer mas tiempo en la zona social (Grossman et al, 2010). Los tanques en las pruebas de agresividad y social fueron llenados con 5 cm de agua con el fin de evaluar únicamente la exploración en las zonas mencionadas.

Se han identificado patrones de conducta específica como la preferencia a reunirse en grupos (conducta social), a unirse a un grupo cuando está separado (coherencia social), conductas de territorialidad y la tendencia a mostrarse agresivo cuando está en grupos pequeños de 2 - 4 individuos, esto último se refleja en el cambio de pigmentación de la superficie corporal y el despliegue de patrones de movimientos complejos que incluyen ondulaciones y la tendencia a atacar y morder al oponente (Eddins et al, 2008; Kalueff et al, 2013).

2.5 Eutanasia

Con el fin de evitar posibles interacciones en la medición del comportamiento y pruebas moleculares, se evitó el uso de tricaína (MS-222) en las pruebas y en la eutanasia, ya que previamente se ha reportado aumento de la actividad locomotora en algunas ocasiones, requiriendo aumento de la dosis para producir una muerte segura (AVMA, 2020). Por otra parte, Strykowski et al. (2013), reportaron una eficacia mayor en la eutanasia a 4°C o menos en comparación con la tricaína, requiriendo un tiempo mayor de exposición, pero evitando introducir una variable adicional en las pruebas. Se usó crioanestesia a 4°C por 20 minutos y posteriormente el pez era transferido a un tubo Eppendorf de 1,5 mL con 700 µl de dH2O y almacenados a -20°C. Posteriormente, con el fin de realizar la extracción de cerebro completo, se procede a decapitación rápida con una hoja de bisturí en la región cefálica posterior (Sterling et al, 2014 y Rico et al, 2011). El peso y la talla de cada pez fue tomado previo a la extracción del cerebro.

2.6 Análisis del comportamiento

Para el análisis del comportamiento se usó inicialmente el software de uso libre ImageJ y el plugin AnimalTracker (Gulyas, 2016) que permiten el análisis automático del comportamiento ajustando los parámetros adecuadamente a cada prueba. Se analizan los 5 minutos de cada video grabado. El software genera datos de la distancia total recorrida, velocidad promedio, tiempo de congelamiento, tiempo de permanencia en regiones de interés (ROI) y el trayecto realizado por el pez. De los softwares disponibles, AnimalTracker cuenta con un análisis locomotor más completo, con una interfaz gráfica de fácil uso y disponible para la mayoría de los sistemas operativos (Tabla 1).

Tabla 1. Software de uso libre para análisis del comportamiento

TABLE 1. OVERVIEW OF FREELY AVAILABLE, OPEN-SOURCE SOFTWARE FOR THE AUTOMATED ANALYSIS BEHAVIOR IN ANIMAL MODELS

Software	*Operative system/program*	*Types of analyses*	*Types of input*	*Types of output*	*Webpage*
wrMTrck	Windows and Mac OS X/ JAVA-ImageJ	Total length, average speed, area, perimeter, and trajectories.	AVI files with jpg compression	txt, xls, tiff files and AVI videos	www.phage.dk/plugins/ wrmtrck.html
Mouse Behavior Tracker	Windows, Mac OS X and Linux/JAVA-ImageJ	Distance and average velocity.	AVI or MPEG-compressed AVI files, Mp4	Txt or xls files	www.BioTechniques.com/ article/114607.
AnimalTracker	Windows, Mac OS X and Linux/JAVA-ImageJ	Total length, average speed, and time spent in ROI.	AVI files with jpg compression	txt, xls, tiff files and AVI videos	animaltracker.elte.hu
idTracker	Windows/MATLAB	Trajectories, identification of one animal in different videos and ROI.	Compatible with MATLAB, uncompressed AVI or MPEG-compressed AVI files	*X* and *Y* coordinates and images files	www.idtracker.es
Mousetracker	Windows, Linux and Mac OS X/Pascal-Delphi-MS Excel	Velocity, acceleration, and time spent in ROI.	AVI format	*XY* coordinates can be copied directly	www.neuro.ufrn.br/ softwares/mouselabtracker
JAABA	Windows, Mac OS X and Linux/MATLAB	Bites, persecution, sexual behavior, angle of turn, grooming, jump, walk, immobilization, and touch. Locomotion and ROI.	Several formats and resolutions. *X* and *Y* coordinates	MATLAB files	http://jaaba.sourceforge.net https://www.janelia .org/lab/branson-lab
Ctrax	Windows and Mac OS X/ Phyton—MATLAB	Trajectories, velocities, speed, position, and turning speed histograms.	Common digital video formats, mainly AVI	csv and mat files. Converts the file to .ann extension	ctrax.sourceforge.net
VideoHacking	Windows, Mac OS X and Linux/Phyton—Open CV	Velocity, acceleration, total length, average speed, and time spent in ROI.	Common digital video formats	Graphical interface to view data summary	faculty.ithaca.edu/iwoods/ docs/
ToxTrack/ToxId	Windows/C++	Total distance, speed, acceleration, time near the walls (measure of anxiety), and ROI.	AVI or MPEG-compressed AVI files	txt, xls, tiff files and AVI videos	https://sourceforge .net/projects/toxtrac/
EthoWatcher	Windows/C++	Frequency, duration, and latency of each behavior.	AVI or MPEG-compressed AVI files	csv files	http://ethowatcher .paginas.ufsc.br
MouseMove	Windows/LabView—ImageJ	Distance, average velocity, acceleration, curvature, stationary fraction, laterality y ROI.	AVI or MPEG-compressed AVI files	csv files	https://www.nature.com/ articles/srep16171#s3, Supplementary File 2
Cowlog	Windows, Mac OS X and Linux/Java—html	Analysis of different behaviors can be set (tapping a button when the event occurs)	Common digital video formats	csv files	cowlog.org

AVI, audio video interleaved; MJPEG, motion joint photographic experts group; ROI, region of interest.

En general, para el procesamiento de los videos se emplean el método clásico de seguimiento de binarización o BS que incluye una transformación de los videos a blanco y negro, donde el objeto/pez a seguir contrasta con el entorno y se generan los datos de locomoción. Usando el método BS más el método de trayectorias densas (flujo óptico) (Wang et al., 2011) fue posible obtener datos de locomoción y mapas de ocurrencia para posición, velocidad y aceleración (Figura 3). En ese sentido, AnimalTracker nos permitió hacer una validación de los datos de locomoción obtenidos frente al método propuesto.

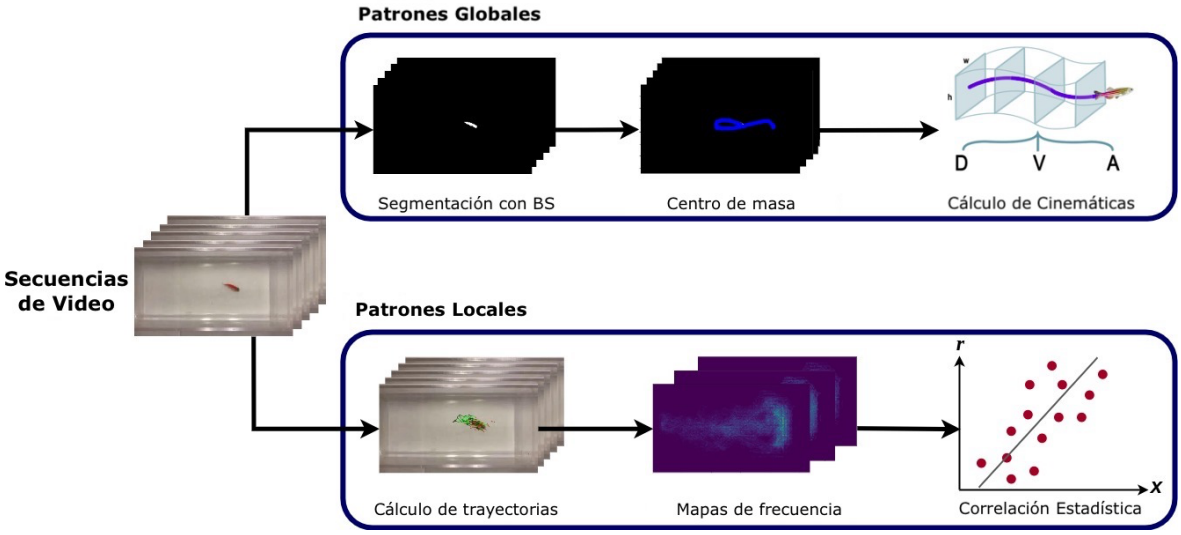

Figura 3. Flujo de trabajo para análisis del comportamiento social.

Con los videos previamente grabados, las secuencias de video son analizadas mediante el método de Background Subtraction (BS) para obtener los datos de locomoción; el flujo óptico permite crear los mapas de ocurrencia y ser analizados mediante correlación estadística.

A partir de los mapas de ocurrencia fue posible hacer una imagen promedio con desviación estándar de cada grupo y mediante correlación estadística comparar para ver las diferencias y similitudes entre los grupos. Como se mencionó en la introducción, evaluar los mapas es una tarea subjetiva y el análisis propuesto permite tener resultados de los mapas de forma cuantitativa. Con la finalidad de hacer esta tarea mas objetiva, se utilizaron herramientas computacionales como el aprendizaje de maquina, redes neuronales convolucionales y aprendizaje profundo con el objetivo de clasificar y agrupar los mapas de ocurrencia generados. Para la validación de esta herramienta se usaron mapas de ocurrencia de peces cebra control, expuestos a estrés y estrés más alcohol o cafeína y mostró una precisión de 84% usando 3 cinemáticas (posición, velocidad, aceleración) (Figura 4).

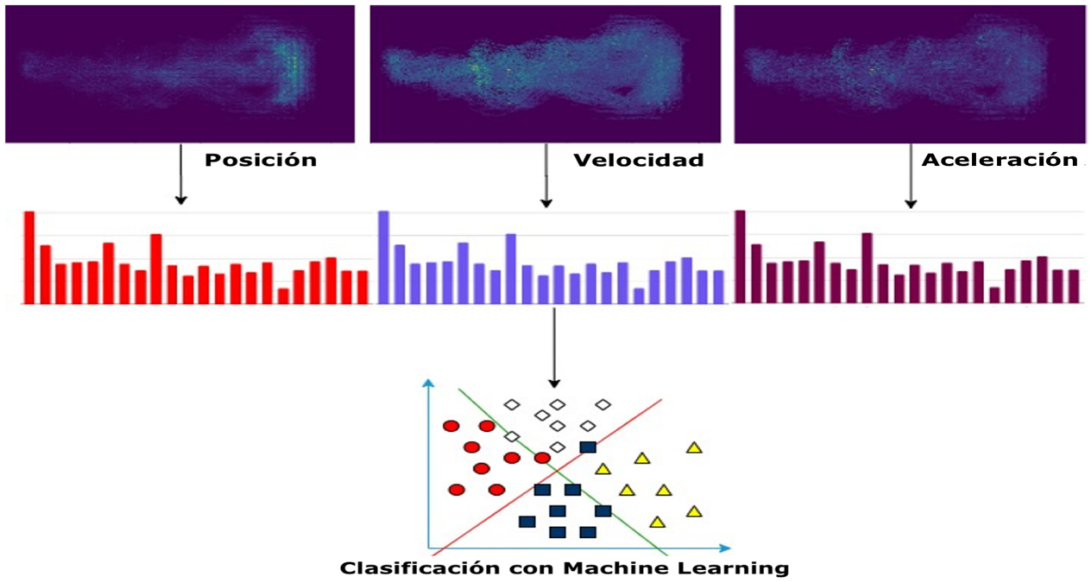

Figura 4. Clasificador de mapas de ocurrencia con aprendizaje de maquina.

Clasificación a partir de los mapas de ocurrencia y con el uso de aprendizaje de maquina.

2.7 Análisis de expresión génica

Uno de los estudios que ha empleado la mayor cantidad de análisis de genes (más de 24.000 genes y secuencias) a nivel cerebral en el pez cebra adulto (6 meses) con exposición a alcohol, demostró un cambio de expresión en múltiples tipos funcionales de genes incluyendo diferencias en la actividad enzimática, moléculas de unión a sitios específicos, transportadores de membrana, actividad de transcripción, hidrolasas, receptores, metabolismo de alcohol entre otros (Pan et al, 2011). En dicho estudio, la exposición a alcohol se realizó de forma continua y gradual por 21 días hasta alcanzar una concentración en el agua de 0.5%. El análisis realizado RT-PCR y microarreglos demostró sobreexpresión del transportador de glucosa dependiente de sodio (slc5a1, 79.34 veces), receptor del factor de crecimiento opioide (ogfr), apolipoproteína A-IV (apoa4, 53.32 veces), carboxipeptidasa B1 (cbp1), alcohol deshidrogenasa (adha8a) y varias familias de citocromo p450, incluyendo 2, 3, 4, 8, 11, 12, 22, 46 y 71. Los genes que demostraron reducción de la expresión fueron el receptor de neuromedina B (nmbr) y el transportador de glicina (slc6a9). Con cambios significativos en la qRT-PCR demostraron disminución de la expresión en el receptor de dopamina (drd3) y el receptor de N-metil D-aspartato (grin1a) (Pan et al, 2011). A pesar de la información existente en este y otros modelos animales, no se conoce a totalidad los mecanismos neuronales moleculares por los cuales el alcohol genera cambios en el comportamiento.

En nuestros resultados, el comportamiento locomotor de los grupos sometidos a estrés y estrés más 0.75% de etanol mostraron diferencias significativas comparado con el grupo control. Por lo tanto, se realizó un estudio piloto de análisis de expresión génica con muestras de tejido cerebral de pez cebra. Los ejemplares fueron sacrificados y se extraía el cerebro después de las pruebas de comportamiento. Para esto se uso crioanestesia a 4°C y se procedió a realizar la disección del cerebro.

Una vez extraído el cerebro se realizó la extracción de RNA (siguiendo el protocolo de TRIzol™ de invitrogen) para posteriormente generar el cDNA (SuperScript® III First-Strand Synthesis System for RT-PCR) y ser procesadas y analizadas por PCR en tiempo real con el equipo CFX96 Touch™ Real-Time PCR Detection System. Un volumen final de 10µL por tubo de PCR fue procesado, usando 2 µL de cDNA, 5 µL de SYBR® Green Master Mix qPCR, 0,8 µL de cada primer y 1,4 µL de agua grado molecular. Los parámetros de la RT-PCR fueron: 95 °C por 2 min, seguido de 40 ciclos a 95°C por 20 segundos y 55 °C por 30 segundos (Adaptado de Theodoridi et al, 2017). Se tomó como gen de referencia el elf1a (factor de elongación 1 alfa) para comparar los niveles de expresión con el método comparativo CT ($2^{-\Delta\Delta CT}$), este gene presenta la menor variabilidad en expresión frente a la exposición a sustancias, etapa del desarrollo y tejidos en pez cebra (McCurley et al, 2008; Parker et al, 2014). Los primers que se usaran para la RT-PCR previamente han sido usado por otros autores (Tabla 2. Producto # 3). Sin embargo, se realizó una comprobación in silico (UCSC in silico PCR) y verificación de homología de los aminoácidos (Clustal Omega).

Tabla 2. Primers usados en RT-QPCR.

Gene	Forward primer	Reverse primer	Reference
slc6a4a	ACTGCACCCACTACCTGTCC	ATGCCAGGAGAACACCAAAG	Emerson et al., 2000
slc6a3	AGACATCTGGGAAGGTGGTG	ACCTGAGCATCATACAGGCG	Barreto et al., 2012
comta	TCACGACCACAGCGCATCT	CCCACATTCATGGCCCATT	Alazizi et al., 2011
bdnf3	GGCGAAGAGCGGACGAATATC	AAGGAGACCATTCAGCAGGACAG	Licino et al., 2002
elf1a	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC	McCurley et al., 2008

Los genes candidatos analizados en el pez cebra por sus funciones neuronales implicadas en mecanismos de adicción, tolerancia al alcohol, cambios en el comportamiento, y para los cuales no hay estudios previos en expresión génica relacionados con la exposición a estrés y sustancias son el transportador y recaptación de serotonina (slc6a4a) (Theodoridi et al, 2017); el transportador de dopamina (scl6a3, previamente conocido como dat) (Huang et al, 2017); el factor neurotrófico derivado del cerebro (bdnf), el cual está relacionado con crecimiento, diferenciación y mantenimiento de las neuronas (Theodoridi et al, 2017); el gen comt que codifica para la enzima catecol -o- metiltransferasa encargado de la degradación de las catecolaminas incluyendo la regulación de los niveles de dopamina (Sagud et al, 2018). El gene elf1a se usó como gen de referencia ya que han demostrado poca variabilidad entre tejidos y con exposición a sustancias (McCurley et al, 2008). Todos los genes mencionados anteriormente expresan proteínas que regulan el comportamiento por diferentes mecanismos moleculares. El alcohol y el ambiente actúan de forma compleja a nivel neuronal y es relevante investigar sus efectos a nivel molecular y relacionarlos con el comportamiento.

2.8 Análisis estadístico

Se aplicó estadística descriptiva a los datos registrados que incluye promedio aritmético y error estándar, en cada grupo. El análisis estadístico se llevó a cabo en el software SPSS statistics 18 de IBM y JASP. Con el fin de evaluar las diferencias en el comportamiento entre grupos se realizo ANOVA de dos o más factores seguido del test de comparaciones múltiples HSD (Honestly-significant-difference) de Tukey con una significancia reportada a partir de $p \leq 0.05$. Se usó t student en un grupo de peces en un diseño pre-post con exposición a 0.75% de etanol para evaluar diferencias significativas. Para la estadística inferencial se empleó el Test de ANOVA de dos vías o más para determinar las diferencias en la expresión de mRNA entre los grupos control y los expuestos a estrés ambiental y sustancias.

Capítulo 3: Justificación

Se plantea que es necesario mejorar las condiciones de salud mental en el país que incluye desafíos en la disminución del consumo de sustancias, violencia intrafamiliar y social, tasa de suicidios y victimas del conflicto armado por mencionar algunos (PDSP). En este contexto, conocer los mecanismos subyacentes al estrés ambiental y la exposición a sustancias es relevante con el fin de mejorar la calidad de vida y los tratamientos de los trastornos psiquiátricos tales como el trastorno depresivo y de ansiedad. A diferencia de, por ejemplo, los cultivos celulares, el pez cebra es un organismo completo que nos brinda la capacidad de analizar su comportamiento, manipular su entorno con factores como el estrés psicosocial y la fácil administración de sustancias.

Los efectos del estrés agudo administrado a los peces cebra evidencia una disminución significativa en patrones locomotores como la aceleración. El estrés asociado a la exposición a alcohol o cafeína acentúa la disminución del comportamiento locomotor afectando la distancia recorrida, velocidad y aceleración. En la prueba con tanque trapezoidal, los peces expuestos a estrés y alcohol aumentan la geotaxis, permaneciendo más en el fondo, posiblemente causado por el efecto depresor del alcohol sumado al estrés.

Los peces expuestos a estrés y cafeína presentaron un comportamiento locomotor similar. La cafeína a altas dosis tiene efecto ansiogénico actuando en receptores A_1 que explica la disminución de la locomoción. En pruebas del comportamiento social, se evidencio un aumento significativo en la cohesión social de los peces expuestos a estrés y dosis más altas de cafeína, permaneciendo mayor tiempo en el área social. El efecto combinado del estrés y la cafeína altera el comportamiento locomotor y la cohesión social.

Desde un punto molecular, se explora como la disminución de la expresión de los genes slc6a3, comt y bdnf alteran la homeostasis sináptica, disminuyendo la biodisponibilidad de neurotransmisores y conduciendo a la neurodegeneración (Figura 5). Sin embargo, se necesitan más estudios moleculares con muestras de tejido cerebral para aclarar los mecanismos agudos del estrés y exposición a sustancias.

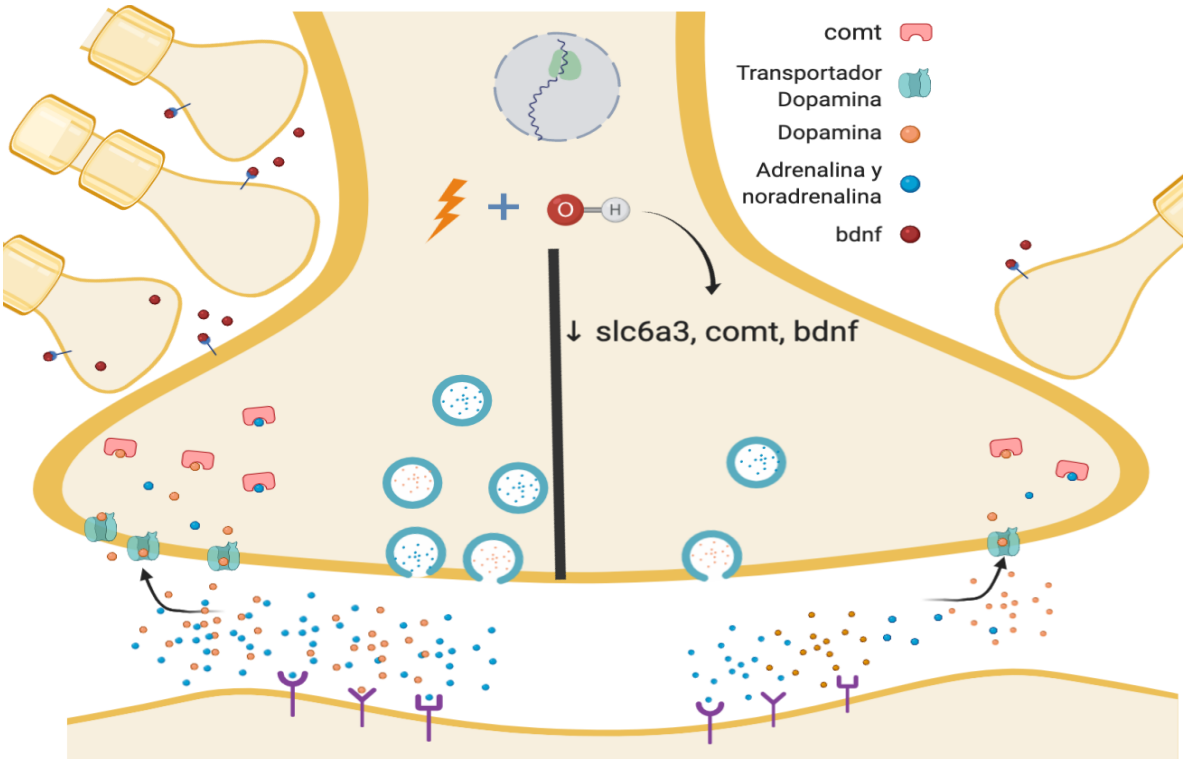

Figura 5. Efecto molecular del estrés y alcohol.

A la izquierda de la línea negra se muestra el funcionamiento normal de una neurona y a la derecha con la expresión disminuida de los genes slc6a3, comt y bdnf por el efecto del estrés y alcohol al 0.75%

El análisis del comportamiento es una labor que toma tiempo y en ocasiones no es objetiva en su totalidad. El uso de herramientas computacionales como la desarrollada en el presente trabajo de tesis disponen de múltiples ventajas tales como ahorro de tiempo en el procesamiento de los videos; libre acceso al algoritmo que se traduce en un impacto económico para los investigadores del comportamiento, neurociencias y farmacología; permite crear mapas de ocurrencia de las principales cinemáticas por individuo y promedios cinemáticos por grupo con desviación estándar; correlación estadística; y clasificar cada individuo según su tratamiento (Figura 6). Este tipo de análisis de clasificación permite visualizar en una gráfica el efecto de un tratamiento administrado con cada uno de los peces.

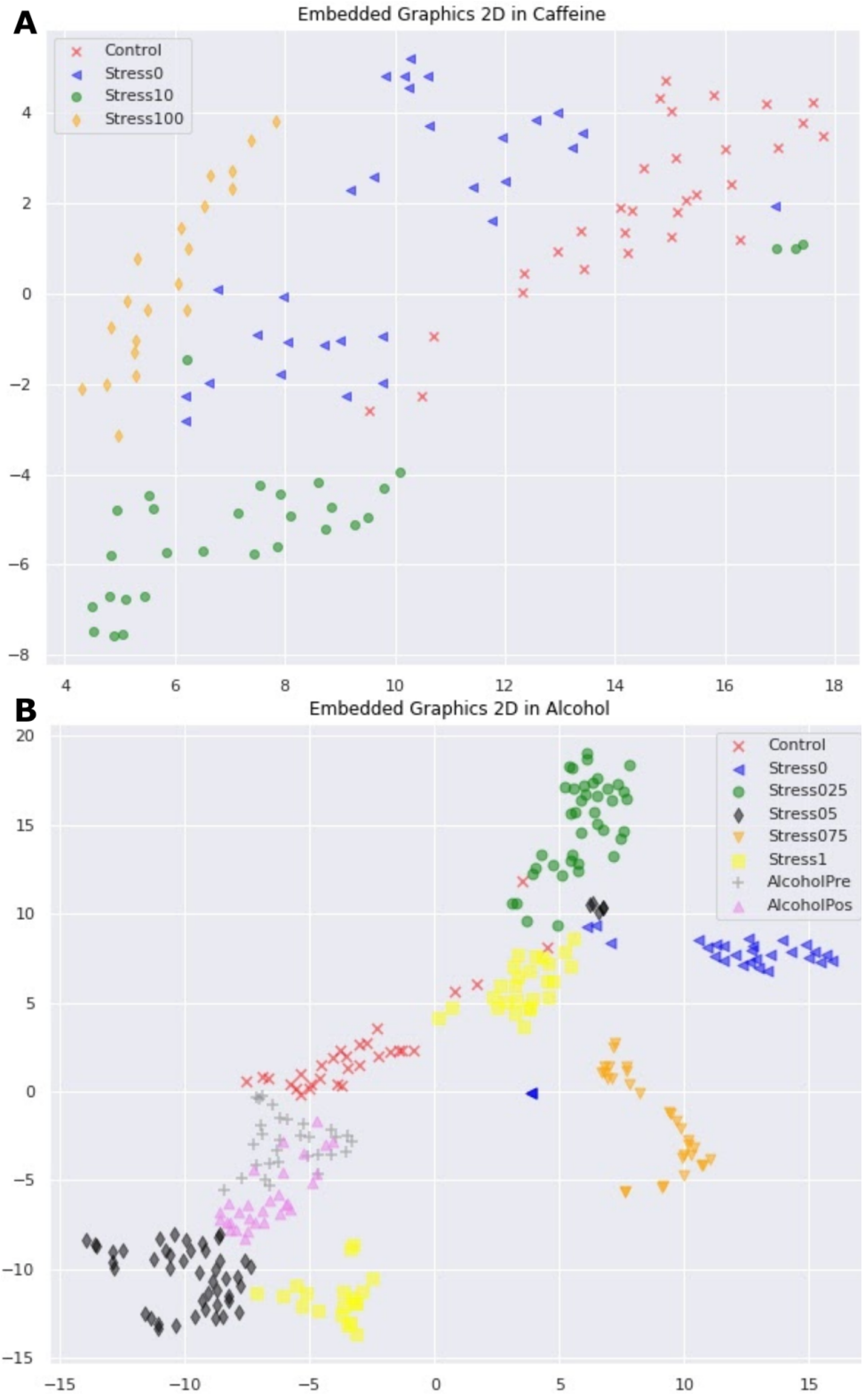

Figura 6. Clasificación de individuos mediante aprendizaje de máquina

A. Clasificación del comportamiento de peces cebra expuestos a estrés y alcohol. **B**. Clasificación en peces cebra expuestos a estrés y cafeína.

Capítulo 4. Producción

Los productos relacionados a continuación están organizados primero los manuscritos y continua con los demás productos en orden cronológico. Los primeros dos manuscritos fueron revisiones, el primero relacionado con la enfermedad de Alzheimer con el fin de comprender y profundizar en los mecanismos genéticos e interacciones a nivel molecular; la segunda revisión es la recopilación de software de uso libre para el análisis de patrones locomotores aplicables al pez cebra y otras especies. Estas dos primeras revisiones fueron la base para realizar los manuscritos originales siguientes. El tercer manuscrito (original) hace parte del análisis de expresión génica y comportamiento de agresividad y locomoción del pez cebra expuesto a estrés y alcohol. El cuarto manuscrito se realizó con datos del comportamiento social del pez cebra sometido a estrés más cafeína y, junto con la colaboración del Dr. Fabio Martínez Carrillo (Biomedical imaging, Vision and Learning Laboratory, Universidad Industrial de Santander), se desarrolló un software de uso libre para el análisis de patrones locomotores a partir de secuencias de video. Con base en el trabajo mencionado anteriormente, se desarrolló un algoritmo de machine learning (quinto manuscrito) para la clasificación de los niveles de estrés y tratamientos farmacológicos en el pez cebra. Adicionalmente, con colaboración internacional del Dr. Gil Rosenthal (Texas A&M University, Faculty of Ecology & Evolutionary Biology, Texas, Unite States), se obtuvieron videos de diferentes especies de peces para verificar la aplicabilidad del algoritmo y se trabajó en conjunto para el desarrollo del quinto producto. Los productos siguientes fueron el entrenamiento en métodos de manejo del pez cebra a nivel de reproducción, mantenimiento y análisis genético. Finalmente, las presentaciones orales y posters en congresos nacionales e internacionales.

4.1 Productos

Clase de Producto	Título	Medio de publicación	Clasificación	Fecha	Estado
1. Manuscrito	APOE Gene and Neuropsychiatric Disorders and Endophenotypes, A Comprehensive Review	American Journal of Medical Genetics, Part B, Neuropsychiatric Genetics	Q1 Impact Factor 2019: 3.123	2016	Publicado
2. Manuscrito	A Review of Freely Available, Open-Source Software for the Automated Analysis of the Behavior of Adult Zebrafish	Zebrafish	Q1 Impact Factor 2018: 1.742	2019	Publicado
3. Manuscrito	Behavioral and Molecular Effects of Alcohol in Tests of Anxiety and	IJPP	Q4 Impact Factor 2018: 0.20	2021	Sometido

	Aggressiveness in a Model of Unpredictable Stress in Adult Zebrafish				
4. Manuscrito	ZebraMov: An automatic strategy to analyze and quantify zebrafish social behaviour from kinematic patterns captured in video sequences.	Zebrafish	Q1 Impact Factor 2019: 1.845	2021	Sometido
5. Manuscrito	Machine learning	?	?	2021	En elaboración
6. Pasantía	Manejo del pez cebra	U. Andes, Facultad de Medicina, Laboratorio de ritmos circadianos	No aplica	14-21 de febrero de 2017	Finalizado
7. Congreso	Presentación oral: Pez cebra (Danio Rerio) como modelo en investigación biomédica, avances en neurobiología	VII Congreso Nacional y III Congreso Internacional de Medicina UAN	No aplica	15/09/2017	Finalizado
8. Seminario	Participante con poster y presentación oral del uso de software en investigación biomédica	III Seminario Internacional Smart Health. Universidad Antonio Nariño	No aplica	02/11/2017	Finalizado
9. Curso	Participante curso	Curso Teórico práctico: Crianza, reproducción y manejo de bioterios de pez cebra. Universidad de Chile y Danio-Biotechnologies	No aplica	27 de noviembre - 1 de diciembre de 2017. 35 horas	Finalizado
10. Congreso	Presentación con poster: Análisis Del Comportamiento De Agresividad Y Ansiedad En Pez Cebra Posterior A	XI Congreso Nacional, XII Seminario Internacional de Neurociencias. Colegio Colombiano de	No aplica	26-28 de abril de 2018	Finalizado

	La Exposición A Estrés Y Etanol	Neurociencias (COLNE)			
11. Congreso	Presentación oral: Análisis del comportamiento en pez cebra posterior a la exposición a estrés ambiental y etanol	International School on Neurogenetics. Colegio Colombiano de Neurociencias (COLNE)	No aplica	2–5 de mayo de 2018	Finalizado
12. Búsqueda pasantía	Presentación oral de avances de tesis doctoral	Departamento de Biología, Laboratorio Dra. Julia Dallman. Universidad de Miami	No aplica	02/11/2018	Finalizado
13. Congreso	Presentación oral: Efectos Comportamentales y Moleculares del Estrés Ambiental y Exposición a Sustancias en el Pez Cebra	Congreso Internacional Virtual de Neurociencias: Cerebro y Comportamiento en Tiempos de COVID-19.	No aplica	26-28/11/2020	Finalizado
14. Pasantía	Análisis de videos del comportamiento y elaboración de manuscrito	Texas A&M University. Pasantía en modalidad virtual.	No aplica	09/2020 01/2021	Finalizado

4.2 Participación en proyectos de investigación

Nombre del proyecto	Entidad	Año	Valor (Especie y contrapartida)
Estudio de los factores ambientales y genéticos para la agresividad, un endofenotipo de la violencia interpersonal y la conducta suicida en una muestra colombiana	VCTI-UAN	2016	10´175.000

Producto 1

REVIEW ARTICLE

AMERICAN JOURNAL OF medical genetics PART B
Neuropsychiatric Genetics

APOE Gene and Neuropsychiatric Disorders and Endophenotypes: A Comprehensive Review

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The Apolipoprotein E (*APOE*) gene is one of the main candidates in neuropsychiatric genetics, with hundreds of studies carried out in order to explore the possible role of polymorphisms in the *APOE* gene in a large number of neurological diseases, psychiatric disorders, and related endophenotypes. In the current article, we provide a comprehensive review of the structural and functional aspects of the *APOE* gene and its relationship with brain disorders. Evidence from genome-wide association studies and meta-analyses shows that the *APOE* gene has been significantly associated with several neurodegenerative disorders. Cellular and animal models show growing evidence of the key role of APOE in mechanisms of brain plasticity and behavior. Future analyses of the *APOE* gene might find a possible role in other neurological diseases and psychiatric disorders and related endophenotypes. © 2016 Wiley Periodicals, Inc.

Key words: Apolipoprotein E; meta-analyses; neuropsychiatric genetics; candidate gene; neurogenetics

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INTRODUCTION

The Apolipoprotein E (*APOE*) gene is one of the main candidates in neuropsychiatric genetics [Villeneuve et al., 2014]. Since the initial findings of an association of the *APOE* gene and Alzheimer's disease (AD) in 1993 [Corder et al., 1993; Strittmatter et al., 1993], hundreds of studies have been carried out in order to explore the possible role of polymorphisms in the *APOE* gene in a large number of neurological diseases, psychiatric disorders, and related endophenotypes [Giau et al., 2015]. In the current article, we provide a comprehensive review of the structural and functional aspects of the *APOE* gene and its relationship with neuropsychiatric disorders and endophenotypes.

AN OVERVIEW OF THE STRUCTURE AND FUNCTION OF THE *APOE* GENE

The *APOE* gene is located on chromosome 19q13.32, with four exons and three introns spanning 3,612 bp; it encodes a protein of 317 amino acids (including a signal peptide region of 18 amino acids) and 36.2 KDa. *APOE* gene is located in a genomic region where other genes encoding apolipoproteins (such *APOC1*, *APOC4*, and *APOC2*) are situated (Fig. 1). The APOE protein aids in lipid transport, facilitating the clearance of triglyceride- and cholesterol-rich lipoproteins from the plasma [Mahley et al., 2009]. As such, the APOE protein has two main functional regions: a

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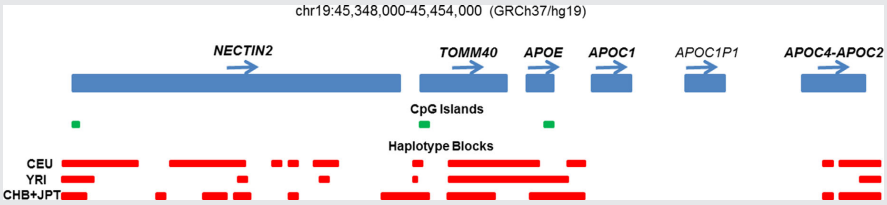

FIG. 1. An overview of the genomic region for the *APOE* gene, highlighting its neighbor genes and their transcriptional directions, locations of the CpG islands, and of the haplotype blocks for three major populations from HapMap. Abbreviations: *NECTIN2*: nectin cell adhesion molecule 2; *TOMM40*: translocase of outer mitochondrial membrane 40; *APOC1*: apolipoprotein C1; *APOC1P1*: apolipoprotein C1 pseudogene 1; *APOC4*: apolipoprotein C4; *APOC2*: apolipoprotein C2; CEU: Utah residents with Northern and Western European ancestry; YRI: Yoruba in Ibadan, Nigeria; CHB + JPT: Han Chinese in Beijing and Japanese in Tokyo. [Color figure can be viewed at wileyonlinelibrary.com].

receptor-binding domain (amino acid residues 154–168) and a major lipid-binding domain (amino acid residues 262–290), connected by a hinge region (amino acid residues 184–242) [Hatters et al., 2006; Frieden and Garai, 2013; Giau et al., 2015]. A CpG island is located on exon 4 of the *APOE* gene [Yu et al., 2013] (Figs. 1 and 2), and no main binding sites for miRNAs have been found on the 3′ untranslated region (UTR) of the *APOE* gene. It has been found that this CpG island is hypermethylated in lymphocytes and postmortem brains of AD patients and controls [Wang et al., 2008]. The rs429358 and rs7412 single nucleotide polymorphisms (SNPs) change the number of CpGs in this CpG island located in an exon, which shows transcriptional enhancer activity in different cell types and regulates the expression of the *APOE* and *TOMM40* genes [Yu et al., 2013]. APOE is expressed in several brain tissues, such as amygdala and prefrontal cortex, in humans and mice [Su et al., 2004] (Fig. 3) and in both neurons and glial cells [Xu et al., 1999]. APOE expression is regulated by complex transcriptional networks [Bekris et al., 2012], including Activating Protein 2 (AP-2) and Activating transcription factor 4 (ATF4) transcription factors and the nerve growth factor-mediated activation of mitogen-activated protein kinase and protein kinase C [Garcia et al., 1996; Geng et al., 2011; Strachan-Whaley et al., 2015]. The nuclear factor-κB (NF-κB) signaling pathway has been shown as involved in the regulation of APOE expression in cells treated with high levels of homocysteine [Trusca et al., 2016].

APOE has a key function in the receptor-mediated endocytosis of lipoproteins in the brain: it has been shown that cholesterol from APOE-containing lipoproteins is important for synaptogenesis and for the maintenance of neuronal connections [Holtzman et al., 2012]. It is known that APOE binds to receptors such as the low-density lipoprotein receptor (LDLR)-related protein 1 (LRP1), very low density lipoprotein receptor (VLDLR) and APOE receptor 2 (APOER2), which are expressed in neurons and glial cells (particularly astrocytes and microglia) [Fan et al., 2001]. The binding of APOE to APOER2 leads to the activation of the reelin pathway and N-methyl-D-aspartate (NMDA) receptors [Chen et al., 2010], among other mechanisms of importance for brain and synaptic plasticity. Among the downstream proteins and pathways of relevance for brain function are: c-Jun N-terminal kinase (JNK), glycogen synthase kinase 3 beta (GSK3B), and the brain-derived neurotrophic factor (BDNF) [Hoe et al., 2005; Herz and Chen, 2006; Bu, 2009; Holtzman et al., 2012] (Fig. 4).

POLYMORPHISMS IN THE HUMAN *APOE* GENE

The two main polymorphisms in the *APOE* gene are two common SNPs located on exon 4 (rs429358 and rs7412), which

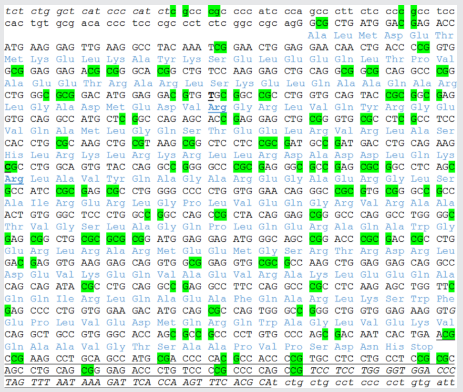

FIG. 2. DNA and protein sequences for the exon 4 of *APOE* gene. CpG dinucleotides are highlighted in green and regions outside the CpG island are highlighted in italic. DNA sequence of the 3′UTR region is shown as underlined. The SNPs and codons 130 and 176 are shown in bold. [Color figure can be viewed at wileyonlinelibrary.com].

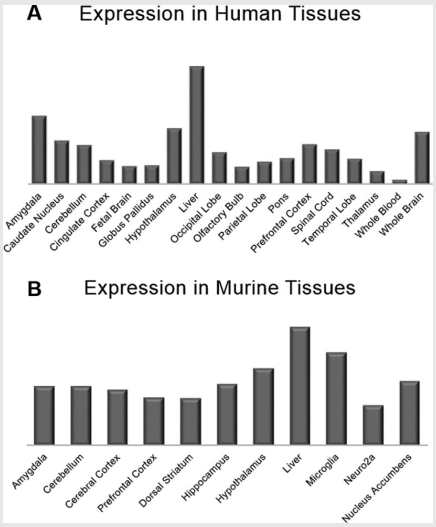

FIG. 3. An overview of the relative expression of APOE in several human and murine brain tissues.

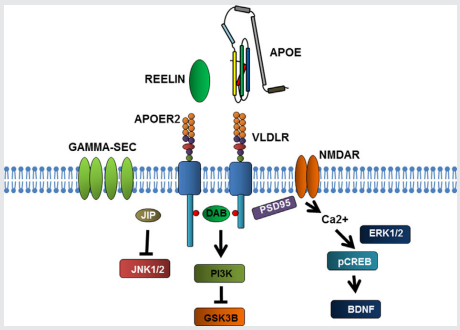

FIG. 4. An overview of functional interactions of APOE in the brain. Abbreviations: APOER2: apolipoprotein E receptor 2; NMDAR: NMDA receptors, VLDLR: very low density lipoprotein receptor; GSK3B: glycogen synthase kinase 3 beta; DAB: disabled; PSD: postsynaptic density protein 95; BDNF: brain-derived neurotrophic factor; CREB: cAMP response element binding protein; ERK1/2: extracellular signal-regulated protein kinases 1 and 2; PI3 K: phosphoinositide 3-kinase; GAMMA-SEC: gamma-secretase complex; JNK1/2: c-Jun N-terminal kinases 1 and 2; JIP: JNK-interacting protein. [Color figure can be viewed at wileyonlinelibrary.com].

lead to the p.Cys130Arg and p.Arg176Cys non-synonymous changes on the encoded protein (Fig. 2). Combinations of these two SNPs generate the commonly defined E2, E3, and E4 alleles, with the E4 being the ancestral allele [Eisenberg et al., 2010]. APOE E3, the most common allele, has the combination Cys/Arg at those two amino acid residues (T/C at DNA level), and APOE E2 and APOE E4 have the Cys/Cys and Arg/Arg combinations, respectively (T/T and C/C, at DNA level) [Hatters et al., 2006]. APOE E3 and E4 have a similar binding affinity to low-density lipoprotein (LDL) receptor but the APOE E2 isoform is less effective in binding to this receptor [Hatters et al., 2006].

There are known ethnic and geographical differences for the frequencies of the *APOE* E2/E3/E4 alleles around the world [Eisenberg et al., 2010] (Fig. 5). Available genotyping methods for *APOE* E2/E3/E4 include multiplex tetra-primer amplification refractory mutation system (multiplex T-ARMS) [Yang et al., 2007], PCR- Restriction Fragment Length Polymorphism (RFLP) [Hixson and Vernier, 1990], TaqMan [Koch et al., 2002], allele-specific amplifications in conventional PCR [Pantelidis et al., 2003], or in qPCR [Calero et al., 2009]. In addition, there are other polymorphisms located in the promoter region, in introns and in exons of the *APOE* gene (Table I). There is a relative low level of linkage disequilibrium in this genomic region [Yu et al., 2007] and a haplotype block covering portions of *TOMM40* and *APOE* genes is found in CEU and YRI populations (Fig. 1).

EVIDENCE OF THE INVOLVEMENT OF THE *APOE* GENE IN NEUROLOGICAL DISEASES AND PSYCHIATRIC DISORDERS AND RELATED ENDOPHENOTYPES

The *APOE* E4 allele is the largest known genetic risk factor for AD. The lifetime risk for AD in *APOE* 4/4 carriers, at age 85, is estimated to be around 51% and 61% in males and females, respectively [Genin et al., 2011]. For *APOE* 3/4 carriers it is estimated to be around 23% and 30% for males and females, respectively. Therefore, *APOE* has been considered a major gene with a semi-dominant inheritance [Genin et al., 2011]. In addition, to the strong and consistent association of the *APOE* E4 allele with the increased risk for AD [Rubinsztein and Easton, 1999], other meta-analyses have found significant evidence for the role of *APOE* in neurodegenerative diseases and psychiatric disorders [Deb et al., 2000; Verpillat et al., 2002; Huang et al., 2004; Sudlow et al., 2006; Lopez-Leon et al., 2008; Peck et al., 2008; Williams-Gray et al., 2009; Xin et al., 2009; Elias-Sonnenschein et al., 2011; Maxwell et al., 2011; Varvarigou et al., 2011; Xuan et al., 2011; Khan et al., 2013; Rannikmae et al., 2013; Rubino et al., 2013; Govone et al., 2014; Wei et al., 2014; Zhang et al., 2014; Feng et al., 2015; Gonzalez-Castro et al., 2015; Li et al., 2015a; Liu et al., 2015; Sun et al., 2015]. Table II lists the candidate gene studies in which a meta-analysis was conducted to evaluate the risk of carrying the different variants in the *APOE* gene. Associations between the *APOE* E4 allele and the following outcomes were reported: late life depression, schizophrenia,

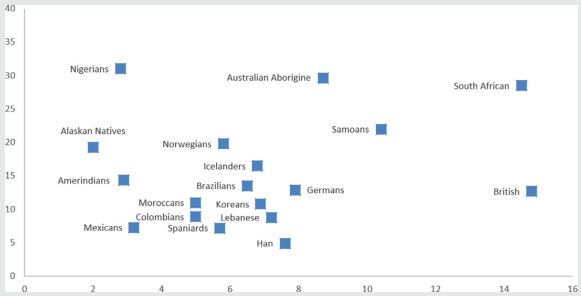

FIG. 5. An overview of frequencies for the commonly studied *APOE* polymorphisms in global populations. Relative frequencies for *APOE* E2 and E4 alleles are shown on the X and Y axis, respectively. [Color figure can be viewed at wileyonlinelibrary.com].

migraine, earlier age of temporal lobe epilepsy, poor outcome of traumatic brain injury, Creutzfeld Jakob and all types of stroke, brain microbleeds, and dementia. *APOE* E2 was associated with frontotemporal dementia, Parkinson disease, and stroke. One meta-analysis reported an association between multiple sclerosis and *APOE* E2/E3 carriers. In addition, genome-wide association studies have identified highly significant associations with SNPs located in the *APOE* genomic region for neurodegenerative diseases (Table III) [Jun et al., 2010; Seshadri et al., 2010; Logue et al., 2011; Wijsman et al., 2011; Hollingworth et al., 2012; Kamboh et al., 2012a,b; Lambert et al., 2013; Miyashita et al., 2013; Reitz et al., 2013; Beecham et al., 2014; Ferrari et al., 2015]. The fact that several of these highly associated SNPs are located on other genes (such as *TOMM40*), could be explained by the linkage disequilibrium with *APOE* and the relative low coverage of this gene in SNP chips [Radmanesh et al., 2014].

APOE E4 has been associated to AD, since it has been proposed that this isoform is related to decrease in synaptic function, glucose metabolism, neurogenesis, β-amyloid (Aβ) clearance, mitochondrial functions, and with increase in neuronal toxicity, Aβ aggregation, among others [Liu et al., 2013]. Study of endophenotypes of relevance for neuropsychiatric disorders (such as cognitive and neuroimaging dimensions and biomarkers) has identified significant associations in both meta-analysis of candidate gene studies and genome-wide association studies (GWAS) (Tables II and III) [Wisdom et al., 2011; De Jager et al., 2012; Cruchaga et al., 2013; Cao et al., 2014; Ramirez et al., 2014; Zhang and Pierce, 2014; Chauhan et al., 2015; Davies et al., 2015; Debette et al., 2015; Liu et al., 2015; Li et al., 2016]. Other neuropsychiatric symptoms and endophenotypes commonly present in Alzheimer's disease patients that have evidence of an association with *APOE* E4 allele are depression, anxiety, apathy, agitation, aggression, hallucinations, and delusions [Borroni et al., 2010; Panza et al., 2011, 2012].

Several studies have explored the possible neuropsychological and functional alterations in young subjects that are carriers of the *APOE* 4 allele [Greenwood et al., 2005; Alexander et al., 2007;

TABLE I. Main Common Genetic Variants Located in the Human *APOE* Gene

dbSNP ID	Genomic position	Alleles	MAF (EA/AA)	Annotation	Protein variant/alias[a]
rs449647	45408564	T > A	0.16/0.33	5′ region	−491
rs405509	45408836	C > A	0.48/0.24	5′ region	Th1/E47cs
rs440446	45409167	G > C	0.36/0.12	Intron	
rs769449	45410002	G > A	0.12/0.00	Intron	
rs769450	45410444	G > A	0.41/0.35	Intron	
rs429358	45411941	T > C	0.16/0.27	Missense	p.(C130R)
rs769455	45412040	C > T	0.00/0.03	Missense	p.(R163C)
rs7412	45412079	C>T	0.06/0.10	Missense	p.(R176C)
rs34078567	45413225	− > G	0.41/0.32	3′ region	
rs75627662	45413576	C > T	0.19/0.16	3′ region	

dbSNP ID, identifier from the database of short genetic variation (dbSNP); MAF, minor allele frequency; EA, European Americans; AA, African Americans.
[a]Numbering for the complete protein of 317 amino acids (UniProt Accession: P02649).

TABLE II. Meta-Analyses of Case-Control Studies Assessing the Association of Neuropsychiatric Disorders and Endophenotypes and *APOE* Gene Polymorphisms

Disease/endophenotype	Author, year	Studies/N	Model	Highest OR /beta/ d (95%CI)	*P*-value
Late life depression	Feng et al. [2015]	20/6,131	E4	**1.30 (1.06–1.59)**	0.01
Major depressive disorder	Lopez-Leon et al. [2008]	7/2,443	E2 vs. E3	**0.51 (0.39–0.68)**	NR
Schizophrenia	Gonzalez-Castro et al. [2015]	28/8,155	E3	**0.73 (0.54–0.98) Asians**	NR
Delirium	Adamis et al. [2016]	8/1,762	E4	Log OR 0.18 (−0.23 to 0.59)	0.38
Late onset Alzheimer's disease	Rubinsztein and Easton [1999]	31/NR	E4	**3.18 (2.93–3.45)**	<0.001
			E4/E4	**11.57 (8.67–15.44)**	
Alzheimer's disease—conversion from mild cognitive impairment	Elias-Sonnenschein et al. [2011]	35/6,095	E4	**2.29 (1.88–2.80)**	NR
Dementia—frontotemporal	Verpillat et al. [2002]	10/5,653	E2 vs. E3	**2.01 (1.02–3.98)**	0.04
Dementia—vascular	Sun et al. [2015]	37/NR	E4	**1.83 (1.56–2.13)**	<0.001
Dementia in Parkinson	Williams-Gray et al. [2009]	10/14,264	E2	**1.16 (1.03–1.31)**	0.02
Ischemic stroke	Khan et al. [2013]	74/91,903	E4/E4	**1.15 (1.09–1.21)**	NR
Hemorrhagic stroke	Peck et al. [2008]	11/4,807	E4 carriers	1.17 (0.98–1.40)	0.08
Subarachnoid hemorrhage	Sudlow et al. [2006]	31/23,926	E4 carriers	**1.42 (1.01–1.99)**	NR
Ischemic stroke				**1.11 (1.01–1.22)**	
Early-onset ischemic stroke	Xin et al. [2009]	26/664	E4 carriers	**2.53 (1.71–3.73)**	NR
Intracerebral hemorrhage	Zhang et al. [2014]	11/4,813	E4	**1.42 (1.21–1.67)**	<0.001
Brain microbleeds	Maxwell et al. [2011]	10/7,351	E4 carriers	**1.22 (1.05–0.144)**	0.01
Creutzfeld Jakob	Wei et al. [2014]	11/2,212	E4/E4	**3.16 (1.37–7.26)**	0.007
Multiple sclerosis	Xuan et al. [2011]	20/10,199	E4	0.99 (0.86–1.16)	>0.05
Amyotrophic lateral sclerosis	Govone et al. [2014]	13/14,646	E4	1.18 (0.91–1.53)	NR
Parkinsońs disease	Huang et al. [2004]	22/9,988	E2	**1.20 (1.02–1.42)**	NR
Unfavorable functional outcome after TBI	Li et al. [2015a]	12/2,628	E4	**1.36 (1.07–1.74)**	0.01
Cerebral amyloid angiopathy	Rannikmae et al. [2013]	24/3,520	E4	**2.67 (2.31–3.08)**	<0.001
Hippocampal volume	Liu et al. [2015]	14/1,628	E4 carriers	**−0.47 (−0.82** to **0.13)**	0.007
Amyloid deposition				**0.62 (0.27–0.98)**	
Frontotemporal lobar degeneration	Rubino et al. [2013]	28/8,370	E4 carriers	**1.94 (1.43–2.64)**	<0.01
Postoperative cognitive dysfunction	Cao et al. [2014]	9/4,046	E4	**1.83 (1.18–2.85)**	NR
Cognitive functioning	Wisdom et al. [2011]	77/40,942	E4 carriers		
Episodic memory				**−0.14 (−0.21, −0.07)**	<0.01
Global cognitive ability				**−0.05 (−0.10, −0.004)**	<0.05
Executive functioning				**−0.06 (−0.12, −0.004)**	<0.05
Perceptual speed				**−0.07 (−0.13, −0.01)**	<0.05

d, standardized difference; LOAD, late onset Alzheimer's disease; NR, not reported; OR, odds ratio; PET, positron emission tomography; WMH, white matter hyperintensity.
Bold $P = 0.05$.

Rusted et al., 2013; Shine et al., 2015; Sinclair et al., 2015; Stening et al., 2016; Su et al., 2016] (Table IV). *APOE* E4 has not only been associated with worse episodic memory but it has also been found that in young adults *APOE* E4 is associated with better performance in episodic and working memory, executive function, and verbal fluency [Hubacek et al., 2001; Bunce et al., 2011; Rusted et al., 2013]. Several of these studies included persons younger than 35 years of age. The study by Alexander et al. [2007] was performed in individuals aged 6–65 and they found that *APOE* E4 carriers in the oldest age group (51–65 years) had a better verbal fluency

TABLE III. Genome Wide Association Studies and the *APOE* Genomic Region

Disease/endophenotype	Author year	N initial/replication	Reported gene (strongest)	Strongest association	OR or beta (95%CI)	*P*-value
LOAD	Kamboh et al. [2012b]	2,229/6,063	TOMM40 APOE APOC1 PVRL2	rs10119	2.12	**3.14E-85**
LOAD	Logue et al. [2011]	1,009	PVRL2 APOE TOMM40	rs6859-A	1.58	5.39E-07
LOAD	Jun et al. [2010]	15,239	APOE	APOE-E4	(1.80–9.05)	**6.10E-94**
LOAD	Seshadri et al. [2010]	17,646/7,360/10,328	TOMM40	rs2075650-G	2.53 (2.41–2.66)	**1.04E-295**
LOAD	Lambert et al. [2013]	54,162/19,884	APOE	NR	NR	**5.00E-28**
LOAD	Miyashita et al. [2013]	1,735 /26,109	APOE PVRL2 TOMM40	rs429358	5.5 (4.4–6.9)	**2.46E-49**
LOAD	Reitz et al. [2013]	5,896	APOE	rs429358	2.31 (2.19–2.42)	**5.50E-47**
LOAD	Wijsman et al. [2011]	3,839/1,093	TOMM40 APOE	rs2075650	NR	**3.20E-81**
Alzheimer's disease with psychosis	Hollingworth et al. [2012]	7,693	TOMM40	rs157582	2.3	**9.28E-52**
Lewy body disease	Beecham et al. [2014]	3,526	APOE	rs429358-C	0.49 (SE: 0.07)	**2.83E-11**
Frontotemporal dementia	Ferrari et al. [2015]	1,456	PVRL2 TOMM40	rs6857-T	1.7 (1.46–1.94)	8.33E-06
Neuritic plaques	Beecham et al. [2014]	4,232	PVRL2	rs6857-T	0.95 (SE: 0.07)	**3.00E-47**
Neurofibrillary tangles	Beecham et al. [2014]	4,707	PVRL2	rs6857-T	0.66 (SE: 0.05)	**4.73E-47**
Age of onset Alzheimer's disease	Kamboh et al. [2012a]	2,222	APOC1	rs4420638	2.2	**1.11E-12**
Age-related cognitive decline	Zhang et al. [2014]	6,655	TOMM40 APOE	rs115881343	0.044	**6.60E-11**
General cognitive function	Davies et al. [2015]	53,949	TOMM40 APOE	rs10119	NR	**5.67E-09**
Verbal declarative memory	Debette et al.[2015]	29,076/14,428	APOC1 PVRL2 TOMM40	rs4420638-A	NR	**1.36E-16**
Neuroimaging measures in AD	Li et al. [2016]	764	APOE	APOE-E4	NR	2.00E-03
Cerebral amyloid angiopathy	Beecham et al. [2014]	2,807	PVRL2	rs6857-T	0.67 SE: 0.07)	**2.92E-21**
Cingulate cortical AB load	De Jager et al. [2012]	749/2,279	APOC1 TOMM40	rs4420638	NR	**3.74E-27**
Hippocampal atrophy	Chauhan et al. [2015]	19,724	TOMM40	rs2075650	0.04 (SE: 1.02)	0.0054
CSF AB1-42 levels	Ramirez et al. [2014]	363/515	APOE	rs429358	0.40 (SE: 0.05)	**4.30E-17**
CSF tau	Cruchaga et al. [2013]	1,278	APOE TOMM40	rs769449-A	0.082	**1.96E-16**

CSF, cerebrospinal fluid; LOAD, late onset Alzheimer's disease; N, number of persons; OR, odds ratio; SE, standard error.
Bold $P = <5E\text{-}08$.

TABLE IV. An Overview of Neuropsychological Studies Carried Out in Young Subjects Carriers of the *APOE* E4 Allele

Neuropsychological measures	Sample size	Age (mean, SD)	Main findings	Reference
Trail making test, Part A; Trail making test, Part B; Letter digit substitution; Mental rotation (episodic and spatial memory); Object location task (episodic and spatial memory)	123	19–35 (14.9, 3.3)	*APOE* E4 carriers showed superior performance in several memory tasks, especifically in spatial function and memory and object location memory.	Stening et al. [2016]
MMSE; MoCA; Visual space/executive ability (score); NCT-A; NCT-B; Vocabulary Learning; Delayed recall; Word fluency; Graph recall; Visual recognition; Digit span; Similarity test; DST	83	18–30 (24.2, 2.4)	All of the neuropsychological tests showed no differences among groups (all $P > 0.05$).	Su et al. [2016]
RVIP (sustained attention); Covert attention task; Iimmediate free recall of word list (episodic memory); PM (Prospective memory)	41	18–20	Individuals with ε4 allele showed better task performance in attention. No significant differences between the groups on measures of verbal IQ or episodic memory	Rusted et al. [2013]
One-back visual working memory task; Visual odd-one-out paradigm	30	College age	*APOE* ε4 carriers showed the worst performance in perceptual task for the recognition of faces and objects, as well as working memory.	Shine et al. [2015]
N-back task (working memory)	2,135	18	A decline in performance was found in several of the groups, including E2/2, E3/4, and E4/4 groups. Faster reaction times were found in E3/4 and E4/4.	Sinclair et al. [2015]
Visual working memory; Word generation (phonetic fluency and semantic fluency); Executive maze task	415	6–65	E4 carriers showed superior performance on phonetic and semantic fluency. E3 subjects showed superior performance in an executive maze task. E4 carriers had better verbal fluency in the oldest age group (51–65 years).	Alexander et al. [2007]
Mattis; Buschke; WMS	177	41–85 (59.9, 0.9)	Accuracy declined and reaction time increased in E4 homozygotes. Inheritance of at least one allele of the *APOE* E4 gene was associated with a specific impairment in visuospatial attention and working memory, ε4 homozygotes showed reduced ability to retain memory for location.	Greenwood et al. [2005]

MMSE, mini-mental state examination; MoCA, montreal cognitive assesment; NCT-A/B, number connection test type A/B; DST, digit symbol test; RVIP, rapid visual information processing; PM, event-related prospective memory; Mattis, Mattis dementia rating scale; Buschke, Buschke selective reminding test; WMS, Wechsler memory scale; SD, standard deviation.

compared to *APOE* E3 carriers. The study by Greenwood et al. [2005] included individuals aged 41–85 and they reported an increase in reaction time for the *APOE* E4 homozygotes.

It has been found that education in young adults might reduce the effects of *APOE* E4 on metabolism independently of amyloid deposition in cognitively normal adults [Arenaza-Urquijo et al., 2015]. Moreover, the education-related increased metabolism in *APOE* E4 carriers was positively associated with episodic memory performance [Arenaza-Urquijo et al., 2015]. The possibility that *APOE* E4 confers cognitive benefits in young adults while becoming a risk factor for cognitive impairment and AD in later life is of considerable theoretical interest [Alexander et al., 2007]. In early life, a particular genotype may have evolutionary benefits (survival and selection), while in old age it may become a risk factor for diseases; this relationship between *APOE* E4 allele and cognition throughout life is an example of antagonistic pleiotropy, a concept of evolutionary biology that suggests that individual alleles have different effects on the performance at different ages [Han and Bondi, 2008], although there are other studies that do not support this association [Mathias and Wheaton, 2015].

A polymorphism located in the promoter region of the *APOE* gene (rs449647) has been associated with AD in meta-analyses [Xin et al., 2010]. There is the possibility that additional rare non-synonymous changes in the *APOE* gene may also be associated with neuropsychiatric disorders [Medway et al., 2014].

AN OVERVIEW OF CELLULAR MODELS FOR THE *APOE* GENE

Taking into account the association between *APOE* gene and neuropsychiatric disorders, several studies have evaluated, using in vitro models, the functional effects of the three APOE isoforms in different types of brain cells (Table V). These studies have used primary cultures, mainly from mice, for neurons [Nathan et al., 1994; Jordan et al., 1998; Tolar et al., 1999; Qiu et al., 2003; Hoe et al., 2005; Ye et al., 2005; Chen et al., 2010; Zhu et al., 2015], astrocytes [Hu et al., 1998; Gong et al., 2002; Koistinaho et al., 2004; Guo et al., 2006; Liu et al., 2012; Cudaback et al., 2015; Chung et al., 2016; Simonovitch et al., 2016], and microglia [Laskowitz et al., 1997; Chen et al., 2005; Brown et al., 2008; Jiang et al., 2008; Cudaback et al., 2011; Li et al., 2015b]. In those experiments, brain cells were purified from transgenic mice for the different APOE isoforms or were exposed to recombinant APOE proteins. It is important to mention that few studies related to APOE function have used human neuronal or glial cell lines, which are commonly employed in neuroscience research [Kovalevich and Langford, 2013].

In neuronal cells, both APOE E3 and APOE E4 have been shown to reduce branching, whereas APOE E3 increased neurite extension and APOE E4 decreased neurite extension, [Nathan et al., 1994]. Moreover, whereas APOE E4 can contribute to neurotoxicity [Tolar et al., 1999; Ye et al., 2005], APOE E3 can be neuroprotective; for example, a study observed that the pretreatment of neurons with the E3 isoform prevented toxicity induced by Aβ a molecule involved in AD) [Jordan et al., 1998]. It has been shown the functional interaction of APOE E4 with several signaling mechanisms, such as the NMDA receptor-dependent and extracellular signal-regulated kinase (ERK) and Akt pathways [Qiu et al., 2003; Hoe et al., 2005; Chen et al., 2010; Zhu et al., 2015] (Fig. 4).

Astrocytes play an important role in the metabolism of APOE, since these cells are the main synthesizers of APOE in the brain, although it has also been demonstrated that astrocytes secrete factors that stimulate expression of APOE in neurons [Gong et al., 2002; Harris et al., 2004]. Cholesterol secreted by glial cells in APOE-containing lipoproteins has been identified as a key factor in a rat model of synaptogenesis in the central nervous system [Mauch et al., 2001]. In astrocytes, it has been shown that APOE regulates their activation after exposure to Aβ peptide [Hu et al., 1998; Koistinaho et al., 2004] and that APOE E4 regulates the expression of other genes, such as monoamine oxidases A and B [Guo et al., 2006; Liu et al., 2012; Cudaback et al., 2015]. More recently, the APOE E4 isoform has been implied in impaired autophagy and lower clearance of Aβ plaques in astrocytes [Simonovitch et al., 2016] and it has been shown that APOE E2 increases phagocytosis of synapses by astrocytes in vitro [Chung et al., 2016].

The APOE E4 isoform has also been shown to modulate the inflammatory response [Brown et al., 2008] in the brain: in microglial cells this isoform stimulates secretion of prostaglandin E2, cyclooxygenase, and interleukin 1 beta [Chen et al., 2005; Li et al., 2015b] and it reduces microglial migration [Cudaback et al., 2011] and Aβ proteolysis [Jiang et al., 2008] (Table V).

Of potential interest for possible therapeutic approaches, almost 104,000 compounds were tested in a high-throughput screening in the human CCF-STTG1 astrocytoma cell line and a chrysanthemic ester was found to increase significantly APOE expression and secretion [Fan et al., 2016]. These cell models have been useful to identify the molecular and cellular effects of the APOE isoforms on the different brain cell types.

AN OVERVIEW OF ANIMAL MODELS FOR THE *APOE* GENE

Animal models for behavioral and functional changes associated to the *APOE* gene have used knockout [Gordon et al., 1995; Masliah et al., 1995; Oitzl et al., 1997; Robertson et al., 2005; Siegel et al., 2011; Lane-Donovan et al., 2016; Lee et al., 2016], transgenic [Raber et al., 1998; Hartman et al., 2001; Harris et al., 2003; Levi et al., 2003; Robertson et al., 2005; Korwek et al., 2009; Nichol et al., 2009; Siegel et al., 2011], and knockin approaches [Grootendorst et al., 2005; Blain et al., 2006; Korwek et al., 2009; Li et al., 2009; Nichol et al., 2009; Tu et al., 2009; Andrews-Zwilling et al., 2010; Chen et al., 2010; Shinohara et al., 2016]. These animal models highlighted the importance of the *Apoe* gene and showed the deleterious effect of the *APOE* E4 allele and the protective effect of the *APOE* E2 allele for a correct neurological function. The majority of these studies focused on memory performance measured by the water maze test and several works have used paradigms for the analysis of exploratory behavior and anxiety [Raber et al., 1998; Hartman et al., 2001; Robertson et al., 2005] (Table VI).

TABLE V. An Overview of Studies in Cellular Models Showing the Functional Effects of the Three APOE Isoforms (E2, E3, and E4)

Cell type	Cell model	Main results	Reference
Neurons	Primary culture of neurons from DRG (rabbit)	APOE E3 increased neurite extension and decreased branching; APOE E4 reduced both neurite extension and branching.	Nathan et al. [1994]
	Primary culture of hippocampal neurons (rat)	APOE E3 treatment prevented the toxicity induced by β-amyloid (Aβ)	Jordan et al. [1998]
	Primary culture of hippocampal neurons (rat)	APOE E4 and synthetic Apoe-related peptide increased the intracellular calcium levels and cell death.	Tolar et al. [1999]
	Primary culture of hippocampal neurons (rat)	APOE E4 increased neurotoxicity by increasing intracellular calcium levels with changes in the calcium response to NMDA	Qiu et al. [2003]
	Neuroblastoma B103 cells (rat)	APOE E4 increased Aβ production more effectively than APOE E3 mediated by the LRP pathway.	Ye et al. [2005]
	Primary culture of cortical neurons (mouse)	APOE E4 induced less phosphorylation of AKT and ERK1/2 and reduces phosphorylation of JNK, APOE-mediated ERK activation depends on calcium influx and NMDA receptor and APOE-mediated JNK inactivation depends on gamma-secretase.	Hoe et al. [2005]
	Primary culture of cortical neurons (mouse)	APOE E4 Led to a decrease of Apoer2 with consequent reduction in the ability to phosphorylate NMDA receptors	Chen et al. [2010]
	Primary culture of neurons (mouse)	APOE E4 induced reduction in PIP2 levels, compared with APOE E3.	Zhu et al. [2015]
Astrocytes	Primary culture of astrocytes (rat)	Exogenous Apoe attenuated astrocyte activation induced by Aβ1-42.	Hu et al. [1998]
	Primary culture of astrocytes (mouse)	APOE E3 astrocytes released more cholesterol than APOE E4 astrocytes.	Gong et al. [2002]
	Primary culture of astrocytes (mouse)	Apoe−/− astrocytes did not degrade Aβ present in Aβ plaque-bearing brain sections.	Koistinaho et al. [2004]
	Primary culture of astrocytes (rat)	APOE E2 had not effect on Aβ-induced MMP-9 expression, whereas APOE E4 decreased MMP-9.	Guo et al. [2006]
	Glioma C6 cell line (rat)	In cells transfected with APOE E4 and APOE E2, the levels of melatonin were higher than in APOE E3 cells. APOE E4 also had lower levels of MAOA and MAOB mRNA expression.	Liu et al. [2012]
	Primary culture of astrocytes (mouse)	APOE E4 modulated secretion of CCL3 by astrocytes.	Cudaback et al. [2015]
	Primary culture of astrocytes (mouse)	APOE E4 showed impaired autophagy and lower elimination of Aβ plaques.	Simonovitch et al. [2016]
	Primary culture of astrocytes (mouse)	APOE E2 increases and APOE E4 decreases phagocytosis of synapses by astrocytes.	Chung et al. [2016]
Microglia	Primary culture of microglia (mouse)	Preincubation with Apoe suppressed the secretion of TNFα by glia after LPS stimulation.	Laskowitz et al. [1997]
	Primary culture of microglia (rat)	APOE E4 stimulated secretion of PGE2 and IL-1β	Chen et al. [2005]
	Primary culture of microglia (mouse)	Apoe promoted the proteolytic degradation of soluble Aβ in microglia. APOE E4 showed impaired ability to promote Aβ proteolysis, compared with the APOE E2 and APOE E3 isoforms.	Jiang et al. [2008]
	Primary culture of microglia (mouse)	Anti-inflammatory response by 17β-Estradiol was reduced in APOE E4 cells	Brown et al. [2008]
	Primary culture of microglia (mouse)	APOE E4 and APOE E2 showed significantly reduced C5a- and ATP-stimulated migration, compared with APOE E3.	Cudaback et al. [2011]
	Primary culture of microglia (mouse)	APOE 4/4 cells treated with PIC and LPS induced higher expression of COX, via TLR3 and TLR4.	Li et al. [2015b]

DRG, dorsal root ganglion; APOE3, apolipoprotein E, isoform 3; APOE4, apolipoprotein E, isoform 4; NMDA, N-methyl-D-aspartate receptor; Aβ, β-amyloid peptide; LRP, LDL receptor-related protein; Apoer2, ApoE receptor-2; PIP_2, phosphoinositol biphosphate; MMP-9, matrix metallopeptidase 9; MAOA, monoamine oxidase A; MAOB, monoamine oxidase B; CCL3, C-C motif chemokine ligand 3; TNFα, tumor necrosis factor-α; LPS, lipopolysaccharide; PGE2, prostaglandin E2; IL-1β, interleukin 1 beta; C5a, complement component C5a; ATP, adenosine triphosphate; PIC, polycytidylic acid; COX, cyclooxygenase; TLR3, toll-like receptor 3; TLR4, toll-like receptor 4.

In the studies with *Apoe* knockout mice, the results showed evidence of the importance of APOE in cognition and neural plasticity [Gordon et al., 1995; Masliah et al., 1995; Oitzl et al., 1997; Siegel et al., 2011]. In *Apoe* knockout mice, exposure to an environmental stress led to an improvement in learning [Grootendorst et al., 2001] and other behavioral dimensions were tested (such as locomotion and anxiety) [Oitzl et al., 1997; Grootendorst et al., 2001]. A recent study used mice that had

TABLE VI. An Overview of Studies in Animal Models Showing the Functional Effects of the Three APOE Isoforms (E2, E3, and E4)

Animal model	Main results	Reference
Apoe knockout mice	Deficits in working memory but not in reference memory. Brain choline acetyltransferase activity was lower in frontal cortex and hippocampus.	Gordon et al. [1995]
Apoe knockout mice	Loss of synaptophysin- inmunoreactive nerve terminals and presence of dendritic alterations.	Masliah et al. [1995]
Male *Apoe* knockout mice	Learning was absent in the water maze. General locomotor activity was not altered.	Oitzl et al. [1997]
NSE-*APOE* E3 and −APOE E4 transgenic mice	NSE-*APOE* E4 mice exhibited impairments in the water maze task and in vertical exploratory behavior, which were found primarily in female animals and increased with age.	Raber et al. [1998]
Apoe knockout mice subjected to stress	Repeated exposure to an environmental stress (contact with rats) led to an improvement in performance in the water maze. Measures of locomotion and anxiety, under basal conditions, were similar.	Grootendorst et al. [2001]
GFAP-*APOE* E3 and −APOE E4 transgenic mice	*APOE* E4 mice group was the most reactive in analysis of locomotor activity, open-field behaviors, acoustic startle/prepulse inhibition, and elevated plus maze. *APOE* E4 mice were impaired on a working memory-based protocol in the radial arm maze. No changes in synaptic, neuronal, or glial markers in neocortex or hippocampus.	Hartman et al. [2001]
Transgenic mice for carboxyl-terminal-truncated *APOE* E4	Transgenic mice for truncated *APOE* E4 showed impaired learning and memory (Morris water maze) at 6–7 months of age.	Harris et al. [2003]
APOE E3 and *APOE* E4 transgenic mice subjected to environmental stimulation	Environmental stimulation in the *APOE* E3- transgenic mice improved learning and memory in a T maze paradigm. In contrast, environmental stimulation had no effect on learning and memory in the *APOE* E4-transgenic mice. The cognitive effects in the *APOE* E3 mice were associated with higher levels of the presynaptic protein synaptophysin in the hippocampus.	Levi et al. [2003]
Apoe knockout mice and GFAP and NSE −APOE E3 and *APOE* E4 transgenic mice	Knockout and *APOE* E4 transgenic mice displayed increased measures of anxiety. These behavioral alterations were correlated with structural changes in the amygdala.	Robertson et al. [2005]
APOE E3 and *APOE* E4 knockin mice	Female *APOE* E4 mice showed deficits in spatial memory performance.	Grootendorst et al. [2005]
APOE E3 and *APOE* E4 knockin mice	An impairment in reactive sprouting was found in *APOE* E4 mice subjected to an entorhinal cortex lesion. Thirty days post lesion, *APOE* E4 mice showed reactive astrocytes and an accumulation of APOE and beta-amyloid.	Blain et al. [2006]
APOE E2, *APOE* E3 and *APOE* E4 knockin mice	Long-term potentiation (LTP) was enhanced in *APOE* E4 animals, in comparison with *APOE* E2 mice. Synaptic transmission was not affected.	Korwek et al. [2009]
APOE E3 and *APOE* E4 knockin mice	In *APOE* E4 transgenic mice, 6 weeks of exercise improved deficits in performance in the radial-arm water maze and increased synaptophysin levels.	Nichol et al. [2009]
APOE E2, *APOE* E3 and *APOE* E4 knockin mice	APOE E4 inhibited reelin-induced enhancement of long-term potentiation.	Chen et al. [2010]
APOE E4 knockin mice	An impaired maturation and dendritic development of newborn hippocampal neurons in *APOE* E4 mice. As a consequence, a GABAergic interneuron dysfunction was observed.	Li et al. [2009]
APOE E4 knockin and *Apoe* knockout mice	In an induced experimental autoimmune encephalomyelitis, *APOE* E4 and *Apoe* knockout mice showed significant deficits in spatial learning and recall.	Tu et al. [2009]
APOE E3 and *APOE* E4 knockin mice	Female *APOE* E4 knockin mice showed an age-dependent decrease in GABAergic interneurons, which correlated with learning and memory deficits.	Andrews-Zwilling et al. [2010]
Apoe knockout mice	Knockout mice showed impaired learning in the five-choice serial reaction time task and a reduced number of cortical and hippocampal muscarinic acetylcholine receptors.	Siegel et al. [2011]
APOE E2, *APOE* E3 and *APOE* E4 knockin mice	Aged *APOE* E2 mice showed a better spatial memory, which has a positive correlation with APOE concentrations in the cortex and hippocampus and a negative correlation with cholesterol levels in the cortex.	Shinohara et al. [2016]
Apoe brain knockout mice (bEKO)	bEKO mice showed synaptic loss and dysfunction but did not show learning impairment. In these mice, the AMPA/NMDA ratio was unchanged.	Lane-Donovan et al. [2016]
Apoe knockout mice	In a metabolomic analyis on brains of young *Apoe* knockout in mice, there were no changes in cholesterol or its metabolites. Subtle alterations in histamine and lysine metabolites were observed in the brain.	Lee et al. [2016]

NSE, neuron specific enolase; GFAP, glial fibrillary acidic protein.

a conditional knockout for the *Apoe* gene in brain and showed that these animals, with normal levels of Apoe and lipids in plasma, had synaptic loss and dysfunction but did not have the impairments in learning and memory found in *Apoe* knockout mice, highlighting the possibility of the independent effects of Apoe expression in brain and plasma lipids on cognitive function [Lane-Donovan et al., 2016].

Transgenic models for *APOE* have used methods for conditional expression in neurons or astrocytes (using neuron specific enolase -NSE- or glial fibrillary acidic protein -GFAP-based vectors, respectively) and have tested the effects of the three APOE isoforms (Table VI). These studies in transgenic mice have found learning deficiencies and other behavioral alterations in *APOE* E4 mice [Raber et al., 1998; Hartman et al., 2001; Robertson et al., 2005], which cannot be ameliorated by environmental stimulation [Levi et al., 2003]. In addition, a study with transgenic mice for carboxyl-terminal-truncated *APOE* E4, which is toxic, also showed impairments in learning and memory [Harris et al., 2003].

In knockin mice models for the different APOE isoforms (inserted in the murine *Apoe* locus), *APOE* E4 mice have shown alterations in learning and memory and in structural and functional correlates, such as long-term potentiation, of neural plasticity [Grootendorst et al., 2005; Blain et al., 2006; Korwek et al., 2009; Li et al., 2009; Tu et al., 2009; Andrews-Zwilling et al., 2010; Chen et al., 2010]. A study found that exercise ameliorated the negative role of APOE E4 on memory and neural plasticity, which could be correlated with an increase in synaptophysin levels [Nichol et al., 2009]. In *APOE* E2 knockin mice, it was found that these animals showed better memory performance, which was correlated with higher Apoe levels in the cortex and hippocampus and with lower cholesterol levels in the cortex [Shinohara et al., 2016].

A study found that the effect of the different APOE isoforms on long-term potentiation is dependent on the NMDA receptors and that the chronic expression of the APOE isoforms alters activation of the ERK1/2 and JNK1/2 signaling pathways [Korwek et al., 2009].

These genetically modified animal models have been useful for a better understanding of the role of the *APOE* gene and its main isoforms on behavior and neural plasticity mechanisms.

Implications for Future Works

It is important that the *APOE* E2/E3/E4 alleles, and epigenetic variants, will be explored in molecular analyses of other neuropsychiatric disorders and endophenotypes of high epidemiological importance, due to the key role of the *APOE* gene in brain homeostasis and neural plasticity. Larger resequencing studies of *APOE* gene will identify novel rare variants potentially associated with different diseases. Cellular studies will help to recognize the additional factors that regulate the expression of the *APOE* gene and its neighbor genes and the potential modification by nutritional or pharmacological approaches. Analysis of different world populations will be useful to identify the possible role of linkage disequilibrium patterns on the association of the *APOE* gene and neuropsychiatric disorders [Forero et al., 2014]. A complete behavioral and functional phenotyping of young subjects that are carriers of the *APOE* E2/E3/E4 alleles will be important to find the earliest neural alterations associated with these functional polymorphisms.

Methods for Data Search and Retrieval

The ALFRED—the ALlele FREquency Database was used to retrieve APOE E2/E3/E4 frequencies in global populations [Rajeevan et al., 2003]. Information about genes and CpG islands was extracted from the UCSC genome browser [Speir et al., 2016]. Haplotype blocks were identified with the Haploview program, version 4.2 [Barrett et al., 2005], visualizing genotype data for three major populations from the HapMap project [International HapMap C, 2003]. Data about relative APOE expression in human and murine tissues were retrieved from the BioGPS database (root squares from the signal intensity of the respective microarray probes were used for Fig. 3) [Su et al., 2004; Wu et al., 2013]. Information for known SNPs in the *APOE* gene were retrieved from the Exome Variant Server, 1000 genomes, dbSNP, HapMap, and Ensembl databases [International HapMap C, 2003; Genomes Project C, 2015; Coordinators, 2016; Yates et al., 2016]. PubMed, the NHGRI GWAS Catalog and the HuGE Navigator [Yu et al., 2010; Welter et al., 2014] were used to identify GWAS and meta-analyses of studies for candidate genes for traits of interest.

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A Review of Freely Available, Open-Source Software for the Automated Analysis of the Behavior of Adult Zebrafish

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Abstract

The analysis of behavior in animal models is an important objective in many research fields, including neuroscience, psychology, toxicology, and neuropsychopharmacology. Animal models have been used for many years, and several behavioral paradigms, such as locomotor activity, social interactions, and cognitive behavior, have been studied in animal models to correlate the behaviors with pharmacological or environmental interventions and with molecular, biochemical, and physiological findings. We reviewed the literature looking for open-source, freely available software to analyze animal behavior and found 12 freely available programs: ToxTrack, EthoWatcher, Mouse Behavior Tracker, Mouse Move, JAABA, wrMTrck, AnimalTracker, idTracker, Ctrax, Mousetracker, VideoHacking, and Cowlog, which were developed with different programs, work on different platforms, and have particular types of inputs and outputs and analysis capabilities. We reviewed some examples of their use, tested some of them, and provided several recommendations for the future development of programs for the automated analysis of behavior in animal models. In conclusion, we show freely available software for the automated analysis of behavior in animal models such as adult zebrafish and provide information for researchers and students looking for quick, easy-to-implement, and inexpensive behavior analysis alternatives.

Keywords: animal behavior, neurobiology, computational neuroscience, automated analysis, open-source software, adult zebrafish behavior

Introduction

HISTORICALLY, ANIMAL MODELS have been used for a long time in medical and biological research, having their origins in ancient Greece with Aristotle (384–322 BC).[1] In 1902, Lucien Cuénot used mice (*Mus musculus*) for the first time to investigate Mendelian inheritance, and in 1903, William Castle used fruit flies (*Drosophila melanogaster*) to study the heritability of albinism.[2] In 1822, zebrafish (*Danio rerio*) were described by Hamilton, and in the 1980s, the zebrafish emerged as a novel organism for research in many fields to study biological phenomena.[3,4] These three animal models have complete genome sequences that make them suitable for behavioral studies that correlate with molecular analyses. In comparison with the human genome sequence, genes from mice have ~80% homology,[5] zebrafish 70%,[4] and fruit flies 60%.[6] These genetic similarities allow the study of human diseases and biological phenomena in animal models, in which it is necessary to perform behavioral analyses, among other systematic studies. The behavior description, or ethography, is a task that requires time by a trained researcher to recognize and analyze behavior patterns in animal species. For example, aggressive behavior may be recognized as follows: chasing and biting patterns in mice and adult zebrafish.[7,8] Traditionally, this type of behavior is recorded, and the results are manually extracted.

Many of the behavioral tests commonly used in several animal models are an adaptation from the scientific literature available in mouse models. The three main types of behavioral domains evaluated in animal models, including adult zebrafish, are emotional behavior (e.g., anxiety or fear), cognitive behavior (e.g., spatial memory, object recognition, or learning), and social interaction (e.g., aggressiveness, social preference, and courtship), as evidenced in several publications.[9–12] For instance, the analysis of locomotor behavior can be explored in different species and with several

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experimental paradigms (e.g., open field in mice, trapezoidal tank in adult zebrafish, and square boxes in flies), and in general, these devices assess the animal's movement in the environment (generating important data, such as total length, velocity, acceleration, and trajectories). On the other hand, there are cognitive tests, such as memory tasks, which assess the number of entries into a region of interest (ROI), time spent in an ROI, and time to reach an ROI. Finally, social interactions can be assessed, and they can have a wide range of presentations; for instance, in larval and adult zebrafish models, it has been found that there are ~190 evaluable behaviors.[8] Of these, more than 20 behaviors represent complex actions that are mostly evaluated manually, such as biting, charging, chasing, and fighting (in aggression), changing body color, swimming in circles, following (in courtship), shoaling, and socially interacting.[8]

Although many animal models have been used in neuroscience research to study animal behavior for several decades, only recently has software for the automated analysis of behavior been developed. Software that adapts to the needs of the researcher allows the design of more complex studies, for example, experiments related to studying the influence of the administration of pharmacological substances, genetic modifications, or physical and/or cognitive impairments in animals, testing their specific behaviors in several domains, making assessments in grouped or individual situations, and conducting these analyses with a low rate of error.

This work is focused on reviewing open-source software oriented to study only adult zebrafish behavior and not larval zebrafish behavior. The zebrafish behavior in larval stages has specific characteristics and has been analyzed using software for video and kinematic analysis.[13,14]

Currently, there are several programs for the automated analysis of animal behavior, and although some of these are commercial, an important number of them are open source and free to use. These developments have provided researchers with many advantages, such as saving time and money, improving accuracy, allowing a larger number of animals to be analyzed, and possibly generating highly quantitative and precise results, such as the total length of displacement, velocity, acceleration, turn angles, track trajectories, and number of entries in a ROI.[15,16]

Some commercial programs for the analysis of animal behaviors are EthoVision (Noldus, Inc., Leesburg, VA), ANY-maze (Stoelting Co., Wood Dale, IL), and TopScan (Clever Sys, Inc., VA). These programs require payment for their use. In this article, we focus on freely available open-source software oriented to video analysis, which permits the automated analysis of behavior. It should be noted that some software also needs C++, MATLAB, LabView (National Instruments, TX) commercially available programs, or Python (which needs advanced computational skills) to work.

Literature Review

For this work, a search was made of the existing literature in PubMed and the academic Google search engine. The keywords for the search included adult zebrafish behavior, animal behavior, automated analysis, and open-source software. Review articles and original articles closely related to the topic were selected and reviewed. Based on this, 12 freely available programs were identified: 3 ImageJ-based programs (wrMTrck, Mouse Behavior Tracker, and Animal-Tracker), 2 MATLAB-based image processing programs (idTracker and JAABA), 2 C++-based image processing programs (ToxTrack and EthoWatcher), 2 python-based image processing programs (Ctrax and VideoHacking), 1 Pascal/Delphi image processing program (Mousetracker), 1 LabVIEW-based image processing program (Mouse Move), and 1 Java- and HTML-based image processing program (Cowlog). These programs work on different operative systems (Windows, Mac OS, and Linux). In some cases, toolboxes, libraries, and licenses from commercial programming languages (MATLAB, C++, and LabVIEW) are required (Table 1).

Open-Source and Freely Available Software

The programs mentioned below have in common the semiautomatic analysis of previously recorded videos or the processing of the behavior in real time. Actually, the analysis of behavior through software is performed semiautomatically because the user must adjust the parameters (distance measurements and ROI, mainly), and the software will be responsible for executing the order to follow a specific object. For practical purposes, we will call it automatic analysis. It is important to keep in mind that depending on the software used, the number of steps needed to obtain the behavioral data may be different.

Image processing algorithms

Processing times may also vary, and it will depend mainly on the video resolution and length, and the software speed. Briefly, the first algorithm commonly used by the software is a segmentation algorithm (i.e., background subtraction followed by threshold definition). It helps to improve animal tracking and to decrease the variations in light in the field (Appendix). After that, animal location (center of mass or animal head) is tracked in *X* and *Y* coordinates, and the results are generated.[15,16] It is also important to note that many algorithms can be customized.

One of the main limitations of the available programs is the difficulty of tracking several animals simultaneously. The main cause for this difficulty is the spatial overlap of animals, which leads to a loss of identity and mixed data. From our point of view, the main advantage and usefulness of tracking multiple individuals are related to the study of locomotor and social behavior, where tracking multiple individuals decreases the amount of time for each analysis. Different techniques, such as the use of visual markers, several cameras, and algorithms based on neural networks, can be used to avoid losing track of the animals; however, it can be invasive, it can be difficult to apply to all animal models, and it increases the complexity of the experiments and the computer memory use.[15,16]

Description of Available Software

wrMTrck

This is a plugin that works with ImageJ. ImageJ is a freely available image processing program developed at the National Institute of Health (NIH). It was written in the Java programming language and run on Windows, Mac OS, and Linux systems in 32-bit and 64-bit modes. For ImageJ installation, you can visit https://imagej.nih.gov/ij/download.html.

Table 1. Overview of Freely Available, Open-Source Software for the Automated Analysis Behavior in Animal Models

Software	*Operative system/program*	*Types of analyses*	*Types of input*	*Types of output*	*Webpage*	*References*
wrMTrck	Windows and Mac OS X/ JAVA-ImageJ	Total length, average speed, area, perimeter, and trajectories.	AVI files with jpg compression	txt, xls, tiff files and AVI videos	www.phage.dk/plugins/ wrmtrck.html	17–19,32
Mouse Behavior Tracker	Windows, Mac OS X and Linux/JAVA-ImageJ	Distance and average velocity.	AVI or MPEG-compressed AVI files, Mp4	Txt or xls files	www.BioTechniques.com/ article/114607.	20,32
AnimalTracker	Windows, Mac OS X and Linux/JAVA-ImageJ	Total length, average speed, and time spent in ROI.	AVI files with jpg compression	txt, xls, tiff files and AVI videos	animaltracker.elte.hu	21,32
idTracker	Windows/MATLAB	Trajectories, identification of one animal in different videos and ROI.	Compatible with MATLAB, uncompressed AVI or MPEG-compressed AVI files	*X* and *Y* coordinates and images files	www.idtracker.es	22
Mousetracker	Windows, Linux and Mac OS X/Pascal-Delphi-MS Excel	Velocity, acceleration, and time spent in ROI.	AVI format	*XY* coordinates can be copied directly	www.neuro.ufrn.br/ softwares/mouselabtracker	23
JAABA	Windows, Mac OS X and Linux/MATLAB	Bites, persecution, sexual behavior, angle of turn, grooming, jump, walk, immobilization, and touch. Locomotion and ROI.	Several formats and resolutions. *X* and *Y* coordinates	MATLAB files	http://jaaba.sourceforge.net https://www.janelia .org/lab/branson-lab	24
Ctrax	Windows and Mac OS X/ Phyton—MATLAB	Trajectories, velocities, speed, position, and turning speed histograms.	Common digital video formats, mainly AVI	csv and mat files. Converts the file to .ann extension	ctrax.sourceforge.net	25
VideoHacking	Windows, Mac OS X and Linux/Phyton—Open CV	Velocity, acceleration, total length, average speed, and time spent in ROI.	Common digital video formats	Graphical interface to view data summary	faculty.ithaca.edu/iwoods/ docs/	26
ToxTrack/ToxId	Windows/C++	Total distance, speed, acceleration, time near the walls (measure of anxiety), and ROI.	AVI or MPEG-compressed AVI files	txt, xls, tiff files and AVI videos	https://sourceforge .net/projects/toxtrac/	27,28
EthoWatcher	Windows/C++	Frequency, duration, and latency of each behavior.	AVI or MPEG-compressed AVI files	csv files	http://ethowatcher .paginas.ufsc.br	29
MouseMove	Windows/LabView—ImageJ	Distance, average velocity, acceleration, curvature, stationary fraction, laterality y ROI.	AVI or MPEG-compressed AVI files	csv files	https://www.nature.com/ articles/srep16171#s3, Supplementary File 2	30
Cowlog	Windows, Mac OS X and Linux/Java—html	Analysis of different behaviors can be set (tapping a button when the event occurs)	Common digital video formats	csv files	cowlog.org	31

AVI, audio video interleaved; MJPEG, motion joint photographic experts group; ROI, region of interest.

wrMTrck is a plugin created by Jesper Søndergaard Pedersen and his team to study how prion-like proteins spread in *Caenorhabditis elegans* and to analyze locomotor behavior (www.phage.dk/plugins/wrmtrck.html).[17] The plugin installation and setup are very easy, and you can read Selvaraj and Santhakumar[18] for more details. wrMTrck generates raw data, a summary, a video of the tracking analysis, and an image of the path of the animal tracking. Raw data include total distance, number of frames tracked, time points analyzed, maximum speed by frame, tracking area by frames, average speed by frame, and location in X and Y coordinates. The data summary includes the number of objects tracked, total frames analyzed, the number of group of frames tracked, total length, average speed, and area and perimeter measures, among others. Furthermore, processed videos can be saved in Audio Video Interleaved (AVI) format and images in tiff, jpeg, gif, and other common formats. wrMTrck is a useful tool to analyze several behaviors of one animal at a time because it tracks only one animal; the superposition of images from several animals can alter the results when they cross each other, and the data are mixed. Another useful plugin to solve contrast issues is DanioJ 1.0, which increases the illumination threshold for the animal and thus makes the animal more visible for tracking.[19] It is mainly useful for analyzing locomotor behavior in several species.

Mouse Behavior Tracker

Mouse Behavior Tracker is a macro developed and implemented in ImageJ and the wrMTrck plugin, which allows automatic tracking of the animal by implementing filters sequentially to maximize the contrast between the animal and the test area (https://www.future-science.com/doi/10.2144/000114607/supplementary material). It works in a similar way to AnimalTracker, but with limitations related to obtaining only data for locomotion. This software generates data for distance traveled, speed, and path graphs. In comparison with EthoVision XT (commercial software), there was a correlation of 0.98 ± 0.03 (mean $\pm$ SEM, $n = 6$) in the data.[20] Mouse Behavior Tracker works in Windows, Mac, and Linux and accepts videos with a resolution of 320×240 pixels, 30 frames per second (FPS), and Motion Joint Photographic Experts Group (MJPEG) compression, in AVI or MP4 format, with a duration of up to 30 min. It was initially implemented in mice, but can be adapted to any species, allowing the analysis of one individual at a time. This software is useful for the analysis of locomotor behavior exclusively (distance and speed parameters), as mentioned above, and may overestimate data if there are minimal variations of light.

AnimalTracker

This is a plugin that works with ImageJ, and for installation and more details, you can visit its website (http://animaltracker.elte.hu/). This plugin has three different modules that work together. First, the Tracker module processes the video and facilitates the binarization process to determine the XY coordinates. The TrackAnalyzer module visualizes the trajectories produced by the Tracker module, and finally, the ZoneDesigner module can be set up to analyze different zones.[21] AnimalTracker generates data such as total time analyzed, immobility times, distances, and velocity vectors. Furthermore, raw data, means and standard deviations, and highest and lowest values can be identified for distance, time, and immobility times among different ROIs defined in the ZoneDesigner module. The results can be saved as txt or spreadsheet files for further analysis. This software is useful for locomotor behavior and some analysis of social and cognitive behavior. The software is easy to use and requires a few steps; however, it only recognizes one animal at a time, and data mixing can occur when using several animals.

idTracker

idTracker is a MATLAB package that can be downloaded with MATLAB runtime add-ons from its website (www.idtracker.es/). This program can identify several animals in one video, and idTracker has several automated steps that include segmentation to normalize fluctuations of illumination in each frame; fragments of trajectories in which it can identify each fragment of frames when an animal is moving; transformation to generate high contrast; and selection of images that belong to one animal. Images are collected frame by frame, each animal in the video is identified, and these steps are computed and automatically analyzed to give individual trajectories for several animals. Therefore, it is possible to identify a single animal in a group.[22] One of the main theoretical advantages of idTracker is the possibility to analyze territoriality, leadership hierarchies, and differences between animals in a group solving a task, and identities can be recovered when an animal is out of sight.[22]

Mousetracker

Mousetracker is an application written in Pascal through a Delphi interface that is able to run on Windows. Microsoft Excel is required to save the results. Up to eight animals can be analyzed at once, and it works through black/white differentiation between the animals and the background, based on a grayscale threshold for animal contrast.[23] The hardware requirements include a 1.6 GHz processor with 512 MB RAM and a webcam with proper video recording software installed. The program has a simple, intuitive interface with buttons and functions. The videos to be used with this software need low resolution (video setting in AVI format at 4 FPS and 320×240 pixels). Mousetracker was created at the Brain Institute at the Federal University of Rio Grande do Norte in Brazil and developed to analyze mainly locomotor activity and ROI (the software can be obtained by contact with the software authors at mousetracker@gmail.com).

Janelia Automatic Animal Behavior Annotator

Janelia Automatic Animal Behavior Annotator (JAABA) is a system that uses machine learning in a similar way to EthoWatcher. It creates a library of learned behaviors by taking a few frames where the event of interest has occurred and the researcher must teach the behavior to the program; it takes 15 to 40 s to teach each behavior. This software is implemented in MATLAB for Windows, Mac, and Linux operating systems (http://jaaba.sourceforge.net/).[24] MATLAB runtime add-ons that are freely available for users must be installed to execute the program. JAABA has a large number of algorithms that process characteristics such as position, locomotion, location, wingspan and movement of the wings (tested in flies), and distances between animals, identities,

and field. The machine learning algorithm requires the graph of the animal's trajectory in *X* and *Y* coordinates to find behaviors (learned algorithms) in the video.[24] Thus, it is necessary to use additional software to create the trajectories. In addition, the user can see the results and identify a behavior that is incorrect. It is applicable to a large number of species and diverse behaviors with an error rate lower than 1%, as tested in flies (adult and larva) and mice. In addition, it was reported that chase behavior analysis by this program had an error rate of 0.6%, while Ctrax showed an error rate of 10%. In the demonstration of its performance, authors processed videos of 1000 s (16:40 min).[24]

From our point of view, it is a very useful tool to evaluate the behavior of multiple animals, offering the possibility of evaluating a large number of social, cognitive, and locomotor behaviors (bites, persecution, sexual behavior, angle of turn, grooming events, jumps, walk, immobilization, touch, etc.) in a large group of animals, saving recording and process ing times in behavioral tests. According to the authors, it is necessary to develop algorithms that encompass other animal species, behaviors, and environments because the JAABA system was focused on flies and mice; however, many behaviors measured by JAABA can be adapted to other species with the same algorithms.

Ctrax

Ctrax is freely available image analysis software. It was written in Python, and the MATLAB runtime add-ons freely available for users are required. Ctrax can be downloaded online and is widely used. More than 40 publications reported using Ctrax, mainly in *D. melanogaster* and adult zebrafish (http://ctrax.sourceforge.net/). This software works similarly to others in that it makes a binarization of the animal and then can recognize frame to frame one or several animals (up to 50 flies).[25] A wide number of analyses can be performed with this software, such as velocity, distance, time spent walking, chase between animals, and a path of several animals in a video. The analysis carried out by the authors included seventeen 30-min trials, each with 20 flies, for a total of 170 flight hours. In addition, similar to JAABA, Ctrax contains a machine learning system that allows the evaluation of additional behaviors, while maintaining the identities of each animal, even over time, with an accuracy of 83.9% for locomotor behavior and more than 95% for other behaviors.[25] This program is the most complete of the software presented in this review. It has the capacity to analyze locomotor behavior (although with less precision than other software) and behavior in the social and cognitive domains as determined by the user.

VideoHacking

This software works with Python and OpenCV, including the Python libraries matplotlib and NumPy. It consists of several computational modules working together to analyze complex behaviors, such as thigmotaxis, ROI analyses, and, in general, locomotor behavior. The first step is delimiting an ROI in a field, followed by quantification of the locomotor behavior frame by frame, and then allowing the user to group ROIs according to treatment and display the results. Moreover, it contains a graphical interface to view summary data.[26] Another advantage of VideoHacking is the ability to analyze live or prerecorded videos of several hours with high-quality tracking and tracking of several animals simultaneously (as long as they do not overlap). Outcome parameters of locomotor activity, such as velocity, acceleration, total length, average speed, and time spent in an ROI (e.g., thigmotaxis behavior), are generated by the software. Details for its installation are found online, and some computational skills might be needed to solve some problems related to the installation of VideoHacking and OpenCV (https://faculty.ithaca.edu/iwoods/docs/pytracker/). The capacity for the analysis of several hours of testing, the simultaneous testing of multiple animals ($n = 48$), and the adaptation to changes in the lighting conditions offered by this software make it ideal for the analysis of locomotor, social, and cognitive behavior. In addition, the use of this software reduces the presence of the researcher, which reduces variables such as noise levels and lighting changes.

ToxTrack

ToxTrack is software for Windows, developed in the C++ language (https://sourceforge.net/projects/toxtrac/), which allows the tracking of several animals simultaneously, preserving their identity, even when there are occlusions (loss of continuity of the image). The user can analyze videos recorded previously or in real time (in high resolution, 2048 × 2048 pixels), and the authors recommend that the animals to be followed should be at least 50 pixels in size, the video should be recorded with at least 25 FPS, and no more than 10–20 animals should be tracked in each experiment.[27] ToxTrack can be used in different animal species, with a tracking efficiency of up to 99.9%. More recently, they developed ToxId (an algorithm implemented in ToxTrack) that identifies animals with 99% accuracy, even with occlusions and overlapping of animals. This improvement decreases the recognition error rate for an individual from one frame to another and enhances tracking in other species, such as adult zebrafish.[27] In comparison with idTracker, ToxId[28] is more efficient in processing videos and decreasing the analysis time, and has an average rate of recognition of several animal models of 97% compared to 99% of idTracker. ToxTrack generates results for the total distance, speed, acceleration, time near the walls, and other parameters related to the ROI.

EthoWatcher

EthoWatcher is a program for Windows, programmed in the C++ language, which allows researchers to make a catalog for the automatic analysis of behavior (http://ethowatcher.paginas.ufsc.br/). It has been used in several species and is useful mainly in rodents. EthoWatcher can evaluate specific behaviors, such as vertical exploration, grooming and sniffing, as well as distance, speed, immobilization, and ROI results (measurement in cm), and it generates a plot of paths, using prerecorded or live videos with a length of up to 3 h, with a minimum resolution of 320 × 240 pixels, MJPEG compression, and AVI format.[29] EthoWatcher does not contain preregistered behavior categories; therefore, the initial ethographic analysis is performed by the researcher and then saved as a catalog to be replicated in future analyses. The program reports the frequency, duration, and latency of each behavior and allows the processing of two behaviors simultaneously, which start and end at different times.[29] The main

advantage offered by EthoWatcher is the detection of the angles generated at the poles of the animal (head or tail movements) compared to its center of mass, which allows the automatic analysis of the specific behaviors mentioned above. However, it limits the analysis of some behaviors and species.

MouseMove

This software was developed in LabVIEW 12.0 Development Systems, and it works in the Windows operating system; however, LabVIEW installation is needed to run MouseMove.exe (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4632026/supplementary file 2). Furthermore, it is necessary to execute several steps: (1) create a folder with the videos to be analyzed, including an exclusive video of the arena (without animals); (2) open ImageJ and execute the algorithm designed by the authors; (3) identify the folder that contains the videos (ImageJ memory restricts the maximum video length to 5000 frames); (4) the files will be automatically saved in txt format; (5) open MouseMove.exe and open the folder with saved results; (6) it is necessary to reduce the resolution of the video to adjust the ROI in Mouse Move, and any adjustment made in ROI will be processed and saved automatically; and (7) the generated results will be saved in the folder that contains the analyzed videos as csv files.[30] Mouse Move accepts videos in AVI format with MJPEG compression at 25 FPS and 640×480 resolution (for optimal performance); however, the macro reduces the resolution to 320×240 pixels. The variables measured by this software are distance, average velocity, acceleration, curvature, stationary fraction, laterality (number of left and right turns and L/R ratio), and the ability to evaluate novel object recognition using ROI analysis, with a precision greater than 96%.[30]

Mouse Move can be used to analyze the behavior of different animal species and basic parameters of a test; moreover, it offers the possibility of analyzing specific areas of the field, which makes it a useful software for the analysis of locomotor behavior and some social and cognitive behaviors. However, it has limitations in the input resolution of the analyzed video and cannot process specific behaviors.

Cowlog

Cowlog is another freely available image analysis software. It was implemented using Java and html (http://188.166.24.93/download/). From all the programs reviewed in this article, Cowlog is the only one that works with a manual analysis; it can be set up for the analysis of up to 24 behaviors. There are three different versions (Cowlog 1, 2. and 3), and more than 30 publications report using it in several species. Cowlog is useful when complex behaviors are analyzed, for example, when an animal lays down, sits down, eats, or does another specific task. Furthermore, it works with two windows: a coding window for selecting the videos, scoring the behaviors and selecting the output format, and another window to watch the video.[31] The generated results are saved each time a behavior is scored and a table with two columns is obtained; the first column lists the behaviors scored, and the second lists the time that the particular behavior occurred. Data can be exported to other programs for further analyses.[31] This software is not for automatic analysis; however, we consider it useful when another software package does not allow the possibility of evaluating a specific behavior.

The programs presented were downloaded and tested on a laptop computer Hewlett Packard, HP Pavilion x360 (processor Intel Core i3, 4 GB RAM, Windows 10, 64 bits). Requirements for some programs are processor Intel Core i5, 8 GB RAM, 64 bits (Toxtrack, IdTracker, Ctrax). In cases where the software worked properly, the programs were tested using recorded videos of 2 min assessing the novel tank test in adult zebrafish (Fig. 1A–D, and Table 1).

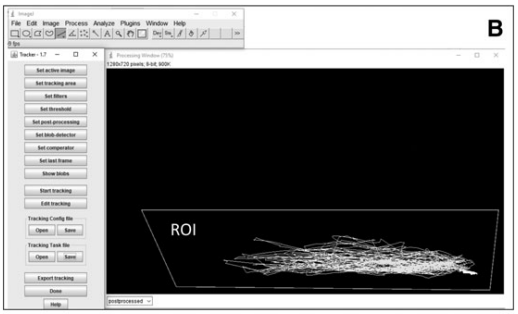
Example of Adult Zebrafish Behavior Analysis with Freely Available Software ImageJ Based

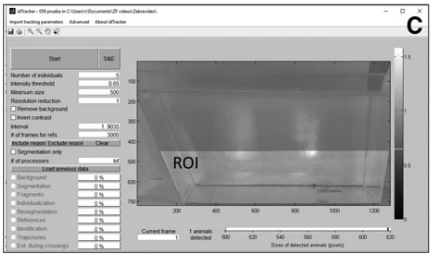
As mentioned before, several programs have been used for the automated analysis of behavior in animal models with *multiple species*. We are interested in ImageJ, a free, public domain, Java-based image processing program developed at the National Institutes of Health (https://imagej.nih.gov/ij/). ImageJ provides extensibility by Java with thousands of plugins and recordable macros that facilitate scientific image analysis. In addition, this tool has been successfully used worldwide by many laboratories.[32] Zebrafish (*D. rerio*), both larvae and adults, have previously been used to analyze locomotor and anxiety behavior when exposed to ethanol and other substances, such as fluoxetine, morphine, and nicotine.[33,34] Briefly, we used two male wild-type adult zebrafish housed in tanks of 7 L at 27°C ± 1°C and acclimated for 2 weeks before any test. Anxiety and aggressive behaviors were evaluated using the novel tank test and the aggressiveness test. For the novel tank test, we employed a trapezoidal tank, and a video was recorded from the lateral view (Figs. 1B and 2A, C). For the aggressiveness test, a rectangular tank with a mirror on one side was used, and a video was recorded from the top (Fig. 2B, D). A commercial camera was used to record videos. Video record settings were AVI format, MJPEG compressed, 30 FPS, resolution of 840×420 pixels, and length of 5 min, and the videos were analyzed with freely available software.

For this example, the software used for the behavior analysis was AnimalTracker, and examples of the results of the paths of the fish for each test are shown in Figure 2C and D. The results for the parameters of total distance traveled, and average speed and time spent in the ROI (bottom in novel tank test and contact zone for aggressiveness test) are shown in Figure 2E–H.

Discussion

Current techniques for automatic behavior assessment provide obvious advantages over a manual analysis, facilitating the research process in several fields. Several programs presented in this study evaluate a wide range of behaviors, and depending on the species, they can be adjusted for different analyses. When a video analysis is performed, you need to consider how most programs work. Few programs, as expected, work exclusively on the Windows operating system, and most are also available for MacOS and Linux. The main format supported by several programs is AVI with MJPEG compression, and problems can occur in several steps, such as software installation, formatting of the input videos, handling the video illumination, and exporting data; however, among the most important factors mentioned are





D

Method for analysis	Distance traveled (cm)	Mean Velocity (cm/s)	Comment
Manually	982	8.20	
AnimalTracker	564	9.39	higher threshold, dropped frames
AnimalTracker	840	7.32	lower threshold
Toxtrack	722	5.5	higher threshold, dropped frames
Toxtrack	965	7.4	lower threshold
IdTracker	789	5.8	higher threshold, dropped frames
IdTracker	872	7.1	lower threshold

FIG. 1. Freely available and open-source video analysis programs. Several programs were evaluated using the same short video (2 min, 30 FPS, and 320×240 pixels) obtained in an anxiety test, using a trapezoidal tank, of an adult zebrafish. Two basic measurements, the total length of displacement and velocity (or average speed), were obtained with each program. **(A)** ToxTrack interface window showing a tracking trace. **(B)** An interface from Animal Tracker, an ImageJ-based program. **(C)** An interface window from IdTracker, a MATLAB-based program. **(D)** Summary of results obtained in the anxiety behavioral tests. We tracked the video on a laptop computer Hewlett Packard, HP Pavilion x360 (processor Intel Core i3, 4 GB RAM, Windows 10, 64 bits).

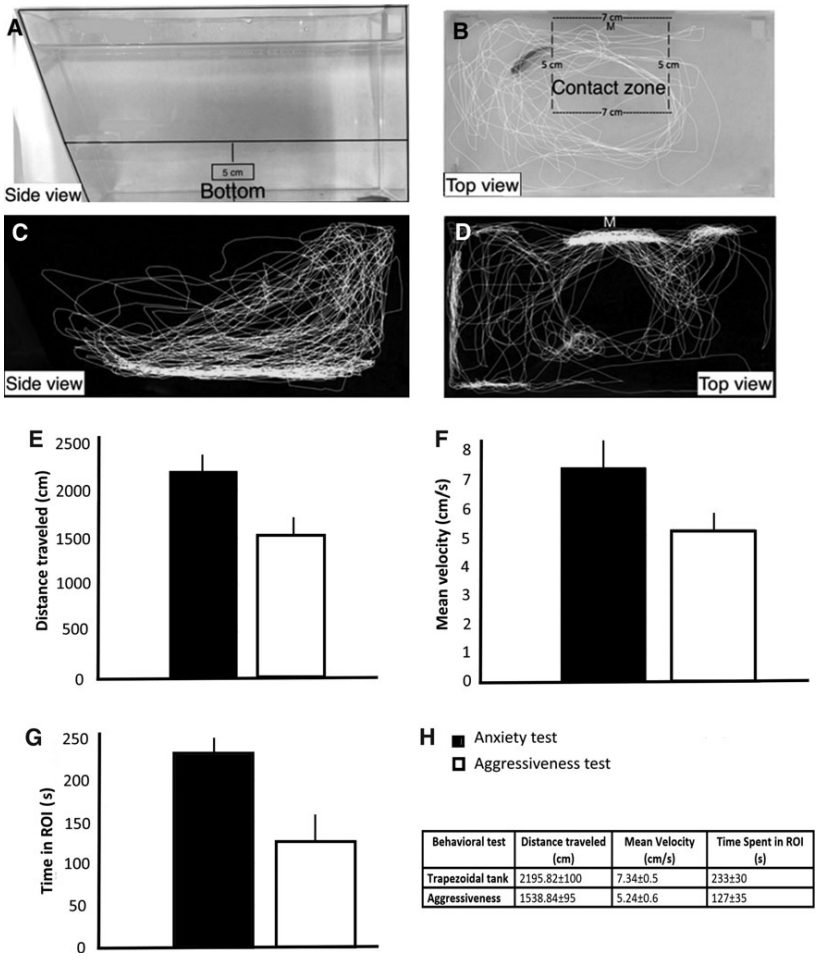
illumination and contrast, which can affect the results depending on the error rate of each program (Appendix).

However, some of the softwares, such as Ctrax, Cowlog, and VideoHacking, do not need special video formats to work. Exported data, in some cases, can result in changes in the content (e.g., due to the misreading of decimal commas), so it is better to save the results in csv or txt files and then import them to the software used for data analysis or a spreadsheet.

As some programs are outdated and therefore do not work on some platforms, it is necessary to have updates for their adequate operation. For example, ZooTracer, another freely available software package developed by Microsoft Research in 2006, is available for download with the last update in 2014 (https://www.microsoft.com/en-us/research/project/zootracer/). However, we did not include it in this review due to problems with the installation. On the other hand, it is important to mention that there are several options for the evaluation of different patterns of behavior and that some programs lack packages or updates for the wider analyses of behavior. Further developments are needed to create more precise and user-friendly free software for the automated analysis of complex and specific behaviors, such as fighting, courtship, and jumps, which might analyze the video by ROI with several animals simultaneously. In the videos analyzed, the resolution had to be downsampled on many occasions to avoid the software failing and closing unexpectedly. Another important limitation is the simultaneous processing capacity of two or more videos, which depends on the computer's RAM, the amount of time or frames processed, and the algorithms of each software. This would save much time, especially when a single subject was tested per video, and a considerable number of videos per experimental group were generated.

For the automated analysis of behavior, we consider it necessary that the minimum data analyzed include the following: distance, speed, freezing time, immobilization time, turn angle (customizable), and number of turns. The ROI should be adjustable to any geometric shape, including asymmetric shapes, and the number of entries, latency, and duration in an ROI should be analyzed (in addition to the variables mentioned above). The *X* and *Y* coordinates and the path graphs are important data for assessing behavior and simplifying the analysis process.

Due to the wide variety of analyses provided by each software package, we recommend using a combination of two, for example, ToxTrack or AnimalTracker plus JAABA or EthoWatcher. The first set can quantify the variables related to locomotion and the analysis of some social and cognitive behaviors, while the second set of programs can



Behavioral test	Distance traveled (cm)	Mean Velocity (cm/s)	Time Spent in ROI (s)
Trapezoidal tank	2195.82±100	7.34±0.5	233±30
Aggressiveness	1538.84±95	5.24±0.6	127±35

FIG. 2. Animal tracker was used to analyze anxiety and aggressiveness in zebrafish. **(A)** *Side view* of the trapezoidal tank used to test anxiety behavior in zebrafish. Two areas were defined: *bottom* and *top* zones. **(B)** *Top view* of the rectangular tank used for the aggressiveness test with a lateral mirror (M) with the contact area delimited by *dotted lines*. **(C)** Representative track of a zebrafish in the anxiety test obtained by AnimalTracker software. It was observed that zebrafish display high activity in the *bottom* area. **(D)** Representative track of a zebrafish in the aggressiveness test obtained by AnimalTracker software. In this test, zebrafish display high activity near the mirror. **(E–H)** Main results generated by AnimalTracker in the anxiety and aggressiveness behavioral tests. **(E)** Distance traveled (cm). **(F)** Mean velocity (cm/s). **(G)** Time spent in the ROI (s). **(H)** Summary of results obtained in the anxiety and aggressiveness behavior tests. Input data were obtained from the experiments shown in Figure 1. Error bars are ±1 SD. ROI, region of interest; SD, standard deviation.

quantify specific behaviors. Finally, something important to include in future reviews will be to contrast the results of the analysis of a single video in different programs, to compare automated analysis to manual analysis, and for some behaviors, to analyze locomotion (and social and cognitive tests) with several individuals with overlapping trajectories.

We think that the information reported in this study could be useful to researchers interested in working in the neuroscience field, especially people interested in a low-cost animal

model, such as zebrafish, in combination with free tools for a behavioral analysis that provide simple, quick, inexpensive or free of cost, and reliable behavior analysis.

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Appendix

Some Recommendations to Obtain High-Quality Video Recording

After testing multiple freely available software packages, we have some suggestions based on our experience. These recommendations could be useful for students and researchers interested in the analysis of adult zebrafish behavior. Considering the following parameters will permit one to obtain high-quality videos and guarantee standardization in video recording.

Illumination is a key factor because it is one of the main factors that generates false recognition in the tracking of animals. Reflections and shadows should be reduced in the field before starting to record videos. To solve this problem, it is important to maintain the same lighting conditions throughout all recordings. The generation of a contrast between the field and the animals is the main objective; therefore, if the animal to be evaluated is dark, the field should be white, and vice versa. If shadows (or artifacts) are generated in the field, the lighting must be improved with LED lights and/or light reflectors. Some software packages have a size filter to track only the animal in the field, even if there are artifacts.

The recording camera used can vary from a simple webcam to a professional camera. In our experience, to maximize accuracy, we recommend a camera that records at least 30 frames per second (FPS) and has a resolution of 840×420 pixels. The accuracy in speed calculations may change because in most algorithms, the position (pixels at X and Y coordinates) is determined in each frame of the video, and therefore, higher resolution and FPS improve the accuracy of the obtained results. However, lower FPS and resolution can produce similar results if there is good contrast (between the field and the animal). It is important to note that some programs allow the video analysis with a resolution of 320×240 pixels, and this is allowed mostly with black and white video due to increases in speed and contrast.

The Audio Video Interleaved format with an MPEG or MJPEG compression is the most common format used by the software tested. There are free programs to convert video from one format to another format, and we recommend Avidemux (Mean.io®; Linnovate, Bnei Brak, Israel) that allows the conversion of formats, changes in compression, size, color, and contrast, and the removal of unwanted regions of the field, among many other utilities. We recommend saving memory and processing time by converting or recording videos in grayscale.

Finally, it is necessary to stabilize the camera with a tripod, maintain the same distance between the lens of the camera and the field during all behavioral tests, and have measurement reference points, such as the length of the field or a ruler outside the arena.

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Abstract:	OBJECTIVES: Stress and anxiety disorders are common health problems and have been related to an increase in the likelihood of developing addictions, with individual and social consequences. Alcohol is one of the substances that, although socially accepted, can generate dependence and abuse. Alcohol misuse, its relationship with stress and its consequences have been studied, but there are multiple limitations on clinical research in humans. In this work, we analyzed the behavioral and molecular effects of the joint exposure to ethanol and an unpredictable stress protocol (USP) in adult zebrafish. MATERIALS AND METHODS: Adult zebrafish behavior was studied employing unpredictable stress and behavioral test. The tests were performed in stressed and non-stressed animals without and with exposed to known concentrations of alcohol. To evaluate the behavior, tracking techniques were used on video recordings and parameters such as distance traveled, swimming speed and place preference, as well as aggression patterns with mirror proximity test, were measured. In control and 0.75% alcohol group expression of candidate stress-related genes (slc6a4a, slc6a3, comta and bdnf3) were analyzed by RT-qPCR. RESULTS: The results showed that concentrations of 0.75% alcohol reduced the activity of the fish, which can be interpreted as an increase in the anxiolytic effect of alcohol in non-stress conditions. Expression for comta, bdnf3 and slc6a3 were reduced in the stress and stress plus 0.75% ethanol groups; slc6a4a expression was increased in the group exposed to stress plus 0.75% alcohol. CONCLUSIONS: Our exploratory work contributes with novel knowledge about the molecular and behavioral effects of the combination of unpredicted stress and alcohol misuse. Future study of other pharmacological compounds and additional genes will be helpful for a deeper understanding of the molecular mechanisms involved in the response to stress and alcohol use.
Keywords:	Neurosciences; Mental Health; Addiction; Anxiety; Animal models
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Behavioral and Molecular Effects of Alcohol in Swimming Deep Test and Mirror Proximity Test in a Model of Unpredictable Stress in Adult Zebrafish

Running title: Alcohol and Stress in Zebrafish

ABSTRACT

OBJECTIVES:

Stress and anxiety disorders are common health problems and have been related to an increase in the likelihood of developing addictions, with individual and social consequences. Alcohol is one of the substances that, although socially accepted, can generate dependence and abuse. Alcohol misuse, its relationship with stress and its consequences have been studied, but there are multiple limitations on clinical research in humans. In this work, we analyzed the behavioral and molecular effects of the joint exposure to ethanol and an unpredictable stress protocol (USP) in adult zebrafish.

MATERIALS AND METHODS:

Adult zebrafish behavior was studied employing unpredictable stress and behavioral test. The tests were performed in stressed and non-stressed animals without and with exposed to known concentrations of alcohol. To evaluate the behavior, tracking techniques were used on video recordings and parameters such as distance traveled, swimming speed and place preference, as well as aggression patterns with mirror proximity test, were measured. In

control and 0.75% alcohol group expression of candidate stress-related genes (*slc6a4a*, *slc6a3*, *comta* and *bdnf3*) were analyzed by RT-qPCR.

RESULTS:

The results showed that concentrations of 0.75% alcohol reduced the activity of the fish, which can be interpreted as an increase in the anxiolytic effect of alcohol in non-stress conditions. Expression for *comta, bdnf3* and *slc6a3* were reduced in the stress and stress plus 0.75% ethanol groups; *slc6a4a* expression was increased in the group exposed to stress plus 0.75% alcohol.

CONCLUSIONS:

Our exploratory work contributes with novel knowledge about the molecular and behavioral effects of the combination of unpredicted stress and alcohol misuse. Future study of other pharmacological compounds and additional genes will be helpful for a deeper understanding of the molecular mechanisms involved in the response to stress and alcohol use.

Keywords: Neurosciences; Mental Health; Addiction; Anxiety; Animal models.

Introduction

Anxiety and stress have been linked to an increased likelihood of developing addictions, such as alcohol use disorders [1]. Known predisposing factors for alcoholism include psychosocial stress and affective disorders, which are also risk factors for chronic diseases. On the other hand, psychosocial stress might potentiate the consumption and the negative effects of alcohol and to reinforce abnormal drinking behavior [2]. In addition, alcohol consumption has been linked to aggressive behaviors, such as violent assault, child abuse, domestic

violence and homicides, among others [3]. Alcohol abuse and its consequences have been studied in humans, there are multiple limitations on clinical research in humans about basic mechanisms [4]. As studies in humans have limited potential to identify basic mechanisms related to the the relationship between stress, anxiety and alcohol misuse, basic studies in animal models are required to investigate its possible pathophysiology [5].

The adult zebrafish is one of the current models used for research in multiple areas of the neurosciences [6]. Aspects related to learning and memory [7], the sleep-wake cycle [8], social behavior [9], stress, anxiety and addictions [10], among others, have been explored in zebrafish models. Acute stress and anxiety have been studied in adult zebrafish using tests similar to those used in rodents [11]. For this purpose, several behavioral tests have been used, such as those based on novel tank, open field, light-dark preference, the predator avoidance and the social preference [12]. Stress has been induced in adult zebrafish models using repetitive stress protocols, which include immobilization, extreme changes in water temperature, net chase, exposure to predators, low water levels, multiple tank changes and social isolation [13]. Unpredictable stress protocols (USP) have been proposed to study the behavioral responses to stress in zebrafish [14, 15], and several investigations have shown responses similar to those observed in other animal models [16]. In general, the zebrafish is useful for studying stress and anxiety because, in addition to the multiple general advantages widely described in multiple publications [17]. The anxiety behavior in the zebrafish is reflected in changes that include: reduction in exploratory behavior, preference for darkness (scototaxis), deep swimming tendency (geotaxis), preference of peripheral zones (thigmotaxis), greater frequency and duration of states of immobility (freezing) and increase in erratic movements [18].

On the other hand, the zebrafish model has been used to study addictions, especially the acute and chronic effects of alcohol intake, including its impacts on behavior [19]. Acute exposure to low and moderate alcohol concentrations in adult zebrafish induced behavioral changes that included decreased anxiety, increased locomotion, increased aggressiveness and changes in place preference and in shoaling behavior [20]. These behavioral variations are associated with increased levels of dopamine and serotonin and decreased levels of glutamate and GABA neurotransmitters [21]. Chronic exposure to ethanol produced tolerance, and abstinence increased anxiety and decreased the tendency to shoaling behavior [22]. Serotonin activity has been linked with regulation of aggressive behavior, stress, arousal, movement among other physiological process in central nervous system in several species; mood (such as anxiety, depression and aggressiveness) and serotonin levels have been shown to be inversely correlated [23]. Furthermore, several studies have found a relationship between gene expression and behavior [24]. In this context, some of the main candidate genes are: The brain-derived neurotrophic factor (*bdnf*), which is related to growth, differentiation and maintenance of neurons [25]; the enzyme catechol-methyltransferase (*comt*) responsible for the degradation of catecholamines, including the regulation of dopamine levels [26]; the dopamine transporter (*scl6a3*) and serotonin transporter (*slc6a4*) are responsible for dopamine and serotonin reuptake from the synaptic gap; it has been shown that their genetic loss or pharmacological blockage alter neurotransmission and behavior [27].

In previous years, research has been carried out in larval and adult stages of zebrafish to determine mRNA expression of candidate genes and their relationship with exposure to substances or stress. To date, there has not been enough research on the combined effects of acute environmental stress and alcohol consumption in adults, therefore there is a need to

investigate the effects of environmental stress and exposure to alcohol in adults, their behavioral changes and its relationship with gene expression. In this pilot work, we explore and analyze the behavioral and molecular effects of the combined exposure to an acute USP, similar to those proposed in other animal models to induce stress [16], and ethanol [22] in adult zebrafish.

Methods and Experimental Design

Animals

Male wild-type short-fin zebrafish of 4-6 months of age were obtained from a local animal store in Bogotá, Colombia. Fishes were kept in 7 L tanks (400 ml per fish) at a temperature of 27 ± 1 °C, with a pH between 6.8 and 7.4, in a room maintained on a 12:12 hour light/dark cycle and fed once a day in the light phase with a TetraMin® standardized diet. Additionally, each tank had an aeration system and filters, guaranteeing adequate water conditions for the animals, which had a two-week adaptation period prior any test. A total of 84 male zebrafish were used for experimental tests, with average weight and size of 362 mg and 2.6 cm, respectively. Only males were used due to differences in tests of aggressiveness and in biochemical markers, in comparison to females [28]. All procedures were carried out based on the ethical principles of the 3 R's and the Guide for the Care and Use of Animals in Neuroscience and in Behavioral Research [29]. All protocols were approved by the ethics committee in animal experimentation of Universidad Antonio Nariño (June 06/2017).

Environmental Stress and Acute Exposure to Ethanol

To induce stress, an acute USP was performed for 3 days (Figure 1). This protocol included on day 1 a tank change with the water temperature at 33 °C for 30 minutes, followed by the water temperature at 23 °C for 30 minutes. On day 2, fish were chased with a net for 10 minutes. On day 3, immobilization was carried out in 2 ml microcentrifuge tubes (open at both sides to allow oxygenated water circulation) for 60 minutes [adapted from 14]. USP was carried out in groups of 6 fish in a different tank from storage tank. On day 4, zebrafish were exposed to ethanol (70% ethyl alcohol, Tecnoquímicas, Bogotá, Colombia) at different concentrations (0, 0.25, 0.5, 0.75 and 1%, n = 12 each group). All dilutions were made in a 1 L tank. Zebrafish were exposed to ethanol at 0.25 and 0.5% for 40 minutes, the minimum time necessary to reach concentrations of alcohol in the blood and brain similar to the concentration in the tank [30]. Zebrafish exposed to ethanol at 0.75 and 1% showed buoyancy and balance loss after 15 to 20 minutes, and an exposure time of 40 minutes was fatal in pilot tests. It was thus decided that zebrafish would be exposed for 20 minutes at these doses (0.75 and 1%). Fish were later transferred to the tanks for behavioral analyses, with fresh water for 5 minutes for recovery and behavioral tests were performed. Additionally, a behavioral test without stress was performed with ethanol exposure at 0.75%. First, the behavior tests were recorded without alcohol and then, the same group was exposed to 0.75% alcohol and a second recording was made (pre-post test). A total of 14 groups with 6 fishes each one was generated, 7 groups (control, stress only, stress plus 0.25, 0.5, 0.75, 1% and pre-post of 0.75% without stress) for swimming deep test and 7 groups for mirror proximity test.

Behavioral Tests and Analysis

To evaluate behaviors, computerized tracking techniques were used with video recordings. The videos were analyzed and parameters such as total distance traveled, swimming speed

and place preference, in the swimming deep test, as well as, the time spent in the region of interest in the mirror proximity test were measured. All behavioral tanks were custom-made in acrylic (Surtiacrylicos, Bogotá, Colombia).

After recovery in a tank with fresh water, zebrafish were transferred to a different tank to assess behavior and a 5-minute video was recorded, with a digital camera (iPhone 6, Apple, California, USA) for further analysis. Only one fish was used in each test at the time. Swimming deep test and mirror proximity test were assessed with two different tanks; the mirror proximity test was evaluated with a rectangular tank, with measurements of 18 X 9 X 10 cm (length, width and height), filled up to 4 cm with fresh water, and a 7 cm long mirror was introduced to assess time spent near its close reflection (contact area of 5 cm with respect to the mirror) and video recording from the top view of the tank was carried out (Figure 2A). The time spent near the mirror could indicate aggressive behavior [31].

Swimming deep test was assessed with a trapezoidal tank, with measures of 23 cm in length at the base and 28 cm in the upper region, 7 cm in width and 15 cm in height, allowing us to assess time spent in different portions (lower third and upper two thirds) as an index of locomotor activity and anxiety, video recording from the lateral view of the tank was carried out (Figure 2B). We used the freely available ImageJ software (NIH, Maryland, USA) [32] with the AnimalTracker plugin (developed by Gulyas et al.) [33], which allows automatic analysis of behavior [34]. Video recording settings were in AVI format, MJPEG compressed, 30 frames per second (FPS) and a resolution of 840 x 420 pixels. Each test was 5 minutes in length and all 5 minutes of recorded video were analyzed. AnimalTracker provided data on total distance traveled, average speed, total freezing time, time in the region of interest (ROI), and total trajectories images. The number of freezing episodes during the test was analyzed manually.

Euthanasia and Brain Extraction

Once the behavior test was completed, cryoanesthesia was performed at -4 °C for 20 minutes and then the fish was transferred to a 1.5 ml microcentrifuge tube with 700 ml of dH2O and stored at -20 °C. To prevent possible interactions in molecular tests, the use of tricaine (MS-222) was avoided. Strykowski and Schech [35] reported a higher efficacy in euthanasia at 4 °C or less compared to tricaine, requiring a longer time of exposure but avoiding the introduction of an additional variable for molecular analyses. Whole brain extraction was performed by removing the skull of the fish and making cuts in spinal cord, optic nerves and cranial nerves, after removing the whole brain, it was stored at -20 ° C until RNA extraction.

Total RNA Extraction and cDNA Synthesis

Once the whole brain sample was extracted, total RNA extraction was performed following the TRIzol protocol (Invitrogen, California, USA) to subsequently generate the first-strand cDNA using the M-MLV Reverse Transcriptase kit (Invitrogen, California, USA). Small adjustments were made to the RNA extraction protocol, due to the weight of the brains (<50 mg) and finally, one brain per tube was used in RNA extraction and in the cDNA generation. Samples were stored at -70 ° C.

Quantitative Real Time PCR

qPCR was performed using BrightGreen 2X qPCR MasterMix (ABM Inc., Vancouver, Canada) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, California, USA). A final volume of 10 µl per PCR tube was used, including 2 µl of cDNA (10 ng), 5 µl of BrightGreen MasterMix, 0.8 µl of each primer and 1.4 µl of molecular grade water [36]. The

parameters of the qPCR were: 95 °C for 2 min, followed by 40 cycles at 95 °C for 20 seconds, 55 °C for 20 seconds and 72 °C for 20 seconds [Adapted from 36]. Samples from three experimental groups were used (control, stress only and stress plus 0.75% ethanol): 3 samples were used per group and each sample was run in triplicate. This was decided due the cost for the molecular tests and because they were the groups with significant statistical differences. The *elf1a* gene (elongation factor 1 alpha 1, like 1) was used as a reference gene, as it has been shown that it has a low expression variability in zebrafish [37, 38]. The mRNA expression of genes encoding sodium-dependent serotonin transporter (*slc6a4a),* sodium-dependent dopamine transporter (*slc6a3),* Catechol -O- Metiltransferase (*comta)* and brain-derived neurotrophic factor, variant 3 (*bdnf3)* was determined in brain samples. These orthologs were selected due to their homology with human genes. Sequences of qPCR primers were taken from previous articles and are available in supplementary Table 1. qPCR data for gene expression were analyzed with the comparative CT method ($2^{-\Delta\Delta CT}$), calculating fold changes for candidate genes in experimental groups, in comparison with the control group and the reference gene [39].

Statistical Analysis

Statistical analyses were carried out using SPSS statistics software V.18 (IBM, New York, USA) and JASP V.0.10.2. To evaluate differences in behavioral parameters between groups, an ANOVA of two or more factors was performed followed by Tukey´s HSD (Honestly-significant-difference) multiple comparison test with a reported level of significance at $p \leq 0.05$. The pre-post group was analyzed using a paired t-test, and a two-way ANOVA was used to determine differences in mRNA expression (fold changes) between groups.

Results

In the present work, we explore and analyze the behavioral and molecular effects of the combined exposure to an unpredicted stress protocol and ethanol in adult zebrafish. The animal exposed to the USP underwent a different type of environmental stress each day and the experimental groups subjected to the combined exposure to stress and to different concentrations of ethanol were compared with control groups of stressed and non-stressed zebrafish. Gene expression was analyzed in animals from three experimental groups (control, stress and stress plus 0.75%), using qPCR in cDNA from brain samples.

Swimming deep tests in zebrafish exposed to both unpredicted stress and ethanol

In the trapezoidal tank, the following parameters were evaluated: total distance traveled, average speed, number of freezing episodes and total duration of freezing. In addition, the exploration of specific tank areas was evaluated by measuring the dwelling time in the lower and upper third parts, as well as the total distance and average speed in each zone.

Adult zebrafish exposed to both unpredicted stress and ethanol had a shorter distance traveled, in comparison with the control group (ctrl). The largest difference in the distance traveled was between the control group and the group exposed to 0.75% ethanol ($p < 0.05$, $F = 10.48$. Figure 3A). Similarly, in terms of average velocity, the differences were mainly between the control group and the group exposed to 0.75% ethanol and USP ($p < 0.05$, $F = 10.38$. Figure 3B). On the other hand, the total freezing time was longer in the group exposed to 0.75% ethanol and USP compared to the other groups, especially with the control group ($p < 0.05$, $F = 9.07$. Figure 3C). There were no significant differences in the number of freezing episodes between groups.

The group exposed to 1% ethanol and USP presented on average a shorter exploration time in the lower third of the tank with 186.1 seconds while animals exposed to 0.75% showed an average of 297.6 seconds; however, there were no statistically significant differences between them ($p = 0.11$) or in contrast to the other groups. Fish exposed to stress plus ethanol (0.25, 0.5, 0.75 and 1%) traveled a smaller distance in the lower third with respect to the control group, having statistically significant differences ($p = 0.014$, $F = 3.35$. Figure 3D). On the other hand, in the upper third portion of the tank there were no statistically significant differences in the time of stay, total distance and average speed.

Mirror proximity tests in zebrafish exposed to both unpredicted stress and ethanol

In the mirror proximity test, the total distance traveled, the average speed and the time spent in the contact area, near the mirror, were evaluated. The distances traveled by the groups exposed to concentrations of 0.25% and 0.75% ethanol, in combination with USP, were shorter (97 cm and 220 cm) in contrast to the control group (463 cm), with a p value <0.05, $F = 4.81$, in both cases (Figure 4A). There were no significant differences in the length of stay in the contact zone between the different groups exposed to stress and ethanol compared with the control group, nor differences between the groups, with a *p* value of 0.46, $F= 0.93$ (Figure 4B).

Overall, these data show that USP modifies the responses in the swimming deep tests and mirror proximity test, especially with alcohol concentrations of 0.75%, where a reduction in activity is observed.

Behavioral tests in animals exposed to ethanol without stress

A pre-post alcohol test was performed for both behavioral tests ($n = 6$ per group) to evaluate differences with those animals exposed to stress. Zebrafish exposed to 0.75 % of alcohol (post) showed a significant decrease in locomotion compared with 0 % (pre) (Figure 5. A,

B), having a reduction in distance traveled and in average speed (t = 2.459, p= 0.032; t = 2.63, p= 0.023, respectively). An analysis of behavioral paradigms (Figure 5. C, D) did not show differences in distance and average speed for the group exposed to alcohol (mirror proximity test: t = 1.639, p= 0.162; t = 1.609, p= 0.169; swimming deep test: t = 1.914 p= 0.114; t = 1.727 p= 0.145). For the swimming deep test, the mean time spent in the lower third showed no differences (t = -0.197 p= 0.852) (Figure 5. C); the mean distance and average speed in the lower third did not show significant differences in comparison with the pre-test (t = 0,812 p= 0.453; t= 1.907 p= 0,115, respectively). For mirror proximity test in the pre-post test, time spent in ROI was not significantly increased in the alcohol exposure group (t = -1.261 p= 0.263) (Figure 5. F); the average speed (t = 2.749 p= 0.040) in the lower third was decreased compared with the pre-test. It is important to mention that the fish did not show freezing in the pre-post tests, in comparison to those under stress plus 0.75% of alcohol.

mRNA Expression

Gene expression for four candidate stress-related genes was analyzed in three experimental groups: control, stress and stress plus 0.75% alcohol. A significant 5-fold down-regulation were observed for *comta* and *bdnf3* in stress and stress plus 0.75% groups compared with the control group (F= 30.17, p= <0.001; F= 12.57, p= 0.007, respectively) (Figure 6. A, B). Compared with the control group, gene expression of *slc6a3* was significant down-regulated in the stress group (F= 7.406, p= 0.024) (Figure 6. C). In contrast, expression of *slc6a4a* gene did not show significant differences (F= 1.325, p= 0.334).

Discussion

In the present exploratory work, we analyzed the behavioral and molecular effects of the combined exposure to unpredicted stress and alcohol in adult zebrafish. Previously, the effects of stress and ethanol have been studied separately and mainly in zebrafish larvae. The novelty in the present work is that we studied the combined effects of acute stress associated with an acute dose of alcohol, using behavioral and molecular tests in adult zebrafish.

In animal models, there are different protocols to induce stress and anxious behavior, including physical stress (single-prolonged and restraint stress, foot shock, stress-enhanced fear learning and underwater trauma), psychological stress (predator-based psychosocial and predator scent stress) and social stress (housing and social instability and early life stress). USP, a type of physical stress, has been proposed as a model to study anxiety, depression and mood disorders [16, 18]. This model has been adapted to the zebrafish model with similar results that have indicated alterations in the physiological and biochemical responses to the stress [14, 15]. In the USP implemented in this work, we changed the stressing factor every day to avoid habituation, as it has been suggested by other researchers [15]. Given the evidence reported, we consider that the use of an anxiogenic conditioning stimulus (manipulation with a net), in addition to the use of environmental stress factors that change between days (temperature, persecution, immobilization) are elements that can lead to anxiety and other behavioral and physiological responses that are common in many mental disorders.

The effect of alcohol on anxiety and aggressiveness in this model was evaluated using concentrations of 0.25, 0.5, 0.75 and 1% alcohol. Blood alcohol levels reached with these concentrations have been measured in previous studies using spectrophotometry techniques and have shown that exposure to concentrations of 0.5% alcohol for 10 minutes led to serum

concentrations of 0.065% and concentrations of 1% led to serum concentrations of 0.1% [20]. In humans, the legally permitted concentrations must be less than 0.08%. However, it is notable that at alcohol concentrations of 0.06% there are already clinical manifestations of toxicity that include neurological alterations with sensorimotor impairment, decreased reflexes, dysmetria, decreased peripheral vision and alterations in reasoning [40]. To ensure adequate serum concentrations in this study, we increased the exposure time to 40 minutes (alcohol 0.25% and 0.5%) and only to 20 minutes with alcohol at 0.75% and 1%, due to the finding that USP and an exposure of 40 minutes at these higher concentrations caused toxicity and mortality in some fish. Exposure to ethanol has previously been carried out without reported mortality, however, in the present work the exposure for 40 minutes was fatal, probably due to the associated stress factor. The causes of death go beyond this work.

In other models, it has been observed that low concentrations of alcohol are associated with a disinhibition of behavior and a tendency to aggression, which is partly explained by the GABAergic effect of alcohol. In contrast, at high concentrations ethanol is a general depressant. In zebrafish, low and intermediate doses of alcohol (0.25-05%) induce a state of arousal and high aggression. Concentrations of alcohol at 1% cause motor alterations and higher doses can cause lethargy [24].

The results presented here show a depressive effect of alcohol on exploratory behavior and a decrease in aggressive responses in a model of US. This effect has previously been described in adult zebrafish under the effect of alcohol but without prior exposure to environmental stress [20]. In our work, the largest effect of alcohol plus stress was achieved with alcohol concentrations of 0.75%, leading to a decrease in total distance traveled and swimming speed and an increase in immobility times. This is contrary to what happens under normal conditions, as observed in the control group and non-stressed zebrafish. A possible

explanation may be related to the effect of stress on neurotransmitters, especially depletion of glutamate and increases in GABA [21, 27, 30]. Alcohol might enhance the inhibitory effect of GABA on stress and anxiety conditions, decreasing exploratory and aggressive behaviors and increasing freezing times. An additional element to be considered is the fact that acute exposure to alcohol has been associated with increased dopamine in a dose-dependent manner and an increase in serotonin at a dose of 1% alcohol, which is associated with a reduction in the tendency toward clumping [27, 30]. Researchers have suggested that dopaminergic reinforcement circuits are involved in the feeling of well-being and protection generated by grouping. In the case of repeated stress, a decrease in 5HIAA is observed and exposure to alcohol concentrations at 1% induced an increase in dopamine, without affecting 5HIAA [38].

Although the results of gene expression presented here did not show differences in the serotonin transporter (*slc6a4a*), previous works found a direct relationship between serotonin levels and transporter expression [41]. Previously, an overexpression of serotonin transporter has been described in 72 hours post-fertilization larvae exposed to ethanol and in adults exposed to ethanol it has been found a reduced expression of serotonin transporter. *bdnf* has been studied in animal models of stress and in postmortem brains of humans with neuropsychiatric disorders. Results presented here showed a decrease for *bdnf3* in the whole brain for stress and stress plus alcohol groups, suggesting that stress plays an important role in the regulation of neuronal maintenance and might be related to the decrease of *bdnf3* expression. On the other hand, as *comt* regulates the levels of catecholamines, its genetic variants and its expression levels have shown a relationship with neuropsychiatric disorders in humans and behavioral changes in animal models [42, 43]. The results shown here show a decrease in expression for *comta*, mainly in the group exposed to stress plus alcohol.

Future molecular analyses would allow the identification of the effects of US and alcohol on additional genes involved in multiple neurotransmission and neuroplasticity mechanisms [44]. It is expected that the information obtained from this study will be useful to advance the knowledge about the factors related to alcohol misuse and its relationship with aggression, anxiety and stress-related disorders. In this context, these results might be of particular relevance for developing countries, in which psychosocial stress and posttraumatic stress are quite common, due to poverty, civil wars and multiple social conflicts [45, 46]. This experimental model will also be helpful for the exploration of novel treatments for stress, anxiety disorders and substance misuse, considering the possibility of exploring the novel effects of known pharmacological compounds [49].

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Supplementary information

Gene	Forward primer	Reverse primer	Reference
slc6a4a	ACTGCACCCACTACCTGTCC	ATGCCAGGAGAACACCAAAG	67
slc6a3	AGACATCTGGGAAGGTGGTG	ACCTGAGCATCATACAGGCG	84
comta	TCACGACCACAGCGCATCT	CCCACATTCATGGCCCATT	83
bdnf3	GGCGAAGAGCGGACGAATATC	AAGGAGACCATTCAGCAGGACAG	73
elf1a	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC	74

Supplementary table 1. Primers used in RT-qPCR.

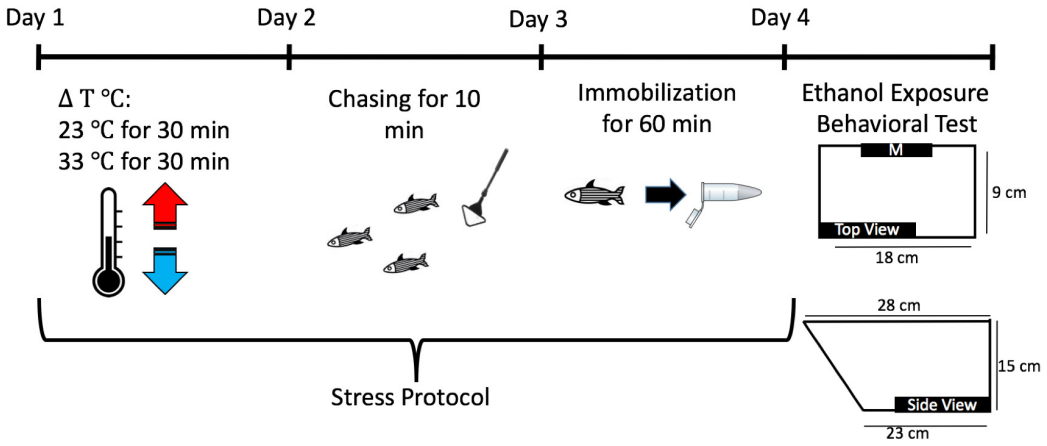

Figure_1

Figure 1. Overview of the USP, ethanol exposure and behavioral assessment. The US protocol was applied for three days. On day one, water in tanks was heated then cooled and zebrafish were exposed for 30 minutes at each temperature. On day two, zebrafish were chased with a net for 10 minutes. On day three, zebrafish were placed in a 2 ml microcentrifuge tube for 60 minutes. Finally, on day four, fish were exposed to ethanol (0, 0.25, 0.5, 0.75 and 1%), and mirror proximity (top) and swimming deep (bottom) behavioral tests were carried out. M: Mirror.

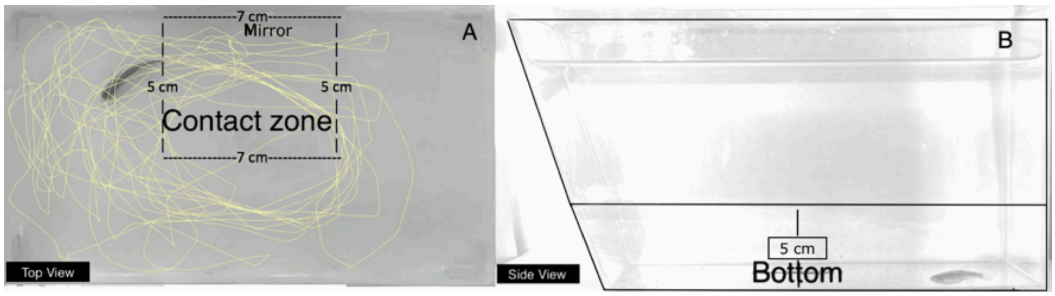

Figure_2

Figure 2. A. Top view of the tank used in the mirror proximity test; the dotted lines limit the defined contact area in relation to the mirror (located in the upper region of the image). Yellow solid lines show an example of tracking with the ImageJ software and the AnimalTracker plugin. B. Side view of the trapezoidal tank used in swimming deep test. The lower third is an area of 5 cm in height, and the upper third is 10 cm in height.

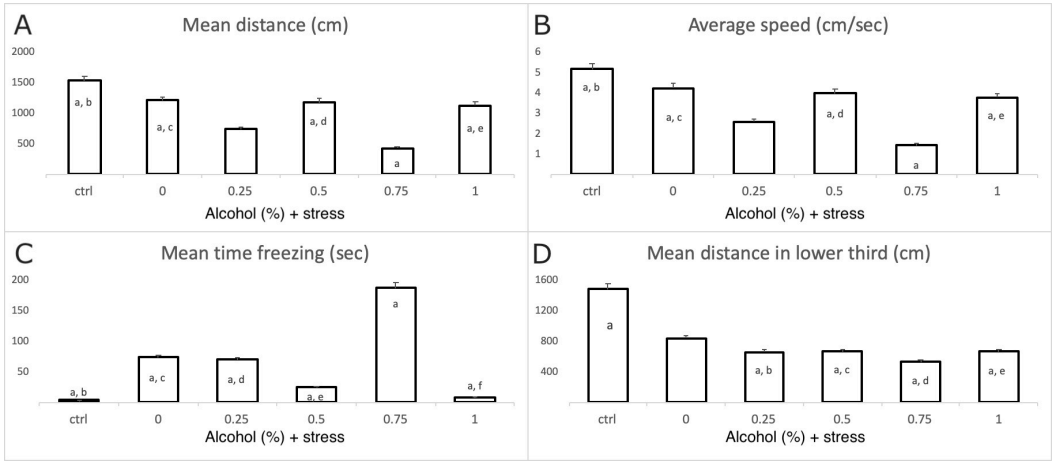

Figure_3

Figure 3. Locomotion parameters and swimming deep test. First bar of each graph corresponds to control group (ctrl), the following bars to the groups exposed to the US protocol plus ethanol doses. The individual letters correspond to the group that presents significant differences in relation to the other groups. A. Mean distance traveled. a, b: p = 5.18 x 10-8; a, c: p = 0.000096; a, d: p = 0.00021; a, e: p = 0.00064. ANOVA p =

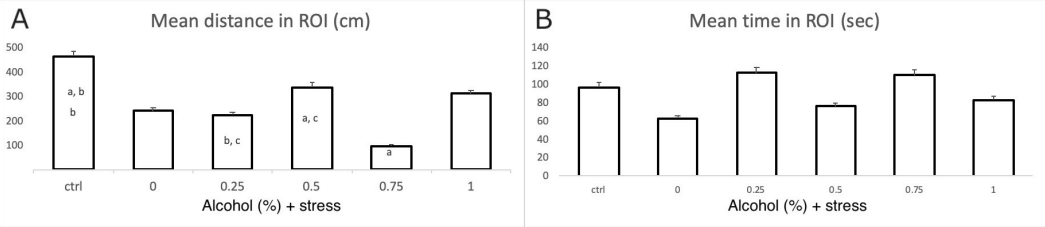

Figure_4

Figure 4. Mirror proximity test parameters. First bar of each graph corresponds to control group (ctrl), the following bars to the groups exposed to the US protocol plus ethanol doses. A. Mean distance in region of interest (ROI) (cm). The individual letters correspond to the group that presents statistically significant differences in relation to the other groups. a, b: $p = 0.00074$; a, c: $p = 0.045$; b, c: $p = 0.048$. ANOVA $p = 0.002$, $F = 4.81$. The bars indicate the means with the standard error (SE), $n = 6$ per group. B. Mean time in ROI. No significant differences were found, ANOVA $p = 0.377$, $F = 1.097$.

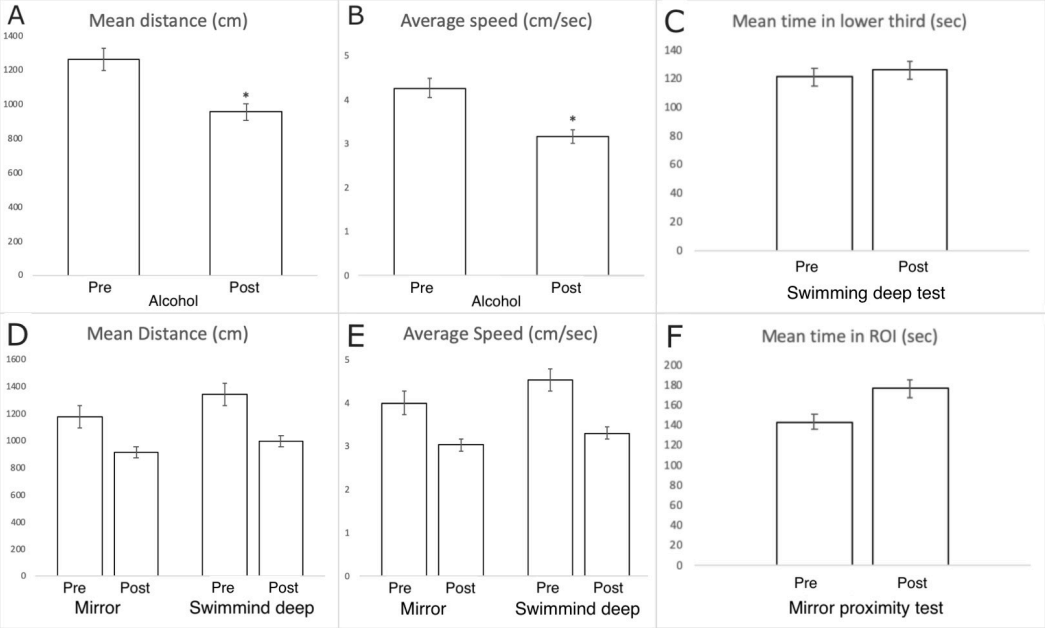

Figure_5

Figure 5. 0.75% pre-post test without stress, locomotion parameters. A and B correspond to mean distance and average speed respectively, Swimming deep test and Mirror proximity test were including in the analysis, n = 12 per group. D and E correspond to mean distance and average speed respectively. C correspond to mean time spend in lower third in Swimming deep test, F correspond to mean time spend in ROI in mirror proximity test. The x-axis indicates the behavioral tests, pre indicates 0% and post, 0.75% of alcohol. n = 6 per group. The bars indicate the means with the standard error (SE). *, P

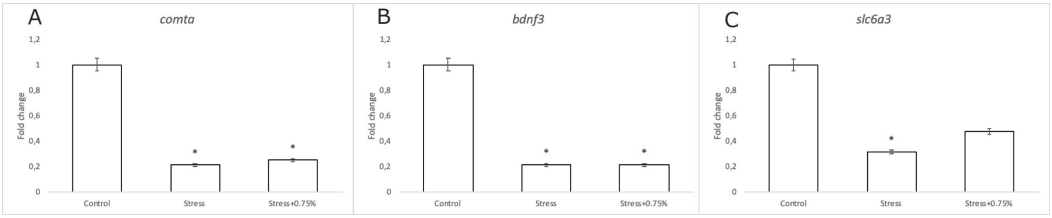

Figure_6

Figure 6. mRNA expression. Fold changes, relative to elf1a. A. comta. Groups exposed to stress and stress plus 0.75% alcohol showed a decrease of 5-fold. B. bdnf3. Groups exposed to stress and stress plus 0.75% alcohol showed a 5-fold. C. slc6a3. Groups exposed to stress and stress plus 0.75% alcohol showed a decrease to one third and half, respectively. The bars indicate the means with the standard error (SE), n = 3 per group, samples were run in triplicate. *, P

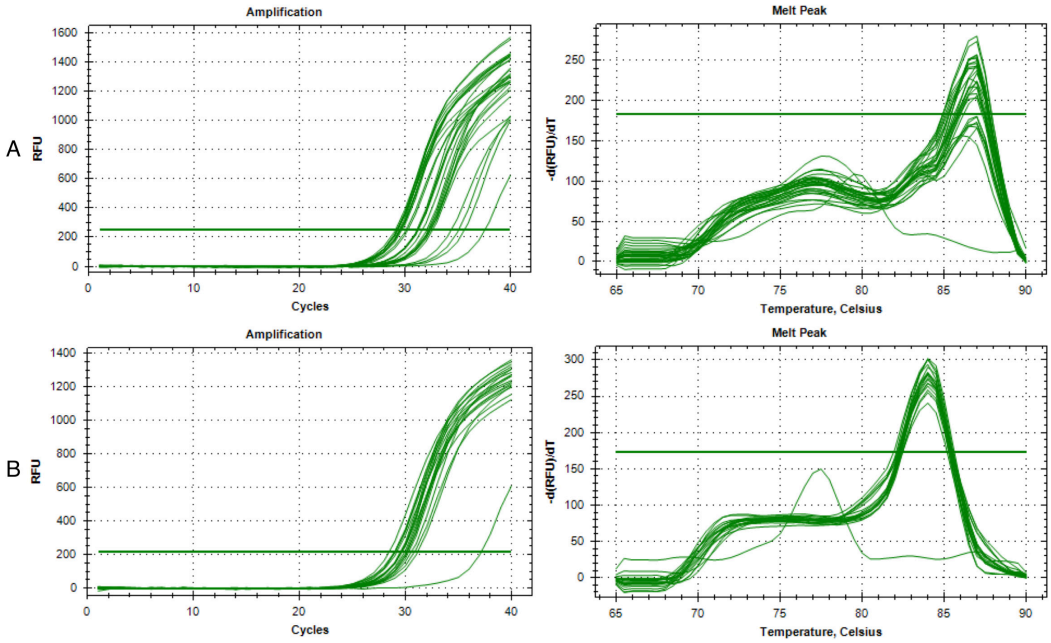

Figure_7

Supplementary figure 1. Representative amplification curves and melt peak for RT-qPCR. A. elf1a amplification curve (left) and melt peak (right). B. comta amplification curve (left) and melt peak (right).

Producto 4

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ZebraMov: An automatic strategy to analyze and quantify zebrafish social behaviour from kinematic patterns captured in video sequences.

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ZebraMov: An automatic strategy to analyze and quantify zebrafish social behavior from kinematic patterns captured in video sequences

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Abstract

The zebrafish (Danio Rerio) is an emerging animal model for studying the effects of chemical compounds, drug discovery, toxicology, neuropsychiatric disorders and disease mechanisms, among many others. Locomotion and behavioral analysis have become an essential test in order to support molecular tests and many other interventions and analysis. Many of the existing computational strategies to analyze motion behavior require many calibration steps and are limited to specific scenarios and many times only compute global motion trajectories of specimens of interest. This work introduces ZebraMov, a novel strategy to automatically measure kinematic occurrence maps that express spatio-temporal patterns of zebrafish, from long-motion trajectories captured into a social behavior experiment. Zebramov is an open-source algorithm available for all operating systems and capable of analyzing any type of video and behavioral tests. In the present work, zebrafish were subject to environmental acute stress and later to 10 or 100 µM of caffeine for 20 minutes and finally to the social behavioral test for 5 minutes. As a main contribution, the proposed strategy returns occurrence kinematic maps that recover locomotion history that allows major description of particular social behavior of each specimen in singular video sequences. These maps can enhance regions of interest and explain behaviors according to velocity and acceleration frequency patterns. In addition, the proposed approach has the capability to track global locomotion of zebrafish

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like standard applications, such as the AnimalTracker (a plugin for behavioral analysis in ImageJ). Locomotor results show a decrease in distance, velocity and acceleration mainly in the group exposed to stress plus 10 µM of caffeine. Besides, the results show a preference for the social zone in all groups which increase with stress plus 100 µM of caffeine exposure, these results were verified with occurrence maps for each group.

Keywords: zebrafish, spatio-temporal patterns, automated analysis, behavior science, caffeine, stress, occurrence kinematic maps.

Introduction

The interaction with other individuals is a fundamental part of behavior in many living beings, including humans and its dynamic alteration may reflect disorders of the nervous system (1). In humans, altered social behavior may reflect neurodevelopmental disorders, such as autism, or acquired diseases, including affective and cognitive disorders, that could come from several diseases (2, 3, 4). The analysis of behavior in animal models has been fundamental to explain and to support diverse phenomena in research areas such as neuroscience, psychology, and neuropsychopharmacology (5, 6, 7). In many animal models, the motion patterns play a fundamental role to correlate emotional, cognitive and social interaction behaviors. Hence, technological tools are key to support the capture and analysis of such locomotion patterns by quantifying kinematic and dynamic patterns in those specimens. For instance, the use of specialized platforms to capture distance, velocity and acceleration measures have been used in animal models such as mice (8), Drosophila (28, 36) or dogs (29). Moreover, some special devices have been attached to body animals to capture kinematic trajectories and correlate neurological and cognitive functions, such as instance muscular contractions and breathing. In addition, in the literature there is evidence

of similar works with smaller experimental individuals such as insects. These methodologies nevertheless could be invasive and alter the natural movement of animals, which are conditioned to specific scenarios of capture.

Nowadays, in preclinical research, the zebrafish has emerged as an alternative model for research because of its remarkable natural social characteristics (9, 10, 11). For instance, the alterations of typical shoal behavior, frequently associated with zebrafish, has been found as reflection of neural functions (12, 13, 14). However, the morphological conditions of the zebrafish limit its analysis in the study of locomotive behavior and classical methodologies could result limited to explore complex social dynamics of the fish. In such underwater scenarios, the movement patterns are usually obtained from computational tools that analyze video sequences. These tools require sophisticated processes of calibration and they are dedicated to capture general kinematic information from global trajectories that describe the motion of a specimen into a controlled scenario (15, 16, 17). In general, these tools involve a segmentation and a tracking of the object of interest using global trajectories. However, this analysis results coarse on dynamic description and lose a lot of relevant information about experiments that could be fundamental to highlight findings or to support the validation of a hypothesis. Current animal motion strategies have complemented such analysis by introducing machine learning concepts to characterize motion patterns from computed motion trajectories. For instance, JAABA or Ctrax calculate motion descriptors from trajectories and generate the behavior analysis data, as it has been tested in Drosophila melanogaster (28).

This article introduces a computational strategy for the analysis of zebrafish social behavior based on the quantification of dense spatial-temporal patterns during locomotion. The

proposed strategy is able to capture the fish motion history, that is strongly correlated with social behaviors, and it is expressed as spatial occurrence kinematic maps1. For this purpose, the top view of a fish in a tank is recorded, with a relatively static camera. The specimen is followed using a global perspective and also a set of dense trajectories, i.e., the local motion for each pixel associated with the fish. At each time, from the resultant trajectories it is computed the velocity and acceleration, preserving the spatial localization of the measures. Then, motion profiles are obtained, which accumulate the kinematics spatially and describe the motion history. With this approach it is possible to make a detailed analysis of the fish behavior and quantitatively compute patterns representing the treatments to which the fish were subjected. During the validation, behavioral tests were performed on zebrafish control and experimental groups to evaluate social behavior. From occurrence maps it was possible to observe and analyze spatial fish patterns, and thus, obtain visual maps that explain behavior of each group with remarkable differences in kinematics for zone frequencies or occurrences and distance using a dataset with zebrafish subjects to acute stress and exposed to caffeine concentrations to validate the utility of our method, thus obtaining a clear classification and segmentation of the data, based on the information captured by neural networks.

Methods and Experimental Design

Subjects

Seven-month-old wildtype short-fin zebrafish were obtained from a local animal store in Bogota, Colombia and acclimatized for a period of at least two weeks before any experiment.

[1] The code is available in https://gitlab.com/bivl2ab/research/2020-2-edgar-zebrafish-behavier-net. The strategy was written in Python supported mainly by the pandas, numpy, matplotlib and OpenCv libraries.

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Fish were kept in 7-liter tanks, 400 ml per fish, at a temperature of 27 ± 1 ° C, pH between 6.8 and 7.4 with a dark light phase of 12:12 hours and fed once a day with a standardized diet from TetraMin®. Each tank had an aeration system and filters to ensure water quality. A total of 22 male and female zebrafish were used. All protocols were approved by the ethics committee in animal experimentation of Universidad Antonio Nariño (June 06/2017).

Treatments and behavioral test

Three groups (n = 6 per group) were initially subjected to an Unpredictable Stress Protocol (USP) for 3 days. This type of acute stress includes on day one, water heating at 33 °C for 30 minutes followed by transfer to a tank with water temperature at 23 °C; on day two, fish were chased with a net in the container tank for 10 minutes and lastly; on day three, fish were immobilized for one hour in 2 ml microcentrifuge tubes, open at both sides to allow oxygenated water circulation (Protocol adapted from Piato et al. 2011) (18). As it can be noted, exposure to stress was increased and changed every day to avoid habituation. USP was carried out in groups of 6-7 zebrafish and always in the afternoon. On day 4, the two groups were exposed to caffeine (100 gr powder, Sigma-Aldrich, St. Louis, Missouri, United States) at concentrations of 10 µM (1,94 mg/L) and 100 µM (19,41 mg/L) for 20 minutes. All dilutions were made in a 1 L tank and an additional group of 6 fish was taken as control. This experimental setup creates four groups: stress, stress plus 10 µM, stress plus 100 µM of caffeine and control group (without stress or caffeine exposure).

Individually, fish were transferred to the social tank (Figure 1) with measures of 30 X 10 cm (length and width), filled with fresh water up to 5 cm. Each end of the tank has a compartment of 10 X 10 cm, one empty and one with a conspecific fish (used to stimulate social behavior and not recorded) that was located at one end (19). A digital camera (iPhone X, Apple,

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California, USA) located 33 cm away from the base of the tank in a top view, was used to record the behavior for 5 minutes to be analyzed offline; the videos were recorded at 720p and 30 frames per second. The camera has a lens aperture of f/1.8 and optical image stabilization that reduces video distortion. Finally, the caffeine used is an antagonist, non-selective competitive adenosine receptor that blocks the A1, A2A, A2B and D2 receptors with mainly effects in the central nervous system (CNS) (20, 21) and changes in zebrafish behavior increasing anxiety, aggressiveness and alertness (21).

Behavior Analysis

In the proposed methodology, we developed two analyses: The first one consisted of a global analysis of the movement in the experimental zebrafish specimens, as in classical frameworks, at each frame, the zebrafish is represented by a point, which is followed during the video. In this approach, the coordinate$(x,y)$of the moving specimen is estimated for each frame, thus, the resulting global trajectory is represented as the set of points and the traditional kinematic variables (position, velocity, acceleration) are calculated using these data. The second analysis recovers a dense motion representation (relative velocity for each pixel) from an optical flow algorithm. The pixel velocities with coherent motion are grouped to form long motion trajectories that represent the specimen motion with larger dynamic information. From such long motion trajectories, it was possible to compute local and differentiable kinematics which also have a spatial reference into the scenario of capture. Then, a spatial 2D frequency histogram was captured from each of the kinematic trajectories that code history of motion patterns, which gives richer information about specific social behavior. As baseline, the analysis of the behavior was initially performed, from only the global perspective, with the free software ImageJ (NIH, Maryland, USA) (22) and the

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AnimalTracker plugin (23) that allows the semiautomatic analysis of the animal conduct. AnimalTracker provided data on total distance traveled, average speed, total freezing time, time in a region of interest (ROI), and total trajectories, the data obtained were subsequently compared with the proposed algorithm.

Statistical Analysis

Statistical analysis was performed using JASP V.0.11.1 (24). In order to determine significant differences in kinematics, multiple group comparisons were made using two-way ANOVA followed by Tukey's HSD test (Honestly-significant-difference) with the groups mentioned before (control, stress, stress with 10 μM and stress with 100 μM of caffeine). Two-way ANOVA followed by Tukey's HSD test (Honestly-significant-difference) multiple comparison test was used to analyze the statistical differences in zones and minutes. Statistical significance was set up at $p \leq 0.05$ in all statistical comparisons.

Kinematic quantification of locomotor patterns in zebrafish

This work bases locomotion analysis on dynamic description from long motion trajectories captured from global and local perspectives. Regarding global trajectories, a recurrent difference of frames was applied to model the static scenario. From such difference, the background is removed and the object in motion (i.e., the zebrafish) is automatically segmented.

Formally, the tank is recorded in a video sequence, described by a sequence of frames $I_t(x)$, whose pixels (x) that temporally remain with same appearance are labelled as background $B($ $x)$. This background estimation is achieved by computing the mean, as: $B(x) = \frac{1}{T}\sum_t^T I_t(x)$ over the whole video sequence. Hence, each frame is subtracted from this background $S_0(x$

) = $|B(x) - I_t(x)|$ to obtain an estimation of changing pixels during time (most of them belong to the fish). A final segmentation is then obtained by applying a threshold function resulting in a binary image $S = \{s \epsilon S_0 \mid s > \tau\}$, where the silhouette corresponds to the segmentation of the fish. This simple heuristic allows a totally automatic, effective and fast segmentation of the specimen without any additional constraint about morphology or motion trend of the fish. An illustration of obtained temporal segmentation is shown in supplementary figure 1.A and all the workflow in figure 2.

From such temporal segmentation, it was possible to capture the center-of-mass trajectory (CoM) of zebrafish, defined as $C_t = (C_x, C_y)_t$, during the experiment (Supplementary figure 1.B). This temporal CoM pattern summarizes the position, movement and musculoskeletal structure of the fish in a single point. The set of CoM points, computed at each frame from the global CoM trajectory that describes the general displacement of zebrafish in study and constitutes the global history of movement throughout the video. From this global motion trajectory (C_t, CoM during time) it was possible to recover kinematic primitives such as velocity components, like the speed $s_t = \|C_{t+1} - C_t\|$ and motion orientation θ_t (supplementary figure 1.C). Additionally, the acceleration was captured as differential kinematic from consecutive velocities.

During a first evaluation, it was found that distance and mean velocity obtained from this automatic approach correspond to the manually annotated by an expert using tools such as AnimalTracker plugin of ImageJ. This fact results in a great alternative to carry out the automatic analysis of individual specimens in multiple experiments including behavioral sciences and tracking of moving objects, avoiding tedious annotation and dependent on an

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expert over long video sequences. Moreover, these subjectivities task can generate inaccurate results.

Analysis of local fish movements

A main contribution of this work is the dense dynamic description of zebrafish behavior, achieved from a set of long motion trajectories that together achieve a better historical description of particular motion patterns at each treatment group. The implemented dense trajectories are recovered from vectorial fields, computed between two consecutive frames, from an optical flow algorithm. In this work was implemented the Farneback multiscale flow that has a proper trade off between accuracy and speed to recover velocity fields in video sequences (36). Velocity patterns from optical flow are relevant to understand the social behavior of the fish but result restrictive to describe complex or temporarily long patterns. Then, from a dense spatial grid of each vector field main velocity patterns are tracked, which along frames, are concatenated to form long motion dense trajectories (MDT) (25). Formally, each trajectory consists of a series of points that describes the temporal path of pixels that conform the fish, denoted as: P(t) = $\{p_1, p_2, p_3, p_4, \ldots p_n\}$ where each value of P represents a coordinate, $p_n = (x_n, y_n)$. Hence, from position p_1 to p_2 there exists a velocity vector that concatenates both positions, and results from optical flow computation between two consecutive frames. Such trajectories allow the quantification of spatio-temporal patterns and enable the computation of kinematics such as distance, speed, acceleration or angular direction, becoming a powerful tool for the description of movement features of the fish (Supplementary figure 1.D). Trajectories with small movement (less than 4 pixels) corresponding to background are removed from analysis to obtain a motion representation focused only on fish dynamics.

MDT works as spatio-temporal descriptors of zebrafish movement with altered behavioral states, characterizing the irregular movements that these specimens may present according to a drug or treatment used (e.g., their level of stress and caffeine). MDT is a great tool in the characterization of zebrafish social dynamics and allows the study of social behavior experiments, regarding the environment with local sensibility of different zebrafish segments.

Creation of Kinematic Maps

From MDT it was possible to summarize historical fish behavior as a map of X occurrences which for the position kinematics calculates the frequency at each coordinate where movement was detected by the following equation: $X(p_x,p_y) = X(p_x,p_y) + 1$ summing the kinematics found within the same location during the experiment. For our other variables of velocity and acceleration we compute the maximum value obtained per coordinate $V(p_x,p_y) = max(\sqrt{(p_{x-1} - p_x)^2 + (p_{y-1} - p_y)^2})$ and $A(v_x,v_y) = max(\sqrt{(v_{x-1} - v_x)^2 + (v_{y-1} - v_y)^2})$ so that we focus on the areas of interest where the most significant changes or behaviors stand out. These frequency distributions are stored on a map (with frame size WxH), where each location will count the particular kinematic that results during the experiment. Hence, at each frame, the map is updated with computed kinematic trajectories being the position, speed and acceleration included in this study (Supplementary figure 1.E-G). At the end of the sequence, the resultant map has an historical occurrence of each kinematic for each of the positions of the frames. This representation allows us to directly visualize the frame regions where the fish has spent more time, or have particular velocity and acceleration profiles, denoting the regions with the highest frequency of positions captured by the fish in relation to the time of the video.

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These occurrence maps are not limited to the comparison of values with frequency positions but also allow the comparison of the behavior of more kinematics allowing to quantify locally data such as velocity or acceleration and as in the previous case you get a representation of the spatial distribution that also takes into account the behavior in time that ultimately describes the movement of the fish. Examples of such maps can be observed on supplementary figure 1. E-G.

Statistical Correlation with Kinematic maps

In order to obtain a statistical comparison, a correlation (R) was performed. The mean position occurrence maps (histograms) of the controls were compared with each occurrence map (5 minutes) of the controls, stress, stress plus 10 µM and stress plus 100 µM of caffeine. First, the data are transformed from a two-dimensional (R^2) into a one-dimensional vector space (R^1). R was calculated from the following equation: $R = \frac{1}{n-1} \frac{\sum_{i=1}^{n}(b_i - \overline{B})(c_i - \overline{C})}{\sqrt{\overline{B}}\sqrt{\overline{C}}}$ Where $B = (b_1, b_2, b_3, \ldots b_n)$ is the mean position occurrence map of the control group and $C = (c_1, c_2, c_3, \ldots c_n)$ corresponds to the data of one histogram of any group. These two unidimensional histograms were compared, generating a value for each comparison between each individual map against the mean control map.

Intersection

Additionally, a comparison was developed by computing the intersection of our occurrence maps in one-dimensional space ($\mathbb{R}1$). To estimate the intersection of the B and C arrangements of n size, the sum of the minimum values between each of the aligned B and C components was calculated with the following equation: $d = \sum_{i=1}^{n} min(b_i, c_i)$, where d

represents a similarity distance between the maps that may change between 0 (Totally different) and 1 (Totally similar). Finally, the control data were compared by intersection against our entire data set (each individual map against the mean control map).

Evaluation and Results

Fish behavior data obtained in social tasks was analyzed comparing the following groups: control, stress (without any substance), stress plus 10µM and stress plus 100 µM of caffeine. Initially, the mean distance, average speed and acceleration were analyzed in a complete tank. Moreover, in order to compare changes in each minute (0-1, 1-2, 2-3 and so on) an analysis minute per minute was performed. Hence, it was analyzed the mean occurrences by zone (lateral, middle and social) and time; and mean trajectories with occurrence maps and its standard deviation in complete tank.

Global patterns results

For the complete tank, groups exposed to acute stress and caffeine presented a significant decrease in locomotor behavior. Two-way ANOVA showed differences in mean distance ($F= 10.04$, $p= <0.001$) and average speed ($F= 10.26$, $p= <0.001$), these were lower compared to the control group (Figure 3.A, B). Acceleration was significantly decreased in the group exposed to acute stress and 10 µM of caffeine ($F= 2.97$, $p= 0.042$) compared to the control group (Figure 3.C). Behavioral locomotor results minute by minute in complete tank showed the same trend mentioned above, Two-way ANOVA showed a significant decrease in distance mainly in stress plus 10 µM at minute 1 ($F= 7.60$, $p= 0.002$), minute 2 ($F= 7.07$, $p= 0.002$), minute 3 ($F= 7.00$, $p= 0.003$), minute 4 ($F= 10.12$, $p= <0.001$) and minute 5 ($F= 6.75$, $p= 0.003$). Tukey's post-hoc test revealed a significant decrease in distance in stress plus 100

μM at minute 1 (p= 0.011), minute 3 (p= 0.040), minute 4 (p= 0.004) and minute 5 (p= 0.016) compared to control; finally, stress group showed a significant decrease in distance at minute 4 (p= 0.030) compared to control group (Figure 3.D).

In order to calculate the permanence of the fish in a given zone, position occurrences maps were used. The results are reported in a range from 0 to 100%. The occurrences analysis by zones showed a significant preference in all the groups for the social zone in comparison to middle and lateral zones (ANOVA, F= 36.04, p= < 0.001) (Figure 4.A). Two-way ANOVA did not show significant differences between zones and experimental groups compared to control in five-minute test; lateral zone (F= 0.608, p= 0.61), middle zone (F= 0.849, p= 0.48) and social zone (F= 0.741, p= 0.54) (Figure 4.B). Noteworthy, there is a preference for social zone, especially for the group subject to stress plus 100 μM of caffeine that remained a mean time of 75%, decreasing exploration in the other zones.

A minute-by-minute analysis (Supplementary figure 2) using two-way ANOVA did not show a significant difference of the zones explored. Anyway, with a similar behavioral patron mentioned above, zebrafish showed a higher frequency in the social zone with some changes per minute and group. At minute one, lateral exploration was higher in the group exposed to stress plus 100 μM of caffeine, but without significant differences in contrast with control group (F= 0.64, p= 0.54); at minute two, there was a behavioral change, lateral exploration was reduced without a significant difference in comparison with control group (F= 1.16, p= 0.35). This behavioral pattern was accentuated at minute four (only in the group stress plus 100), prevailing exploration in social zone and lower exploration in lateral and middle zone (F= 50.19, p= 0.004, p= 0.002 respectively), in contrast, control group did not show significant differences in lateral and middle zone (p= 0.6, p= 1 respectively) compared with

social zone (Supplementary figure 2). These results suggest that both acute stress and 10 µM of caffeine are able to reduce locomotor behavior without changes in social behavior. Moreover, acute stress associated with exposure to 100 µM of caffeine, in addition to reduced locomotor behavior, significantly increases social cohesion with effect over time, with the first minute as a recognition environment phase and later the marked preference for social zone.

To validate the obtained results, a comparison of distance and speed was made with AnimalTracker results. As can be seen in supplementary figure 3, both results are similar, however, due to manual calibration of some steps for image processing in AnimalTracker, the fish/object may get lost in some frames and therefore information is lost. It is important to keep in mind that some possible variations may correspond to the calibration of the pixels per cm, for this reason the standardization of some parameters is emphasized to minimize the error when processing the videos (see discussion).

Local patterns results

We were able to extract the characteristic patterns of each treatment administered by processing the accumulator maps (or average map) for each group. The average map per group was calculated and with it, its respective standard deviation. The results show the characteristic regions with the highest concentration of movement summarized in a single map (Figure 5). The left column shows the mean position occurrence maps by groups, the intensity values that compose the strongest shades of white represent the most explored areas while the blacks represent those of absence of movement. The control group had a greater exploration in middle and lateral zones, but with preference for social zone; the stress group shows a similar pattern to control, but with the social zone less explored; the group exposed

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to stress and 10 µM shows a reduced exploration which suggest higher stress compared to control, this groups presents a decreased behavior called thigmotaxis (related to the tendency of an animal to remain close to the walls), but paradoxically this behavior is contrary to what is expected in stressed animals, however, the debate still persists because it may be an exploratory response (35). Finally, the group exposed to stress and 100 µM showed a decreased thigmotaxis, decreased exploration in the lateral zone and a marked preference for the social and middle zone of the tank compared to the control. In order to quantify these results, the frequency histograms were analyzed by statistical correlation and intersection. These results showed that the mean occurrence position maps of the controls compared with each video of the controls, have a significant correlation ($R = 0.81$, $p=0.025$) and intersection of 0.69; the mean occurrence position maps of the stress group showed a moderate correlation ($R= 0.75$, $p=0.042$) and intersection of 0.62; the group exposed to stress plus 10 µM showed a weak correlation ($R= 0.51$, $p=0.15$) and intersection of 0.56; and the group exposed to stress plus 100 µM showed a moderate correlation ($R= 0.67$, $p=0.16$) and intersection of 0.58, all compared to control group (Table 1). This function makes it easier for the researcher to see the differences or similarities between the groups; it is very useful for studies in pharmacology, in which it is necessary to analyze the biological and behavioral effect of a drug.

The kinematics generated from a single fish change slightly. As can be seen in figure 5, the velocity of an individual in the control group shows an area of greater occurrence compared to its position and acceleration. This pattern appears similarly in the maps of the other groups. However, their spatial patterns change between groups due to the applied treatment. The control map presented more exploration in the middle and lateral zones compared to the other

groups; the stress map shows an exploration in the middle of the tank, decreasing thigmotaxis; the stress plus 10µM map shows a marked preference for the social zone, with less exploration in the middle and lateral zones; finally, the stress plus 100µM shows a pattern similar to the group of stress plus 10µM, but with less exploration in the lateral zone.

Discussion

The proposed algorithm in the present work aims to provide analysis of additional locomotor patterns that can be useful to understand behavior of experimental animals. Traditionally, only the occurrence map is acquired for distance and per animal. In addition to distance and velocity, behavioral patterns acquired were acceleration, occurrence maps of position, velocity, acceleration, its mean and standard deviation and zone frequencies; these can be analyzed in any behavior test carried out. The main contribution of occurrence maps of position, speed, acceleration, its mean and standard deviation (occurrence maps) is that are useful to understand behavioral phenomena, for example, discriminate between areas of greater permanence and those with greater acceleration or distance. In a novel object test, for example, these measures can discriminate if the animals have a lower speed and acceleration near the novel object but more distance traveled near the object. One characteristic of the proposed algorithm is a great proximity to what would be expected in the physical sense, where zones of low or high occurrence allows to develop more representative visual analysis of the physical movement of the fish. Therefore, it allows statistical correlation analysis to be carried out, comparing positions and locomotion histograms (maps) directly. Additionally, other advantages are shorter data extraction time, an open-source algorithm, additional behavioral parameters, and can be implemented with any type of recorded video. However,

minimum recording parameters are highly recommended such as camera stability and contrast between animal and environment (15).

As in classical approaches some limitations related with video recording may affect the automatic data analysis. For example, light reflections are the most frequent problem in loss of tracking; fisheye lens creates space distortion, making measurement calibration difficult and inaccurate; low video resolution produces loss of information due to a low number of frames per second (FPS) processed; and camera instability produces erroneous data of locomotor behavior. Therefore, we recommend recording the videos by reducing the amount of direct light on the surface of the water or aquarium, to achieve this you can use mirrors or use a translucent material such as white sheet of paper between the light source and the aquarium; avoid the use of distortion lens; use cameras capable of recording video at least 30 fps and video resolution of 720p (1280 x 720 pixels); finally, maintaining camera stability with a tripod or a flat, transparent surface (such as acrylic) is highly recommended.

Previously, it has been shown that high caffeine doses are able to reduce exploration and increase whole body cortisol in zebrafish, which can be translated into an anxiogenic effect (26) and it has been shown that after acute stress, cortisol levels can be reduced using fluoxetine or bromazepam and therefore, the measurement of locomotor behavior does not show significant differences with the control (30, 31). The affinity for caffeine is higher for A1 and A2A receptors, but its anxiogenic effect is mainly caused by A1 receptors (20, 27), which in turn is related to reduced locomotor activity. On the other hand, acute stress such as netting zebrafish from their home tanks and car transportation for 30 minutes, can produce a reduced locomotor activity and exploration in wild type zebrafish in novel tank diving test and light/dark box test (32). Similar approaches have been performed in zebrafish to study

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gender differences in aggressive behavior (33), locomotion, shoal cohesion (18), showing reduced exploration, more freezing bouts and tendency to social cohesion. Previously, the social behavioral effects of stress and caffeine combined have not been studied in zebrafish. With the results obtained in this work, the combination of acute environmental stress associated with caffeine exposure at high doses, increases social cohesion, possibly caused by the sum of the anxiogenic effects, which in turn explains the decrease in exploration in other areas of the tank. However, both stress and caffeine mechanisms in the CNS remain unclear and there is a need for further research in many fields such as physiology, psychopharmacology, neurogenetics, ethology, among others.

Conclusions

The results show the utility of an automated and objective method in the analysis of complex behavioral patterns in zebrafish, which can be extrapolated to other animal models and humans. The necessary data are only the recorded videos of the experimental specimen; the format and size of the video does not affect the methodology since our algorithm adjusts the size to our worked standard (150x268 px). The fundamental advantage of ZebraMov is in the application of dense trajectories, this large number of close and compact points on the moving objects show a more characteristic form of the movement close to the real behavior. With this set of points, we acquire frequency images similar to thermal maps evidencing directly the main areas or regions of greatest interest, decrease of movement, long displacements and abrupt changes. Our mean occurrence maps obtained in the calculation of average maps and standard deviation show the tendency to social behavior in all groups of stress plus caffeine doses. Furthermore, the group exposed to stress plus 10 μM of caffeine reduces its locomotor behavior. We can affirm that acute stress decreases average speed but does not affect the

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tendency of social behavior and caffeine affects the locomotor behavior, decreasing movement in the zebrafish.

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ZebraMov: An automatic strategy to analyze and quantify zebrafish social behavior from kinematic patterns captured in video sequences

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Figures Legends

Figure 1. Top view of social tank. Total length of the tank (50 cm) is divided at each end (10 cm) with acrylic that allows the fish to see through the divisions. The central zone (30 cm) is divided in three equal virtual zones of 10 x 10 cm, from left to right, lateral, middle and social zone. Recordings of the central area were made from a top view of the tank.

Figure 2. Zebramov algorithm workflow. At the top is represented the video sequences that are analyzed using the Background Subtraction (BS) method to obtain locomotion data; at the bottom, the optical flow allows to create the occurrence maps and to be analyzed by means and statistical correlation.

Figure 3. Locomotor parameters (or behavior). Groups from left to right, control, stress (without any substance), stress plus 10μM and stress plus 100 μM of caffeine (Ctrl, S, S10, S100, respectively). **A**. Mean distances traveled. Distance was lower for groups S10 and S100 compared to Ctrl ($p = <0.001$, $p = 0.006$ respectively, $F = 10.04$) **B**. Average Speed. Speed was lower in S, S10 and S100 compared to Ctrl ($p = 0.05$, $p = <0.001$, $p = 0.008$ respectively, $F = 10.26$). **C**. Acceleration was lower in S10 ($p=0.042$, $F = 2.97$). **D**. Distance by minute. The squares correspond to the control group, the dots to the stress group, the X to stress plus 10 and the triangles to stress plus 100. Distance was significantly lower in group S10 at minute

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1 to 5 (p= 0.002, p= 0.001, p= 0.002, p= <0.001, p= 0.003 respectively); in group S100 at minute 1, 3, 4 and 5 (p= 0.011, p= 0.04, p= 0.004, p= <0.016 respectively); and lower in S group at minute 4 (p= 0.030). All compared to the Ctrl group. Groups marked with asterisk indicate p value ≤ 0.05. The bars indicate the means with the standard error (SE), n= 6 per group. Statistical significance was determined using two-way ANOVA followed by Tukey's HSD test (Honestly-significant-difference) multiple comparison test.

Figure 4. Mean position occurrence for Social behavioral test. Groups in x axis from left to right, control, stress (without any substance), stress plus 10µM and stress plus 100 µM of caffeine (Ctrl, S, S10, S100, respectively), y axis show the percentage for each zone (lateral, middle and social). **A**. Mean occurrences in all groups, social zone was preferred compared to lateral and middle zones (p= <0.001, p= <0.001 respectively, F= 36.04), n=22 per group. **B**. Mean occurrences by group and zone in 5 minutes. Social zone was preferred for the S100 group, but without statistical differences (F= 0.741, p= 0.54). Groups marked with asterisk indicate p value ≤ 0.05. The bars indicate the means with the standard error (SE), n= 6 per group.

Figure 5. Characteristic occurrence maps of a single fish. The rows represent the group (Ctrl, S, S10 and S100) and the columns represent the kinematic (position, velocity and acceleration). The less occurrence regions are in black and the most occurrence regions in white. Note that the intensity may change depending on the kinematics analyzed, even if it belongs to a single individual.

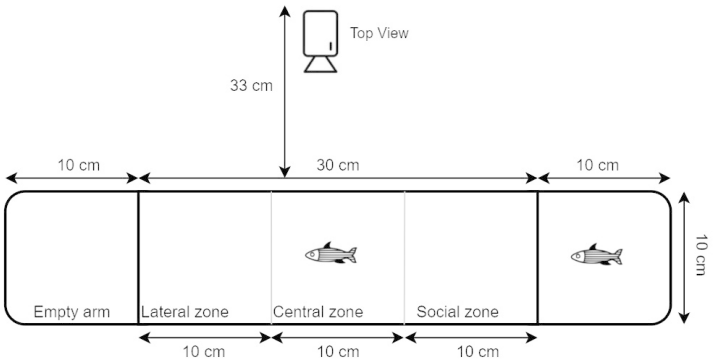

Figure 1. Top view of social tank.

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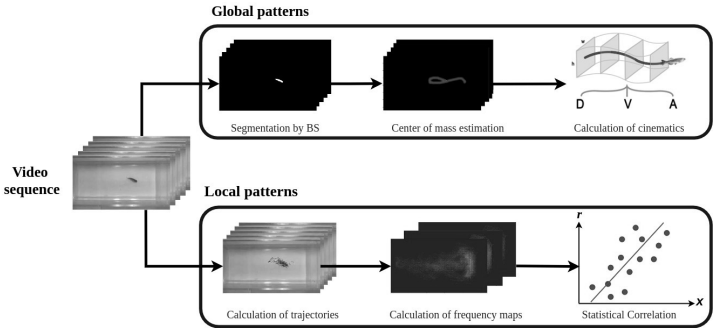

Figure 2. Zebramov algorithm workflow

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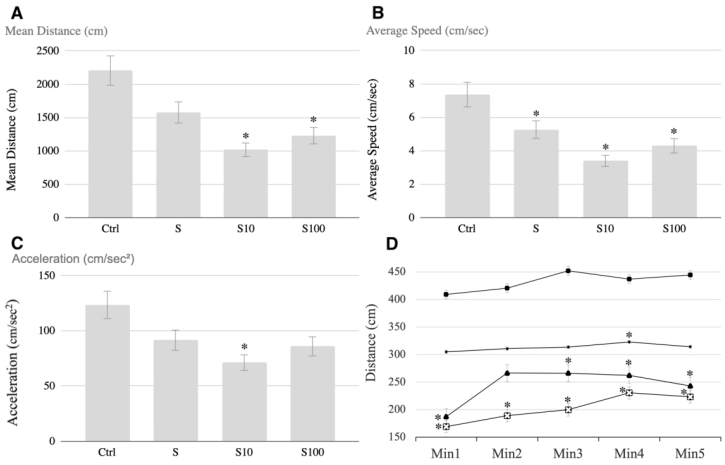

Figure 3. Locomotor parameters (or behavior)

620x397mm (72 x 72 DPI)

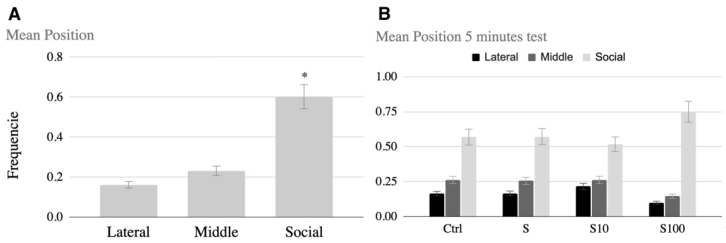

Figure 4. Mean position occurrence for Social behavioral test

605x196mm (72 x 72 DPI)

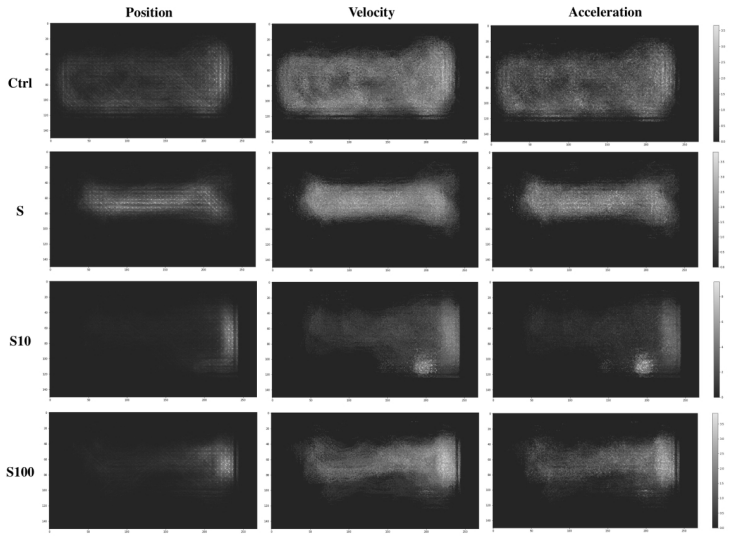

Figure 5. Characteristic occurrence maps of a single fish

845x623mm (72 x 72 DPI)

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Tables

Correlation

	Position	*p*	*Velocity*	*p*	*Acceleration*	*p*
	Ctrl		**Ctrl**		**Ctrl**	
Ctrl	0.81	0.025	0.86	0.014	0.83	0.020
S	0.75	0.042	0.82	0.022	0.78	0.03
S10	0.51	0.15	0.55	0.12	0.51	0.15
S100	0.67	0.16	0.76	0.12	0.70	0.15

Intersection

	Position	*Velocity*	*Acceleration*
	Ctrl	**Ctrl**	**Ctrl**
Ctrl	0.69	0.75	0.68
S	0.62	0.72	0.64
S10	0.56	0.62	0.55
S100	0.58	0.65	0.54

Table 1. Correlation and intersection results. At the left, the correlation between control group and stress (S), stress plus 10µM (S10) and stress plus 100µM (S100). At right, intersection between control group and S, S10 and S100. Kinematic for position, velocity and acceleration were used to create the comparisons.

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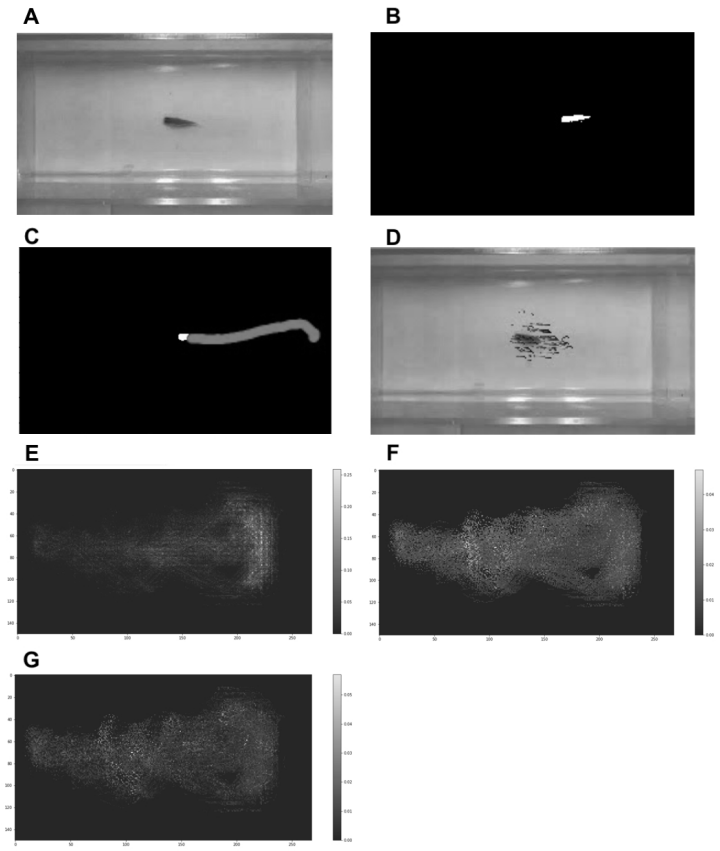
Supplementary information

Supplementary figure 1. Kinematic patterns acquisition. **A**. Original image. **B**. Fish segmentation. **C**. Global fish trajectory (CoM) computation. **D**. Optical flow and dense trajectories, its corresponding motion dense trajectories. **E**. Motion distribution. **F**. Velocity distribution. **G**. Acceleration distribution. The gray scale on figures E-G shows the regions with no occurrence in black and regions with the highest occurrence in white.

Supplementary figure 2. Mean occurrences minute by minute and zone. Groups in x axis from left to right, control, stress (without any substance), stress plus 10µM and stress plus 100 µM of caffeine (C, S, S10, S100, respectively), y axis show the percentage for each zone (lateral, middle and social). Groups marked with asterisk indicate p value ≤ 0.05. The bars indicate the means with the standard error (SE), n= 6 per group.

Supplementary Figure 3. Results compared with AnimalTracker. The dark gray columns represent the data obtained with Zebramov and the light gray columns by AnimalTracker. X axis shows each video/animal analyzed and Y axis for distance (**A**) and velocity (**B**).



Supplementary figure 1. Kinematic patterns acquisition

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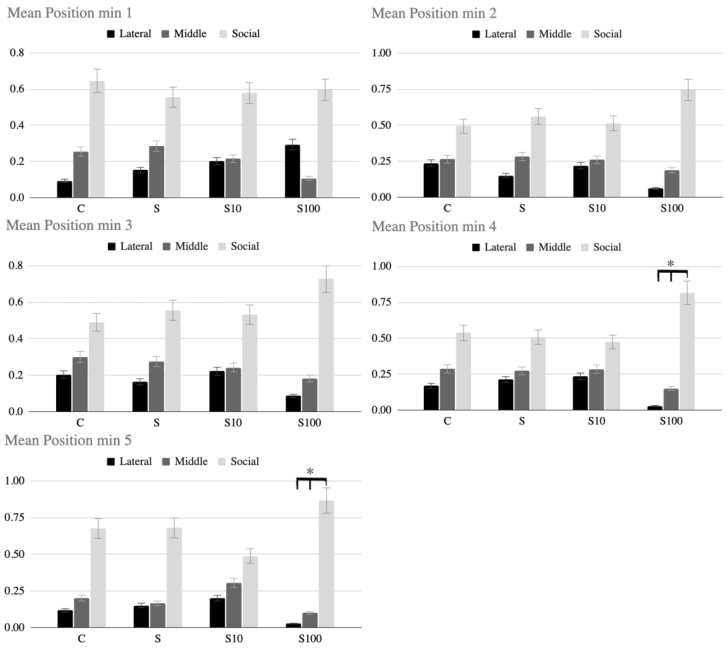
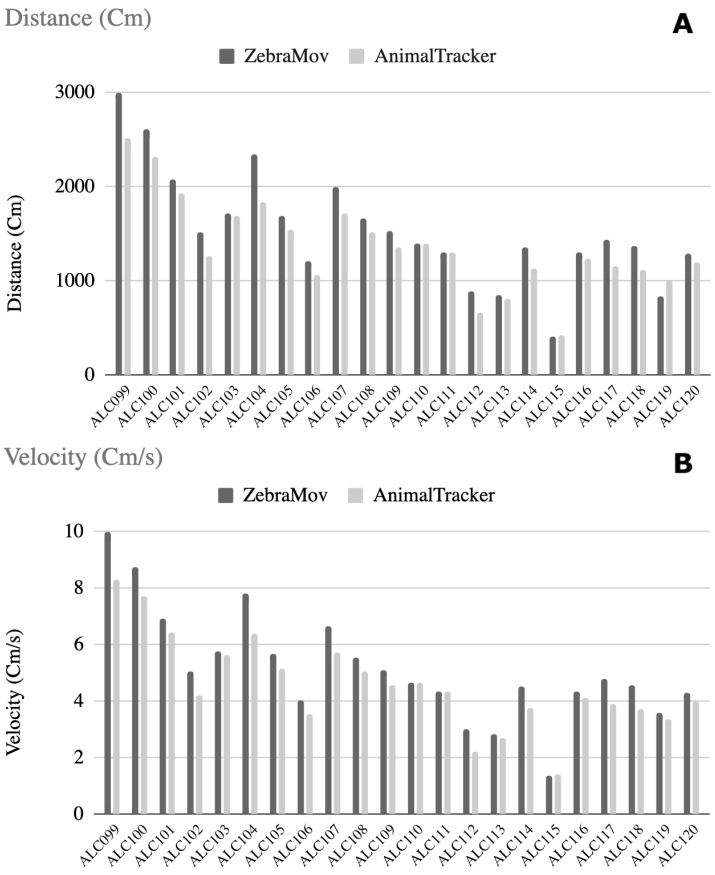


Supplementary figure 2. Mean occurrences minute by minute and zone

561x502mm (72 x 72 DPI)



Supplementary Figure 3. Results compared with AnimalTracker

440x536mm (72 x 72 DPI)

Capítulo 5. Conclusiones

El presente trabajo explora los efectos del estrés y alcohol en el pez cebra en términos del comportamiento y expresión de genes relacionados con los posibles mecanismos iniciales de la enfermedad en los trastornos mentales como el depresivo y de ansiedad. El pez cebra es un modelo idóneo para la investigación en ciencias de salud y la medicina traslacional, permitiendo la manipulación de múltiples variables, la correlación de datos del comportamiento con pruebas moleculares. El uso de herramientas computacionales como la propuesta en el presente trabajo hace rápido, fácil, económico y amplio el análisis del comportamiento del pez cebra y otras especies. Se publicaron varios artículos en revistas internacionales indexadas como resultado del presente trabajo de tesis. Teniendo en cuenta la causa multifactorial de los trastornos mentales, es necesario realizar más estudios en ciencias básicas que ayuden a aclarar los efectos neuronales del estrés psicosocial. Un modelo animal de estrés permite a los investigadores administrar moléculas que pueden resultar más efectivas que la polimedicación de pacientes psiquiátricos. Actualmente existen múltiples moléculas probadas en estudios clínicos que han demostrado ser efectivas y es necesario comprender sus efectos moleculares. Finalmente, se espera continuar con esta línea de investigación y extendiendo la información existente.

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