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CÁSSIO MORAIS LOSS

Hipoativação de receptores NMDA ao longo do desenvolvimento: consequências comportamentais e influência do enriquecimento ambiental

Porto Alegre, abril de 2017.

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica, como requisito parcial para obtenção do título de Doutor em Ciências Biológicas – Bioquímica.

Orientador: Professor Dr. Diogo Losch de Oliveira

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ALMA LEVE
Este dia está tão lindo
que me traz uma calma
para a alma,
que faz música,
do canto da natureza,
que faz do feio,
beleza, sem tristeza...
Alma é isso:
pura leveza.

VENTOS DE AGOSTO
Agosto, mês do desgosto
e dos assovios dos ventos.
Mas os sopros dos ventos de agosto
levam para longe os frios dos invernos
e acenam com os ares da primavera,
que se avizinha, que vem de mansinho,
abrindo as primeiras florzinhas,
pintando o mundo de cores.
E os ventos de agosto
acolhem os sóis de setembro,
com novo alento,
embora, ainda com vento.
E vai-se o agosto,
que não significa desgosto,
mas simplesmente,
mais um tempo que se vai
e não volta nunca mais...

EM BUSCA DE BONS TEMPOS
Neste caminho da vida,
sigo sempre a procurar
mais flores, menos dores,
mais belezas, menos tristezas...
Enxotando os invernos,
anseio por verões e primaveras
e vou, pela vida seguindo,
chorando menos, mais sorrindo...
Quando as labutas me abatem,
quando me entrego ao cansaço,
amenizo com abraços
e o que parecia inútil,
passa a ter outro valor.
A vida tem outro sentido
sempre que a alimentamos
com a harmonia das cores,
e o colorido das flores.

Maria Lêda Lóss dos Santos

(In: RUMOS DE MIM – Palavras e Imagens)

Esta Tese é dedicada a meus pais Jesus Ivonei e Marizete por todo o carinho e incentivo.

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APRESENTAÇÃO

Esta Tese é constituída de seis partes:

Parte I. Resumo, Abstract, Lista de Abreviaturas, Introdução e Objetivos;

Parte II. Os resultados que fazem parte desta Tese estão apresentados em quatro capítulos, sendo os três primeiros sob a forma de artigos científicos, subdivididos em: Introdução, Procedimentos Experimentais, Resultados, Discussão e Referências Bibliográficas; o quarto capítulo está apresentado na forma de resultados parciais contendo: Racional do Estudo, Material e Métodos e Resultados Parciais;

Parte III. Discussão e Conclusão Geral;

Parte IV. Perspectivas;

Parte V. Referências bibliográficas referentes à Parte I e Parte III;

Parte VI. Anexos relacionados à Tese;

SUMÁRIO

PARTE I

RESUMO

A estimulação da reserva cognitiva e encefálica (BCR), através do enriquecimento ambiental (EA), por exemplo, influencia positivamente funções cognitivas e induz alterações neuroanatômicas, sendo sugerido muitas vezes como uma terapia complementar aos tratamentos farmacológicos convencionais em pacientes com distúrbios neurológicos. A esquizofrenia é um distúrbio neurológico o qual tem sido associado a um estado de hipoativação de receptores NMDA (NMDAR). O bloqueio de NMDAR durante períodos iniciais do desenvolvimento encefálico induz alterações duradouras nas redes neurais. Estas alterações estão associadas a prejuízos no aprendizado espacial, memórias de trabalho, memórias de curta e longa duração, comportamento exploratório e emocionalidade, apresentando alta relevância para o estudo da esquizofrenia. Durante estágios precoces do desenvolvimento encefálico a subunidade GluN2 predominantemente expressa nos NMDAR é a subunidade GluN2B. Desta forma, torna-se de extrema importância investigar se a hipoativação seletiva de NMDAR contendo a subunidade GluN2B durante períodos iniciais do desenvolvimento é capaz de induzir alterações comportamentais causando um fenótipo tipo-esquizofrênico similar as induzidas pela hipoativação de toda a população de NMDAR. Na presente Tese, investigamos se o EA durante a infância é capaz de prevenir a evolução das alterações comportamentais induzidas pela hipoativação de NMDAR durante o neurodesenvolvimento. Para atingir esses objetivos, primeiramente nós propusemos a utilização de uma ferramenta estatística alternativa, a Análise de Componentes Principais (PCA), para investigar se o período do dia em que os testes eram realizados afetava o comportamento de animais *naive* (Capítulo I) ou submetidos ao EA (Capítulo II). No Capítulo I nós validamos a utilização da PCA para o estudo do comportamento, a qual revelou que além do padrão de atividade dos animais, o período do dia também alterou a micro estrutura do comportamento exploratório. No Capítulo II, nós mostramos que algumas alterações comportamentais induzidas pelo EA (tais como diminuição na exploração e na auto-exposição a ambientes potencialmente perigosos) são observadas durante o período diurno, mas não durante o período noturno. Contudo, o EA melhorou o desempenho dos animais em uma tarefa que avalia a formação de memórias episódicas independentemente do período em que foram testados. Após validar a PCA e estabelecer os períodos do dia ideais para o estudo dos comportamentos de interesse, nós avaliamos os efeitos do EA durante a infância e do bloqueio neonatal de NMDAR sobre o comportamento e também sobre o metabolismo encefálico de glicose em animais adultos. No Capítulo III, nós mostramos que bloquear seletivamente NMDAR contendo a subunidade GluN2B durante períodos iniciais do desenvolvimento altera o comportamento dos animais de maneira diferente do bloqueio não-seletivo de NMDAR, sugerindo diferentes funções das diferentes populações de NMDAR nesta etapa do desenvolvimento. Além disso, nós observamos que o EA durante a infância é capaz de prevenir as alterações comportamentais induzidas pelo bloqueio de NMDAR contendo a subunidade GluN2B. Resultados parciais do Capítulo IV indicam que a hipoativação neonatal de NMDAR não induz macro-alterações na captação encefálica de glicose. Contudo, alterações no padrão regional de utilização de glicose pelo encéfalo não podem ser descartados. Além disso, animais submetidos ao EA apresentaram uma tendência a aumentarem a captação de glicose encefálica, o que pode indicar um aumento de atividade encefálica. Contudo, novos estudos são necessários para melhor elucidação deste fenômeno. Como conclusões da presente tese, nossos resultados permitem sugerir que diferentes populações de NMDAR estão envolvidas em diferentes processos de maturação encefálica, e que interferências no funcionamento das distintas populações de NMDAR durante períodos críticos de desenvolvimento podem alterar o funcionamento encefálico gerando adaptações nas comunicações neurais e modificando o fenótipo comportamental de maneira duradoura. A estimulação da BCR apresenta potencial para prevenir estas alterações, contudo, novos estudos são necessários para completa elucidação deste tema.

ABSTRACT

Brain and cognitive reserve (BCR) stimulation has been suggested as a complementary therapy to conventional pharmacological treatments in patients with neurological disorders since BCR stimulation, through environmental enrichment (EE) for instance, positively influences cognitive functions and induces neuroanatomical changes. Schizophrenia is a neurological disorder which has been associated with a NMDA receptor (NMDAR) hypoactivation state. NMDAR hypoactivation during early periods of brain development induces long-lasting changes in neural networks. These brain alterations are associated with impairments in spatial learning, working memory, short- and long-term memories besides exploration and emotionality, presenting high relevance to schizophrenia. Since GluN2B subunit is the predominant GluN2 subunit expressed during early stages of brain development, it becomes of extreme importance to investigate whether GluN2B-containing NMDAR hypoactivation during early periods of brain development is sufficient to induce schizophrenic-like behavioral changes similar to those induced by the entire NMDAR population hypoactivation. In the present Thesis, we investigated whether early life EE may prevent the evolution of schizophrenic-like behavioral changes induced by NMDAR hypoactivation during early periods of brain development. In order to reach these objectives, we first proposed the use of an alternative statistical tool, the Principal Component Analysis (PCA), to investigate whether the time-of-day in which tests were performed affected the behavior of naive animals (Chapter I) or EE submitted animals (Chapter II). In Chapter I, we validated the use of PCA to study animal behavior. PCA revealed that time-of-day influenced activity pattern of animals and altered the micro structure of exploratory behavior. In Chapter II, we have shown that some behavioral changes induced by EE (such as decreased exploitation and self-exposure to potentially dangerous places) are observed during the light period but not during the dark period of light/dark photoperiod cycle. However, EE improved the performance of animals in a task that evaluates the formation of episodic memories regardless of the time-of-day they were tested. After validating PCA and establishing the ideal time-of-day for investigation of the behaviors of interest, we evaluated the effects of neonatal NMDAR blockade and early life EE on behavior and brain glucose metabolism in adult animals. In Chapter III, we showed that selectively blocking GluN2B-containing NMDAR during early periods of brain development alters animals' behavior differently from neonatal non-selective NMDAR blockade, suggesting different functions for the different NMDAR populations at this stage of development. In addition, we have observed that early life EE is able to prevent the behavioral changes induced by neonatal GluN2B-containing NMDAR blockade. Partial results from Chapter IV indicate that neonatal NMDAR hypoactivation does not induce macro-alterations in brain glucose uptake. However, changes in the regional pattern of glucose utilization by the brain cannot be ruled out. In addition, animals submitted to EE presented a trend to increase the brain glucose uptake, which may indicate an increase brain activity in these animals. However, additional studies are needed to complete elucidation of this phenomenon. As conclusions of the present Thesis, our results allow us to suggest that different populations of NMDAR are involved in different processes of brain maturation. We can also suggest that interferences in the different NMDAR populations' activity during critical periods of brain development can alter brain functioning, which can generates adaptations in the neural communications and induce long-lasting behavioral alterations. BCR stimulation presents potential to prevent these alterations. However, additional studies are necessary to complete elucidation of this theme.

LISTA DE ABREVIATURAS

SNC	**Sistema Nervoso Central**
AMPA	**α-amino-3-hidroxi-5-metil-4-isoxazol propionato**
NMDAR	**receptores N-metil-D-aspartato (NMDA)**
NTD	**domínio N-terminal (ou amino-terminal) do NMDAR**
ABD	**domínio de ligação do agonista do NMDAR**
TMD	**domínio transmembranar do NMDAR**
CTD	**domínio Carboxi-terminal intracelular do NMDAR**
BCR	**reserva cognitiva e encefálica (*Brain and Cognitive Reserve*)**
EA	**enriquecimento ambiental**
AP	**ambiente padrão**
PCA	**Análise de Componentes Principais**
CA	**campo aberto**
LCE	**labirinto em cruz elevado**
SAL	**solução salina (0,9%)**
KET	**cetamina**
CI	**CI-1041**
18F-FDG	**[18F]-fluorodesoxiglicose**

1. SISTEMA GLUTAMATÉRGICO

O sistema de neurotransmissão glutamatérgica é considerado o principal sistema de neurotransmissão excitatória do Sistema Nervoso Central (SNC) de mamíferos, onde participa de inúmeros eventos fisiológicos e plásticos tais como: memória e aprendizado (Izquierdo e Medina, 1997; Riedel, Platt e Micheau, 2003; Peng *et al.*, 2011), desenvolvimento e envelhecimento (Segovia *et al.*, 2001; Menard e Quirion, 2012), adaptação ambiental (Ozawa, Kamiya e Tsuzuki, 1998; Ernst e Chang, 2008; Martinez-Rivera *et al.*, 2013), proliferação e migração celular (McDonald e Johnston, 1990; Jansson e Akerman, 2014; Luhmann, Fukuda e Kilb, 2015).

Para que a neurotransmissão glutamatérgica seja possível, primeiramente é necessário que ocorra a síntese do aminoácido glutamato no SNC, uma vez que a barreira hematoencefálica possui uma baixa permeabilidade a este aminoácido (Hertz *et al.*, 1999). Desta forma, praticamente todo o glutamato presente no encéfalo é sintetizado a partir do α-cetoglutarato e/ou glutamina tanto em neurônios quanto em glia. Nas células gliais, o glutamato é convertido à glutamina através da enzima glutamina sintetase e, então, liberado no meio extracelular (Schousboe *et al.*, 1997; Leke e Schousboe, 2016; Sonnewald e Schousboe, 2016). A glutamina é captada pelos neurônios, convertida a glutamato pela ação da enzima glutaminase, e posteriormente o glutamato é armazenado nas vesículas pré-sinápticas onde sua concentração pode chegar a 100 mM (Nedergaard, Takano e Hansen, 2002). A exocitose destas vesículas provoca a liberação deste *pool* de glutamato para o meio extracelular onde a concentração de glutamato é altamente regulada e mantida a níveis muito baixos (concentrações nanomolares), especialmente na fenda sináptica onde a neurotransmissão acontece (Benveniste *et al.*, 1984; Anderson e Swanson, 2000). Após a exocitose das vesículas glutamatérgicas, a concentração de glutamato aumenta na

fenda sináptica, provocando a estimulação de receptores glutamatérgicos (Fonnum, 1984). A neurotransmissão glutamatérgica é terminada com a remoção do glutamato da fenda sináptica através da ação de transportadores de glutamato presentes tanto em neurônios quanto em astrócitos, os quais restauram a concentração de glutamato a níveis abaixo de 1 µM (Auger e Attwell, 2000) e permitem a síntese *de novo* do glutamato no meio intracelular.

1.1 Receptores glutamatérgicos

As diversas ações do glutamato, tanto fisiológicas quanto patológicas, resultam da ativação de receptores de membrana, tanto neuronais como gliais (Ozawa, Kamiya e Tsuzuki, 1998), os quais são divididos em duas classes: metabotrópicos (mGluRs) e ionotrópicos (iGluRs) (Tanabe *et al.*, 1992; Bear, Connors e Paradiso, 2002).

Os mGluRs estão acoplados ao mecanismo de transdução de sinal via proteínas G modulando a produção intracelular de segundos mensageiros (Ozawa, Kamiya e Tsuzuki, 1998). Já os iGluRs, os quais são funcional e farmacologicamente distintos dos mGluRs, contêm um canal iônico cátion-específico e, quando estimulados, permitem a passagem de íons através de seu canal induzindo um potencial de ação no neurônio pós-sináptico (Cotman, Monaghan e Ganong, 1988). Esta classe de receptores é responsável pela iniciação, propagação e ampliação do sinal glutamatérgico, e é subdividida em três subtipos: cainato, α-amino-3-hidroxi-5-metil-4-isoxazol propionato (AMPA) e N-metil-D-aspartato (NMDA).

Tanto receptores AMPA quanto cainato são permeáveis aos íons sódio (Na^{+}) e potássio (K^{+}), mas a maioria deles é impermeável ao íon cálcio (Ca^{+2}) (Cotman, Monaghan e Ganong, 1988; Bear, Connors e Paradiso, 2002). Já os receptores NMDA (NMDAR) são altamente permeáveis a Ca^{+2} e possuem como co-agonistas glicina e D-

serina (Johnson e Ascher, 1987; Kemp e Leeson, 1993; Schell, Molliver e Snyder, 1995; Mothet *et al.*, 2000; Henneberger *et al.*, 2013). Enquanto receptores AMPA quando ativados causam uma despolarização rápida e intensa devido à entrada de Na^{+} na célula, os NMDAR são dependentes de voltagem e necessitam de uma pré-despolarização da membrana. Desta forma, os receptores AMPA são normalmente co-expressos com os NMDAR em muitas sinapses no encéfalo, de forma que muitos potenciais excitatórios pós-sinápticos (PEPSs) mediados por glutamato possuem componentes formados por ambos (Cotman, Monaghan e Ganong, 1988).

1.2 Receptores NMDA

Desde o seu descobrimento na década de 1980, os NMDAR tem fascinado neurocientistas por causa de seus papéis centrais na função do SNC. Esses canais iônicos ativados por glutamato são mediadores essenciais da plasticidade encefálica e são capazes de converter padrões específicos de atividade neuronal em mudanças de longo prazo na estrutura e função da sinapse que se pensa estar subjacentes a funções cognitivas superiores (Traynelis *et al.*, 2010). Essas mudanças na estrutura e função celular são resultantes do influxo de Ca^{+2} no neurônio através do canal formado pelas subunidades destes receptores. O Ca^{+2} intracelular, proveniente da abertura do canal dos NMDAR, funciona como um segundo mensageiro e ativa mecanismos específicos da célula, os quais estão relacionados com mudanças na fisiologia do neurônio (Petralia, 2012).

Muitos estudos demonstraram que disfunções na atividade dos NMDAR estão relacionadas a vários distúrbios neurológicos e psiquiátricos (Lau e Zukin, 2007; Mony *et al.*, 2009; Traynelis *et al.*, 2010; Loss, Cordova e de Oliveira, 2012), incluindo acidente vascular encefálico, dor patológica, doenças neurodegenerativas, epilepsias e esquizofrenia. A hiperestimulação dos NMDAR pode levar a um excessivo influxo de

Ca^{+2} no neurônio, causando um evento patofisiológico denominado de excitotoxicidade neurodegenerativa, o qual é capaz de promover a morte da célula (Nicoletti *et al.*, 1996). Por outro lado, uma vez que a neurotransmissão mediada pelos NMDAR é vital para as computações corticais subjacentes à cognição, a hipoestimulação dos NMDAR pode causar défices cognitivos e alterações na emocionalidade, os quais são observados em transtornos psiquiátricos como esquizofrenia, transtornos de ansiedade e humor (Anticevic *et al.*, 2012; Dawson *et al.*, 2012). Desta forma, muitos estudos têm visado aumentar a compreensão dos diferentes papéis dos NMDAR na neurotransmissão glutamatérgica, de forma que há um interesse crescente na busca por novas estratégias que revertam os efeitos deletérios da função desregulada dos NMDAR, e ajude no desenvolvimento de novos fármacos que visam modular estes receptores.

1.3 Estrutura e composição dos NMDAR

NMDAR são proteínas integrais de membrana que incorporam quatro grandes subunidades formando um poro no centro do canal iônico, o qual apresenta permeabilidade altamente seletiva para os cátions Na^{+}, K^{+} e Ca^{+2} (Paoletti, 2011). Cada uma das subunidades GluN compartilham uma arquitetura modular que é composta por quatro domínios distintos: o domínio N-terminal (NTD); o domínio de ligação do agonista (*agonist-binding domain* - ABD); o domínio transmembranar (TMD) contendo o canal iônico; e um domínio C-terminal intracelular (CTD) (Monaghan e Jane, 2009; Williams, 2009; Paoletti, Bellone e Zhou, 2013) (Figura 1).

Dentre os diferentes tipos de iGluRs, os NMDAR aparentemente são os que apresentam subunidades mais longas, sendo que o número total de aminoácidos por subunidade varia de 900 a mais de 1.480 unidades (Paoletti, 2011; Paoletti, Bellone e Zhou, 2013). A diferença no tamanho da subunidade é explicada quase inteiramente por

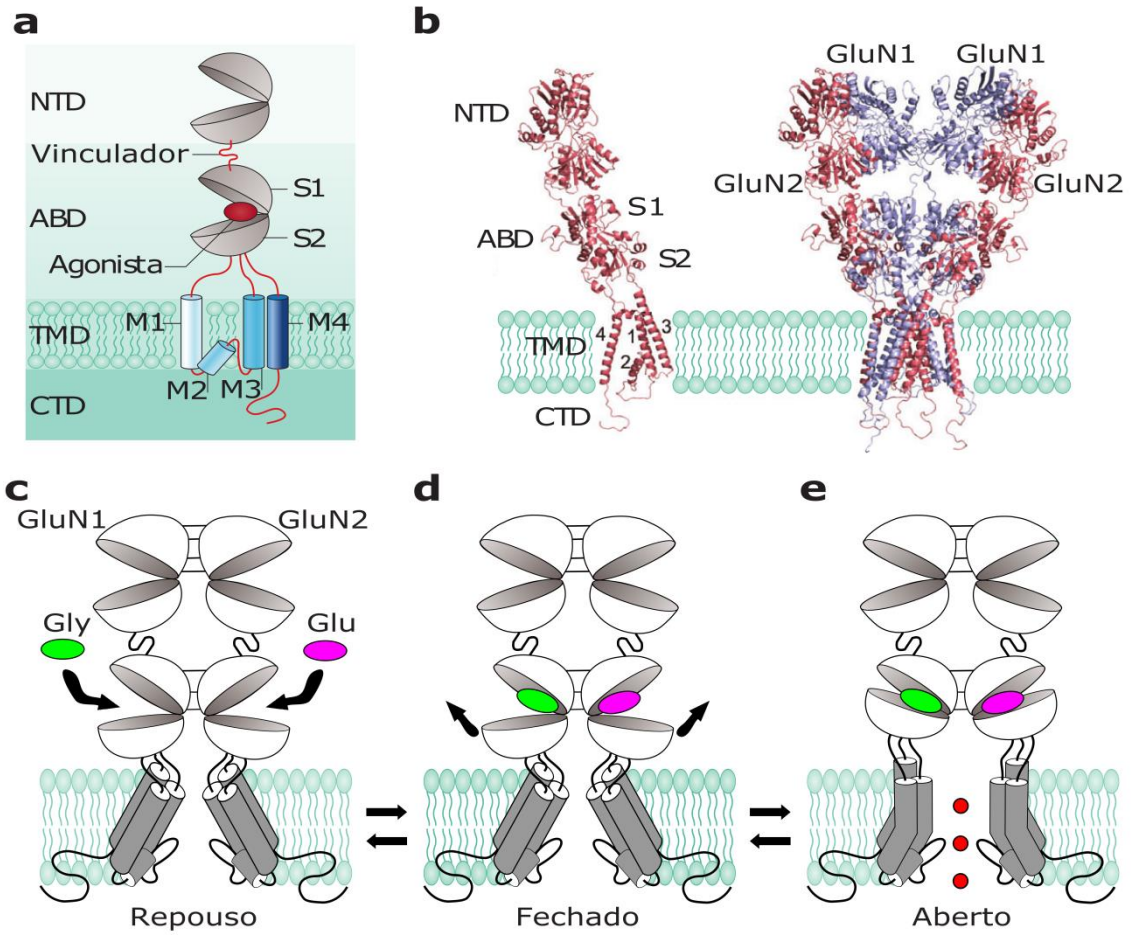

Figura 1- Estrutura e mecanismo de ativação dos NMDAR. (a) Todas as subunidades de GluN compartilham uma arquitetura modular que é feita de quatro domínios distintos: um domínio N-terminal (NTD) na porção extracelular e um domínio C-terminal (CTD) na porção intracelular, sendo estas as regiões mais divergentes entre subunidades; o domínio de ligação do agonista (ABD) que se liga a glicina ou d-serina em GluN1 e GluN3 e ao glutamato em GluN2; o domínio transmembrana (TMD) contendo o canal iônico. **(b)** Arquitetura do NMDAR; à esquerda, organização dos domínios de uma única subunidade do NMDAR; à direita, vista de lado da disposição das subunidades de um receptor contendo uma subunidade GluN1 (em azul) e uma subunidade GluN2 (em vermelho). **(c-e)** Esquema demonstrando como o receptor passa de um estado de repouso para um estado ativado com o poro aberto. Tanto a glicina (Gly) quanto o glutamato (Glu) são necessários para que os canais NMDAR sejam abertos e permitam a passagem de íons (representado por círculos vermelhos). No esquema, apenas duas subunidades estão representadas no complexo do NMDAR, porém, um NMDAR completo é um tetrâmero formado por 4 subunidades. Fonte: Adaptado de Paoletti, Bellone e Zhou (2013); Vyklicky *et al.* (2014); e Paoletti (2011).

diferenças no comprimento do CTD, uma região que está envolvida no tráfego de receptores e contém diversos sítios de fosforilação que acoplam os receptores as cascatas de sinalização (Petralia, Al-Hallaq e Wenthold, 2009; Traynelis *et al.*, 2010).

Embebido na membrana celular está o canal iônico que é formado por um domínio de membrana composto de três segmentos transmembranares (M1, M3 e M4), além de um alça reentrante curta (P *loop* ou M2) (Paoletti, 2011). Nesta região estão localizados os sítios de ligação da cetamina, da memantina, do MK-801 e do Mg^{+2} (Orser, Pennefather e MacDonald, 1997; Monaghan e Jane, 2009; Williams, 2009; Limapichat *et al.*, 2013; Paoletti, Bellone e Zhou, 2013). Durante o potencial de repouso, os canais acoplados ao NMDAR estão normalmente bloqueados por íons Mg^{+2} (Riedel, Platt e Micheau, 2003), o que impede a passagem de outros íons. Para que o Mg^{+2} desobstrua o poro e ocorra a ativação do NMDAR, é necessário ocorrer a despolarização da membrana neuronal. Quando a membrana está despolarizada, o Mg^{+2} sai do poro, o que normalmente ocorre após a ativação de canais AMPA presentes na mesma sinapse ou em sinapses vizinhas (Bear, Connors e Paradiso, 2002). Por este motivo, a entrada de íons através do canal do NMDAR é considerada dependente de voltagem, uma vez que depende tanto do glutamato, para ativação de NMDAR, quanto da despolarização da membrana, para saída do Mg^{+2} e desobstrução do canal (Bear, Connors e Paradiso, 2009).

Na região extracelular, existe um tandem de grandes domínios globulares semelhantes a garras, o NTD, o qual engloba ~380 aminoácidos e está envolvido na montagem das subunidades, e o ABD (de ~300 aminoácidos), o qual contém o sítio de ligação da glicina nas subunidades GluN1 e GluN3, e o sítio de ligação do glutamato nas subunidades GluN2 (Paoletti, Bellone e Zhou, 2013). Os NMDAR contêm vários sítios de ligação para antagonistas que podem servir como alvos para farmacoterapias

(Bigge, 1993). A maioria dos sítios de ligação de reguladores alostéricos presentes nos NMDAR (tais como os sítios de ligação das poliaminas, do ifenprodil, do zinco, entre outros) está localizada nestes dois domínios situados na região extracelular do receptor (Monaghan e Jane, 2009; Williams, 2009; Paoletti, Bellone e Zhou, 2013). Desta forma, a presença destes sítios modulatórios tanto na região extracelular (NTD e ABD) quanto na região intracelular (CTD) irá depender da composição e arranjo das subunidades dentro do complexo do NMDAR. Portanto, tanto as funções e propriedades farmacológicas bem como a localização (tanto a nível anatômico quanto a nível celular) e as cascatas de sinalização as quais os NMDAR estão acoplados variam de acordo com as subunidades que os compõem (Chatterton *et al.*, 2002; Prybylowski e Wenthold, 2004).

Os NMDAR são complexos heterotetraméricos formados por quatro subunidades que em conjunto formam um canal para a passagem de íons. Até o presente momento foram identificadas 3 famílias de subunidades dos NMDAR: GluN1, GluN2 e GluN3 (Paoletti, 2011; Paoletti, Bellone e Zhou, 2013) (Tabela 1). A família de subunidades GluN1é formada por oito variantes de subunidades (GluN1 1a-4a; 1b-4b) as quais são geradas por processamento (*splicing*) alternativo do RNA mensageiro (mRNA) de um único gene (Dingledine *et al.*, 1999; Watkins e Jane, 2006). Já a família de subunidades GluN2 é composta por quatro subunidades (GluN2A-D) codificadas por 4 diferentes genes (GRIN2A-D), enquanto que a família GluN3 é composta por duas subunidades (GluN3A-B) codificadas por 2 diferentes genes (GRIN3A-B). Desta forma, NMDAR podem existir como tetrâmeros formados como GluN1/GluN2, GluN1/GluN2/GluN3 ou GluN1/GluN3 (Henson *et al.*, 2010; Paoletti, 2011).

Em acordo com o grande número de subunidades, muitos subtipos de NMDAR diferentes coexistem no SNC. Levando em conta as variantes de *splicing* da subunidade GluN1, pelo menos uma dúzia de subtipos de NMDAR funcionalmente distintos foram

Tabela 1- Família de subunidades que compõem os NMDAR. Baseado em Paoletti, Bellone e Zhou (2013); e Paoletti (2011).

GluN1		GluN2		GluN3	
subunidade	gene	subunidade	gene	subunidade	gene
GluN1 1a	*GRIN1*	GluN2A	*GRIN2A*	GluN3A	*GRIN3A*
GluN1 1b		GluN2B	*GRIN2B*	GluN3B	*GRIN3B*
GluN1 2a		GluN2C	*GRIN2C*		
GluN1 2b		GluN2D	*GRIN2D*		
GluN1 3a					
GluN1 3b					
GluN1 4a					
GluN1 4b					

descritos (Cull-Candy e Leszkiewicz, 2004; Paoletti, 2011), porém o número exato pode ser significativamente maior. Imagina-se que todos os subtipos de NMDAR são compostos pela combinação de duas cópias da subunidade obrigatória GluN1 somados a duas subunidades GluN2 e/ou GluN3 (Paoletti, Bellone e Zhou, 2013). Contudo, um NMDAR funcional, responsivo ao glutamato e à glicina, necessariamente requer a presença de, pelo menos, uma subunidade GluN2 (Monaghan e Jane, 2009), uma vez que, conforme citado anteriormente, o glutamato liga-se a subunidades GluN2, enquanto glicina ou D-serina ligam-se a subunidades GluN1 e GluN3 (Henson *et al.*, 2010). Mesmo os NMDAR formados como GluN1/GluN2 podem apresentar diferenças entre si, tais como farmacocinética, condutância e permeabilidade a íons diferenciada, uma vez que a existência de um grande repertório de subunidades para o NMDAR permite várias combinações de montagem dando origem a uma multiplicidade de

receptores funcionalmente distintos (Ogden e Traynelis, 2011; Paoletti, 2011). Receptores compostos pelas subunidades GluN2A ou GluN2B exibem alta condutâncias e elevada sensibilidade ao bloqueio por Mg^{+2}, enquanto que os NMDAR que contêm subunidades GluN2C ou GluN2D apresentam condutâncias menores e uma menor sensibilidade para Mg^{+2} (Paoletti, 2011). Além disso, sendo a subunidade GluN2 responsável pela modulação do influxo de íons ativada pelo glutamato, NMDAR formados como GluN1/GluN2/GluN3 exibem baixa condutância de Ca^{+2} e permeabilidade reduzida, e NMDAR formados como GluN1/GluN3 são virtualmente impermeáveis a Ca^{+2} e não são responsivos ao glutamato, sendo assim receptores de glicina (Henson *et al.*, 2010; Pachernegg, Strutz-Seebohm e Hollmann, 2012).

Além disso, exemplos de receptores com duas isoformas de GluN1 dentro do mesmo complexo do receptor já foram reportados (Blahos e Wenthold, 1996; Chazot e Stephenson, 1997). De forma semelhante, a coexistência de dois tipos diferentes assim como dois tipos idênticos de subunidades GluN2 dentro de um único receptor também foi descrita (Sheng *et al.*, 1994; Petralia, Al-Hallaq e Wenthold, 2009). Desta forma, os NMDAR podem existir tanto como di-heterômeros GluN1/GluN2 assim como tri-heterômeros GluN1/GluN2/GluN2 (Sheng *et al.*, 1994; Paoletti, Bellone e Zhou, 2013) (Figura 2 a). Os receptores di-heteroméricos GluN1/GluN2B e GluN1/GluN2A representam uma fração importante de NMDAR. Contudo, os receptores tri-heteroméricos GluN1/GluN2A/GluN2B também povoam muitas regiões no cérebro adulto, particularmente no hipocampo e córtex, com estimativas de abundância variando de 15% a >50% da população total de receptores (Al-Hallaq *et al.*, 2007; Gray *et al.*, 2011; Rauner e Kohr, 2011). Apesar disso, embora NMDAR di-heteroméricos tenham sido extensivamente estudados em sistemas de expressão recombinantes, muito pouco é

conhecido sobre as propriedades funcionais de NMDAR tri-heteroméricos (Paoletti, Bellone e Zhou, 2013).

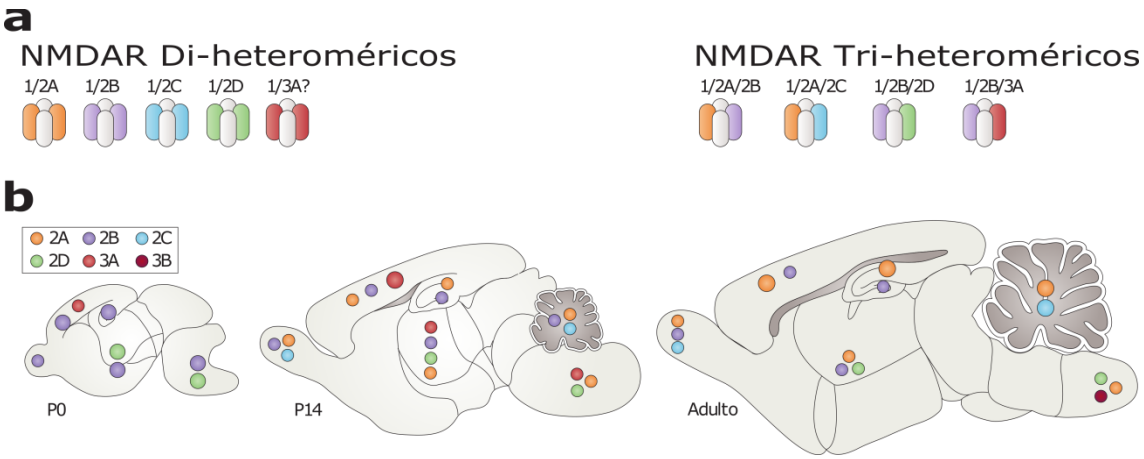

Figura 2- Composição e ontogenia dos NMDAR. (a) Representação de combinações de subunidades (dentre inúmeras outras combinações possíveis) que podem compor os NMDAR de forma a formar NMDAR Di-heteroméricos ou Tri-heteroméricos. (b) Representação da distribuição das diferentes subunidades dos NMDAR em diferentes fases do desenvolvimento encefálico. (P0 indica dia do nascimento; P14 indica 14 dias pós-natal). Fonte: Adaptado de Paoletti, Bellone e Zhou (2013).

1.4 Ontogenia dos NMDAR

Todos os membros da família de NMDAR são caracterizados por padrões de expressão regional e de desenvolvimento distintos, mas sobrepostos, indicando que a expressão de NMDAR nas populações individuais de neurônios é idade dependente. Isto potencialmente sugere papéis particulares para subtipos de NMDAR durante o desenvolvimento e em diferentes áreas do encéfalo (Cull-Candy e Leszkiewicz, 2004; Ewald e Cline, 2009).

Os NMDAR são amplamente distribuídos no SNC, sendo sua expressão máxima na região CA1 do hipocampo (Monaghan e Cotman, 1985). De acordo com esta ampla distribuição, em roedores, a subunidade GluN1 é expressa de forma ubíqua desde o período embrionário (por volta do 14° dia embrionário - E14) até à idade adulta, atingindo um pico de expressão em torno da terceira semana pós-natal (Watanabe *et al.*,

1992; Laurie e Seeburg, 1994; Paupard, Friedman e Zukin, 1997). A variante GluN1-2 é amplamente e mais ou menos homogeneamente expressa em todo o encéfalo, enquanto que as isoformas GluN1-1 e GluN1-4 formam padrões quase complementares, sendo GluN1-1 restrito a regiões mais rostrais (córtex, hipocampo, caudado) e GluN1-4 a regiões mais caudais (tálamo, cerebelo, colículo) do SNC (Ewald e Cline, 2009; Paoletti, 2011; Paoletti, Bellone e Zhou, 2013). De modo geral, o padrão aproximado da expressão das subunidades GluN1 é GluN1-2 > GluN1-1 > GluN1-4 >> GluN1-3 (Ewald e Cline, 2009). Esse padrão de expressão parece não mudar significativamente durante o desenvolvimento, uma vez que os padrões de expressão são estabelecidos em torno do nascimento. No entanto, o significado funcional da expressão diferencial das isoformas GluN1 ainda não está bem esclarecido (Ewald e Cline, 2009; Paoletti, 2011; Paoletti, Bellone e Zhou, 2013).

As quatro subunidades GluN2, que são as principais determinantes da heterogeneidade funcional do receptor, mostram perfis de expressão espaço-temporal muito diferentes (Figura 2 b). Assim como a subunidade GluN1, a expressão das subunidades GluN2 começa em níveis baixos por volta do E14 e torna-se progressivamente mais enriquecida à medida que o desenvolvimento embrionário continua. Contudo, durante este período, somente as subunidades GluN2B e GluN2D são expressas (Ewald e Cline, 2009; Paoletti, 2011; Paoletti, Bellone e Zhou, 2013). É durante as 2 primeiras semanas pós-natal que ocorrem as principais alterações nos padrões de expressão das subunidades GluN2. A subunidade GluN2B continua sendo a subunidade predominante durante estágios iniciais do desenvolvimento pós-natal. Esta subunidade atinge o pico máximo de expressão no hipocampo e no córtex por volta da terceira semana pós-natal, e depois declina a níveis moderados durante a idade adulta, tornando-se progressivamente restrita ao prosencéfalo (Ewald e Cline, 2009; Paoletti,

2011; Paoletti, Bellone e Zhou, 2013). Já a expressão da subunidade GluN2A começa logo após o nascimento e aumenta de forma constante para se tornar ampla e abundantemente expressa em praticamente todo o SNC adulto. Semelhante a subunidade GluN2B, a subunidade GluN2A atinge um pico de expressão na terceira semana pós-natal antes de diminuir para níveis adultos (Ewald e Cline, 2009; Paoletti, 2011; Paoletti, Bellone e Zhou, 2013). Neste período, as subunidades GluN2A e GluN2B se tornam as subunidades GluN2 predominantemente encontradas no córtex cerebral e no hipocampo do encéfalo maduro (Monyer *et al.*, 1994; Sheng *et al.*, 1994; Cull-Candy, Brickley e Farrant, 2001) indicando que estas duas subunidades possuem um papel central na função sináptica e plasticidade (Paoletti, Bellone e Zhou, 2013). Já a subunidade GluN2D, a qual é ausente no telencéfalo mas abundante no diencéfalo, mesencéfalo e medula espinhal durante o período pré-natal, aumenta a expressão durante a primeira semana pós-natal atingindo um pico de expressão por volta do 7° dia pós-natal (P7), quando também pode ser detectada em níveis muito baixos no córtex, hipocampo e septo. Após este período, a expressão de GluN2D cai acentuadamente sendo encontrada, no encéfalo adulto, principalmente no diencéfalo e no mesencéfalo com níveis de expressão muito baixos (Ewald e Cline, 2009; Paoletti, 2011; Paoletti, Bellone e Zhou, 2013). A expressão da subunidade GluN2C aparece tarde no desenvolvimento, sendo muito baixa em P7, mas torna-se marcadamente aumentada e restrita ao cerebelo por volta de P12. Sua expressão está principalmente confinada ao cerebelo e ao bulbo olfatório no encéfalo adulto (Ewald e Cline, 2009; Paoletti, 2011; Paoletti, Bellone e Zhou, 2013).

As subunidades GluN3A e GluN3B também exibem perfis ontogenéticos diferenciados. Em ratos, a subunidade GluN3A também é expressa durante o período embrionário (por volta de E15) em algumas regiões como medula espinal, hipotálamo e

tálamo (Ewald e Cline, 2009). Seu pico de expressão ocorre em torno de P8 e depois diminui rapidamente, sendo que já em P20 são encontrados níveis de expressão similares aos encontrados no encéfalo adulto, idade em que sua expressão também inclui o hipocampo, amigdala e partes do córtex (Wong *et al.*, 2002; Ewald e Cline, 2009; Henson *et al.*, 2010; Low e Wee, 2010; Paoletti, Bellone e Zhou, 2013). Em contraste, a subunidade GluN3B é restrita a motoneurônios do tronco encefálico e medula espinhal. Seu pico de expressão ocorre em torno de P14 e permanece elevado até a idade adulta em motoneurônios (Nishi *et al.*, 2001; Matsuda *et al.*, 2002; Ewald e Cline, 2009; Paoletti, Bellone e Zhou, 2013) e possivelmente outras regiões (Wee *et al.*, 2008; Paoletti, Bellone e Zhou, 2013).

Durante estágios iniciais do desenvolvimento encefálico as conexões sinápticas ainda não estão bem estabelecidas e, portanto, os NMDAR são predominantemente não-sinápticos. Nesta etapa do desenvolvimento, neurônios corticais e hipocampais expressam principalmente a subunidade GluN2B enquanto que a subunidade GluN2A começa a ser expressa mais tardiamente, e aumenta sua expressão ao longo do desenvolvimento (Li *et al.*, 2002).

Trabalhos tem sugerido que em sinapses maduras, a subunidade GluN2A é expressa principalmente no botão sináptico (NMDAR sinápticos) enquanto que a subunidade GluN2B é expressa na extra-sinapse (NMDAR extrasinápticos) (Petralia, 2012). Além disso, estudos têm sugerido que as diferentes localizações dos NMDAR refletem diferentes funções destes receptores, as quais são influenciadas não somente pelo local onde se encontram (compartimentalização), mas também pelas subunidades que os compõem (Hardingham, Fukunaga e Bading, 2002; Li *et al.*, 2002; Petralia, 2012). Estas diferentes funções dos NMDAR tem sido relatada estar envolvida tanto nos processos fisiológicos quanto nos processos patológicos do SNC.

1.5 Distúrbios do neurodesenvolvimento e consequências comportamentais

A modulação da neurotransmissão mediada por receptores excitatórios glutamatérgicos, principalmente do tipo NMDA, é conhecida por ter importantes implicações para a origem da lesão e morte celular. Isto é observado numa variedade de condições diferentes, incluindo acidente vascular encefálico, hipóxia, isquemia, epilepsia entre outras (Flores-Soto *et al.*, 2012). Estas condições compartilham características patológicas comuns, incluindo perda neuronal gradual e seletiva, principalmente devido à hiperatividade de NMDAR. Embora os grupos neuronais afetados variem de acordo com a doença, e as causas da morte neuronal sejam desconhecidas, a hiperestimulação dos NMDAR geralmente conduz a um aumento na concentração de Ca^{+2} no citoplasma e a geração de espécies reativas de oxigênio (Di Maio *et al.*, 2011; Flores-Soto *et al.*, 2012) levando a oxidação lipídica, proteica e de DNA. Esses fatores parecem desempenhar um papel muito importante na neurodegeneração em patologias tais como as epilepsias.

Por outro lado, conforme já citado anteriormente, a hipoativação de NMDAR também pode desencadear alterações na funcionalidade dos neurônios (e das redes a ele vinculadas) podendo causar morte neuronal e, consequentemente, alterações comportamentais duradouras (Kaindl *et al.*, 2008; Loss, Cordova e de Oliveira, 2012; Turski e Ikonomidou, 2012). Estes eventos de hipoativação de NMDAR são ainda mais graves quando ocorridos durante períodos iniciais (e críticos) do desenvolvimento encefálico devido ao fato de que durante este período ocorrem as principais alterações na expressão de NMDAR, as quais estão envolvidas com a maturação do SNC. Alguns estudos demonstraram que roedores expostos a repetidos episódios de hipofunção de NMDAR durante períodos iniciais do desenvolvimento apresentaram, quando adultos, alterações comportamentais similares às observadas em pacientes esquizofrênicos

(Latysheva e Raevskii, 2003; Kawabe, Iwasaki e Ichitani, 2007; Akillioglu *et al.*, 2012; Akillioglu, Binokay e Kocahan, 2012), tais como alterações na atividade locomotora exploratória, nos níveis de ansiedade, e comprometimento das habilidades cognitivas.

De fato, a hipótese de que a esquizofrenia é um distúrbio do neurodesenvolvimento causada pela hipofunção de NMDAR tem sido proposta (du Bois e Huang, 2007; Insel, 2010). Alguns estudos propuseram que uma disfunção dos NMDAR durante o desenvolvimento induz um "estado de hipofunção de NMDAR" que se instala no encéfalo como uma condição latente com potencial de desencadear manifestações psicóticas na idade adulta, mas não na adolescência (Olney, Newcomer e Farber, 1999). A hipótese de que é necessária que algumas circuitarias encefálicas tenham atingido a maturação antes que sintomas psicóticos sejam expressos (Olney, Newcomer e Farber, 1999) pode explicar a ausência de manifestações psicóticas na adolescência. Essa hipótese postula que interferências em um primeiro sistema de neurotransmissão afeta o funcionamento de um segundo sistema de neurotransmissão que influência um terceiro sistema e assim por diante, até atingir um mecanismo chave que envolve a hiperativação e um impacto neuropsicotóxico sobre os neurônios que estão no final da série sináptica. Embora o mecanismo pelo qual o "estado de hipofunção de NMDAR" desencadeia estas alterações não esteja bem elucidado, estudos sugerem o envolvimento de diversos sistemas de neurotransmissão, tais como o glutamatérgico, colinérgico, adrenérgico, gabaérgico, serotoninérgico e dopaminérgico (Olney, Newcomer e Farber, 1999; Bondi, Matthews e Moghaddam, 2012), o que destaca a complexidade da esquizofrenia e a dificuldade que a comunidade científica tem em estudar esse distúrbio neurológico.

2. RESERVA COGNITIVA E ENCEFÁLICA

O termo "reserva encefálica" foi utilizado pela primeira vez por Katzman et al. (1988), após observarem que indivíduos apresentando características da Doença de Alzheimer Leve (como presença de elevada quantidade de placas neocorticais) não apresentavam declínio cognitivo. Os autores relacionaram esse efeito ao encéfalo mais pesado e ao maior número de neurônios observado nesses indivíduos quando comparado a indivíduos controle (indivíduos que não apresentavam Doença de Alzheimer), e propuseram que essas pessoas podem ter tido uma Doença de Alzheimer incipiente, sem perda de grande número de neurônios, ou, alternativamente, começaram com encéfalos maiores e maior número de neurônios, ou seja, tiveram uma maior reserva encefálica. Desde então o termo "reserva" tem sido utilizado para explicar a disjunção entre o grau de lesão encefálica e seu desfecho clínico (Stern, 2009).

Atualmente, diversos subconceitos de reserva encefálica emergiram (Stern *et al.*, 2005; Stern, 2009), tais como "reserva cognitiva" (diferenças individuais em como as pessoas processam as tarefas permitem que alguns indivíduos lidem melhor do que outros com as patologias encefálicas); "reserva neural" (variabilidade interindividual nas redes encefálicas ou paradigmas cognitivos que subjazem o desempenho das tarefas no encéfalo saudável); "compensação neural" (variabilidade interindividual na capacidade de compensar a interrupção da patologia encefálica de redes de processamento padrão usando estruturas encefálicas ou redes que não são normalmente usadas por indivíduos com encéfalos intactos). Ao que diz respeito a presente Tese, utilizarei o termo "reserva cognitiva e encefálica" (do inglês *Brain and Cognitive Reserve* – BCR) com o intuito de abranger todos os tipos de reserva acima descritos. Desta forma, para esta Tese, o conceito de BCR postula que indivíduos saudáveis com elevados níveis de reserva encefálica apresentam melhor desempenho cognitivo através

do uso de redes neurais subjacentes ao desempenho das tarefas de maneira mais eficiente, além de apresentarem maior resistência a danos e patologias encefálicas (serem resilientes) do que outros indivíduos com menores níveis de BCR.

2.1 Estimulação da Reserva Cognitiva e Encefálica

O conceito de BCR é baseado na coleta extensiva de dados epidemiológicos, os quais indicam que aqueles indivíduos que possuem um estilo de vida com níveis mais elevados de envolvimento social, atividade física e estimulação cognitiva têm um menor risco de desenvolver demência mesmo apresentando características de patologia encefálica (Fratiglioni, Paillard-Borg e Winblad, 2004; Nithianantharajah e Hannan, 2009). Uma vez que este conceito é estabelecido, diferentes intervenções cognitivas emergiram como uma potencial estratégia não farmacológica para o tratamento de patologias encefálicas (ou prevenção da evolução do quadro desta patologia) (Nithianantharajah e Hannan, 2009; Gates e Sachdev, 2014; Gehres *et al.*, 2016). Dentre estas diferentes intervenções cognitivas pode-se citar o treinamento cognitivo (Clare *et al.*, 2003; Bahar-Fuchs, Clare e Woods, 2013), a estimulação cognitiva (Woods *et al.*, 2012) e a reabilitação cognitiva (Clare *et al.*, 2003; Bahar-Fuchs, Clare e Woods, 2013), as quais, embora baseadas em diferentes constructos teóricos, não possuem distinção clara em testes clínicos (Gehres *et al.*, 2016), e por este motivo, ao que diz respeito a presente Tese, serão tratadas como estimulação da BCR.

As principais estratégias de estimulação da BCR são o engajamento em atividades de exercício físico (tais como o treinamento físico regular), estimulação intelectual (tais como leitura, jogos, aprender alguma tarefa nova tal como tocar um instrumento, por exemplo), e o convívio social (tais como visitar amigos ou familiares, e fazer atividades em grupo) (Scarmeas *et al.*, 2001; Nithianantharajah e Hannan, 2009; Stern, 2009). Diversos estudos demonstraram que a estimulação da BCR induz

alterações em diversas classes de moléculas como neurotrofinas, receptores de neurotransmissores, proteínas de vias de sinalização sináptica, e reguladores da proliferação celular, por exemplo (van Praag, Kempermann e Gage, 2000; Rossi *et al.*, 2006; Nithianantharajah e Hannan, 2009; Nithianantharajah e Hannan, 2011). Estas alterações são acompanhadas por um aumento na neurogênese hipocampal (van Praag, Kempermann e Gage, 2000; Brown *et al.*, 2003; van Praag, 2008), arborização dendrítica (Faherty, Kerley e Smeyne, 2003), além de um aumento na sinaptogênese (Mohammed *et al.*, 2002; Zhu *et al.*, 2009), o que leva a um aumento nas conexões sinápticas e reorganização das redes neuronais (Vivar *et al.*, 2012).

Em conjunto com as alterações acima citadas (e como consequência delas), a estimulação da BCR induz uma melhora cognitiva tanto em indivíduos saudáveis (Nithianantharajah e Hannan, 2009; Viola *et al.*, 2009; Loss *et al.*, 2015) quanto em indivíduos que são acometidos por distúrbios encefálicos, sendo que nestes últimos, a estimulação da BCR induz uma redução, atraso, ou até mesmo prevenção dos sintomas neurológicos e cognitivos decorrentes da doença (Scarmeas *et al.*, 2001; Nithianantharajah e Hannan, 2009; Stern, 2009; Gehres *et al.*, 2016).

2.3 Enriquecimento Ambiental

O enriquecimento ambiental (EA), ou refinamento ambiental, pode ser definido como qualquer modificação no ambiente de animais em cativeiro que procure melhorar o bem-estar físico e psicológico dos animais, fornecendo estímulos que satisfaçam as necessidades específicas de cada animal (Baumans e Van Loo, 2013). Apesar de os efeitos benéficos do EA serem muito claros (Marquez-Arias *et al.*, 2010; Gross *et al.*, 2012; Meagher e Mason, 2012), existe uma crescente preocupação a respeito da manutenção de animais de cativeiro (incluindo animais de laboratório) em ambientes empobrecidos, tais quais as caixas moradia “padrão de laboratório” (Van de Weerd *et*

al., 2002; Baumans, 2005; Baumans e Van Loo, 2013). O fato de que o fracasso de uma grande quantidade de testes pré-clínicos são, potencialmente, devidos ao alojamento pobre em que os animais de laboratório são criados (Akkerman *et al.*, 2014; Freedman, Cockburn e Simcoe, 2015) evidencia ainda mais os atuais problemas a respeito da manutenção de animais de cativeiro. Pensando em um aumento na qualidade de vida e do bem-estar de animais de laboratório, atualmente, a União Européia preconiza a utilização de um ambiente enriquecido, no qual "*todos os animais devem ter espaço de complexidade suficiente para permitir a expressão de uma ampla gama de comportamentos naturais. Deve ser fornecido um grau de controle e escolha sobre seu ambiente para reduzir o comportamento induzido pelo estresse*" (EC, 2010). Baseado neste conceito, para o que diz respeito a presente Tese, ambiente padrão (AP) será definido como (i) todo alojamento que possua o mínimo de complexidade (ou enriquecimento) permitindo a expressão dos comportamentos naturais de cada espécie (por exemplo, sociabilidade em animais naturalmente sociáveis, evitação de espaços abertos em animais naturalmente predados, aumento da atividade física em animais com elevados níveis basais de atividade), além de (ii) alojamentos "empobrecidos", tais quais aqueles contendo apenas maravalha (sendo os animais mantidos isoladamente ou não) (Figura 3). Por outro lado, EA será definido como alojamentos complexos (podendo os animais serem mantidos de maneira contínua ou não) capazes de fornecer estimulação das funções sensoriais, cognitivas e motoras através de adaptações tanto do ambiente físico quanto social. Estes ambientes podem ser compostos pela combinação de diferentes objetos tais como rodas de correr, abrigos e vários outros objetos com diferentes texturas, cores, formas e tamanhos (Figura 3).

O paradigma de EA tem sido amplamente utilizado tanto como uma estratégia de estimulação da BCR em animais de cativeiro quanto como uma estratégia de

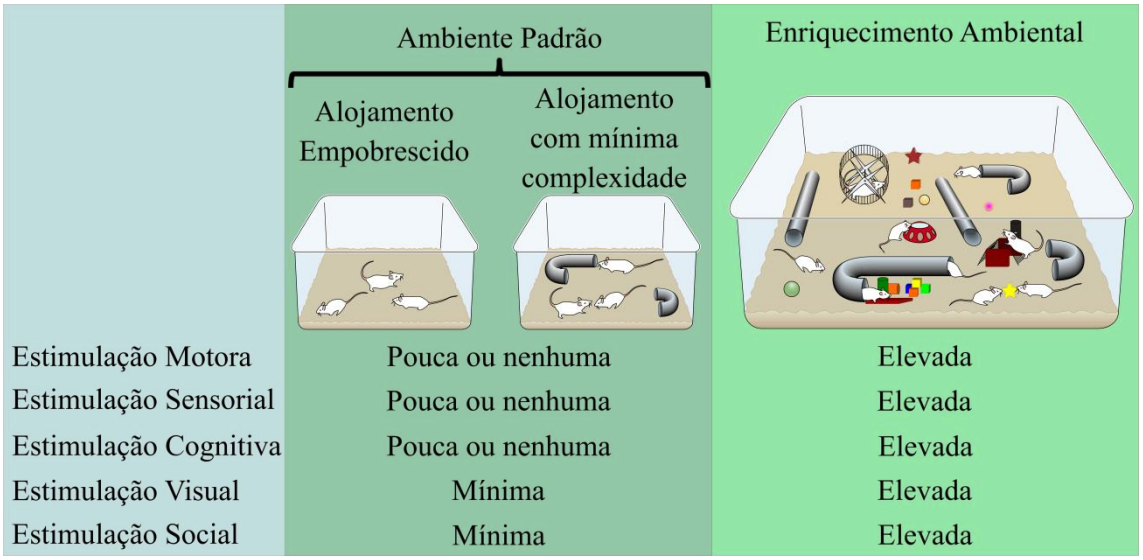

Figura 3- Tipos de alojamento. Esquema representando os tipos de alojamentos considerados como Ambiente Padrão ou Enriquecimento Ambiental, e os tipos de estimulação que eles propiciam. Modificado de Nithianantharajah e Hannan (2006) e van Praag, Kempermann e Gage (2000).

intervenção não farmacológica no tratamento de distúrbios do SNC (Mohammed *et al.*, 2002; Nithianantharajah e Hannan, 2006; Viola *et al.*, 2010; Nithianantharajah e Hannan, 2011; Monteiro *et al.*, 2014; Burrows *et al.*, 2015; Loss *et al.*, 2015; Huttenrauch, Salinas e Wirths, 2016). Estes estudos têm observado que o EA induz alterações bioquímicas, morfológicas e anatômicas, as quais são muitas vezes acompanhadas por alterações comportamentais, tais como melhora do desempenho cognitivo e alterações da emocionalidade (Nithianantharajah e Hannan, 2009; Viola *et al.*, 2009; Diniz *et al.*, 2010; Segovia *et al.*, 2010; Viola *et al.*, 2010; Vazquez-Sanroman *et al.*, 2013; Sampedro-Piquero *et al.*, 2014; Loss *et al.*, 2015; Huttenrauch, Salinas e Wirths, 2016). Contudo, uma variedade de protocolos de EA pode ser encontrada na literatura, o que dificulta, muitas vezes, a replicação de resultados. Essas diferenças na aplicação de protocolos podem ocorrer desde a idade inicial de exposição ao EA (por exemplo, imediatamente após o desmame ou durante a vida adulta), duração

total de enriquecimento (por exemplo, 4 semanas, 8 semanas, 9 semanas), ambiente físico (por exemplo, presença ou ausência de roda de correr), tempo de exposição ao EA (por exemplo, 24h/dia, 3h/dia ou 1h/dia) (Rampon *et al.*, 2000; Brown *et al.*, 2003; Pereira *et al.*, 2009; Kobilo *et al.*, 2011; Bezzina *et al.*, 2015; Burrows *et al.*, 2015; Loss *et al.*, 2015).

Embora o EA tenha efeitos benéficos mesmo que iniciados em períodos de vida mais tardios, como por exemplo, durante o início da vida adulta ou durante o envelhecimento (Diniz *et al.*, 2010; Sampedro-Piquero *et al.*, 2016), evidências têm sugerido que iniciar a estimulação da BCR durante períodos precoces do desenvolvimento encefálico podem acelerar a maturação física e aumentar o desempenho cognitivo de maneira duradoura (Cancedda *et al.*, 2004; Sale *et al.*, 2004; Sale *et al.*, 2007; Baldini *et al.*, 2013). A criação de roedores desde o nascimento em um ambiente enriquecido acelera o desenvolvimento do sistema visual como verificado a nível comportamental, eletrofisiológico e molecular (Cancedda *et al.*, 2004) além de melhorar a acuidade visual e as habilidades de aprendizado e diminuir os níveis de ansiedade destes animais quando adultos (Sale *et al.*, 2004; Baldini *et al.*, 2013). Estes benefícios podem ser atribuídos a diferentes níveis de atenção materna entre diferentes condições ambientais (EA e AP) os quais podem atuar como mediadores indiretos para os primeiros efeitos do EA no desenvolvimento do sistema visual e cognitivo (Sale *et al.*, 2004; Baldini *et al.*, 2013) uma vez que mesmo um protocolo de massagem (o qual mimetiza estimulação tátil fornecida pela mãe) em períodos iniciais do desenvolvimento é capaz de produzir efeitos benéficos duradouros sobre o comportamento de maneira similar ao EA (Baldini *et al.*, 2013). Além disso, efeitos benéficos do EA sobre o sistema visual podem ainda ser observados na prole mesmo quando a intervenção ocorre durante o período gestacional (Sale *et al.*, 2007). Desta forma, visto a gama de

benefícios que o EA promove tanto para indivíduos adultos quanto para animais jovens e filhotes de progenitores submetidos ao EA, é de se esperar que o EA ocorrido durante períodos precoces do desenvolvimento promova alterações encefálicas duradouras que não se restrinjam ao sistema visual, mas que promovam benefícios cognitivos tanto em animais saudáveis quanto em animais suscetíveis ao desenvolvimento de distúrbios do SNC.

3. OBJETIVOS

3.1 Objetivo geral

Investigar se a estimulação da reserva cognitiva e encefálica durante estágios iniciais do desenvolvimento, através do enriquecimento ambiental, é capaz de atenuar as alterações encefálicas e cognitivas induzidas tanto pelo antagonismo neonatal não-seletivo de NMDAR quanto pelo antagonismo neonatal seletivo de NMDAR contendo a subunidade GluN2B em ratos.

3.2 Objetivos específicos

Validar o uso de uma ferramenta estatística alternativa, a Análise de Componentes Principais (PCA), para a análise de comportamentos complexos visando à identificação tanto de macro quanto de micro alterações na estrutura do comportamento animal.

Investigar se o horário do dia em que os animais são testados afeta a atividade locomotora e exploratória, a distribuição espacial e a organização temporal do comportamento exploratório, e os níveis de ansiedade de ratos adultos.

Investigar se os efeitos do EA sobre o repertório comportamental de camundongos *Swiss* é afetado pelo horário do dia em que os animais são testados.

Investigar se o antagonismo transiente de NMDAR e o antagonismo transiente seletivo de NMDAR contendo a subunidade GluN2B durante períodos precoces do desenvolvimento encefálico afeta similarmente o comportamento de roedores adultos.

Investigar se o EA ocorrido durante períodos precoces do desenvolvimento encefálico é capaz de prevenir ou reverter as alterações comportamentais duradouras

induzidas pelo antagonismo transiente de NMDAR durante períodos precoces do desenvolvimento encefálico.

Investigar se o antagonismo transiente de NMDAR e o antagonismo transiente seletivo de NMDAR contendo a subunidade GluN2B durante períodos precoces do desenvolvimento encefálico afetam de maneira duradoura a captação de glicose encefálica em roedores.

Investigar se o EA ocorrido durante períodos precoces do desenvolvimento encefálico é capaz de prevenir ou reverter às alterações na captação de glicose encefálica induzidas pelo antagonismo transiente de NMDAR durante períodos precoces do desenvolvimento encefálico.

PARTE II. RESULTADOS

CAPÍTULO I

Artigo publicado

Time-of-day influence on exploratory behaviour of rats exposed to an unfamiliar environment

Cássio M. Loss*, Sandro D. Córdova, Sidia Maria Callegari-Jacques e Diogo L. de Oliveira.

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Justificativa: Embora seja bem descrito que ratos exibem níveis elevados de atividade durante a fase escura e níveis reduzidos durante a fase clara do ciclo claro/escuro, pouco é sabido a respeito da influência do período-do-dia sobre as estratégias utilizadas por ratos para explorar o ambiente.

Objetivo geral: Investigar a influência do período-do-dia sobre as estratégias exploratórias e a organização espaço-temporal do comportamento exploratório de ratos expostos a ambientes desconhecidos.

Objetivo específico: Validar o uso de uma ferramenta estatística alternativa (Análise de Componentes Principais) para análise e interpretação do comportamento de forma integrada.

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Time-of-day influence on exploratory behaviour of rats exposed to an unfamiliar environment

Cássio M. Loss [a,*], **Sandro D. Córdova** [a], **Sidia Maria Callegari-Jacques** [b] **and Diogo L. de Oliveira** [a]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil
[b] Departamento de Estatística, Instituto de Matemática, Universidade Federal do Rio Grande do Sul, Rio Grande do Sul, Brazil
[*]Corresponding author's e-mail address: cassio.m.loss@gmail.com

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Abstract

It is well known that rats exhibit elevated levels of activity during the dark phase and reduced levels during the light phase of the photoperiod cycle. However, the information about the influence of the time-of-day on the strategies used to explore the environment is still not understood. Here we tested the hypothesis that time-of-day influences the fine-scale exploratory behaviour of rats, measured in the open field (OF) test, and emotionality of rats, measured in the elevated plus maze (EPM) test. Adult male Wistar rats were subjected to the OF and EPM tests during Morning, Afternoon, or Evening sessions. In the OF, a principal component analysis (PCA) revealed that the Evening group exhibited longer duration of locomotion and rearing, and also higher distance travelled, trip length, inter-stop distance, number of stops and stops per trip compared to other groups. PCA also revealed that the Evening group exhibited shorter time spent at the home base, duration of locomotion along the perimeter and distance travelled along the perimeter compared to other groups. In the EPM test, there was no difference between the groups in any of the parameters evaluated. Our results indicate that the time-of-day may influence the spatio-temporal organization of exploration of rats subjected to unfamiliar environments. These alterations appear to be unrelated to differences in the emotional state of the animals.

Keywords

circadian rhythm, exploration, locomotion, spatio-temporal organization, open field, home base, principal component analysis.

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1. Introduction

The term 'circadian rhythm' is widely used to describe the oscillations of several physiological functions that occur in a 24-h cycle. In mammals, a central pacemaker situated in the suprachiasmatic nucleus is the major regulator of this endogenous rhythm that associates the daily fluctuations in environmental cues with output pathways that modulate animal physiology and behaviour (Moore & Eichler, 1972; Stephan & Zucker, 1972; Yao et al., 2006; Panksepp et al., 2008). This circadian time-keeping system plays a pivotal role in regulating key biological processes, such as sleep (Saper et al., 2005), feeding (Kavaliers & Hirst, 1985), thermogenesis (Huang et al., 2011), reward behaviour (Abarca et al., 2002; Mcclung et al., 2005), depression/anxiety-like behaviours (Verma et al., 2010), learning and memory (Chaudhury et al., 2002) and exploratory behaviour (Easton et al., 2003).

To date, studies have analysed the light/dark photoperiod cycle (LDPC) effects on the activity of rodents in the open field (OF) task. Hostetter (1966) found increased activity of DBA/1J mice during the light phase, whereas C57BL/6 mice presented increased activity during the dark phase. In rats, Gentsch et al. (1982) found clear day/night variations in locomotion in the OF test. More recently, Smith et al. (2007) and Verma et al. (2010) observed that rats travelled longer distances and reared more often during the dark phase of the LDPC when compared with animals tested during the light phase. Despite the significantly efforts of the above-mentioned studies to investigate the effects of the light/dark photoperiod cycle on locomotor and exploratory behaviours, there are no studies investigating if LDPC may affect the fine structure of locomotion and exploration of rodents in the OF task.

In this context, recent studies have employed video tracking systems to perform more detailed analyses of the behaviour of rodents in the OF. This kind of analysis has allowed researchers to measure the distribution of locomotion across the duration of test, the places more often visited by animals in the arena, the key locations established as a reference for organizing the exploration (e.g., home base), and the temporal sequence in which each event have occurred (Eilam, 2003; Zadicario et al., 2005; Leke et al., 2012). This spatio-temporal analysis has allowed researchers to identify behavioural patterns which cannot be detected using only the end-point parameters. In the OF test, animals can use similar strategies to acquire information about the environment even presenting different levels of activity. For example, animals with distinct total distance travelled may travel the same proportion of

their locomotion in the different periods of the test (e.g., they travel 50% of their total locomotion during the first half of the test). On the other hand, animals can use different strategies to acquire information about the environment even presenting similar levels of activity. This phenomenon has already been observed by Yaski et al. (2011) which demonstrated that, even if there was no differences in total distance travelled among individuals, spatial distribution of locomotion could vary. Some individuals remained closer to the arena wall than others.

Emotional state may also affect exploratory behaviour of rats (Ramos, 2008). Several studies have demonstrated that anxiety-like behaviours may fluctuate throughout the day (for review see Ramos, 2008) and some compounds that modulate anxiety also affect the circadian system (Gannon et al., 2011). These evidences raise the possibility that LDPC-dependent differences in exploratory behaviour of rats could reflect the fluctuations of the emotional state of animals throughout the day.

We tested the hypothesis that the LDPC affects the fine-scale patterns of exploratory behaviour in the OF test and emotionality in the elevated plus maze (EPM) test. Our first working hypothesis was that time-of-day would not affect only activity of the rats, but also the spatio-temporal organization pattern of exploratory behaviour; we expected that rats tested during the dark phase would be more active, and that they would explore the different places of the arena more times and during longer periods. We also expected that more active rats would use different exploratory strategies than those used by less active rats. Our second working hypothesis was that the anxiety state of animals would affect the exploratory pattern; we expected to observe rats more exposed during the dark phase due to reduced anxiety levels during this phase.

2. Material and methods

2.1. Subjects

Thirty-three male Wistar rats (60 days old, body weight 180–240 g) provided by the Department of Biochemistry of Universidade Federal do Rio Grande do Sul were used. Rats were housed (4–5 animals/cage) in standard polypropylene cages (405 × 335 × 180 mm) and provided with food and water ad libitum. Animals were maintained under a 12 h controlled LDPC (lights on at 7:00 a.m.), and the room temperature was adjusted to 21 ± 2°C.

The handling and care of the animals were conducted according to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (2011). All procedures were approved by the Committee of Ethics at Universidade Federal do Rio Grande do Sul (protocol number 2008058).

2.2. Time-of-day

To evaluate the influences of time-of-day on behaviour, animals were randomly separated into three groups: the Morning group (tested between 8 a.m. and 10 a.m.; $N = 11$), the Afternoon group (tested between 3 p.m. and 5 p.m.; $N = 12$), and the Evening group (tested between 8 p.m. and 10 p.m.; $N = 10$). All the groups were body weight- and age-matched. The testing times were determined based on the fluctuations in plasma levels of corticosterone throughout the day (Moore et al., 1972; Yao et al., 2006). For rats, the lowest plasma corticosterone concentration occurs in the early morning and progressively enhances toward the evening, reaching a peak around the onset of darkness (Yao et al., 2006).

2.3. Behavioural procedures

Animals were handled individually for two minutes for three consecutive days prior to the initiation of the behavioural tasks. The handling procedure was performed to allow the animals to habituate to the researchers and the testing room (Eilam, 2003; Walf & Frye, 2007). The OF and EPM tests were performed when animals were 60 and 67 days old, respectively. To acclimate with the testing conditions, the animals were placed in the behavioural testing room for 1 h preceding the start of the tasks; the same temperature (21 ± 2°C) and illumination settings (see below) were used for each test. Regardless of the period in which the animals were tested (light or dark), the aforementioned acclimation was initiated during the light period of the LDPC; i.e., the animals in the Evening group were removed from their housing rooms 5 min before the time at which the lights were turned off. The ANY-Maze video-tracking system (Stoelting) was used to automatically record and collect the behavioural data.

2.4. Open field task

The OF test consisted of a 60×50 cm circular arena constructed from plywood and homogeneously painted black in order to allow the software identify animals by contrasting their white body with the dark background

of the floor. The testing room was illuminated using two white-lamps directed toward the walls of the room to obtain a 15 Lux light intensity that was uniformly distributed throughout the arena. The illumination settings during the test were the same for all of the experimental groups. Each animal was individually placed on the same peripheral starting square and was allowed to freely explore the arena for 15 min. The apparatus was cleaned using a 70% ethanol solution between trials to eliminate odour cues. In the ANY-Maze software the floor of the apparatus was virtually separated into 28 squares (12 central and 16 peripheral). We followed Eilam (2003) and Li et al. (2010) to define the spatio-temporal organization of locomotion and exploration. We defined locomotor and exploratory behaviours as: (a) duration of rearing: the total time individuals spent on their hind legs with forelimbs off the floor; (b) duration of grooming: the total time individuals performed strokes along the snout, semi-circular movements over the top of the head and behind the ears, licking the tail, the body fur, the genital area or scratching the body with the hind paws; (c) distance travelled: the overall distance that each animal travelled during the 15-min observation period; (d) duration of locomotion: the accumulated time of the locomoting periods (data were calculated as the duration of locomotion divided by the total test time multiplied by 100); (e) average speed: the distance travelled divided by the duration of locomotion; (f) number of stops: the number of immobile episodes with duration equal to or greater than 2 s; and (g) inter-stop distance: the total distance travelled divided by the total number of stops.

To determine the strategies employed by animals to explore the environment, we analysed the distance (as a percentage of the total distance travelled during the test) that animals travelled in each 5-min period. Thus, the percentage of the total distance travelled in each 5-min period was calculated as the absolute distance travelled in each 5-min period divided by the total distance travelled during the entire test multiplied by 100. We analysed the duration of rearing and grooming in the same manner.

We quantified the spatial distribution of behaviours based on: (a) distance travelled along the perimeter: the total distance travelled in the vicinity of the arena walls divided by the total distance travelled during the test multiplied by 100; (b) duration of locomotion spent along the perimeter: the duration of locomotion in the vicinity of the arena walls divided by the total duration of locomotion during the test multiplied by 100; (c) time spent at the home base: the time spent immobile in the home base zone divided by the total time

of the test multiplied by 100. The 'home base' was defined as the square (zone) with the highest rank after ranking all of the squares based on the accumulated 'non-locomoting' intervals (Eilam & Golani, 1989, 1990).

We quantified the fine-scale temporal organization of locomotion and exploration based on: (a) number of trips: the number of times an individual moved out and returned to the home base; (b) trip length: the average metric distance travelled per trip calculated as the total distance travelled divided by the total number of trips; and (c) stops/trip: the average number of stops per trip, calculated as the total number of stops divided by the number of trips.

2.5. Elevated plus maze test

The EPM test was performed to assess anxiety-like behaviours. This task was designed to induce a conflict between the rodents' preference for protected areas and their innate motivation to explore novel environments (Pellow et al., 1985; Walf & Frye, 2007). As the OF apparatus, the maze was constructed from wood and the walls and floor were painted black to allow the software identify the animals. The maze was shaped like a '+' with four arms (50 cm long by 10 cm wide) extending from a 10 cm by 10 cm square central platform. Two of the arms were 'open' (i.e., without walls or ceiling) and had only a 1 cm high railing running along their length. The other two arms were 'closed' with 40 cm high walls. The arms were arranged around the central platform so that open and closed arms opposed each other. This +-shaped form of the maze aims to obtain a symmetrical maze, facilitating the interpretation of results when compared to the previous Y-shaped maze described by Montgomery (Handley & Mcblane, 1993). The maze was elevated 50 cm from the floor. During all tests, the testing room was illuminated using two red lamps directed toward the walls of the room to obtain a uniform red-light intensity of 5 Lux. This illumination pattern has been shown to reduce the preference of animals for closed arms and consequently to facilitate the detection of ansiogenic-like behaviours without affecting the detection of anxiolytic-like behaviours (Handley & Mcblane, 1993; Jones & King, 2001; Loss et al., 2012). At the start of each test, a rat was placed in the centre of the maze facing an open arm and allowed to freely explore the apparatus for 5 min. The maze was cleaned using a 70% ethanol solution between trials to eliminate odour cues. We quantified the anxiety-like behaviours based on: (a) time spent in the open arms; (b) time spent in the closed arms; and (c) number of risk assessment behaviours: the number of explorations of the

open arm in a stretch-attend posture (when the rodent was motionless in the centre or in a closed-arm zone but stretched its body forward into the open arms by moving some, but not all, paws and then returning to the previous position) (Walf & Frye, 2007).

We considered as exploratory activity in the apparatus the following parameters: (a) number of entries in the open arms: an arm entry was recorded when the animal entered the arm using all four paws; (b) number of entries in the closed arms; and (c) total number of entries in either the open or closed arm.

2.6. Statistics

Data that displayed a Gaussian distribution and equal variances across groups are presented as the mean ± the standard error of the mean (SEM) and were analysed using one-way analysis of variance (ANOVA) followed by Tukey's tests for multiple comparisons. We used two-way ANOVA with repeated measures (within-subject factor: time period of the behavioural test; between-subject factor: the time-of-day) followed by Bonferroni post-hoc procedure when the distance travelled, the duration of rearing, and the duration of grooming were evaluated across the test. Data that did not display a Gaussian distribution or equal variances between the groups are expressed as medians (p_{50}) and 25 and 75 percentile ranges (p_{25}, p_{75}) and were analysed using the Kruskal–Wallis (KW) test followed by Dunn's multiple comparisons test. Pearson correlation was performed to quantify relationships between distance travelled, duration of locomotion, duration of rearing, and the duration of grooming, which are the major parameters typically measured on the OF task (Gould et al., 2009). Principal component analysis (PCA) was performed to address the expected correlation between the OF task variables. PCA was used to convert a set of possibly OF correlated variables into a set of values of linearly uncorrelated variables called principal components, without significant loss of information regarding variability among individuals (Jolliffe, 1986; Sanguansat, 2012). The first principal component (PC1) explains the largest proportion of the variance in the data (quantified based on eigenvalue), usually expressed as a percentage of the total variance. Subsequent components (PC2, PC3, PC*n*) display, in turn, decreasing amounts of variation. If the analysis is successful, the data may be represented by a smaller number of PCs than the number of original variables (Jolliffe, 1986; Sanguansat, 2012). Therefore, it provides a way to understand and visualize the structure of complex data sets, such the data collected

during the OF test. Furthermore, it helps us identify new meaningful underlying variables (Sanguansat, 2012). A significance level of 0.05 was set for all analyses.

3. Results

3.1. Open field task

The time-of-day influenced the duration of locomotion ($F_{2,30} = 9.95$, $p = 0.0005$), total distance travelled ($F_{2,30} = 7.97$, $p = 0.0017$), and the inter-stop distance ($F_{2,30} = 5.15$, $p = 0.0119$, Figure 1). Duration of locomotion was longer in the Evening group compared to the Morning and Afternoon groups. The distance travelled and the inter-stop distance were greater in rats tested in the evening compared to those tested in the morning and the afternoon. We found no difference between groups for the number of stops ($F_{2,30} = 3.12$, $p = 0.0586$); Figure 1c or the average speed ($F_{2,30} = 0.31$, $p = 0.733$, data not shown).

The duration of rearing was longer in the Evening group than the Morning group (Figure 2), but did not differ from the Afternoon group ($F_{2,30} = 3.58$, $p = 0.0403$). No difference between the groups was detected in the duration of grooming ($F_{2,30} = 2.11$, $p = 0.1394$).

Animals from the Evening group travelled longer distances during the first and second 5-min periods of the test compared to the Morning and Afternoon groups ($F_{2,30} = 7.97$, $p = 0.0017$, Figure A1a). Moreover, the Evening group also spent more time rearing in the first and second 5-min periods compared to the Morning group ($F_{2,30} = 3.58$, $p = 0.0403$, see Figure A1b in the Appendix). We found no difference between groups in the duration of grooming ($F_{2,30} = 2.11$, $p = 0.1391$, Figure A1c). These habituation profiles are illustrated minute-by-minute in Figure A1d–f.

During the second and third 5-min periods of the test, the percentage of the total distance travelled by the animals in the Morning group was lower than in the first 5-min period ($F_{2,60} = 202.6$, $p < 0.0001$; Figure 3a). For animals in the Afternoon and Evening groups, the percentage of the total distance travelled in the first 5-min period was higher than in the second and third 5-min periods of the test. In addition, the percentage of the total distance travelled in the second 5-min period was higher compared to the third 5-min period of the test. With respect to rearing behaviour, the percentage

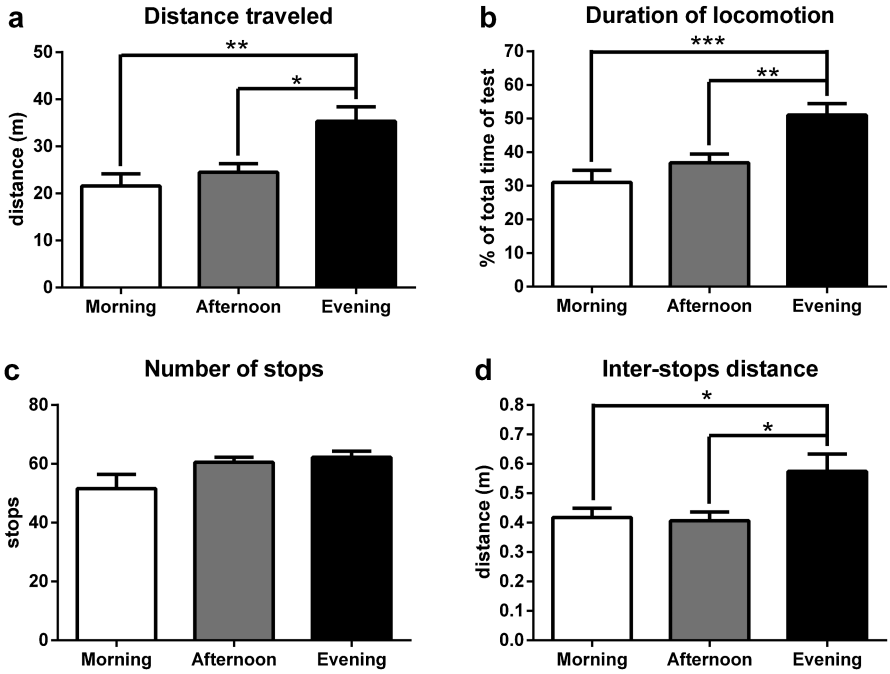


Figure 1. Locomotor activity. The end-point results of (a) distance travelled, (b) duration of locomotion, (c) number of stops and (d) inter-stop distance. The data are expressed as the means ± SEM. $N = 10$–12 animals per group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. All data were analysed using one-way ANOVA followed by Tukey post hoc analysis.

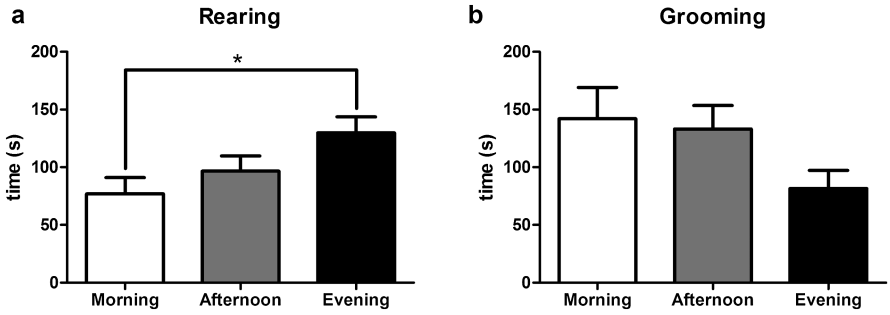


Figure 2. Exploratory activity. The end-point results of accumulated (a) duration of rearing and (b) duration of grooming. The data are expressed as the means ± SEM. $N = 10$–12 animals per group. $^{*}p < 0.05$. All data were analysed using one-way ANOVA followed by Tukey post hoc analysis.

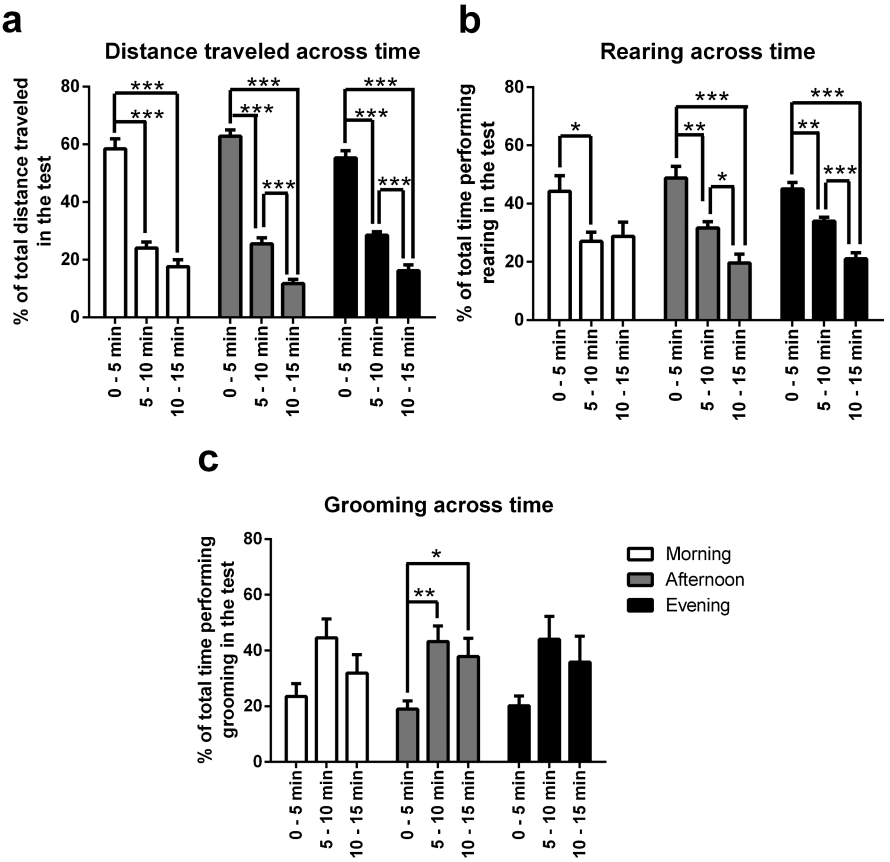


Figure 3. Time-course analyses of the behavioural profile within each group. (a) The distance travelled, (b) duration of rearing, and (c) duration of grooming are represented as the percentage of each behaviour during each 5-min period (for example, the percentage of the distance travelled was calculated as the distance travelled in each 5-min period divided by the accumulated distance travelled during the entire test multiplied by 100). The data are expressed as the means ± SEM. $N = 10$–12 animals per group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Data were analysed using two-way ANOVA with repeated measures (within-subject factor: time period of the test; between-subject factor: time-of-day) followed by the Bonferroni post hoc test.

of duration of rearing decreased significantly across the 5-min periods in the Afternoon and Evening groups. For animals in the Morning group, the percentage of duration of rearing was higher in the first 5-min period compared to the duration of rearing in the second and third 5-min periods of the test ($F_{2,60} = 22.36$, $p < 0.0001$; Figure 3b). Across time, differences in the

duration of grooming were detected only in the Afternoon group, as during the first 5-min period of the test, the percentage of duration of grooming was lower compared to the percentage of duration of grooming during the second and third 5-min periods ($F_{2,60} = 6.93$, $p = 0.0020$; Figure 3c).

All of the groups spent a similar amount of time ($F_{2,30} = 0.46$, $p = 0.6383$), and travelled similar distances ($F_{2,30} = 0.78$, $p = 0.4693$), along the perimeter of the apparatus (Table 1). Moreover, approximately 80–95% of the total time spent at the home base was during the last 10 min of the test (data not shown). Taking this result into account, all of the analyses based on the home base were evaluated only for the last 10 min of the test. During this period, the animals in the Afternoon group spent more time at the home base than the animals in the Evening group but did not spend a different amount of time at the home base compared to the rats in the Morning group $F_{2,30} = 3.92$, $p = 0.0306$; Figure 4.

The Evening group travelled longer distances per trip than the Afternoon group (Figure 5b) but did not travel different distances per trip compared to the Morning group (KW = 6.85, $p = 0.0325$). No difference between the groups was detected in the number of trips (KW = 4.37, $p = 0.1126$; Figure 5a) or the number of stops/trip (KW = 4.77, $p = 0.0921$; Figure 5c).

Principal component analysis (PCA) was performed on all behavioural variables assessed in the OF task. We used the Jolliffe (1986) cut-off value (0.7 for our study) to determine which principal components (PC) to consider significant. This left four PCs which, cumulatively, explained 85.6% of the total variability of the data (Table 2). Consequently, the variance of the sample is summarized very well by these four components. Correlation coefficients between each variable and each specific PC demonstrate that PC1, which accounted for more than 46% of the total variability of the data, was highly influenced by nearly all of the OF variables (except for the number of trips and the average speed). The duration of locomotion, the distance travelled, stops/trip, the trip length, the duration of rearing, the inter-stop distance and the number of stops positively correlated with PC1, while the total time spent at the home base, the duration of locomotion spent along the perimeter and the distance travelled along the perimeter negatively correlated with PC1 (Figure 6a). Differences between the groups were detected for PC1, as the Evening group exhibited higher values on the PC1 compared to the other groups (KW = 9.82, $p = 0.0074$; Figure 6d). No difference between the groups was detected on PC2 ($F_{2,30} = 1.94$, $p = 0.1618$; Figure 6e), PC3

Table 1.
Anxiety levels of animals of the Morning, Afternoon and Evening groups both in OF and EPM.

	Morning		Afternoon		Evening		*F*	*p*
	Mean	SEM	Mean	SEM	Mean	SEM		
OF								
Distance travelled along the perimeter [a]	84.13	3.97	82.54	1.83	79.34	1.73	0.78	0.469
Duration of locomotion spent along the perimeter [b]	93.11	2.45	93.68	1.29	91.37	1.23	0.46	0.638
EPM								
Time spent in the open arms (s)	80.25	12.49	78.49	10.52	71.45	9.05	0.17	0.842
Time spent in the closed arms (s)	162.30	14.01	161.50	12.75	162.10	11.32	0.01	0.999
Number of risk assessment behaviours	4.54	0.98	5.17	1.09	5.50	0.91	0.22	0.803
Number of entries in open arms	10.09	0.98	8.75	1.26	8.00	0.98	0.89	0.423
Number of entries in closed arms	11.73	1.08	10.92	1.14	11.80	1.20	0.19	0.827
Total number of entries in both open and closed arms	21.82	1.37	19.67	1.88	19.80	1.77	0.51	0.608

One-way ANOVA results. $N = 10–12$ animals per group.
[a] Distance travelled along the perimeter was expressed as percentage of the total distance travelled in the test.
[b] Duration of locomotion spent along the perimeter was expressed as percentage of the total duration of locomotion in the test.

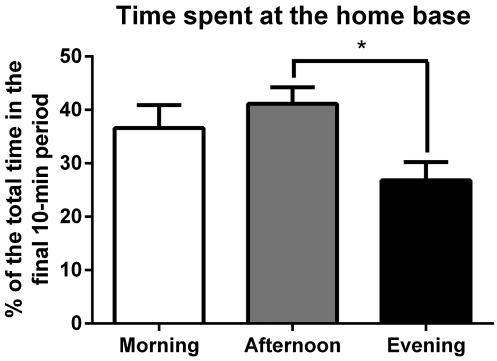


Figure 4. Comparison between the groups with respect to time spent at the home base zone during the final 10 min of testing. The data are expressed as the means ± SEM. $N = 10$–12 animals per group. $^{*}p < 0.05$. Data were analysed using one-way ANOVA followed by Tukey post hoc analysis.

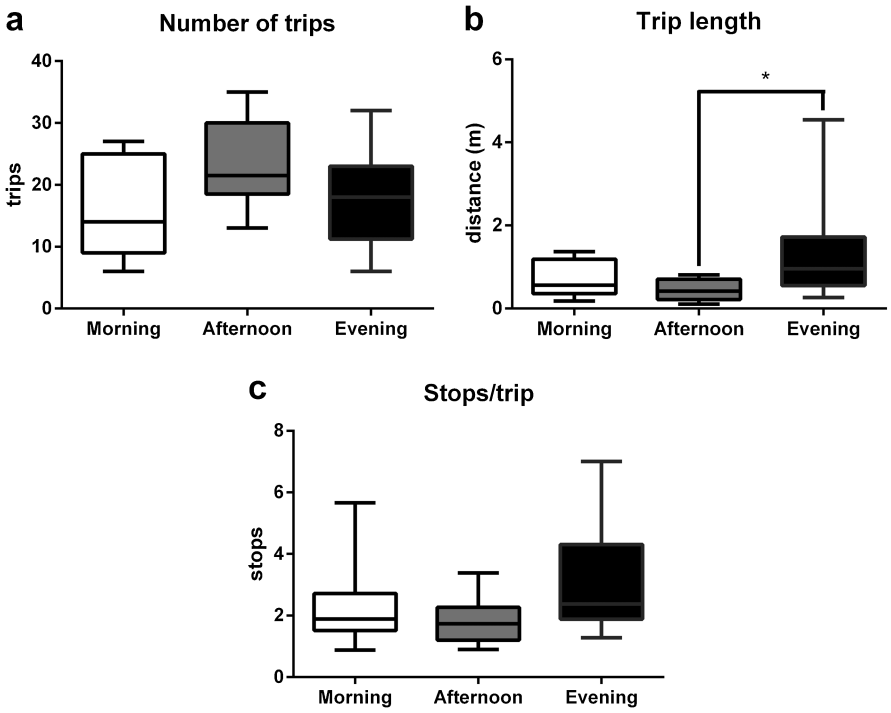


Figure 5. Temporal organization of exploration. (a) The number of trips, (b) the trip length and (c) the average number of stops during each trip. Data are expressed as the medians and the interquartile ranges. $N = 10$–12 animals per group. $^{*}p < 0.05$. All data were analysed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test.

Table 2.
Principal component analysis using data of 33 rats in the OF task: correlation coefficients between each principal component (PC) and each behavioural variable representing locomotor and exploratory activity, as well as eigenvalues (variances) and percentage of variance explained by each PC.

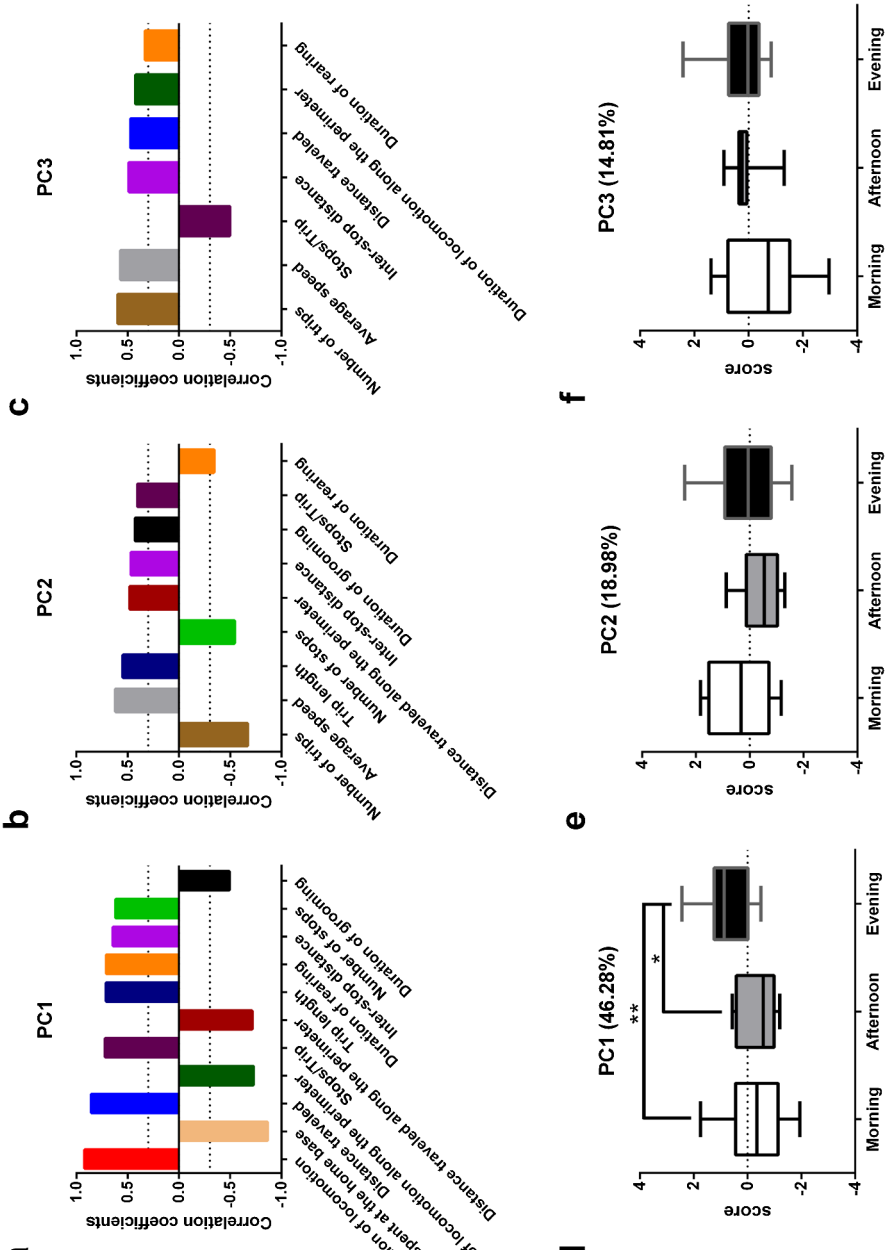
	PC1	PC2	PC3	PC4
Correlation coefficient				
Distance travelled	0.858	0.142	0.473	0.031
Duration of locomotion	0.923	−0.059	0.298	−0.002
Average speed	−0.037	0.621	0.573	0.208
Number of stops	0.619	−0.540	0.095	0.131
Inter-stop distance	0.646	0.468	0.491	−0.012
Duration of rearing	0.710	−0.340	0.330	0.000
Duration of grooming	−0.491	0.429	−0.048	0.693
Distance travelled along the perimeter	−0.712	0.481	0.298	−0.267
Duration of locomotion spent along the perimeter	−0.725	0.292	0.424	−0.277
Time spent at the home base	−0.862	−0.146	0.141	−0.045
Number of trips	−0.279	−0.668	0.597	0.053
Trip length	0.712	0.547	−0.180	−0.156
Stops/trip	0.722	0.405	−0.495	−0.076
Eigenvalues	6.016	2.467	1.925	0.724
% of variance	46.28	18.98	14.81	5.57
Cumulative variance (%)	46.28	65.26	80.07	85.64

The Jolliffe cut-off value was 0.7.

(KW = 1.577, $p = 0.45$; Figure 6f) or PC4 ($F_{2,30} = 0.98$, $p = 0.3872$) (data not shown).

Pearson correlation was performed on a set of four OF variables (the total distance travelled, the total duration of locomotion, the total duration of

Figure 6. Principal component analysis. (a–c) The matrix correlation between the OF task variables and each individual PC (PC1, PC2 and PC3). (d–f) Comparison between the groups with respect to the PC1, PC2 and PC3 values. Dashed lines in panels a–c (values 0.3 and −0.3 on the *y*-axis) indicate the cut-off points (i.e., the variables which presented correlation values > -0.3 and <0.3 are not represented in the graphs). Data in panels d–f are expressed as the medians and the interquartile ranges. $^{*}p < 0.05$; $^{**}p < 0.01$. Data were analysed using one-way ANOVA followed by Tukey post hoc analysis or using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x.


a
PC1
Correlation coefficients
1.0
0.5
0.0
-0.5
-1.0
Duration of locomotion
Time spent at the home base
Distance traveled
Trip length
Stops/Trip
Time spent at the perimeter
Number of stops
Inter-stop distance
Duration of roaming
Duration of grooming
Distance traveled along the perimeter
Duration of locomotion along the perimeter
b
PC2
Correlation coefficients
1.0
0.5
0.0
-0.5
-1.0
Number of trips
Average speed
Trip length
Number of stops
Inter-stop distance
Duration of grooming
Stops/Trip
Duration of roaming
Distance traveled along the perimeter
c
PC3
Correlation coefficients
1.0
0.5
0.0
-0.5
-1.0
Number of trips
Average speed
Inter-stop distance
Stops/Trip
Distance traveled
Time spent at the perimeter
Duration of roaming
Duration of locomotion along the perimeter
d
PC1 (46.28%)
score
4
2
0
-2
-4
**
*
Morning
Afternoon
Evening
e
PC2 (18.98%)
score
4
2
0
-2
-4
Morning
Afternoon
Evening
f
PC3 (14.81%)
score
4
2
0
-2
-4
Morning
Afternoon
Evening

rearing, and the total duration of grooming) (Figure 7). The distance travelled and duration of locomotion correlated positively for all groups (Figure 7c). A positive correlation between the duration of rearing and the distance travelled, as well as between the duration of rearing and the duration of locomotion, was detected only among the groups tested during the light phase (Figure 7a and 7d). In addition, negative correlations between the total duration of grooming and the variables representing exploration (the distance travelled, the duration of locomotion and the duration of rearing) were detected only for the Morning group (Figure 7b, 7e and 7f).

3.2. Elevated plus maze test

We found no difference between groups for any of the parameters evaluated for the EPM test (Table 1).

4. Discussion

As we predicted, the time-of-day affected both activity and the spatio-temporal organization of exploratory behaviour (see PC1 results in Figure 6). However, despite exhibiting two distinct exploratory strategies during the OF test, all groups used the two strategies in the same order. Rats first patrolled the perimeter of the arena and then exhibited a home base strategy. Together these findings support our first hypothesis that LDPC affects fine-scale patterns of exploration in the OF. We did not find support for our second hypothesis. The distinct exploratory patterns observed in the OF were not associated with fluctuations in the anxiety state of animals, since time-of-day did not alter anxiety-like behaviours (Table 1).

4.1. Influences of the time-of-day on the exploratory profile of rats

Consistent with previous studies (Smith & Morrell, 2007; Verma et al., 2010) the time-of-day significantly affected both locomotor and exploratory activities (Figures 1 and 2). Animals in the Evening group travelled longer distances, moved for more time and spent more time rearing compared to rats in the light phase groups. The average speed and the total number of stops performed by the animals was similar for all groups, indicating that the differences in locomotor activity between groups is better described by the greater inter-stop distance of the animals in the Evening group compared to the animals in the light phase groups. Differences in vertical scanning, such as increased time performing rearing, were also detected for animals in the

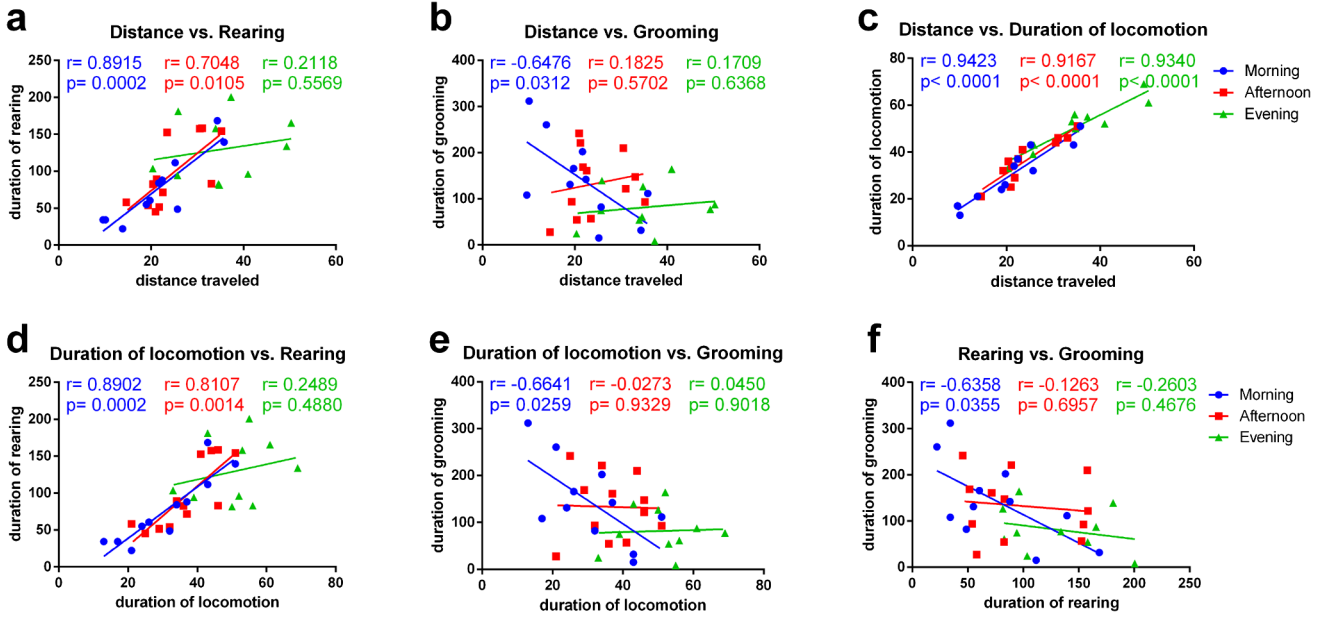


Figure 7. Pearson correlation between (a) the distance travelled and the duration of rearing, (b) the distance travelled and the duration of grooming, (c) the distance travelled and the duration of locomotion, (d) the duration of locomotion and the duration of rearing, (e) the duration of locomotion and the duration of grooming, and (f) the duration of rearing and the duration of grooming. The blue, red and green lines represent the correlation between the variables corresponding to the Morning, Afternoon and Evening groups, respectively. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x.

dark phase group (Figure 2a). These findings indicate that animals tested in our study behaved similarly as those previously studied, exhibiting increased locomotion and exploration during the nocturnal period (Smith & Morrell, 2007).

While the standard end-point parameters we recorded for locomotor and exploratory activity of rats were similar to those observed in previous studies, a finer-scale temporal and spatial analysis of behaviour revealed important, new patterns. We found that the animals in the Morning group exhibited decreased locomotion and exploration compared to the other groups during the final 10 min of the OF test (Figure A1d). This reduction may be due to a lack of motivation (Chkhartishvili et al., 2011) of animals during the light period, which is the less active period for rats (Kelliher et al., 2000). Nevertheless, better learning performance during the light phase (Chaudhury & Colwell, 2002) and/or the use of distinct exploratory strategies, such as locomotion-independent visual exploration, cannot be excluded. The total time spent at the home base was also affected by the time-of-day. Animals in the Evening group spent less time at the home base than those in the Afternoon group. This difference was likely due to the greater distances travelled by the animals in the Evening group (Figure 1). Therefore, although the number of trips was not different between the groups, the rats in the Evening group travel farther from the home base during each trip (Figure 8c).

Despite different patterns of locomotion and exploration during the final 10 min of testing, the exploratory profile of the animals during the first 5-min period was similar across groups (Figure 3a and 3b). All groups travelled about 60% of their total distance, and spent approximately 40% of their total time rearing, during the first 5-min period of the test. Together our data suggest that two distinct strategies were employed by the animals during the

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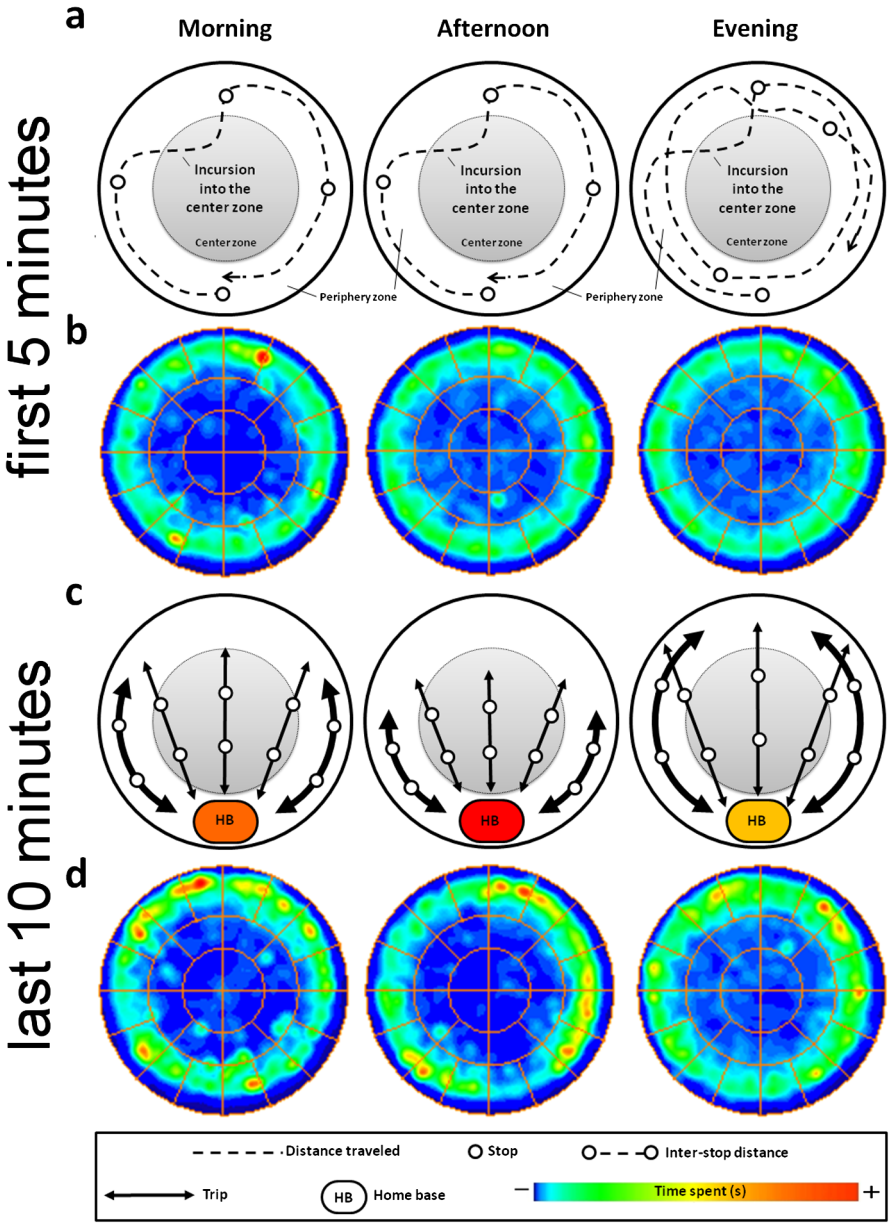
Figure 8. Microstructure of the behaviour. Schematic ethograms (a and c) and occupancy plots of each group (b and d) representing the behaviour of the animals during the first 5 min (perimeter patrolling phase) and the final 10 min (home base phase) of observation. In the home base phase an organized strategy is performed, in which the rats established a safe zone and performed exploratory tours through the arena starting from the home base and returning to it. A higher incidence of yellowish-red points during the final 10 min compared to the first 5 min indicates that little or no home base was established during the first 5 min of the test. In c, the length of the arrows represents the distance travelled during each trip, while the width of the arrows represents the frequency in which the trips occurred along each trail. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x.



test. The perimeter patrolling strategy (Figure 8a and 8b), characterised by a high amount of locomotion near the walls of the arena, was performed to gather information regarding the unfamiliar environment before establishing

trails and a home base (Avni & Eilam, 2008). When the environment became familiar to the animal (approximately 5 min after the start of the test) the perimeter patrolling strategy shifted to a home base strategy, represented by a higher incidence of yellowish-red points during the last 10 min of the test (Figure 8d). This hypothesis is reinforced by the increased percentage of time spent at the home base during the final 10 min of the test (approximately 80% of the total time spent at the home base) compared to the first 5 min. As discussed above, the animals in the Morning group exhibited reduced exploration earlier than the animals in the Afternoon and Evening groups; thus, the animals in the Morning group tended to establish a home base earlier than the other groups (depicted in Figure 8b as yellowish-red points during the first 5-min period).

Although animals from distinct groups used the two strategies in a same order of sequence, they presented different patterns of locomotion and exploration. We tested whether these differences were only due to alterations in their activity, or if they also reflect differences in the way rats explore the environment. Since exploration is a complex combination of multiple factors aiming to gather spatial information, the investigation of this phenomenon requires obtaining a large set of variables. Nevertheless, this large set of variables should be analysed in an integrated manner to generate a more accurate interpretation of behaviour of the rats. For example, number of crossings when analysed individually indicates only the distance travelled, but when the traveling time and average speed are added to the analysis, a representation of locomotor activity can be observed. In the same context, when other variables (e.g., rearing, grooming, trips, time spent at home base and time spent in perimeter) are merged with locomotion parameters, a more robust representation of exploratory behaviour can be perceived. Since PCA provides a way to simplify the structure of complex data sets (Sanguansat, 2012), we used it to combine the 13 original parameters reducing them to a single 'new' variable (PC1) which represented exploration. PCA results corroborate our hypothesis that the way rats explore the environment is also influenced by time-of-day (Figure 6a). Rats that were more active (i.e., the Evening group) also appeared more meticulous, stopping more often during longer trips. Besides, they also spent less time at home base and moved less along the perimeter, expanding their exploration towards the centre of the arena more than Afternoon and Morning groups (Figure 8b and 8d). These subtle behavioural differences were only identified by employing PCA.

Our correlation analysis also revealed effects of the time-of-day on the fine-scale patterns of OF behaviour (Figure 7). Grooming behaviour was strongly correlated with activity and was reduced in the Evening group. In addition, although horizontal and vertical activities were closely related, the vertical scanning did not seem to follow the increase in locomotor activity when activity became very high (Evening group). Based on these data, we speculate that high activity levels and hyperactivity lead to dissociation of exploration and locomotion. In other words, not all bouts of locomotion reflect exploration, especially in a hyperactive state. This phenomenon was also observed by others researchers in a model of brain lesion (Gaffori et al., 1980).

Taken together, our results indicate that the spatio-temporal organization of exploratory behaviour is significantly affected by the time-of-day. In methodological terms, this highlights the importance of testing all experimental groups for a given study at the same phase of the LDPC. However, depending on the working hypotheses, it may also be important to perform all trials at the same time-of-day, because some subtle changes in behavioural pattern may exist even if the animals are tested during the same phase. In addition, when researchers are attempting to evaluate effects of drug-induced behavioural alterations using the OF task, our data suggest that the dark phase of the LDPC is the more appropriate period for such investigation. This is supported by the finding that either a reduction or elevation in drug-induced activity could be observed if the animals are tested during the dark phase (Figure A1d). On the other hand, when the animals are tested during the morning, a putative reduction in drug-induced activity could be masked by the reduced basal activity of the rats (Figure A1d). Furthermore, because the animals in the Afternoon group behaved similarly to those in the Morning group with respect to some parameters (e.g., rearing) and similarly to those in the Evening group with respect to others (e.g., grooming) (Figure 7), we suggest that testing animals during this time-of-day should be avoided.

Our second working hypothesis was that the LDPC-dependent differences in the exploratory behaviour would be related to the anxiety state of the animals. Our results demonstrated that the LDPC did not alter anxiety-like behaviours using either the OF or the EPM test (Table 1). Therefore, the differences in the exploratory profiles on the OF task were not consequences of fluctuations in the emotionality of the rats throughout the day.

In summary, by analysing OF locomotor and exploratory behaviour on finer-scale we have demonstrated that the time-of-day influences not only activity, but also the way rats explore an unfamiliar environment. We also demonstrated that these differences in the exploratory profiles appear to be unrelated to alterations in anxiety-like behaviour throughout the day. Moreover, our results indicated that rats use different exploratory strategies across time during the OF test regardless of the phase of the LDPC. As a final consideration we suggest that behavioural experiments should be done during the dark phase of LDPC to avoid a reduction in exploratory motivation, and, that a fine-scale assessment of behaviour should be done in order to examine the spatio-temporal profile of exploratory behaviour which can help to identify subtle alterations among populations.

Acknowledgements

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References

Abarca, C., Albrecht, U. & Spanagel, R. (2002). Cocaine sensitization and reward are under the influence of circadian genes and rhythm. — Proc. Natl. Acad. Sci. USA 99: 9026-9030.

Avni, R. & Eilam, D. (2008). On the border: perimeter patrolling as a transitional exploratory phase in a diurnal rodent, the fat sand rat (*Psammomys obesus*). — Anim. Cogn. 11: 311-318.

Chaudhury, D. & Colwell, C.S. (2002). Circadian modulation of learning and memory in fear-conditioned mice. — Behav. Brain Res. 133: 95-108.

Chkhartishvili, E., Maglakelidze, N., Babilodze, M., Chijavadze, E. & Nachkebia, N. (2011). Changes of open field behavior in animal model of depression. — Georgian Med. News 11: 107-112.

Easton, A., Arbuzova, J. & Turek, F.W. (2003). The circadian Clock mutation increases exploratory activity and escape-seeking behavior. — Genes Brain Behav. 2: 11-19.

Eilam, D. (2003). Open-field behavior withstands drastic changes in arena size. — Behav. Brain Res. 142: 53-62.

Eilam, D. & Golani, I. (1989). Home base behavior of rats (Rattus norvegicus) exploring a novel environment. — Behav. Brain Res. 34: 199-211.

Eilam, D. & Golani, I. (1990). Home base behavior in amphetamine-treated tame wild rats (*Rattus norvegicus*). — Behav. Brain Res. 36: 161-170.

Gaffori, O., Le Moal, M. & Stinus, L. (1980). Locomotor hyperactivity and hypoexploration after lesion of the dopaminergic-A10 area in the ventral mesencephalic tegmentum (VMT) of rats. — Behav. Brain Res. 1: 313-329.

Gannon, R.L., Lungwitz, E., Batista, N., Hester, I., Huntley, C., Peacock, A., Delagrange, P. & Millan, M.J. (2011). The benzodiazepine diazepam demonstrates the usefulness of Syrian hamsters as a model for anxiety testing: evaluation of other classes of anxiolytics in comparison to diazepam. — Behav. Brain Res. 218: 8-14.

Gentsch, C., Lichtsteiner, M. & Feer, H. (1982). Behavioural comparisons between individually- and group-housed male rats: effects of novel environments and diurnal rhythm. — Behav. Brain Res. 6: 93-100.

Gould, T., Dao, D. & Kovacsics, C. (2009). The open field test. — In: Mood and anxiety related phenotypes in mice, Vol. 42. Humana Press, p. 1-20.

Handley, S.L. & Mcblane, J.W. (1993). An assessment of the elevated X-maze for studying anxiety and anxiety-modulating drugs. — J. Pharmacol. Toxicol. Methods 29: 129-138.

Hostetter, R.C. (1966). Time of day effects on learning and open field activity. — Psychon. Sci. 5: 257-258.

Huang, W., Ramsey, K.M., Marcheva, B. & Bass, J. (2011). Circadian rhythms, sleep, and metabolism. — J. Clin. Invest. 121: 2133-2141.

Jolliffe, I.T. (1986). Principal component analysis. — Springer, New York, NY.

Jones, N. & King, S.M. (2001). Influence of circadian phase and test illumination on pre-clinical models of anxiety. — Physiol. Behav. 72: 99-106.

Kavaliers, M. & Hirst, M. (1985). The influence of opiate agonists on day-night feeding rhythms in young and old mice. — Brain Res. 326: 160-167.

Kelliher, P., Connor, T.J., Harkin, A., Sanchez, C., Kelly, J.P. & Leonard, B.E. (2000). Varying responses to the rat forced-swim test under diurnal and nocturnal conditions. — Physiol. Behav. 69: 531-539.

Leke, R., De Oliveira, D.L., Mussulini, B.H., Pereira, M.S., Kazlauckas, V., Mazzini, G., Hartmann, C.R., Silveira, T.R., Simonsen, M., Bak, L.K., Waagepetersen, H.S., Keiding, S., Schousboe, A. & Portela, L.V. (2012). Impairment of the organization of locomotor and exploratory behaviors in bile duct-ligated rats. — PLoS One 7: e36322.

Li, C.R., Huang, G.B., Sui, Z.Y., Han, E.H. & Chung, Y.C. (2010). Effects of 6-hydroxydopamine lesioning of the medial prefrontal cortex on social interactions in adolescent and adult rats. — Brain Res. 1346: 183-189.

Loss, C.M., Cordova, S.D. & De Oliveira, D.L. (2012). Ketamine reduces neuronal degeneration and anxiety levels when administered during early life-induced status epilepticus in rats. — Brain Res. 1474: 110-117.

Mcclung, C.A., Sidiropoulou, K., Vitaterna, M., Takahashi, J.S., White, F.J., Cooper, D.C. & Nestler, E.J. (2005). Regulation of dopaminergic transmission and cocaine reward by the Clock gene. — Proc. Natl. Acad. Sci. USA 102: 9377-9381.

Moore, R.Y. & Eichler, V.B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. — Brain Res. 42: 201-206.

Panksepp, J.B., Wong, J.C., Kennedy, B.C. & Lahvis, G.P. (2008). Differential entrainment of a social rhythm in adolescent mice. — Behav. Brain Res. 195: 239-245.

Pellow, S., Chopin, P., File, S.E. & Briley, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. — J. Neurosci. Methods 14: 149-167.

Ramos, A. (2008). Animal models of anxiety: do I need multiple tests? — Trends Pharmacol. Sci. 29: 493-498.

Sanguansat, P. (2012). Principal component analysis. — InTech, Rijeka.

Saper, C.B., Cano, G. & Scammell, T.E. (2005). Homeostatic, circadian, and emotional regulation of sleep. — J. Comp. Neurol. 493: 92-98.

Smith, K.S. & Morrell, J.I. (2007). Comparison of infant and adult rats in exploratory activity, diurnal patterns, and responses to novel and anxiety-provoking environments. — Behav. Neurosci. 121: 449-461.

Stephan, F.K. & Zucker, I. (1972). Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. — Proc. Natl. Acad. Sci. USA 69: 1583-1586.

Verma, P., Hellemans, K.G.C., Choi, F.Y., Yu, W. & Weinberg, J. (2010). Circadian phase and sex effects on depressive/anxiety-like behaviors and HPA axis responses to acute stress. — Physiol. Behav. 99: 276-285.

Walf, A.A. & Frye, C.A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. — Nature Protocols 2: 322-328.

Yao, Z., Dubois, D.C., Almon, R.R. & Jusko, W.J. (2006). Modeling circadian rhythms of glucocorticoid receptor and glutamine synthetase expression in rat skeletal muscle. — Pharm. Res. 23: 670-679.

Yaski, O., Portugali, J. & Eilam, D. (2011). City rats: insight from rat spatial behavior into human cognition in urban environments. — Anim. Cogn. 14: 655-663.

Zadicario, P., Avni, R., Zadicario, E. & Eilam, D. (2005). 'Looping' — an exploration mechanism in a dark open field. — Behav. Brain Res. 159: 27-36.

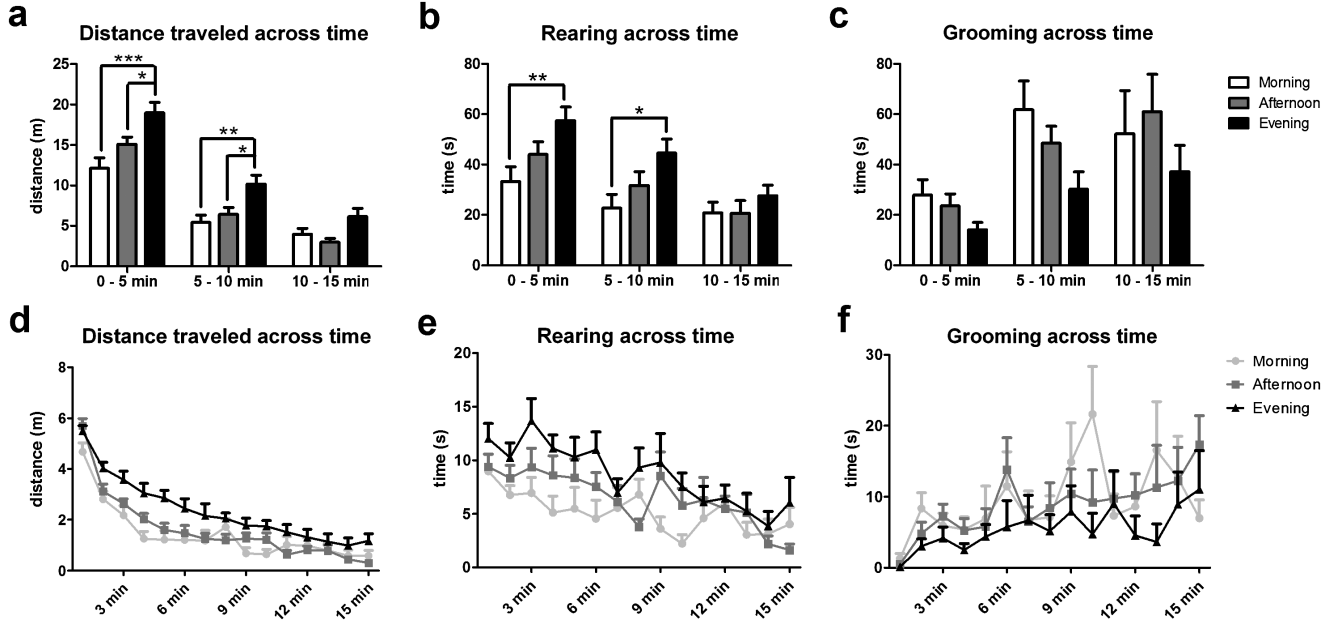

Figure A1. Time-course analyses of the behaviour profile of each group with respect to the distance travelled (a and d), the duration of rearing (b and e), and the duration of grooming (c and f). The animals in the Evening group travelled a longer distance during the first and second 5-min periods of the test compared to the Morning and Afternoon groups. The Evening group exhibited a longer duration of rearing during the first and second 5-min periods than the Morning group. We did not detect any difference between the groups in the duration of grooming. The minute-by-minute habituation profile of the distance travelled, the duration of rearing, and the duration of grooming is illustrated in d–f, respectively. The data are expressed as the means ± SEM. $N = 10$–12 animals per group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Data were analysed using two-way ANOVA with repeated measures (within-subject factor: time period of the test; between-subject factor: time-of-day) followed by the Bonferroni post hoc test.

CAPÍTULO II

Artigo publicado

Influence of environmental enrichment vs. time-of-day on behavioral repertoire of male albino Swiss mice

Cássio Morais Loss*, Luisa Bandeira Binder, Eduarda Muccini, Wagner Carbolin Martins, Paulo Alexandre de Oliveira, Samuel Vandresen-Filho, Rui Daniel Prediger, Carla Ines Tasca, Eduardo Rigon Zimmer, Luiz Ernesto Costa-Schmidt, Diogo Losch de Oliveira, Giordano Gubert Viola.

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Justificativa: É sabido que tanto o enriquecimento ambiental quanto o ritmo circadiano influenciam o comportamento de roedores. Contudo, pouco se sabe a respeito do efeito que o ritmo circadiano exerce sobre o repertório comportamental de animais criados em ambientes enriquecidos.

Objetivo geral: Investigar a influência do período-do-dia sobre o repertório comportamental de camundongos Swiss criados em ambientes enriquecidos.

Objetivo específico: Investigar a influência do período-do-dia sobre o comportamento exploratório e os níveis de ansiedade de camundongos Swiss criados em ambientes enriquecidos; Investigar a influência do período-do-dia sobre o desempenho cognitivo de camundongos Swiss criados em ambientes enriquecidos em uma tarefa de avaliação de memória episódica.

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Cássio Morais Loss [a], Luisa Bandeira Binder [b], Eduarda Muccini [b], Wagner Carbolin Martins [c], Paulo Alexandre de Oliveira [d], Samuel Vandresen-Filho [c,e], Rui Daniel Prediger [b], Carla Inês Tasca [b,c], Eduardo R. Zimmer [a], Luiz Ernesto Costa-Schmidt [f,g], Diogo Losch de Oliveira [a], Giordano Gubert Viola [c,h,i,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Departamento de Bioquímica, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[c] Programa de Pós-Graduação em Neurociências, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[d] Departamento de Farmacologia, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[e] Departamento de Ciências Básicas em Saúde, Faculdade de Medicina, Universidade Federal de Mato Grosso, Cuiabá, MT, Brazil
[f] Laboratorio de Biología Reproductiva y Evolución, Instituto de Diversidad y Ecología Animal – IDEA/CONICET, Universidad Nacional de Córdoba – UNC, Vélez Sarsfield 299, 5000 Córdoba, Argentina
[g] Programa de Pós-Graduação em Biologia, Universidade do Vale do Rio dos Sinos – UNISINOS, São Leopoldo, RS, Brazil
[h] Universidade Federal de Santa Catarina, Campus Curitibanos, Curitibanos, SC, Brazil
[i] Programa de Pós-Graduação em Ciências Fisiológicas, Centro de Ciências Biológica, Universidade Federal do Sergipe, São Cristóvão, SE, Brazil

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ABSTRACT

Environmental enrichment (EE) is a non-pharmacological manipulation that promotes diverse forms of benefits in the central nervous system of captive animals. It is thought that EE influences animal behavior in a specie-(strain)-specific manner. Since rodents in general present different behaviors during distinct periods of the day, in this study we aimed to investigate the influence of time-of-day on behavioral repertoire of Swiss mice that reared in EE. Forty male Swiss mice (21 days old) were housed in standard (SC) or enriched conditions (EC) for 60 days. Behavioral assessments were conducted during the light phase (in presence of light) or dark phase (in absence of light) in the following tasks: open field, object recognition and elevated plus maze. First, we observed that the locomotor and exploratory activities are distinct between SC and EC groups only during the light phase. Second, we observed that "self-protective behaviors" were increased in EC group only when mice were tested during the light phase. However, "less defensive behaviors" were not affected by both housing conditions and time-of-day. Third, we showed that the performance of EE animals in object recognition task was improved in both light and dark conditions. Our findings highlight that EE-induced alterations in exploratory and emotional behaviors are just evident during light conditions. However, EE-induced cognitive benefits are remarkable even during dark conditions, when exploratory and emotional behaviors were similar between groups.

1. Introduction

Environmental enrichment (EE) is a form of manipulation in which captive animals are exposed to complex conditions through adaptations in the physical and social environment. This complex environment is composed by running wheels, shelters and various other objects with different textures, colors, shapes and sizes, which can be changed of place for stimulating sensory, cognitive and physical functions (Girbovan & Plamondon, 2013). In fact, it is well-established that EE promotes diverse forms of benefits in a large number of animal species, such as reduction in signs of boredom in caged mink (Meagher & Mason, 2012), reduction of stereotyped movements in mice (Gross, Richter, Engel, & Wurbel, 2012) and stress-related behaviors in macaque (Marquez-Arias,

Abbreviations: EE, environmental enrichment; SC, standard conditions; ORT, object recognition task; EC, enriched conditions; OFT, open field task; EPM, elevated plus maze; TN, time exploring the novel object; TF, time exploring the familiar object; AICc, Akaike's Information Criterion corrected for small samples; PCA, principal component analysis; PC, principal component.

* Corresponding author at: Programa de Pós-graduação em Ciências Fisiológicas, Departmento de Fisiologia, Universidade Federal de Sergipe, Cidade Universitária Prof. José Aloísio de Campos, Av. Marechal Rondon, s/n Jardim Rosa Elze, 49100-000 São Cristóvão, SE, Brazil.

E-mail addresses: cassio.m.loss@gmail.com (C.M. Loss), binder.luisa@gmail.com (L.B. Binder), dudamuccini@hotmail.com (E. Muccini), wagnerberserk@gmail.com (W.C. Martins), paulofarm@gmail.com (P.A. de Oliveira), samuelvandresen@yahoo.com.br (S. Vandresen-Filho), ruidsp@hotmail.com (R.D. Prediger), tasca@ccb.ufsc.br (C.I. Tasca), eduardo.zimmer@ufrgs.br (E.R. Zimmer), luizernesto@gmail.com (L.E. Costa-Schmidt), diogolosch@gmail.com (D.L. de Oliveira), giorgviola@gmail.com (G.G. Viola).

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Santillan-Doherty, Arenas-Rosas, Gasca-Matias, & Munoz-Delgado, 2010). Recently, a study in humans suggested that EE at work has potential for reducing the risk for developing dementia (Then et al., 2013). Hence, it is evident that EE promotes an enhancement in the quality of life and welfare of the animals (EC, 2010).

Interestingly, brain regions associated with behavioral phenotype undergoes substantial neuroplastic change in rats or mice allowed to rear in enrich environments (Diniz et al., 2010; Sampedro-Piquero et al., 2014; Segovia, Del Arco, De Blas, Garrido, & Mora, 2010; Vazquez-Sanroman et al., 2013; Viola et al., 2009). In a recent study, Leger et al. (2012) observed an enhancement in the episodic-like memory associated with distinct brain activation profile in the EE mice after object recognition paradigm, if compared to mice that reared in a poor environment (standard condition – SC). Furthermore, Bonaccorsi et al. (2013) observed a faster neuronal recruitment in the prefrontal cortex, and activation of a large number of cortical neurons in the EE mice after Morris Water Maze exposure. These findings provide further evidences that EE is capable of modulating brain plasticity, including morphological, physiological, neurochemical and behavioral features.

Importantly, the effects of EE on behavioral tasks are typically dependent on the animals' species or strain. In the object recognition task (ORT) for example, the EE exposure increases objects exploration in Berkeley S1 rats (Renner, 1987), but did not affect object exploration in Sprague Dawley rats (Bruel-Jungerman, Laroche, & Rampon, 2005), and decreases objects exploration in CF1 mice (Viola et al., 2010). A similar pattern can be found in terms of emotionality, since EE exposure increases anxiogenic-like behaviors in BALB/c mice (van de Weerd, Baumans, Koolhaas, & van Zutphen, 1994), but did not affect anxiety-like behaviors in C57BL/6 mice (Abramov, Puussaar, Raud, Kurrikoff, & Vasar, 2008; van de Weerd et al., 1994) or even promotes an anxiolytic effect in Long–Evans hooded rats (Baldini et al., 2013) and Hsd:ICR mice (Friske & Gammie, 2005). Conflicting results can also be found regarding EE effects over aggressiveness (Abramov et al., 2008; Haemisch, Voss, & Gartner, 1994; Marashi, Barnekow, & Sachser, 2004; Mesa-Gresa, Perez-Martinez, & Redolat, 2013; Pietropaolo et al., 2004) reinforcing the hypothesis that EE effects vary according to the animal species (strain), by accentuating their intrinsic features (Toth, Kregel, Leon, & Musch, 2011; van de Weerd et al., 1994). In addition, distinct EE effects are not exclusively for behavioral parameters since conflicting results can be found also in morphological parameters, even in studies using the same strain. For example, Monteiro, Moreira, Massensini, Moraes, and Pereira (2014) observed increased neurogenesis in Swiss albino mice that reared in EE while Silva, Duarte, Lima, and de Oliveira (2011) observed no effect of EE in the neurogenesis process using the same mice strain. It is important to emphasize the between-laboratory inter-variability effects on behavioral profile, which could explain these distinct phenotypes in the same species (strains) (Krackow et al., 2010). One could argue that a combination of different species (or strains), inter-laboratory variability, EE protocols, age or time-period of analysis during the protocol application accounts for these discrepancies (Viola & Loss, 2013).

Another important player is the endogenous circadian system that can modulates animal physiology and behavior. In fact, time-of-day affects diverse behavioral features such as emotionality (Verma, Hellemans, Choi, Yu, & Weinberg, 2010), locomotion and exploration (Loss, Córdova, Callegari-Jacques, & de Oliveira, 2014; Panksepp, Wong, Kennedy, & Lahvis, 2008) as well as their performance on particular learning and memory tasks (Chaudhury & Colwell, 2002). By contrast, in some cases a given treatment can be powerful enough to affect animal's behavior independently of the circadian rhythm. For example, ethanol administration had potent intoxicating effects, impairing mice behavior regardless of circadian phase in which the animals were tested (Munn et al., 2011; Zhou et al., 2015). In keeping with the above-stated, becomes crucial to understand whether different times of the day (i.e. light or dark phase) have an impact in the behavior profile after EE exposure.

Here, we aimed to investigate the influence of environmental conditions and the time-of-day on the behavioral repertoire of albino Swiss mice, which seems to be an EE effects-resistant strain (Silva et al., 2011). For this we have used alternative statistical analyses for identifying fine scale patterns of behavior that could not be detected by using the traditional methods. Our experimental design was based in two independent hypotheses: (i) EE will have a direct impact on individual performance in exploratory- and anxiety-like tasks, which will resembles wild life reactions of the species along their circadian rhythm (less exposition during the day; more exposition during the night). We expected that animals reared in the EE will show a higher exploratory activity than those reared in standard environments when tested during the evening (dark phase). The opposite effect is expected for those animals tested during the day (light phase); (ii) EE will have a direct influence over individual performance in an episodic-like memory task, which will not be affected by the circadian rhythm of the species. More specifically, we expect that EE effects will likely be potent enough for improving mice performance in the ORT independently of the time of the day.

2. Material and methods

2.1. Animals

Forty male albino Swiss mice (21 days old) were obtained from Central Animal Facility of Universidade Federal de Santa Catarina. Mice were maintained with free access to water and food, under a 12:12 h controlled light/dark photoperiod cycle (lights on at 7:00 a.m.) and room temperature adjusted to 21 ± 1 °C. All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals, and approved by the Ethical Committee of the Universidade Federal de Santa Catarina (UFSC), under the protocol number 00795. All efforts were made in order to minimize the number of animals and their suffering.

2.2. Housing conditions

Mice were weaned when they were 21 days old, and then assigned randomly in two housing conditions: standard condition (SC) or enriched condition (EC). Mice were housed in groups of 10 animals per apparatus for 60 days. Standard housing consisted in an apparatus of Plexiglas box (38 cm × 32 cm × 16 cm) containing just sawdust. Enriched housing apparatus consisted of an Plexiglas box (38 cm × 32 cm × 16 cm) connected to a three-story metal cage (28 cm × 21 cm × 50 cm) containing sawdust, two running wheels and a variety of objects, including wood and plastic objects, nesting material and hiding places, such tunnels for instance, in order to represent eco-ethological expansions for mice including the sense of security and to provide a place where they could avoid open spaces and luminosity, a natural behavior of wild mice. Additional cognitive stimulation regarding the formation of spatial mapping was provided by changing the objects or by shifting their positions in the enriched housing twice a week (Amaral, Vargas, Hansel, Izquierdo, & Souza, 2008; Viola et al., 2009). Due to the territorial features of mice, we did not change all objects at the same time for avoiding an increase in the aggressiveness of the animals.

2.3. Behavioral procedures

From 81st to 86th postnatal days a battery of behavioral tasks, which encompass open field task (OFT), object recognition task (ORT) and elevated plus maze (EPM), was performed with an interval of 24 h between each task (Fig. 1). In order to acclimate, animals were put on behavioral testing room one hour before the beginning of tasks at controlled temperature (21 ± 1 °C) and illumination (see below in Time-of-day session) used during the tests. The ANY-Maze video-tracking system (Stoelting, CO) was used to automatically recording and collects the behavioral data.

2.4. Time-of-day

Animals from both housing conditions were divided in two groups: Light (mice tested between 1 and 6 p.m.; $n = 10$/per group), or Dark (mice tested between 7 and 12 p.m.; $n = 10$/per group). Behavioral tasks performed during the light phase were conducted under a white-light illumination (200 Lux), while behavioral procedures performed during the dark phase were conducted under a red-light illumination (4 Lux). These conditions were used in order to avoid the following interfering factors: (i) masking effects (such an increasing in activity when the lights are switched off or a decreasing in activity when the lights are switched on); and (ii) phase shifting along the days (due the lights being kept on during dark phase or the lights being kept off during light phase).

2.5. Open field

The OFT task was performed in two sessions, when animals were 81 and 82 days old. The apparatus was a square transparent Plexiglas box with dimensions of 50 × 50 × 50 cm. The floor of apparatus was virtually divided into 16 squares (4 central and 12 peripheral). Animals were housed in two different conditions (SC and EC), and thereafter tested in different times-of-day (SC/Light, EC/Light, SC/Dark, and EC/Dark groups). Each animal was individually placed in the arena center and it was left free to explore it for 10 min. The apparatus was cleaned with a 70% ethanol solution between trials to eliminate odor cues. Based on studies performed by Eilam (2003) and Loss et al. (2014), locomotor activity and spatial distribution were quantified as described below:

– Locomotor activity:

(a) Distance traveled: overall distance traveled during the 10 min observation; (b) locomoting time: duration of locomoting periods, expressed as percentage of total time of test; (c) average speed: distance traveled divided by locomoting time; (d) number of stops: the incidence of "non-locomoting" intervals that were bounded by "locomoting" intervals. A "non-locomoting" interval was registered when an animal stayed immobile by a period equal or greater than 2 s; (e) inter-stops distance: average metric distance traveled between two consecutive stops (total distance traveled divided by the total number of stops).

– Spatial distribution:

(a) Distance traveled along the perimeter: the total distance traveled in the vicinity of the arena walls divided by the total distance traveled during the test multiplied by 100; (b) locomoting time along the perimeter: the locomoting time in the vicinity of the arena walls divided by the total locomoting time during the test multiplied by 100; (c) time spent on perimeter: the time in which the mouse was in the perimeter divided by the total time of the test multiplied by 100.

2.6. Object recognition task

ORT was performed in the same apparatus used in OFT task. Each mouse was subjected to three distinct sessions; (1) presentation session (performed 24 h after OFT), (2) novel object discrimination session, and (3) place discrimination session. The ORT protocol was based in Borsoi et al. (2014) and it was adapted for mice according to Viola et al. (2010). The inter-session interval was of 24 h.

The presentation session consisted into placing a mouse in the apparatus containing two similar objects (A and A′) and allowed it to explore for 10 min. The objects were fixed on the floor of the apparatus equidistant from two corners, 12 cm apart the wall. Each mouse was always placed individually in the apparatus center, facing the wall, which was opposite to the objects. At the end of the test mice were immediately put back in their home cage. The novel object discrimination session was similar to presentation session, but at this time, two dissimilar objects were presented, a familiar and a novel one (A and B, respectively). We used glass objects presenting the same texture and size (13 cm height) but with different shapes and colors (transparent and amber). In the place discrimination session, mice were allowed to explore the same objects used in the novel objects discrimination session for 10 min. However, in this session, the place of object B was changed to an adjacent corner. No one object used in ORT was previously presented to mice from EC group. The apparatus and the objects were cleaned using a 70% ethanol solution between trials to eliminate odor cues. In all three sessions, the following parameters were analyzed:

(a) Exploration of object A: object exploration was recorded when a mouse was directing the nose to the object at a distance ⩽2 cm and/or touching the object with the nose or forepaws; sitting on the object was not recorded as object exploration. (b) Exploration of object A′ (or B in the discrimination sessions). (c) Exploration of both objects.

Discrimination ratio for each mouse was expressed by TN/(TN + TF) ratio [TF = time spent exploring familiar object; TN = time spent exploring the novel object].

2.7. Elevated plus maze

EPM was performed in order to assess anxiety-like behaviors. The apparatus was made of a black-painted wood, consisting of

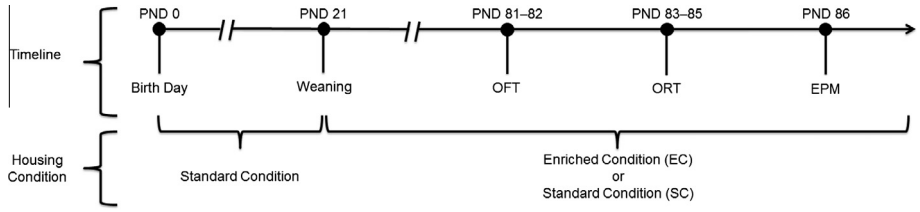

Fig. 1. Experimental design. Diagram depicting the timeline in which the experiments were performed and the periods when the mice were exposed to the different housing conditions.

two open arms (60 × 5 cm) and two closed arms (60 × 5 × 15 cm), with the arms of each type opposite to each other, and a central platform (5 × 5 cm) between arms. The maze was 40 cm elevated from the floor. Each mouse was individually placed into the maze center facing to the open arm (Kazlauckas et al., 2005) and were left free to explore the apparatus for 5 min. The maze was cleaned with a 70% ethanol solution between each trial to eliminate odor cues. The following parameters were analyzed:

– Anxiety levels:

(a) Time spent in the open arms. (b) Time spent in the closed arms. (c) Number of entries into the open arms: an arm entry was defined as all four paws in an arm. (d) Number of entries into the closed arms. (e) Distance traveled in the open arms. (f) Distance traveled in the closed arms. (g) Time spent in the central area. (h) Number of risk assessment behaviors: number of exploration of the open arm through stretch-attend posture (when the rodent is motionless in center- or closed-zone, but has its body stretched forward into the open arms by placing some but not all paws, returning then to the same position) (Walf & Frye, 2007).

– Exploratory activity in the apparatus:

(a) Total distance traveled in both open and closed arms. (b) Total time immobile.

2.8. Statistics

All data are presented as mean ± standard error (SEM). Two-way ANOVA (factor 1: housing condition; factor 2: time-of-day) followed by Bonferroni's multiple comparisons test were employed to analyze the OFT and EPM data.

The analysis of the ORT data was done with a model selection procedure in order to infer which hypothesis (model) best explains the observed variation in the exploration patterns of each individual. The dependent variable was the logarithm of the absolute time invested by the animal in exploring the two objects, while the predictive variables were: the housing condition in which the animal was maintained (SC or EC), the time-of-day (light or dark), and the correspondent trial session (presentation, novel object discrimination, and place discrimination). A fourth variable was considered in order to control de random effect associated to the repeated measures resulting from the session variable. The combination of the predictor variables resulted in 10 concurrent models (Supplementary Table S1), and their maximum likelihood was inferred using the Akaike's Information Criterion corrected for small samples (AICc). We ran all the models using the function 'lmer' from the package 'lme4' (Bates, Maechler, & Bolker, 2013) for R software (R Development Core Team, 2013).

In addition, two-way ANOVA with repeated measures (within-subject factor: ORT sessions; between-subject factor: housing condition + time-of-day) followed by Bonferroni's multiple comparisons test was used to analyze object discrimination index between sessions.

Principal component analysis (PCA) was performed to address the expected correlation between variables from OFT, ORT and EPM. PCA was used to convert a set of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components, without significant loss of information regarding variability among individuals (Jolliffe, 1986; Sanguansat, 2012). The first principal component (PC1) explains the largest proportion of the variance in the data (quantified based on eigenvalue), usually expressed as a percentage of the total variance. Every next component (PC2, PC3, PCn) displays, in turn, decreasing amounts of variation. If the analysis is successful, the data may be represented by a smaller number of PCs than the number of original variables (Jolliffe, 1986; Sanguansat, 2012). Therefore, it provides a way to understand and visualize the structure of complex data sets, such the data collected during the behavioral tasks in our study. Furthermore, it helps us identify new meaningful underlying variables (Sanguansat, 2012). We ran PCA using the software SPSS 19 (IBM SPSS Statistics, version 19). PCs that presented eigenvalues lower than 1 were disregarded. A significance level of 0.05 was adopted for all analyses.

3. Results

3.1. Housing condition and time-of-day impact on behavioral repertoire in the open field task

Data from both days of the OFT were analyzed and compared with each other. Since no differences were observed between the two days (data not shown), only the data of locomotor activity and spatial distribution of the second day are described below. Regarding locomotor activity (Fig. 2), two-way ANOVA indicated a significant influence of housing conditions on locomoting time [$F(1,35) = 5.33$; $P = 0.027$] and on distance traveled [$F(1,35) = 20.39$; $P < 0.001$]. In addition, a significant influence of time-of-day was observed on locomoting time [$F(1,35) = 57.29$; $P < 0.001$], distance traveled [$F(1,35) = 8.91$; $P = 0.005$], locomoting average speed [$F(1,35) = 49.28$; $P < 0.001$], number of stops [$F(1,35) = 66.28$; $P < 0.001$] and inter-stops distance [$F(1,35) = 12.70$; $P = 0.001$]. Animals from both housing conditions tested in the dark have reduced locomoting time when compared to their respective housing conditions tested in the light ($P < 0.001$ to SC; $P < 0.001$ to enriched condition – EC). Moreover, animals from EC/Light demonstrated reduced locomoting time when compared with animals from SC/Light ($P = 0.025$). However, no difference was found between EC/Dark and SC/Dark groups ($P > 0.999$). In addition, mice from SC/Dark demonstrated a reduction in the distance traveled as compared to SC/Light ($P < 0.001$), but not to EC/Dark ($P = 0.77$). Similarly, EC/light group presented reduced distance traveled as compared to SC/light ($P = 0.004$), but not when compared to EC/Dark ($P = 0.125$). Animals tested in the dark presented greater locomoting average speed ($P < 0.001$ to SC; $P < 0.001$ to EC) and number of stops ($P < 0.001$ to SC; $P < 0.001$ to EC) when compared with their respective housing conditions tested in the light. Finally, SC/Dark presented minor inter-stop distance than mice from SC/Light ($P = 0.003$).

In relation to spatial distribution (Fig. 3 and Supplementary Fig. S1 – Top), no significant effect for both housing condition as well as for time-of-day was found in the time spent in perimeter [$F(1,35) = 0.84$; $P = 0.366$ to housing condition; $F(1,35) = 1.01$; $P = 0.321$ to time-of-day], locomoting time along the perimeter [$F(1,35) = 0.15$; $P = 0.703$ to housing condition; $F(1,35) = 0.58$; $P = 0.453$ to time-of-day], and distance traveled along the perimeter of apparatus [$F(1,35) < 0.01$; $P = 0.973$ to housing condition; $F(1,35) = 2.46$; $P = 0.126$ to time-of-day].

3.2. Housing condition and time-of-day impact on behavioral repertoire in the object recognition task

A mathematical modeling using the ORT data indicated two concurrent models. Both models encompass the additive effect of all predictor variables (housing conditions, time of the day, and trial session), and one of them also includes the interaction term between the time-of-day and trial session (Supplementary Table S1). The biological hypothesis that supports these models is the independent effect of each variable, i.e., each of them

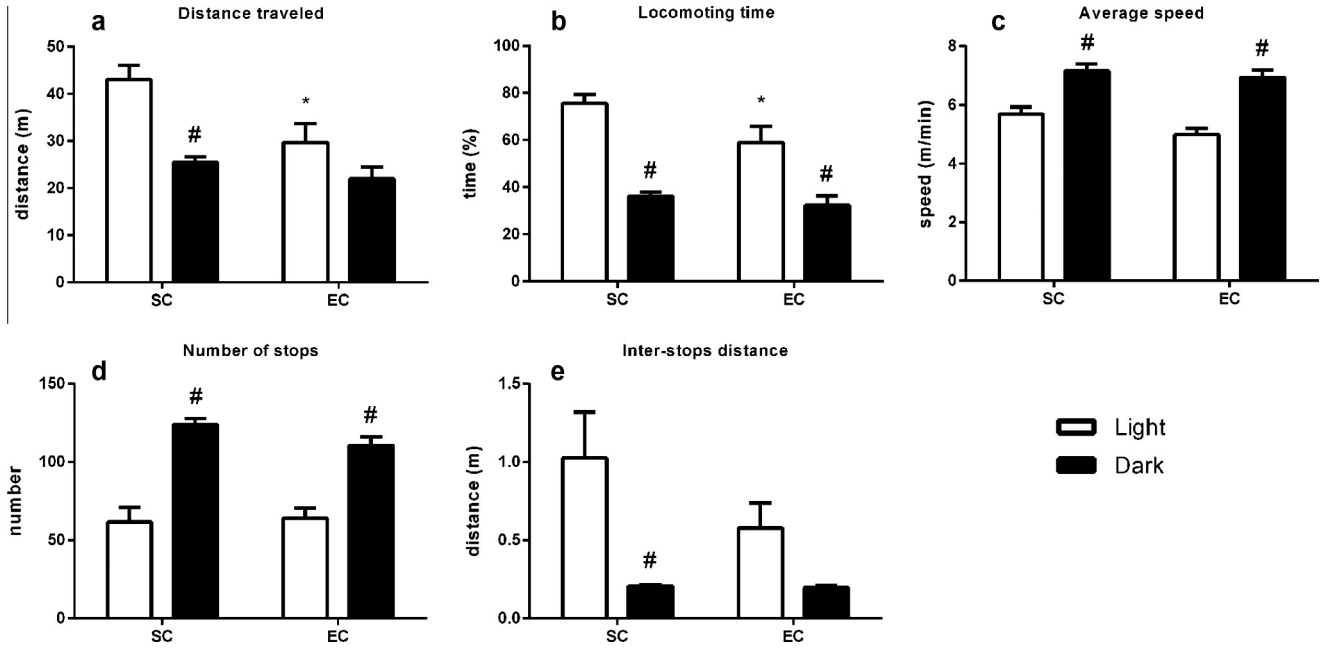

Fig. 2. Locomotor activity. The end-point results of (a) distance traveled, (b) locomoting time, (c) average speed, (d) number of stops and (e) inter-stop distance. The data are expressed as the means ± sem. $n = 9–10$ animals per group. * indicates $p < 0.05$ when compared with SC group in the same time-of-day; # indicates $p < 0.05$ when compared with the same housing condition tested in Light. All data were analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test.

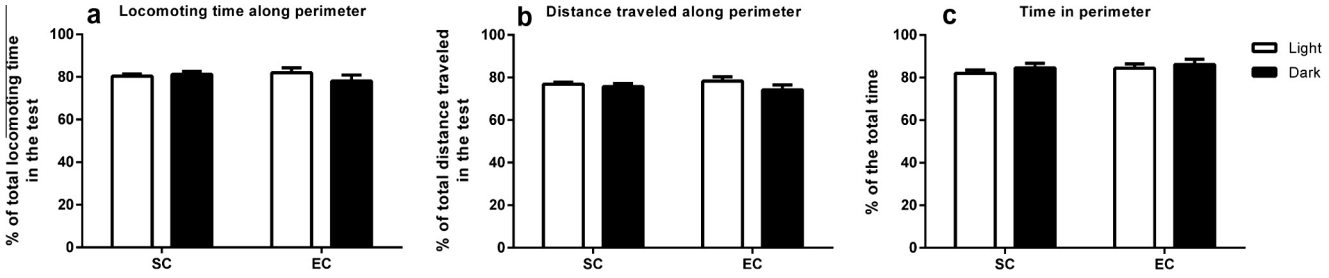

Fig. 3. Spatial distribution. The end-point results of (a) distance traveled along the perimeter, (b) locomoting time along the perimeter and (c) time spent on perimeter. The data are expressed as the means ± sem. $n = 9–10$ animals per group. All data were analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test.

explains a parcel of the overall information contained within the dependent variable. The alternative model also provides additional information regarding the interaction observed between the time-of-day and trial sessions.

A graphical analysis of the data clearly explains the relationship between variables in the selected models (Fig. 4a–c). There is a tendency for a decrease in the total time of objects exploration for trials conducted with individuals raised in the EC for each time-of-day and trial session. Trials executed in the dark phase showed a decrease in the total time of objects exploration for each housing condition and trial session. Lastly, there was a decrease in the total time of objects exploration along the trial sessions for each housing condition and time-of-day. For the alternative model, the graphical analysis showed a decrease in the total time of objects exploration for trials conducted during the dark phase in the first session, but not in the following sessions.

Given all the subtleties that this experimental design may provide, we have performed the following analyses for each trial session.

3.2.1. Object exploration

In the presentation session of ORT, two-way ANOVA indicated a significant influence of both housing conditions [$F(1,34) = 37.57$; $P < 0.001$] and time-of-day [$F(1,34) = 12.22$; $P = 0.001$] in the total time of objects exploration (A + A′). For the novel object discrimination and the place discrimination sessions, two-way ANOVA indicated a influence of housing conditions [$F(1,34) = 62.28$; $P < 0.001$; $F(1,34) = 27.59$; $P < 0.001$, respectively], but not of time-of-day [$F(1,34) = 2.40$; $P = 0.131$; $F(1,34) = 2.96$; $P = 0.095$, respectively], in the total time of objects exploration (A + B) (Fig. 4a–c).

During the presentation session (Fig. 4a), animals from both housing conditions tested in the dark presented reduced time of exploration of objects when compared to their respective housing conditions tested in the light ($P = 0.033$ to SC; $P = 0.042$ to EC). In addition, mice from EC (both EC/Light and EC/Dark) presented reduced exploration of objects when compared to their respective SC group ($P < 0.001$ to Light; $P < 0.001$ to Dark). Moreover, EC animals have shown reduced exploration of objects when compared to their respective SC group in the novel object discrimination ($P < 0.001$ to Light; $P < 0.001$ to Dark – Fig. 4 b) and in the place discrimination sessions ($P < 0.001$ to Light; $P = 0.014$ to Dark – Fig. 4c).

3.2.2. Object discrimination

In relation to object discrimination ratios, it was not found any influence of both housing conditions and time-of-day in all three sessions (Fig. 4). More specifically, animals from both housing conditions tested in both time-of-day were capable of discriminating between the novel and the familiar object in the novel object discrimination session ($P = 0.001$ to SC/Light; $P = 0.002$ to EC/Light;

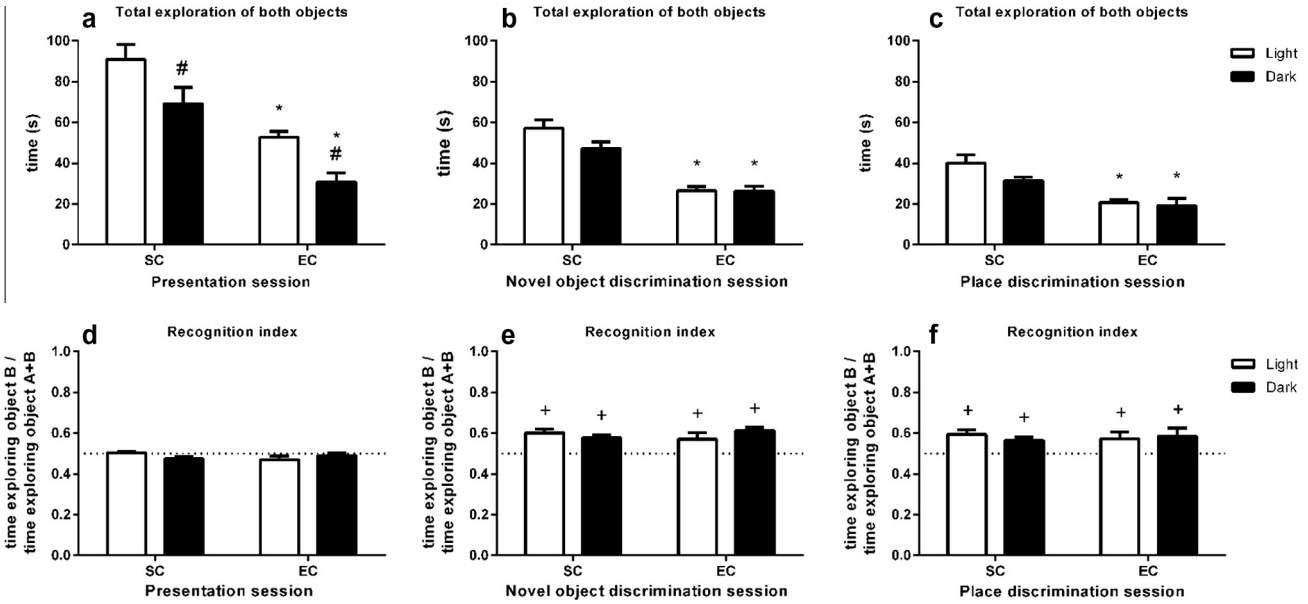

Fig. 4. Object recognition task. End-point results of exploration of both objects during: (a) the presentation session; (b) novel object discrimination session; and (c) place discrimination session. The object discrimination index between groups during: (d) the presentation session; (e) novel object discrimination session; and (f) place discrimination session. The data are expressed as the means ± sem. $n = 9–10$ animals per group. Data presented in (a–f) were analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test. Two-way ANOVA with repeated measures (within-subject factor: ORT sessions; between-subject factor: housing condition + time-of-day) followed by Bonferroni's multiple comparisons test was used to analyze object discrimination index between sessions. * indicates $p < 0.05$ when compared with SC group in the same time-of-day; # indicates $p < 0.05$ when compared with the same housing condition tested in Light; + indicates $p < 0.05$ when compared with the same group during the presentation session.

$P < 0.001$ to SC/Dark; $P < 0.001$ to EC/Dark). Also, the same profile was obtained during the place discrimination session ($P = 0.032$ to SC/Light; $P = 0.022$ to EC/Light; $P = 0.040$ to SC/Dark; $P = 0.038$ to EC/Dark).

3.3. Elevated plus maze

Elevated plus maze (EPM) was used in this study to investigate the influence of housing conditions and time-of-day on anxiety-like behaviors. Two-way ANOVA indicated an influence of time-of-day [$F(1,35) = 6.33$; $P = 0.017$], but not of housing condition [$F(1,35) = 0.50$; $P = 0.486$] in the number of risk assessment behaviors. Animals from EC/Dark presented higher number of risk assessment behaviors if compared to animals from EC/Light ($P = 0.016$) (Table 1). We did not find any difference with respect to total distance traveled [$F(1,35) = 1.31$; $P = 0.259$ to housing condition; $F(1,35) = 0.49$; $P = 0.490$ to time-of-day], total time immobile [$F(1,35) = 0.06$; $P = 0.809$ to housing condition; $F(1,35) = 0.87$; $P = 0.358$ to time-of-day], time spent in the central area [$F(1,35) = 0.15$; $P = 0.705$ to housing condition; $F(1,35) = 3.26$; $P = 0.080$ to time-of-day], distance traveled into the closed arms [$F(1,35) = 2.02$; $P = 0.165$ to housing condition; $F(1,35) < 0.01$; $P = 0.946$ to time-of-day], time spent into the closed arms [$F(1,35) < 0.01$; $P = 0.930$ to housing condition; $F(1,35) = 0.06$; $P = 0.803$ to time-of-day], entries into the closed arms [$F(1,35) = 2.82$; $P = 0.102$ to housing condition; $F(1,35) = 1.99$; $P = 0.167$ to time-of-day], distance traveled in the open arms [$F(1,35) = 0.35$; $P = 0.556$ to housing condition; $F(1,35) = 0.61$; $P = 0.439$ to time-of-day], time spent in the open arms [$F(1,35) = 0.08$; $P = 0.776$ to housing condition; $F(1,35) = 1.89$; $P = 0.178$ to time-of-day], and entries into the open arms [$F(1,35) = 0.05$; $P = 0.831$ to housing condition; $F(1,35) = 2.00$; $P = 0.166$ to time-of-day], as observed in Table 1 and Supplementary Fig. S1 (on bottom).

Table 1
Elevated plus maze task.

	SC		EC	
	Light	Dark	Light	Dark
Distance traveled (m)	4.91 ± 0.32	5.16 ± 0.29	4.62 ± 0.33	4.78 ± 0.23
Time immobile (%)	70.69 ± 2.06	68.60 ± 1.66	70.71 ± 1.99	69.45 ± 1.41
Number of risk assessment behaviors	12.60 ± 1.54	13.80 ± 1.23	9.89 ± 0.70	14.80 ± 1.15#
Time spent in the central area (%)	10.46 ± 2.07	7.53 ± 1.87	12.01 ± 2.31	7.55 ± 1.96
Distance traveled in the closed arms (%)	85.84 ± 1.88	86.37 ± 3.81	82.14 ± 2.66	82.00 ± 2.61
Time spent in the closed arms (%)	78.64 ± 3.29	77.17 ± 5.52	78.58 ± 3.31	77.96 ± 3.81
Number of entries in closed arms	5.90 ± 0.85	4.80 ± 0.77	7.11 ± 0.72	6.10 ± 0.62
Distance traveled in open arms (%)	2.61 ± 1.25	4.88 ± 2.70	4.51 ± 1.96	5.32 ± 1.63
Time spent in the open arms (%)	2.55 ± 1.18	6.55 ± 3.19	3.32 ± 1.24	4.67 ± 1.23
Number of entries in open arms	0.90 ± 0.32	1.70 ± 0.68	1.11 ± 0.42	1.70 ± 0.45

Data are presented as mean ± SEM; two-way ANOVA results. $N = 9–10$ animals per group.
# Indicates that $P < 0.05$ when compared with the same housing condition tested in the Light.

3.4. Principal component analysis

The investigation of animal behavior requires the analyses of a large set of variables in an integrated manner for generating more accurate interpretation (Loss et al., 2014). For reducing these variables to a smaller set of "new" variables – created from a combination of the original variables – we used a mathematical tool, termed principal component analysis (PCA), which consists in a feasible way for simplifying the structure of complex data sets (Sanguansat, 2012). We used a set of eighteen behavioral variables (eight from OFT, one from ORT and nine from EPM – Supplementary Table S2), which resulted in seven principal components (PC). The accumulated eigenvalues of these principal components (PC1–PC7) was capable of explaining 74.62% of the total data variability (Supplementary Table S3).

Correlation coefficients between behavioral measures and each specific PC are depicted in Supplementary Table S3, and suggest that PC1 was associated with locomotor- and exploratory-like behaviors (been positively correlated to locomoting time in OFT, distance traveled in OFT, inter-stop distance, and objects exploration, and also negatively correlated to number of stops, average speed, locomoting time along the perimeter, risk assessment behavior, and time spent in perimeter), whereas PC2 and PC3 were related to anxiety-like behaviors. PC2 was related to the expression of anxiolytic-like behaviors, which seems to indicate less defensive behaviors (been positively correlated to entries into the open arms, distance traveled in EPM, entries into the closed arms, time spent in the open arms, and distance traveled in the open arms, and also negatively correlated to distance traveled in the closed arms and time spent in the perimeter). PC3 was related to the expression of anxiogenic-like behaviors, which seems to indicate self-protective behaviors (been positively correlated to time spent in the closed arms, locomoting time along the perimeter, entries into the closed arms, and time spent in the perimeter, and also negatively correlated to average speed, time spent in the open arms, and distance traveled in OFT).

When groups were analyzed by their PC1 scores, two-way ANOVA revealed differences for housing condition [$F(1,35) = 6.38$; $P = 0.016$] and for time-of-day [$F(1,35) = 101.5$; $P < 0.001$]. Animals from both housing conditions tested in the dark presented lower scores for PC1 than their respective housing conditions tested in light ($P < 0.001$ to SC; $P < 0.001$ to EC). Moreover, animals from EC/Light group presented lower scores for PC1 than animals from SC/Light group ($P = 0.022$). No difference was found between EC/Dark and SC/Dark groups ($P > 0.999$) (Fig. 5). Regarding PC2, no differences were found neither to housing condition [$F(1,35) = 0.10$; $P = 0.755$] nor to time-of-day [$F(1,35) = 0.36$; $P = 0.554$]. With respect to PC3, two-way ANOVA revealed an interaction between housing condition and time-of-day [$F(1,35) = 10.34$; $P = 0.003$]. Animals from EC/Light group presented higher scores than SC/Light and EC/Dark groups ($P = 0.019$ and $P = 0.016$, respectively). No differences were observed between SC/Dark and SC/Light ($P > 0.999$), or SC/Dark and EC/Dark ($P > 0.999$) (Fig. 5). Regarding PC4–PC7, there were no differences either to housing condition or to time-of-day (data not shown). PC1 vs. PC3 score plots shows an interesting pattern, which suggests that animals from both housing conditions behaved similar in the dark phase, but in the light phase, animals from SC presented higher exploratory-like profile associated with less self-protected behavior.

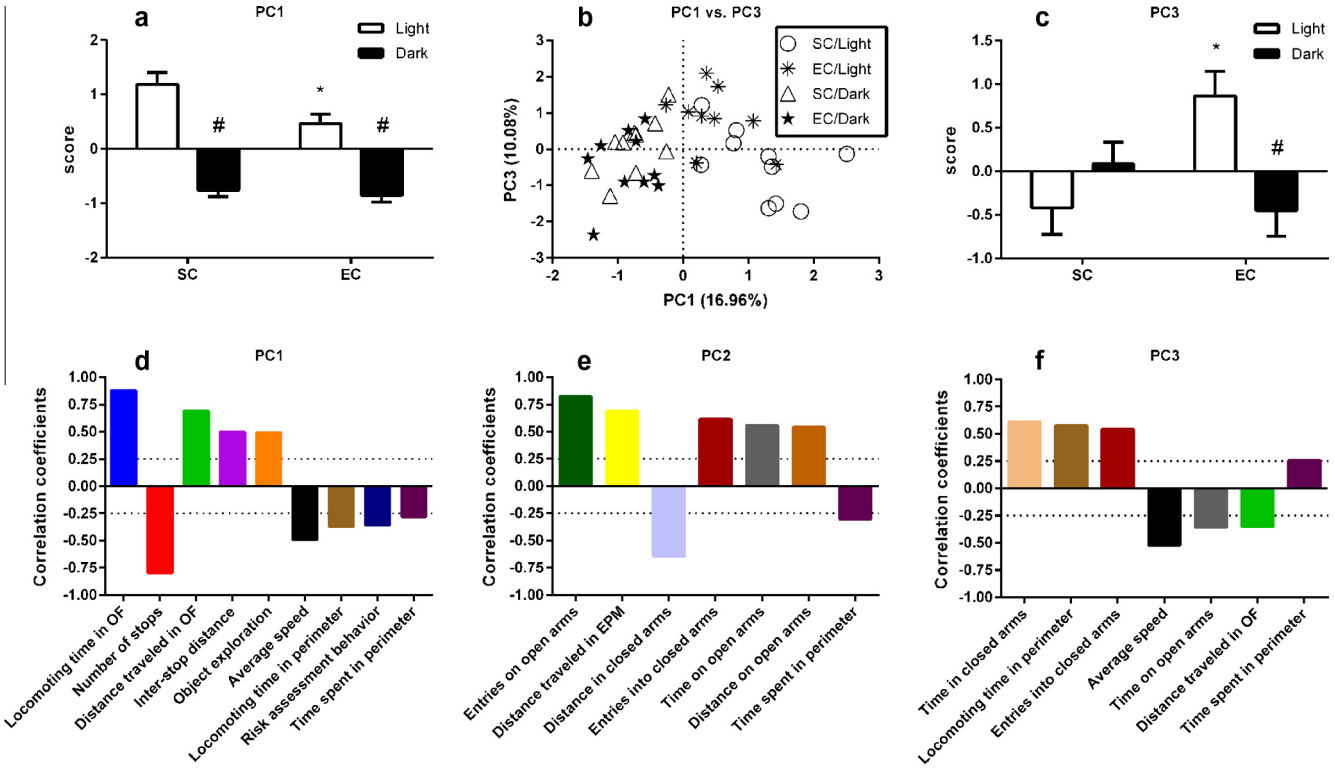

Fig. 5. Principal component analysis. Comparison between groups with respect to the PC1 and PC3 values are presented in (a and c, respectively). A scatter plot of PC1 and PC3 individual values are presented in (b). The matrix correlation between the behavioral variables and each individual PC (PC1, PC2, and PC3) are presented in (d–f). Dashed lines in (d–f) (values 0.25 and −0.25 in the *Y*-axis) indicate the cutoff points (i.e., the variables which presented correlation values >−0.25 and <0.25 are not represented in the graphs). Data from (a–c) are expressed as the means ± sem and were analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test. $n = 9$–10 animals per group. * indicates $p < 0.05$ when compared with SC group in the same time-of-day; # indicates $p < 0.05$ when compared with the same housing condition tested in Light.

4. Discussion

Here we demonstrated that the housing conditions and time-of-day have distinct impact on behavioral repertoire of male albino Swiss mice. As predicted by our first working hypothesis, animals from EC tested in light phase showed less exploratory behaviors, which were associated with decreased exposure to potentially dangerous environment. In contrast to our first hypothesis, there was no difference between animals from both housing conditions when they were tested in the dark phase. Moreover, these animals demonstrated lower locomotor and exploratory profiles. The second working hypothesis was corroborated, since animals from EC tested both in dark and light phase presented a better performance in ORT than SC animals. In the following sections, we provide experimental evidence supporting our findings and propose putative mechanisms involved in the expression of behavioral repertoire of Swiss male mice.

4.1. Exploratory- and anxiety-like tasks

Consistent with previous studies, EE significantly affected both locomotor and exploratory profiles (Amaral et al., 2008; Brenes, Padilla, & Fornaguera, 2009; Leger et al., 2012; Viola et al., 2010; Vivinetto, Suarez, & Rivarola, 2013). Nevertheless, this effect was only observed when the animals were tested during the light phase. The EC/Light group traveled shorter distances and moved less time if compared to the SC/Light group. These differences were not observed when the tasks were performed during the dark phase (Fig. 5). Surprisingly, both housing conditions when tested in the dark traveled shorter distances and moved less time than animals tested in the light phase. In contrast, previous studies demonstrated that animals tested during the dark phase were more active than animals tested during the light phase (Loss et al., 2014; Smith & Morrell, 2007; Verma et al., 2010). However, in our study, mice tested in the dark moved faster, stopped more and presented shorter inter-stop distance than those tested in the light phase. In fact, differences found between light and dark phases in our study are very likely not related to the circadian rhythm *per se*, but to the presence (or absence) of light. Indeed, previous studies (Avni, Zadicario, & Eilam, 2006; Zadicario, Avni, Zadicario, & Eilam, 2005) demonstrated that rodents exposed to the OFT presents a different pattern of behavior in the presence or absence of light. Although there are discrepancies in the distance traveled and traveling speed if compared to our data, the intrinsic characteristics of each species (*Meriones tristrami* and *Mus musculus*) may explain these conflicting results (Viola & Loss, 2013). Therefore, we believe that data regarding both groups tested in dark do not represent activity properly, but a behavioral pattern that is modulated by a combination of illumination and time of the day (see PC1).

Providing further support to our claim, an exacerbation in the self-protective behavior was found in mice that reared in EE and were tested in the presence of light if compared to animals that reared in SC and were tested in the same conditions (Fig. 5c). A similar effect was observed when EC/Light group was compared to EC/Dark group. We propose that the reduction of neophobic responses and the decline of novelty seeking induced by EE (Walker & Mason, 2011; Zambrana et al., 2007) accelerates the process of habituation stimulating the expression of more evolutionary propitious behaviors in mice, such as defensive behaviors. Indeed, the use of less risky exploratory strategies and the enhancement of anti-predatory behaviors were observed in both fishes and mammals exposed to EE (Jule, Leaver, & Lea, 2008; Roberts, Taylor, & de Leaniz, 2011). The increase in self-protective behaviors (as indexed by PC3) in EC/Light group and the lack of differences between groups with respect to less defensive behaviors (as indexed by PC2) give further support to this hypothesis. EE animals reduced their exposition to potentially dangerous environments during the light phase (the less active period in normal conditions), but did not alter their behavior during the dark phase (the period in which rodents usually increase their activity to foraging for food, sexual partners and to explore novel environments). Importantly, these differences represent subtle alterations in the Swiss mice behavioral pattern and were only identified by employing the PCA. Thus, it does not indicate a behavioral impairment or even an anxiety-like disorder. The absence of difference in data from EPM and OFT in our study, and also data from other study using the same strain (Silva et al., 2011) give further support to this statement and highlight the hypothesis of EE affecting rodents behavior in a species(strain)-specific manner.

4.2. Episodic-like memory task

As expected, we demonstrated that EE influences behavior of animals in the ORT (Bruel-Jungerman et al., 2005; Leger et al., 2012; Viola et al., 2010). In addition, Leger et al. (2012) reported that NMRI male mice that reared in the EE for 3 weeks have improved long-term memory in ORT task performed in a Y-maze apparatus. They observed that EE induces a reduction in the exploratory activity, leading to very poor exploration of objects if compared to animals reared in SC. In fact, our data using Swiss albino mice reared in EE for 8 weeks indicate that the object exploration act seems to be a composite of housing conditions, time-of-day, and trial session, i.e., each of these predictor variables explain a parcel of the overall information contained within the dependent variable (Supplementary Table S1). These data suggest that all groups were able to recognize the context of the task (an environment containing two objects without any relevance to the mice) since all of them decreased objects exploration along the sessions (i.e. have habituated). Furthermore, our data suggest that one of the housing conditions habituates faster than the other. In addition, an interaction between the time-of-day and trial session indicates that the behavior of animals when challenged with novelty was influenced in a distinct way by light and dark conditions (Supplementary Table S1 and Fig. 4a). However, when they became familiarized, there were no differences between these two conditions. By contrast, the housing condition was the major factor influencing behavioral repertoire of animals in the familiar context (Fig. 4b and c). Here we demonstrated that mice that reared in EE spent less time exploring the objects, which could be related to reduced motivation, curiosity or interest for the objects. It is important to underline that this putative reduced motivation for exploring the objects does not necessarily implicate in less motivation to perform other behaviors (Franks, Champagne, & Higgins, 2013). In fact, our data suggest a faster habituation to novelty (objects) in EE animals, probably since they have experienced more stimulating environmental conditions (learning, social and physical) than SC animals (Leger et al., 2012; Viola et al., 2010). Therefore, the degree of novelty seems to be distinct, being a considerable novelty to SC groups and just subtle novelty to EC groups.

In relation to the cognitive performance, our data reinforce the cognitive benefits of EE (Green, Melo, Christensen, Ngo, & Skene, 2006; Karelina et al., 2012; Kobayashi, Ohashi, & Ando, 2002; Kotrschal & Taborsky, 2010). Despite no difference was found between groups in the discrimination ratio, EC animals were able to discriminate the objects (and the location of the objects) even exploring them for less time. These data plus the faster habituation of the EC groups suggested by the model selection (AIC) indicate that to discriminate one object from the other the SC animals needed to explore the objects for more time than EC animals. Thus, based on the premise that the performance of an individual

can be measured by the time it takes to achieve a certain outcome (discriminate the objects in this case), our data suggest that regardless the time-of-day, mice reared in EE presented an improved performance in both object and local discriminations if compared to SC mice.

5. Conclusions

Accordingly to the Directive 2010/63/EU of the European Parliament and of the Council of the European Union (EC, 2010), "All animals shall be provided with space of sufficient complexity to allow expression of a wide range of normal behavior. They shall be given a degree of control and choice over their environment to reduce stress-induced behavior". This environment proposed by the Council of the European Union—an EE environment—leads to the enhancement of cognitive abilities. In this study we showed that the time-of-day influences the behavioral repertoire of Swiss mice that reared in EE. First, we showed that the locomotor and exploratory activities are distinct between SC and EC groups only during the light phase. We postulated that EE reinforces the innate behavioral features of animals, e.g., EE seems to triggers animals wild behavior. In accordance, EE animals have reduced locomotor and exploratory activities in the light phase, which seems to follow natural conditions, where rodents are less active and do not have to expose themselves during this phase. Second, we showed that mice rearing in both SC and EC were capable of discriminating objects and its localization in the dark condition, which indicates that the recognition process may include non-visual signals (Albasser et al., 2013), and the information is probably captured by whiskers stimulation (tactile system) (Alwis & Rajan, 2013). Moreover, EE animals have improved performance in objects and place discrimination in both light and dark conditions. These findings underscore that even presenting similar behavioral repertoire, the cognitive performance of EE animals was improved. Thus, seems plausible to propose that the improvement of somatosensory system induced by EE is tied involved in this process (Guic, Carrasco, Rodriguez, Robles, & Merzenich, 2008). Piecing together, our data provide behavioral support to the concept of EE promoting neuroplastic changes in several brain regions and reinforce the need of better understanding of the circadian behavioral patterns of rodents.

Authors' contributions

GGV was responsible for the design of the study. GGV, LBB, EM, WCB, PAO and SVF were responsible for the acquisition of data. CML and LECS were responsible for the analysis of the data. CML, LECS and GGV were responsible for interpretation of data. CML, LECS, ERZ, DLO and GGV were responsible for the design, drafting and revising the manuscript. DLO, RDP and CIT revised and have given final approval of the version to be published. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2015.07.016.

References

Abramov, U., Puussaar, T., Raud, S., Kurrikoff, K., & Vasar, E. (2008). Behavioural differences between C57BL/6 and 129S6/SvEv strains are reinforced by environmental enrichment. *Neuroscience Letters, 443*, 223–227.

Albasser, M. M., Olarte-Sanchez, C. M., Amin, E., Horne, M. R., Newton, M. J., Warburton, E. C., et al. (2013). The neural basis of nonvisual object recognition memory in the rat. *Behavioral Neuroscience, 127*, 70–85.

Alwis, D. S., & Rajan, R. (2013). Environmental enrichment causes a global potentiation of neuronal responses across stimulus complexity and lamina of sensory cortex. *Frontiers in Cellular Neuroscience, 7*, 124.

Amaral, O. B., Vargas, R. S., Hansel, G., Izquierdo, I., & Souza, D. O. (2008). Duration of environmental enrichment influences the magnitude and persistence of its behavioral effects on mice. *Physiology & Behavior, 93*, 388–394.

Avni, R., Zadicario, P., & Eilam, D. (2006). Exploration in a dark open field: A shift from directional to positional progression and a proposed model of acquiring spatial information. *Behavioural Brain Research, 171*, 313–323.

Baldini, S., Restani, L., Baroncelli, L., Coltelli, M., Franco, R., Cenni, M. C., et al. (2013). Enriched early life experiences reduce adult anxiety-like behavior in rats: A role for insulin-like growth factor 1. *Journal of Neuroscience, 33*, 11715–11723.

Bates, D., Maechler, M., & Bolker, B. (2013). *lme4: Linear mixed-effects models using S4 classes. R package version 0.999999-2*. <http://CRAN.R-project.org/package=lme4>.

Bonaccorsi, J., Cintoli, S., Mastrogiacomo, R., Baldanzi, S., Braschi, C., Pizzorusso, T., et al. (2013). System consolidation of spatial memories in mice: Effects of enriched environment. *Neural Plasticity, 2013*, 956312.

Borsoi, M., Antonio, C. B., Viana, A. F., Nardin, P., Goncalves, C., & Rates, S. M. (2014). Immobility behavior during the forced swim test correlates with BNDF levels in the frontal cortex, but not with cognitive impairments. *Physiology & Behavior, 140C*, 79–88.

Brenes, J. C., Padilla, M., & Fornaguera, J. (2009). A detailed analysis of open-field habituation and behavioral and neurochemical antidepressant-like effects in postweaning enriched rats. *Behavioural Brain Research, 197*, 125–137.

Bruel-Jungerman, E., Laroche, S., & Rampon, C. (2005). New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment. *European Journal of Neuroscience, 21*, 513–521.

Chaudhury, D., & Colwell, C. S. (2002). Circadian modulation of learning and memory in fear-conditioned mice. *Behavioural Brain Research, 133*, 95–108.

Diniz, D. G., Foro, C. A., Rego, C. M., Gloria, D. A., de Oliveira, F. R., Paes, J. M., et al. (2010). Environmental impoverishment and aging alter object recognition, spatial learning, and dentate gyrus astrocytes. *European Journal of Neuroscience, 32*, 509–519.

EC (2010). Directive 2010/63/EU of the European Parliament and the Council of 22 September on the protection of animals used for scientific purposes. *Official Journal of the European Union, 276*, 33–79.

Eilam, D. (2003). Open-field behavior withstands drastic changes in arena size. *Behavioural Brain Research, 142*, 53–62.

Franks, B., Champagne, F. A., & Higgins, E. T. (2013). How enrichment affects exploration trade-offs in rats: Implications for welfare and well-being. *PLoS One, 8*, e83578.

Friske, J. E., & Gammie, S. C. (2005). Environmental enrichment alters plus maze, but not maternal defense performance in mice. *Physiology & Behavior, 85*, 187–194.

Girbovan, C., & Plamondon, H. (2013). Environmental enrichment in female rodents: Considerations in the effects on behavior and biochemical markers. *Behavioural Brain Research*.

Green, R. E., Melo, B., Christensen, B., Ngo, L., & Skene, C. (2006). Evidence of transient enhancement to cognitive functioning in healthy young adults through environmental enrichment: Implications for rehabilitation after brain injury. *Brain and Cognition, 60*, 201–203.

Gross, A. N., Richter, S. H., Engel, A. K., & Wurbel, H. (2012). Cage-induced stereotypies, perseveration and the effects of environmental enrichment in laboratory mice. *Behavioural Brain Research, 234*, 61–68.

Guic, E., Carrasco, X., Rodriguez, E., Robles, I., & Merzenich, M. M. (2008). Plasticity in primary somatosensory cortex resulting from environmentally enriched stimulation and sensory discrimination training. *Biological Research, 41*, 425–437.

Haemisch, A., Voss, T., & Gartner, K. (1994). Effects of environmental enrichment on aggressive behavior, dominance hierarchies, and endocrine states in male DBA/2J mice. *Physiology & Behavior, 56*, 1041–1048.

Jolliffe, I. T. (1986). *Principal component analysis*. New York: Springer New York.

Jule, K. R., Leaver, L. A., & Lea, S. E. G. (2008). The effects of captive experience on reintroduction survival in carnivores: A review and analysis. *Biological Conservation, 141*, 355–363.

Karelina, K., Hansen, K. F., Choi, Y. S., DeVries, A. C., Arthur, J. S., & Obrietan, K. (2012). MSK1 regulates environmental enrichment-induced hippocampal plasticity and cognitive enhancement. *Learning & Memory, 19*, 550–560.

Kazlauckas, V., Schuh, J., Dall'Igna, O. P., Pereira, G. S., Bonan, C. D., & Lara, D. R. (2005). Behavioral and cognitive profile of mice with high and low exploratory phenotypes. *Behavioural Brain Research, 162*, 272–278.

Kobayashi, S., Ohashi, Y., & Ando, S. (2002). Effects of enriched environments with different durations and starting times on learning capacity during aging in rats assessed by a refined procedure of the Hebb-Williams maze task. *Journal of Neuroscience Research, 70*, 340–346.

Kotrschal, A., & Taborsky, B. (2010). Environmental change enhances cognitive abilities in fish. *PLoS Biology, 8*, e1000351.

Krackow, S., Vannoni, E., Codita, A., Mohammed, A. H., Cirulli, F., Branchi, I., et al. (2010). Consistent behavioral phenotype differences between inbred mouse strains in the IntelliCage. *Genes, Brain and Behavior, 9*, 722–731.

Leger, M., Quiedeville, A., Paizanis, E., Natkunarajah, S., Freret, T., Boulouard, M., et al. (2012). Environmental enrichment enhances episodic-like memory in association with a modified neuronal activation profile in adult mice. *PLoS One, 7*, e48043.

Loss, C. M., Córdova, S. D., Callegari-Jacques, S. M., & de Oliveira, D. L. (2014). Time-of-day influence on exploratory behaviour of rats exposed to an unfamiliar environment. *Behaviour, 151*, 1943–1966.

Marashi, V., Barnekow, A., & Sachser, N. (2004). Effects of environmental enrichment on males of a docile inbred strain of mice. *Physiology & Behavior, 82*, 765–776.

Marquez-Arias, A., Santillan-Doherty, A. M., Arenas-Rosas, R. V., Gasca-Matias, M. P., & Munoz-Delgado, J. (2010). Environmental enrichment for captive stumptail macaques (*Macaca arctoides*). *Journal of Medical Primatology, 39*, 32–40.

Meagher, R. K., & Mason, G. J. (2012). Environmental enrichment reduces signs of boredom in caged mink. *PLoS One, 7*, e49180.

Mesa-Gresa, P., Perez-Martinez, A., & Redolat, R. (2013). Environmental enrichment improves novel object recognition and enhances agonistic behavior in male mice. *Aggressive Behavior, 39*, 269–279.

Monteiro, B. M., Moreira, F. A., Massensini, A. R., Moraes, M. F., & Pereira, G. S. (2014). Enriched environment increases neurogenesis and improves social memory persistence in socially isolated adult mice. *Hippocampus, 24*, 239–248.

Munn, E., Bunning, M., Prada, S., Bohlen, M., Crabbe, J. C., & Wahlsten, D. (2011). Reversed light-dark cycle and cage enrichment effects on ethanol-induced deficits in motor coordination assessed in inbred mouse strains with a compact battery of refined tests. *Behavioural Brain Research, 224*, 259–271.

Panksepp, J. B., Wong, J. C., Kennedy, B. C., & Lahvis, G. P. (2008). Differential entrainment of a social rhythm in adolescent mice. *Behavioural Brain Research, 195*, 239–245.

Pietropaolo, S., Branchi, I., Cirulli, F., Chiarotti, F., Aloe, L., & Alleva, E. (2004). Long-term effects of the periadolescent environment on exploratory activity and aggressive behaviour in mice: Social versus physical enrichment. *Physiology & Behavior, 81*, 443–453.

R Development Core Team (2013). *R: A language and environment for statistical computing*. 3-900051-07-0. Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org/.

Renner, M. (1987). Experience-dependent changes in exploratory behavior in the adult rat (*Rattus norvegicus*): Overall activity level and interactions with objects. *Journal of Comparative Psychology, 101*, 94–100.

Roberts, L. J., Taylor, J., & de Leaniz, C. G. (2011). Environmental enrichment reduces maladaptive risk-taking behavior in salmon reared for conservation. *Biological Conservation, 144*, 1972–1979.

Sampedro-Piquero, P., De Bartolo, P., Petrosini, L., Zancada-Menendez, C., Arias, J. L., & Begega, A. (2014). Astrocytic plasticity as a possible mediator of the cognitive improvements after environmental enrichment in aged rats. *Neurobiology of Learning and Memory, 114C*, 16–25.

Sanguansat, P. (Ed.). (2012). *Principal component analysis*. Rijeka, Croatia: InTech.

Segovia, G., Del Arco, A., De Blas, M., Garrido, P., & Mora, F. (2010). Environmental enrichment increases the in vivo extracellular concentration of dopamine in the nucleus accumbens: A microdialysis study. *Journal of Neural Transmission, 117*, 1123–1130.

Silva, C. F., Duarte, F. S., Lima, T. C., & de Oliveira, C. L. (2011). Effects of social isolation and enriched environment on behavior of adult Swiss mice do not require hippocampal neurogenesis. *Behavioural Brain Research, 225*, 85–90.

Smith, K. S., & Morrell, J. I. (2007). Comparison of infant and adult rats in exploratory activity, diurnal patterns, and responses to novel and anxiety-provoking environments. *Behavioral Neuroscience, 121*, 449–461.

Then, F. S., Luppa, M., Schroeter, M. L., Konig, H. H., Angermeyer, M. C., & Riedel-Heller, S. G. (2013). Enriched environment at work and the incidence of dementia: Results of the Leipzig longitudinal study of the aged (LEILA 75+). *PLoS One, 8*, e70906.

Toth, L. A., Kregel, K., Leon, L., & Musch, T. I. (2011). Environmental enrichment of laboratory rodents: The answer depends on the question. *Comparative Medicine, 61*, 314–321.

van de Weerd, H. A., Baumans, V., Koolhaas, J. M., & van Zutphen, L. F. (1994). Strain specific behavioural response to environmental enrichment in the mouse. *Journal of Experimental Animal Science, 36*, 117–127.

Vazquez-Sanroman, D., Sanchis-Segura, C., Toledo, R., Hernandez, M. E., Manzo, J., & Miquel, M. (2013). The effects of enriched environment on BDNF expression in the mouse cerebellum depending on the length of exposure. *Behavioural Brain Research, 243*, 118–128.

Verma, P., Hellemans, K. G. C., Choi, F. Y., Yu, W., & Weinberg, J. (2010). Circadian phase and sex effects on depressive/anxiety-like behaviors and HPA axis responses to acute stress. *Physiology & Behavior, 99*, 276–285.

Viola, G. G., Botton, P. H., Moreira, J. D., Ardais, A. P., Oses, J. P., & Souza, D. O. (2010). Influence of environmental enrichment on an object recognition task in CF1 mice. *Physiology & Behavior, 99*, 17–21.

Viola, G. G., & Loss, C. M. (2013). Letter to Editor about: "Physical exercise increases GFAP expression and induces morphological changes in hippocampal astrocytes". *Brain Structure and Function*.

Viola, G. G., Rodrigues, L., Americo, J. C., Hansel, G., Vargas, R. S., Biasibetti, R., et al. (2009). Morphological changes in hippocampal astrocytes induced by environmental enrichment in mice. *Brain Research, 1274*, 47–54.

Vivinetto, A. L., Suarez, M. M., & Rivarola, M. A. (2013). Neurobiological effects of neonatal maternal separation and post-weaning environmental enrichment. *Behavioural Brain Research, 240*, 110–118.

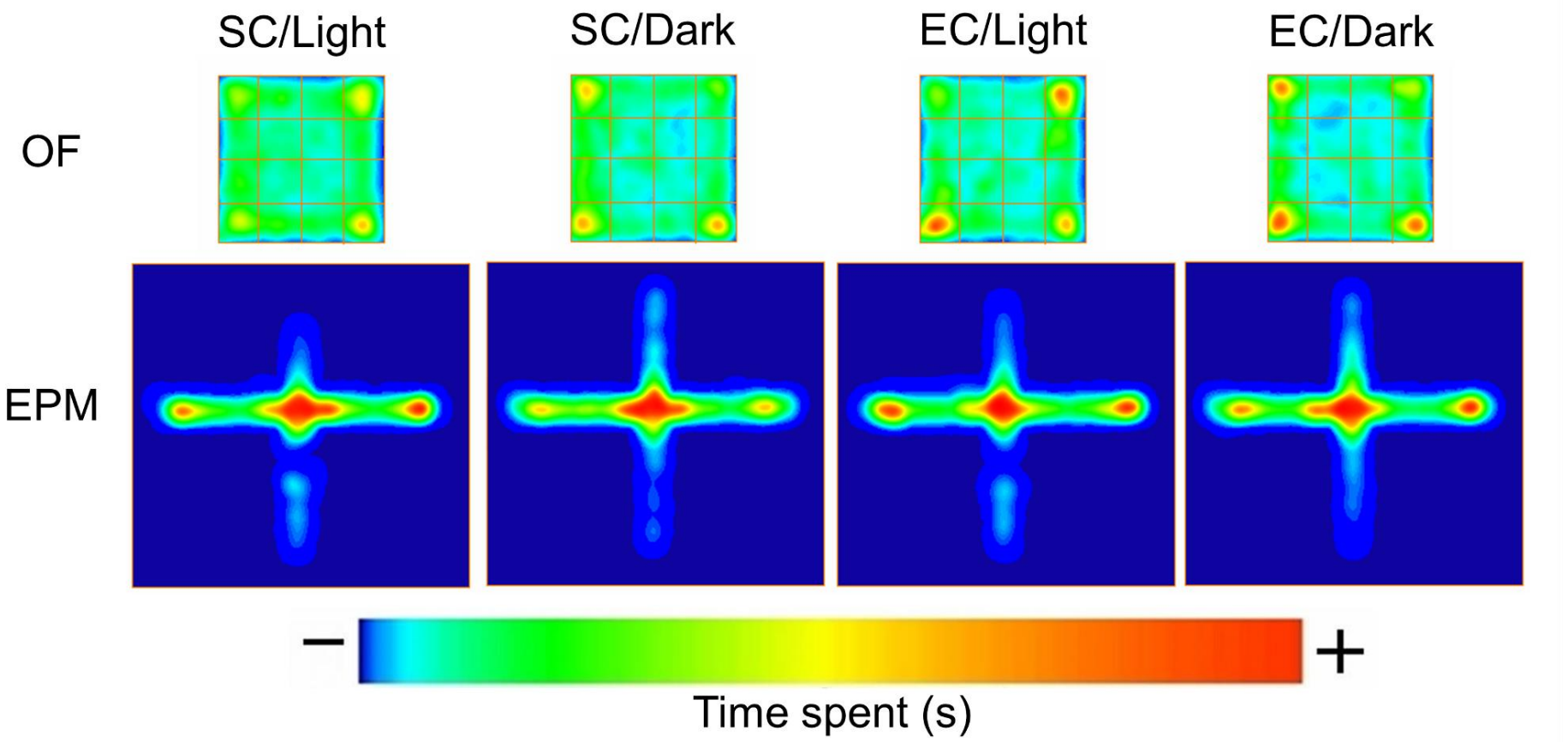
Walf, A. A., & Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols, 2*, 322–328.

Walker, M. D., & Mason, G. (2011). Female C57BL/6 mice show consistent individual differences in spontaneous interaction with environmental enrichment that are predicted by neophobia. *Behavioural Brain Research, 224*, 207–212.

Zadicario, P., Avni, R., Zadicario, E., & Eilam, D. (2005). 'Looping' – An exploration mechanism in a dark open field. *Behavioural Brain Research, 159*, 27–36.

Zambrana, C., Marco, E. M., Arranz, L., de Castro, N. M., Viveros, M. P., & de la Fuente, M. (2007). Influence of aging and enriched environment on motor activity and emotional responses in mice. *Annals of the New York Academy of Sciences, 1100*, 543–552.

Zhou, P., Werner, J. H., Lee, D., Sheppard, A. D., Liangpunsakul, S., & Duffield, G. E. (2015). Dissociation between diurnal cycles in locomotor activity, feeding behavior and hepatic PERIOD2 expression in chronic alcohol-fed mice. *Alcohol*.



Supplementary Fig. S1. Occupancy plots representing mice behavior during the OFT (on Top) and the EPM (on Bottom). The yellowish-red points in the center of the EPM indicate that mice were probably immobile in the center to assessing a potential risk of the open areas. The higher incidence of yellowish-red points in the corners of the OFT and inside the closed arms of the EPM, in contrast with the green-blue areas in the open spaces (center zone of the OFT and open arms of the EPM), indicate that mice prefer to stay immobile in safe areas.

Supplementary Table S1- Summary of the models fitted to data on the total time of objects' exploration (T).

Model	AICc	k	ΔAICc	w_i
log(T) ~ HC + TD + S + (S|Id)	112.3	12	-	0.4278
log(T) ~ HC + TD + S + TD:S + (S|Id)	112.3	14	0.0	0.4262
log(T) ~ HC + TD + S + HC:TD + (S|Id)	116.0	13	3.7	0.0688
log(T) ~ HC + TD + S + HC:TD + TD:S + (S|Id)	116.1	15	3.8	0.0652
log(T) ~ HC + TD + S + HC:S + (S|Id)	121.0	14	8.6	0.0057
log(T) ~ HC + TD + S + HC:S + TD:S + (S|Id)	121.4	16	9.1	0.0046
log(T) ~ HC + TD + S + HC:TD + HC:S + (S|Id)	124.7	15	12.4	<0.001
log(T) ~ HC + TD + S + HC:TD + HC:S + TD:S + (S|Id)	125.3	17	12.9	<0.001
log(T) ~ HC + (S|Id)	137.3	9	25.0	<0.001
log(T) ~ HC + TD + (S|Id)	140.2	10	27.9	<0.001
log(T) ~ S + (S|Id)	142.1	10	29.7	<0.001
log(T) ~ HC + TD + HC:TD + (S|Id)	143.8	11	31.4	<0.001
log(T) ~ 1 + (S|Id)	170.1	8	57.8	<0.001
log(T) ~ TD + (S|Id)	173.4	9	61.1	<0.001

AICc: Akaike's Information Criterion corrected for small sample size; k: number of parameters in the models; DAICc: change in AICc for each model in relation to the best-fitted model; wi: Akaike weight; HC: housing condition; TD: time-of-day; S: trial session; '+': additive effects; ' : ' : interaction effects. For all models it was considered an additive error effect term relative to the trial session and the individual identification (S:Id).

Supplementary Table S2- Meaning of the parameters used in the PCA.

Parameter	Behavioral category			
	Locomotor activity	Exploratory activity	Spatial distribution	Anxiety-like behavior
Open Field				
Distance traveled	X	X		
Locomoting time	X	X		
Average speed	X	X		
Stops	X	X		
Inter-stop distance	X	X		
Distance traveled in perimeter			X	X
Locomoting time in perimeter			X	X
Time spent in perimeter			X	X
Object Recognition				
Object exploration		X		
Elevated Plus Maze				
Distance traveled	X	X		
Time immobile	X	X		
Risk assessment behavior		X		X
Distance in closed arms			X	X
Time in closed arms			X	X
Entries into closed arms	X			X
Distance on open arms			X	X
Time on open arms			X	X
Entries on open arms	X			X

PCA was performed on a set of eighteen behavioral variables (eight from OF, one from OR and nine from EPM). Each parameter represents one or more behavioral category.

Supplementary Table S3 - Principal component analysis using data of 39 rats in the OF, ORT and EPM.

		PC1	PC2	PC3	PC4	PC5	PC6	PC7
Correlation coefficients	Distance traveled in OF	0.690	0.130	-0.351	-0.206	0.077	-0.227	0.297
	Locomoting time in OF	0.879	0.040	-0.042	0.042	0.080	0.184	0.177
	Average speed	-0.489	0.035	-0.522	0.404	-0.300	0.020	0.251
	Number of stops	-0.794	0.099	-0.037	-0.176	0.026	-0.164	-0.315
	Inter-stop distance	0.497	-0.096	0.189	0.207	0.066	0.468	-0.365
	Distance traveled in perimeter	-0.050	-0.218	0.101	0.800	0.172	0.132	0.168
	Locomoting time in perimeter	-0.368	-0.005	0.580	0.097	0.351	-0.090	0.492
	Time spent in perimeter	-0.281	-0.302	0.259	0.183	-0.537	0.181	0.024
	Object exploration	0.491	0.003	-0.073	0.177	-0.248	-0.359	-0.387
	Distance traveled in EPM	-0.023	0.691	0.026	0.238	-0.404	0.302	0.110
	Time immobile	-0.126	-0.207	-0.103	0.178	0.737	-0.143	-0.135
	Risk assessment behavior	-0.357	0.044	-0.188	-0.205	0.321	0.563	-0.242
	Distance in closed arms	0.050	-0.643	0.162	-0.350	-0.090	-0.004	0.390
	Time in closed arms	0.149	-0.235	0.613	0.352	-0.061	-0.408	-0.248
	Entries into closed arms	0.179	0.614	0.544	-0.110	0.138	0.376	0.031
	Distance on open arms	-0.019	0.541	0.241	-0.130	-0.040	-0.366	-0.068
	Time on open arms	0.046	0.558	-0.357	0.431	0.291	-0.157	0.069
	Entries on open arms	-0.163	0.824	0.173	-0.105	-0.001	-0.170	0.083
	Eigenvalues	3.053	2.829	1.814	1.599	1.537	1.444	1.155
	% of variance	16.96	15.72	10.08	8.88	8.54	8.02	6.42
	Cumulative variance (%)	16.96	32.68	42.76	51.64	60.18	68.20	74.62

Correlation coefficients between each principal component (PC) and each behavioral variable representing locomotor and exploratory activity, spatial distribution, and anxiety-like behaviors as well as eignevalues (variances) and percentage of variance explained by each PC. PCs which presented eigenvalues lower than 1 were disregarded.

CAPÍTULO III

Artigo em processo de escrita para publicação

Effects of early life transient NMDAR and GluN2B-containing NMDAR antagonisms in adult behavior: interaction with rearing environments

Cássio Morais Loss*, Natã Sehn da Rosa, Natividade de Sá Pereira, Fabrício Figueiró, Giordano Gubert Viola, Diogo Losch de Oliveira.

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Justificativa: Evidências crescentes têm fortalecido a hipótese glutamatérgica de hipofunção de NMDAR da esquizofrenia. Estudos tem demonstrado que o bloqueio neonatal de NMDAR desencadeia fenótipos esquizofrênicos na idade adulta. A subunidade GluN2B é altamente expressa em períodos precoces do desenvolvimento, e, portanto, NMDAR contendo essa subunidade podem ser responsáveis pelas alterações comportamentais induzidas pela hipofunção de NMDAR na infância.

Objetivo geral: Investigar a influência do bloqueio neonatal de NMDAR contendo a subunidade GluN2B e da estimulação da reserva cognitiva e encefálica durante a infância sobre comportamento de animais adultos.

Objetivo específico: Investigar se o bloqueio neonatal de NMDAR induz alterações comportamentais tipo-esquizofrênicas; Investigar se o enriquecimento ambiental durante a infância é capaz de prevenir as alterações comportamentais induzidas pelo bloqueio de NMDAR durante idades precoces do desenvolvimento encefálico.

Effects of early life transient NMDAR and GluN2B-containing NMDAR antagonisms in adult behavior: interaction with rearing environments

Cássio Morais Loss[1,a,*], Natã Sehn da Rosa[1,a], Natividade de Sá Pereira[1], Fabrício Figueiró[1], Giordano Gubert Viola, Diogo Losch de Oliveira[1]

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rio Grande do Sul – Brazil

[a]These authors have equally contributed to this work.

* Corresponding author: Cássio Morais Loss, Departamento de Bioquímica, ICBS, UFRGS. Rua Ramiro Barcelos 2600-Anexo. Zip code: 90035-003. Porto Alegre, RS, BRAZIL. Phone: +55 51 33085555. Fax: +55 51 33085540. E-mail: cassio.m.loss@gmail.com

Abstract

Early development NMDA receptor (NMDAR) signaling disruption can cause long-term behavioral abnormalities with relevance to schizophrenia. Brain and cognitive reserve (BCR) stimulation positively modulates brain disorders susceptibility and age-dependent dysfunctions, via neuroprotective and/or compensatory mechanisms. Here, we studied if neonatal GluN2B-containing NMDAR blockade or neonatal full NMDAR population blockade causes similar long-lasting behavioral abnormalities and if these behavioral changes are prevented by early life BCR stimulation. Pregnant female Wistar rats were kept in standard (SH) or enriched housing (EH). From PND5 to PND10 pups received two daily i.p. injections of saline 0.9% (SAL), ketamine (25 mg/kg - KET) or CI-1041 (10 mg/kg – CI). At PND30, all pups were kept in standard housing. Elevated plus maze (EPM), open field (OF) and inhibitory avoidance tasks were conducted from PND60 to PND66. Data were analyzed by Principal Component Analysis. Although full NMDAR population blockade did not induce any behavioral alteration when compared to SAL groups, animals from SH+CI group presented better habituation performance than SH+SAL and SH+KET groups, and maintaining animals in EH reversed this effect. Also, EH animals presented reduced locomotion in the EPM related to decreased exposure to potentially unsafe places as the environment becomes familiar and reduced neophobic response to alternate between two distinct environments. Our results indicate that different neonatal NMDAR populations blockade lead to distinct expression of behavior during adulthood. Our data gives further support to the concept of BCR stimulation promoting neuroplastic changes in the brain and reinforce the need of using tools that allow the identification of subtle alterations in behavioral patterns of rodents.

Keywords: schizophrenia, ketamine, CI-1041, environmental enrichment, development, behavior.

Introduction

Glutamatergic abnormality in schizophrenia was first reported by Kim and colleagues (1980) based on reduced glutamate levels in the cerebrospinal fluid of individuals with schizophrenia. Since then, several indications that NMDA receptor (NMDAR)-mediated glutamate neurotransmission is reduced in this brain disorder were reported (Javitt and Zukin 1991; Moghaddam, Adams et al. 1997; Olney, Newcomer et al. 1999; Javitt 2007; Bondi, Matthews et al. 2012; Poels, Kegeles et al. 2014) strengthened the NMDAR hypofunction hypothesis of schizophrenia (Olney and Farber 1995; Moghaddam, Adams et al. 1997; Adams and Moghaddam 1998; Olney, Newcomer et al. 1999; Hashimoto and Iyo 2002; Hashimoto, Fukushima et al. 2003; Bondi, Matthews et al. 2012). While acute NMDAR hypofunction was observed to induce a schizophrenia-like psychosis in adult human and non-human individuals, transient NMDAR blockade during early development has been demonstrated to induce long-lasting behavioral abnormalities that mimic schizophrenia symptoms and endophenotypes (Wang, McInnis et al. 2001; Sircar 2003; Stefani and Moghaddam 2005; du Bois and Huang 2007; Baier, Blume et al. 2009; Lu, Mamiya et al. 2010; Uehara, Sumiyoshi et al. 2010; Akillioglu, Binokay et al. 2012).

Functional NMDAR are heterotetrameric complexes containing at least one GluN1 subunit and some combination of GluN2 and/or GluN3 subunits (Di Maio, Mastroberardino et al. 2011). The glutamate-binding site is located in the GluN2 subunits being GluN2A and GluN2B the Central Nervous System (CNS)-predominant expressed GluN2 subunits (Cull-Candy, Brickley et al. 2001; Paoletti 2011). The expression pattern of these two subunits differs strikingly in both time (during development) and space (from brain region until cell location), in which drastic changes occur during the first 2 weeks following birth (Paoletti 2011). GluN2A expression

(which is abundantly found in the entire adult CNS) gradually increases after birth while GluN2B expression (which occurs since embryonic development) peaks around postnatal day (P) 7–10, a stage at which GluN2A expression is rising sharply. After that, GluN2B expression progressively becomes restricted to forebrain areas (cortex, hippocampus, striatum, olfactory bulb), where it remains expressed at quite high levels (Monyer, Burnashev et al. 1994; Sheng, Cummings et al. 1994; Cull-Candy, Brickley et al. 2001; Paoletti 2011). This prevalence of GluN2B-containing NMDAR during early stages of brain development is consistent with its high expression in early postnatal synapses (Petralia, Sans et al. 2005; Petralia, Al-Hallaq et al. 2009) and axonal growth cones (Ehlers, Fung et al. 1998; Herkert, Rottger et al. 1998; Wang, Petralia et al. 2011) since it is crucial for neuritogenesis and fasciculation of young neurons (Georgiev, Taniura et al. 2008). Thus, interference in the GluN2B-containing NMDAR functionality during childhood could often be the basis of cognitive deficits and changes in emotionality observed in psychiatric disorders, including schizophrenia.

Growing attention is being paid to the concept of brain and cognitive reserve (BCR) stimulation as an alternative non-pharmacological strategy for the treatment and prevention of CNS disorders (Stern 2002; Nithianantharajah and Hannan 2009; Nithianantharajah and Hannan 2011; Gehres, Rocha et al. 2016). BCR concept is based on the fact that individuals with higher lifetime levels of social, physical, and cognitive engagement present increased cognitive function, and enhanced complex mental activity. Thus, BCR stimulation positively modulates brain disorders susceptibility and age-dependent dysfunction, via neuroprotective and/or compensatory mechanisms (Nithianantharajah and Hannan 2009). In animal models, BCR stimulation can be accomplished through an environmental manipulation termed “environmental enrichment” (EE) (van Praag, Kempermann et al. 2000; Nithianantharajah and Hannan

2006; Viola, Botton et al. 2010; Loss, Binder et al. 2015) which consists in provides to animals a high social, physical, and cognitive stimulating environment (Girbovan and Plamondon 2013). Despite the evidences pointing to the benefits of BCR stimulation, Akillioglu and co-workers (Akillioglu, Babar Melik et al. 2012) observed that keeping post-weaning animals in enriched environments did not reverse the lasting emotional and cognitive impairments caused by transient NMDAR blockade during early development. Nevertheless, some evidences suggest that more potent BCR neuroprotective effects can be observed when pups (and their mothers) are exposed to EE during a pre-weaning period due to both enhanced maternal care and increased social, physical, and cognitive stimulation (Cancedda, Putignano et al. 2004; Sale, Putignano et al. 2004; Sale, Cenni et al. 2007; Baldini, Restani et al. 2013).

Here, we aimed to investigate the influence that early exposition to EE exerts on schizophrenic phenotypes induced by transient NMDAR blockade. Our experiment was designed to answer two independent questions: (1) are the GluN2B-containing NMDAR involved in the long-lasting behavioral abnormalities that mimic schizophrenia symptoms induced by transient NMDAR blockade during early development? We expected that animals which were subjected to GluN2B-containing NMDAR antagonism will present a schizophrenic-like phenotype since GluN2B subunit is highly expressed during development; (2) is pre-weaning BCR stimulation, through EE manipulations, capable to prevent or ameliorate the putative schizophrenia-like phenotype induced by transient NMDAR blockade during early development? We expected that animals subjected to EE will present at least a reduction in the schizophrenic-like behavioral abnormalities.

Material and methods

Animals

Twenty pregnant female Wistar rats (3 months old) were kept in standard housing (SH) or in enriched housing (EH). The lodging occurred immediately after mating (ED0) and the mothers stayed in those environments until weaning. After birth, the puppies were kept with their mothers in the same housing condition until they reached 21-days-old (PND21 – weaning day). After weaning, the offspring stayed in the same housing condition until they were 30-days-old. From PND30 until the end of the experiment, all the animals were kept in standard housing in groups of 2-3 animals/cage. All animals were maintained under a 12:12 h controlled light/dark photoperiod cycle (lights on at 7:00 AM) and room temperature adjusted to 21±1 ºC, with unlimited access to water and standard rodent food during the entire experiment. Animals in each experimental group always came from different litters, with a minimum of nine litters even for the smallest groups, to prevent litter effects from occurring.

Transient NMDAR blockade

Administration of NMDAR antagonists were based on studies of Akillioglu and colleagues (Akillioglu, Babar Melik et al. 2012; Akillioglu, Binokay et al. 2012). Rat pups (male and female) were injected from PND5 to PND10 with (i) ketamine (a non-selective NMDAR antagonist) at a dose of 25 mg/kg; (ii) CI-1041 (a GluN2B-containing NMDAR-selective antagonist) at a dose of 10 mg/kg; or (iii) saline (0.9% NaCl). The volume of injection was 5 ml/kg, and the drugs were administered twice a day (11:00 AM.–6:00 PM.), intraperitoneally.

Housing conditions

Enriched housing apparatus consisted of three Plexiglas box (55.5 x 41.5 x 40 cm each) interconnected by 10 cm diameter plastic tubes (please see Figure 1). Each cage was divided in two floors, except during the second gestational and second postnatal weeks (as described below). In the two boxes of the corners the floor was always covered with sawdust, while in the middle box the floor topping was changed once a week. The different toppings consisted of sawdust, small stones, falcon tubes, plastic chips, plastic bottle caps, small marbles and paper towels. The base of the second floor was always a metal grid. All cages contained a variety of objects with distinct sizes and shapes, including wood, plastic, metal and glass made objects, nesting material and hiding places (such tunnels for instance), in order to represent eco-ethological expansions for rats including the sense of security and to provide a place where they could avoid open spaces and luminosity (a natural behavior of wild rodents). Running wheels (for adults) were just available during the second gestational and second postnatal weeks. During these periods the second floor of one of the cages was removed to accommodate the wheel. Because not all the rats run when running wheels are available (Novak, Burghardt et al. 2012), we choose not to maintain the running wheels available for the entire housing to minimize the data variability without depriving animals from running. Small running wheels (for pups) were available from PND17 to PND30. Additional cognitive stimulation regarding the formation of spatial mapping was provided by changing half of the objects and by shifting their positions twice a week. In addition, although water and food were available out of the cages (through the top metal grid), food pellets were also available inside the cages to stimulate the natural rodents' behavior of stocking and hoarding food. Based in previous studies (Cancedda, Putignano et al. 2004; Sale, Putignano et al. 2004), female rats were

housed in groups of four (being 2-3 rats pregnant). The non-pregnant rats provide both social stimulation for the mothers and increased pups' care by acting like "babysitters" in the periods in which the mothers are absent from the nest foraging for water and food. At ED21, the mothers were isolated in one of the EH cages and stayed there with their own offspring until PND4, when they were putted back together with free access to all the EH compartments.

Standard housing consisted of one pregnant rat kept alone with their own offspring during the entire experiment in a Plexiglas box (40.5 x 33.5 x 18 cm) containing just sawdust.

Battery of behavioral tests

Behavioral and observational tests to assess maturation and development of offspring were conducted in female pups during the period leading up to weaning (PND21), which include sensory-motor reflexes, neuromotor behaviors, and physical development stages (Chen, Tang et al. 2012; Juliano, Sosunov et al. 2015) (Table 1). Care was taken to minimize the duration of separation from the dam. In addition, during adulthood, male rats were subjected to behavioral tests to evaluate the emotionality and cognition (PND60 to PND66) (Table 1).

Developmental Landmarks

Based in Chen et al. (2012), the emergence of physical maturation landmarks was noted each day, including incisor eruption (the first appearance of upper incisors), eye opening (when both eyelids were completely separated) and development of fur (the entire body appeared to be covered in white fur). All female pups in each litter were assessed every scheduled day even after attaining each milestone.

Neonatal Sensory and Motor Development

Surface righting reflex test, Negative geotaxis test, Cliff aversion test, and grip strength test were measured based in Chen et al. (2012).

In the surface righting reflex test, pups were gently removed from the litter and placed on a warming pad (34 ºC). Pups were timed from the moment of being placed in supine position until it had righted itself and all four feet were in contact with the surface. Pups were tested just once in PND12.

In the negative geotaxis test, pups were timed for completing a 180º turn when placed in a head down position on a 25º inclined surface. Latency to rotate 180º on the inclined surface was measured during a 120-s test. If the pup fell, crawled off the plane, or made no movement, the rat was considered to have failed the task, and a maximum time of 120s was assigned. Pups were tested just once in PND12.

In the cliff aversion test, pups were placed with their heads and forepaws over the edge of a table. The latency to retract their body (to turn the body or to crawl away) 1 cm from the edge was recorded. Pups were tested just once in PND12.

In the grip strength test, pups were allowed to grip on a metal grid which initially was in a vertical position and then was gently moved to a horizontal position. The moving time from vertical to horizontal position was 10s. Once horizontal position was achieved, the grid stayed immobile until the pup drop the grid. The length of time (including the moving grid time) the pup was able to hold on the bar unaided before dropping was recorded. Pups were tested three times on PND12 with a 30s intertrial interval.

Emotionality and cognition

Animals were handled individually for two minutes for three consecutive days prior to the initiation of the behavioral tasks. The handling procedure was performed to allow the animals to habituate to the researchers. In order to acclimate, animals were put on behavioral testing room one hour before the beginning of tasks at controlled temperature (21 ± 1 ºC) and illumination (see below the illumination settings for each behavioral task) used during the tests.

The elevated plus maze (EPM) task was performed according to Loss et al. (2014). The maze was constructed from wood and the walls and floor were painted black. The maze consisted of two open arms (50 x 10 cm surrounded by 1 cm high railing running along their length), two closed arms (50 x 10 x 46 cm), with the arms of each type opposite to each other, and a central platform (10 x 10 cm) between arms. The maze was 50 cm elevated from the floor. EPM task was performed during the night period (between 7:00 PM and 12:00 PM). The testing room was illuminated using a red lamp located 2m above the central platform of the apparatus in order to obtain a uniform red-light intensity of 30 Lux on open arms. Each rat was individually placed into the maze center facing to one of the closed arms and was left free to explore the apparatus for 5 min. The maze was cleaned with a 70% ethanol solution between each trial to eliminate odor cues. The ANY-Maze video-tracking system (Stoelting, CO) was used to automatically recording and collecting the behavioral data. The following parameters were analyzed: (1) Anxiety levels: (a) Time spent in the open arms. (b) Time spent in the closed arms. (c) Number of entries into the open arms: an arm entry was defined as all four paws in an arm. (d) Number of entries into the closed arms. (e) Distance traveled in the open arms. (f) Distance traveled in the closed arms. (g) Time spent in the central area. (h) Number of risk assessment behaviors: number of exploration of the open arm through stretch-attend posture (when the rodent is motionless in center- or

closed-zone, but has its body stretched forward into the open arms by placing some but not all paws, returning then to the same position); (2) Exploratory activity in the apparatus: (a) Total distance traveled in both open and closed arms. (b) Total time immobile.

The open field (OF) task was performed according to Loss et al. (2014). As the EPM apparatus, the OF apparatus was constructed from wood and the walls and floor were painted black. The maze was a square box with dimensions of 50 x 50 x 50 cm. The floor of apparatus was virtually divided into 16 squares (4 central and 12 peripheral). The testing room was illuminated using two white-lamps directed toward the ceiling of the room to obtain a 5 Lux light intensity that was uniformly distributed throughout the arena. The illumination settings during the test were the same for all of the experimental groups. Each animal was individually placed in the arena center and it was left free to explore it for 10 min. The apparatus was cleaned with a 70% ethanol solution between trials to eliminate odor cues. The ANY-Maze video-tracking system (Stoelting, CO) was used to automatically recording and collecting the behavioral data. The following parameters were analyzed: (1) Locomotor activity: (a) distance traveled: overall distance traveled during the 10 min observation; (b) locomoting time: duration of locomoting periods, expressed as percentage of total time of test; (c) average speed: distance traveled divided by locomoting time; (d) number of stops: the incidence of ‘‘non-locomoting’’ intervals that were bounded by ‘‘locomoting’’ intervals. A ‘‘non-locomoting’’ interval was registered when at least 65% of animal’s body stayed immobile by a period equal or greater than 1 s; (e) inter-stops distance: average metric distance traveled between two consecutive stops (total distance traveled divided by the total number of stops). (2) Spatial distribution: (a) distance traveled in the center: the total distance traveled in the center of the arena divided by the total distance traveled

during the test multiplied by 100; (b) locomoting time in the center: the locomoting time in the center of the arena divided by the total locomoting time during the test multiplied by 100; (c) time spent on center: the time in which the rat was in the center divided by the total time of the test multiplied by 100; (d) time spent at the home base: the time spent immobile in the home base zone in the last five minutes of test divided by 300 multiplied by 100. The "home base" was defined as the square (zone) with the highest rank after ranking all of the squares based on the accumulated "non-locomoting" intervals (Eilam and Golani 1989; Eilam and Golani 1990). In the cases in which the difference between the highest ranked zone and the subsequent zones was equal to or less than 5 seconds, the home base was considered as the zone with the highest number of stops (trips) between that zones. (3) Temporal organization of locomotion and exploration: (a) number of trips: the number of times an individual moved out and returned to the home base; (b) trip length: the average metric distance travelled per trip calculated as the total distance travelled divided by the total number of trips; and (c) stops/trip: the average number of stops per trip, calculated as the total number of stops divided by the number of trips. Based on Loss et al. (2014), parameters related to home base (i.e. time spent at the home base, number of trips, trip length and stops/trip) were not evaluated in the first five minutes of test because home base was probably not yet defined by the animal during this period.

Inter-session habituation to the OF was analyzed by dividing the amount of a given behavior in OF1 (distance traveled, locomoting time and number of stops for locomotor activity; distance traveled in the center, locomoting time in the center and time spent on center for spatial distribution and anxiety-like behaviors) by the same behavior in OF2.

To evaluation of aversive long-term associative memory rats were trained in one-trial step-down inhibitory avoidance task based on Vianna et al. (2001). The apparatus consisted of a box (50.5 cm long x 25 wide x 29 cm high) containing a 9.5 cm long x 25 cm wide wood platform elevated 5 cm from a 40.5 x 25 cm grid of bronze bars spaced 1 cm apart. The testing room was illuminated using a white lamp located in the ceiling of the room in order to obtain a white light intensity of 200 Lux inside the inhibitory avoidance box. During the training session, the rats were gently placed on the platform and maintained there for 3s. After that, rats were allowed to free explore the box. When the rat stepped down with their four paws onto the grid, three intermittent 0.6-mA (1s each) scrambled foot shocks were immediately delivered to the grid. Immediately after this the rats were gently conducted back to the platform, maintained there for 3s and taken back to their home cages. A test session was performed 24h later. This session was procedurally identical to the training session except that the foot shocks were omitted. Latency to step down with their four paws onto the grid was measured in both sessions. If a rat did not step down onto the grid during a 180s period, a maximum time of 180s was assigned.

Statistics

Data of body weight are expressed as mean ± S.E.M. and were analyzed by Two-way ANOVA with repeated measures (within-subjects factor: age; between-subjects factor 1: Housing Condition; between-subjects factor 2: NMDAR antagonist) followed by Sidak's multiple comparisons post hoc test. Data of neurodevelopment were first analyzed by Principal Component Analysis (PCA) with Varimax Rotation and Kaizer Normalization. Principal Components (PC) scores were extracted by regression

method and analyzed by Two-way ANOVA (between-subjects factor 1: Housing Condition; between-subjects factor 2: NMDAR antagonist) followed by Sidak's multiple comparisons post hoc test.

Aversive long-term associative memory in the inhibitory avoidance task (comparison of latency to step-down in training session and latency to step-down in testing session for each individual group) was analyzed by Wilcoxon matched-pairs signed rank test. In addition, the latency to step-down the platform in testing session was compared between group trough Two-way ANOVA (between-subjects factor 1: Housing Condition; between-subjects factor 2: NMDAR antagonist).

PCA with Varimax Rotation and Kaizer Normalization was also performed in data from adult emotional and cognition behaviors (Loss, Córdova et al. 2014; Loss, Binder et al. 2015). Principal Components (PC) scores were extracted by regression method and analyzed by Two-way ANOVA (between-subjects factor 1: Housing Condition; between-subjects factor 2: NMDAR antagonist) followed by Sidak's multiple comparisons post hoc test. A significance level of 0.05 was set for all analyses.

Results

Effect of housing conditions and transient NMDAR blockade on weight gain of the offspring

Since birth weight (PND3) was minor in EH animals (7.4±0.19) compared to SH animals (8.0±0.17) [$t(22)= 2.15$; $P= 0.042$], offspring weight gain was analyzed as PNDn-PND5, being PNDn the day in which body weight was measured while PND5

was the first day of NMDAR antagonist treatment. Two-way ANOVA with repeated measures revealed a significant Time x Housing Conditions within-subjects effect [$F(1.157)= 5.95$; $P< 0.014$], but not a significant Time x NMDAR antagonist effect [$F(2.313)= 0.85$; $P= 0.445$] neither a Time x Housing Condition x NMDAR antagonist within-subjects effect [$F(2.313)= 1.01$; $P= 0.927$] on offspring weight gain. This indicates that EH offspring gained weight more slowly along the time than did SH offspring (Figure 2). Moreover, significant NMDAR antagonist [$F(2,63)= 9.44$; $P< 0.001$] and Housing Condition [$F(1,63)= 17.33$; $P< 0.001$] but not NMDAR antagonist x Housing Condition between-subjects effects [$F(2,63)= 0.56$; $P= 0.575$] were observed. This indicates that in general, body weight of SH are greater than EH animals, and that in general, body weight of SAL are greater than KET and CI animals.

Effect of housing conditions and transient NMDAR blockade on neurodevelopment of the offspring

To evaluation of neurodevelopment we performed a PCA with the variables measured during development, i.e. the emergence of physical maturation landmarks (incisor eruption, eye opening and development of fur) and sensory and motor development (negative geotaxis, cliff aversion and grip strength). Surface righting reflex test was not included in the analysis because all animals reached the criterion in 1s (data not shown). PCA with the six original measures resulted in two principal components (PC). The accumulated eigenvalues of these principal components (PC1 and PC2) was capable of explaining 52.49% of the total data variability. Correlation coefficients between measures and each specific PC are depicted in Figure 3, and suggest that PC1 was associated with physical maturation and PC2 with sensory and motor development.

When groups were analyzed by their PC1 scores, Two-way ANOVA revealed a

significant NMDAR antagonist effect [F(2,74)= 4.37; P= 0.016] but not Housing Condition [F(1,74)= 3.30; P= 0.073] neither NMDAR antagonist x Housing Condition effects [F(2,74)= 0.06; P= 0.946]. Animals which were injected with CI developed more slowly than animals injected with SAL (P= 0.01). Regarding PC2 scores, Two-way ANOVA also revealed a significant NMDAR antagonist effect [F(2,68)= 15.63; P< 0.0001] but not Housing Condition [F(1,68)= 1.81; P= 0.183] neither NMDAR antagonist x Housing Condition effects [F(2,68)= 2.18; P= 0.121]. Animals from CI groups presented higher latency to retract their body away from the cliff's edge in the cliff aversion test and to drop the bar in the grip strength test when compared to SAL and KET groups (P< 0.0001).

Effect of housing conditions and transient NMDAR blockade on emotionality and cognition

In relation to evaluation of the aversive long-term associative memory in the inhibitory avoidance task, Two-way ANOVA did not point significant effect of NMDAR antagonist [F(2,57)= 0.13; P= 0.883], Housing Condition [F(1,57)< 0.01; P= 0.992] nor NMDAR antagonist x Housing Condition interaction [F(2,57)= 2.64; P= 0.08]. In addition, Wilcoxon matched-pairs signed rank test revealed significant differences between the first (training) and second (testing) sessions for all groups (P< 0.01 for all groups – see Supplementary Table S1).

In addition, adult emotional and cognitive behaviors were also evaluated through PCA (Loss, Córdova et al. 2014; Loss, Binder et al. 2015). We used a set of forty-three behavioral variables (thirty-two from OFT day one (OF1) and day two (OF2), ten from EPM and one from inhibitory avoidance task (testing session) – Supplementary Table S1 and Supplementary Table S2), which resulted in eleven PCs (Figure 4). The

accumulated eigenvalues of these principal components (PC1–PC11) was capable of explaining 86.95% of the total data variability. Interpretation of the meaning of each PC was based on the correlation coefficients between the original behavioral measures and each specific PC (Table 2).

When groups were analyzed by their PC1 scores, Two-way ANOVA revealed significant effect of NMDAR antagonist [$F(2,59)= 4.49$; $P= 0.015$], Housing Condition [$F(1,59)= 5.10$; $P= 0.028$] and NMDAR antagonist x Housing Condition interaction [$F(2,59)= 4.52$; $P= 0.015$]. Animals which reared in EH presented elevated locomotor activity in OF2 (have habituated less) when compared to animals reared in SH (main effect). In addition, animals injected with CI presented reduced locomotor activity in OF2 (have habituated more) when compared to animals injected with KET (main effect; $P= 0.045$). Moreover, SH+CI group presented a reduced locomotor activity in OF2 (have habituated more) when compared with SH+SAL and SH+KET ($P< 0.01$), while no differences were observed between EH+SAL, EH+KET and EH+CI (Figure 5). Regarding, PC6 scores, Two-way ANOVA revealed significant effect of Housing Condition [$F(1,61)= 7.96$; $P= 0.007$] but not of NMDAR antagonist [$F(2,61)= 1.27$; $P= 0.289$] nor NMDAR antagonist x Housing Condition interaction [$F(2,61)= 0.52$; $P= 0.598$]. Animals which reared in EH presented reduced locomotor activity in EPM associated to greater habituation of central exploration of the OF (i.e. decreased exploration of center zone in OF2 in relation to OF1) when compared to animals reared in SH (main effect). With respect to PC11 scores, Two-way ANOVA revealed significant effect of Housing Condition [$F(1,62)= 16.01$; $P< 0.001$] but not of NMDAR antagonist [$F(2,62)= 0.45$; $P= 0.639$] nor NMDAR antagonist x Housing Condition interaction [$F(2,62)= 0.12$; $P= 0.889$]. Animals which reared in EH presented reduced scores for wariness when compared to animals reared in SH (main effect). No

significant effect of NMDAR antagonist, Housing Condition or NMDAR antagonist x Housing Condition interaction were observed to PC2, PC3, PC4, PC5, PC7, PC8, PC9 and PC10 (data not shown).

Discussion

Here we demonstrated that interferences in the functionality of different NMDAR subtypes during early development lead to distinct expression of behavior during adulthood. Also, we confirm that animals reared in enriched environments behave different from animals reared in impoverished environments. Nevertheless, our hypothesis that early exposition to EE could slow down (or even stop) the evolution of schizophrenia-like phenotype induced by transient NMDAR blockade during early development could not be confirmed. In the following sections we discuss in detail experimental evidence which give support to our findings.

Neurodevelopment is affected by early life transient NMDAR antagonism and housing condition

Consistent with other finds, kept the pregnant mother and their offspring in EH significantly affected body weight of the offspring (Van de Weerd, Van Loo et al. 1997; Tsai, Oppermann et al. 2003; Sale, Cenni et al. 2007). However, in contrast with these studies, we found a reduction in birth weight of the offspring from EH mothers when compared to pups from SH mothers. Also, in accordance to others (Fiala, Snow et al. 1977; Moncek, Duncko et al. 2004; Pena, Prunell et al. 2006; Pena, Prunell et al. 2009; Hughes and Collins 2010), we observed a more slowly pattern of weight gain in EH pups when compared to SH pups. Although we cannot attribute the weight gain reduction to physical activity in running wheels because running wheels were not

significantly used for rat pups to actually running in it (earliest running-burst observed in the wheels occurred at PND26), enhanced physical activity cannot be overlooked since animals can exercising by other ways (e.g. running in the large size home cage, climbing, gripping, "playing of fight"). Also, a reduction in eating time was already reported in EH animals (Fiala, Snow et al. 1977), and so, it is possible that EH rats decreased time spent eating because they were engaged in other activities. Therefore, we believe that this reduced weight gain could be explained by increased physical activity (Moncek, Duncko et al. 2004) related to higher energy expenditure/energy intake ratio and reduced stress levels in EH rats since kept animals in EE increases energy expenditure, decreases adiposity, reduce the chance of developing obesity and induces a white to brown fat phenotypic switch by activating the hypothalamic-sympathoneural-adipocyte axis (Cao, Liu et al. 2010; Cao, Choi et al. 2011; Slater and Cao 2015).

In addition, a surprisingly lack of effect of housing condition on physical maturation and sensory and motor development was observed. Previous studies (Cancedda, Putignano et al. 2004; Sale, Putignano et al. 2004; Sale, Cenni et al. 2007) demonstrated an accelerated maturation of visual system (assessed by measuring not only physical maturation through eye opening timing, but also by measuring visual acuity behaviorally and electrophysiologically). So, we were expecting that pre- and post-natal exposure to EE would accelerate physical maturation and sensory and motor development in our study. This conflicting results between studies could be explained by the intrinsic characteristics of each species(strain), the components of the EE or the duration of the experiments (Akillioglu, Babar Melik et al. 2012; Viola and Loss 2014).

On the other hand, as expected, transient NMDAR antagonism also altered body weight of animals (Stefani and Moghaddam 2005; Kawabe, Iwasaki et al. 2007).

Animals which received KET or CI presented reduced weight gain when compared to SAL injected animals. Our data suggest that NMDAR antagonism during early development affect weight gain irrespective if the full NMDAR population or just GluN2B-containing NMDAR population is blockade. Also, early life transient GluN2B-containing NMDAR blockade, but not full NMDAR population blockade, altered physical maturation besides sensory and motor development. Animals injected with GluN2B-containing NMDAR antagonist presented a delayed development when compared to SAL and KET injected animals. These data are supported by the fact that different locations and compositions of NMDAR reflect different physiological functions of these receptors (Hardingham, Fukunaga et al. 2002; Li, Chen et al. 2002; Petralia 2012). However, we were expecting that both KET and CI would affect neurodevelopment in a similar way since GluN2B was the predominant expressed NMDAR subunit at the age in which NMDAR antagonism occurred (PND5-PND10) (Monyer, Burnashev et al. 1994; Sheng, Cummings et al. 1994; Cull-Candy, Brickley et al. 2001; Paoletti 2011). Further investigation is needed for better elucidation of this issue.

Adult emotional and cognitive behaviors

As previous commented, our hypothesis that early exposition to EE could slow down (or even stop) the evolution of schizophrenia-like phenotype induced by transient NMDAR blockade during early development could not be confirmed. This occurred because different from others (Stefani and Moghaddam 2005; Kawabe, Iwasaki et al. 2007; Akillioglu, Babar Melik et al. 2012; Akillioglu, Binokay et al. 2012), in our study full NMDAR population blockade did not induce any behavioral alteration (including aversive long-term associative memory in the inhibitory avoidance task) when compared to SAL injected animals. In fact, Kawabe et al. (2007) and Stefani and

Moghaddam (2005) demonstrated that transient NMDAR blockade during early development induced spatial and working memory impairments during adulthood without locomotor activity alterations in OF task. In contrast, by comparing two different mice strains (BALB/c and C57BL/6), Akillioglu et al. (2012) observed that transient NMDAR blockade during early development induced alterations in both BALB/c and C57BL/6 mice locomotor activity and anxiety-like behaviors in OF and EPM tasks. Nevertheless, these alterations occurred in a strain-dependent manner, being that just one anxiety-like variable in OF (only in C57BL/6 mice but not in BALB/c mice) was influenced by chronic neonatal MK-801 administration. These results were not replicated in a second study of the same group (Akillioglu, Babar Melik et al. 2012) using the same mice strain (BALB/c) where MK-801 treated animals did not presented the same pattern of alteration either in time spent in the center of the OF (SAL = MK-801 in the former and SAL > MK-801 in the second study) or in time spent in the open arms of the EPM (SAL < MK-801 in the former and SAL > MK-801 in the second study). Despite that, spatial impairment in MK-801 treated animals was also observed by Akillioglu et al. (2012). These contrasting results give further support for the species(strain)-specific effects of early life transient NMDAR blockade on animal behavior and highlights the necessity of further investigation of these phenomenon.

In addition, an unexpected difference in adult behavior of animals which had the full NMDAR population blockade in relation to rats which had just the GluN2B-containing NMDAR population blockade was observed. Since GluN2B was the predominant expressed NMDAR subunit at the age in which NMDAR antagonism occurred, we expected that KET and CI groups would present similar behaviors. However, animals from SH+CI group presented better habituation memory performance than SH+SAL and SH+KET groups, and maintaining animals in EH reversed this

effect. It was already shown that GluN2B-containing NMDAR plays key role on learning and memory (Tang, Shimizu et al. 1999; Brim, Haskell et al. 2013; Hanson, Weber et al. 2013; Hanson, Meilandt et al. 2014). GluN2B-containing NMDAR blockade was demonstrated to disrupt learning (Brim, Haskell et al. 2013; Davies, Greba et al. 2013; Hanson, Weber et al. 2013; Hanson, Meilandt et al. 2014) while the enhancement of GluN2B expression improved learning in both young and aged animals (Tang, Shimizu et al. 1999; Cao, Cui et al. 2007; Brim, Haskell et al. 2013). A possible explanation for our finds is that transient blocking GluN2B-containing NMDAR during early periods of brain development induces long-term enhancement of GluN2B subunit expression through a negative-feedback mechanism, which may be related to a better habituation performance during adulthood. However, further investigation is needed.

In relation to the effects of Housing Condition on animals behavior, we observed that animals which reared in EH presented elevated locomotor activity in OF2 (have habituated less) when compared to animals reared in SH. This result is mostly due to the reversion of CI effects observed in EH+CI group, since no differences were found either between SH+SAL and EH+SAL or SH+KET and EH+KET groups. In fact, negative effects of EE on animal cognition, in general, are not reported (van Praag, Kempermann et al. 2000; Nithianantharajah and Hannan 2006; Nithianantharajah and Hannan 2009; Viola, Botton et al. 2010; Nithianantharajah and Hannan 2011; Loss, Binder et al. 2015). Therefore, although a negative interaction between early life NMDAR blockade and EE on spatial learning was already reported (Akillioglu, Babar Melik et al. 2012) we believe that data regarding diminished habituation in EH animals do not represent cognitive disarrangement in our study. Nevertheless, a negative interaction between early life GluN2B-containing NMDAR blockade and EE exposure cannot be discarded. Moreover, we found that animals reared in EH presented a reduced locomotion in the

EPM related to decreased exposure to potentially dangerous environments in familiar environments when compared to unfamiliar environments (see PC6). In addition, a reduced neophobic response to alternate between two distinct environments (see PC11) was also observed in EH animals. These data give further support to the already proposed hypothesis (Loss, Binder et al. 2015) that keeping animals in EE lead to reduction of neophobic responses and accelerates the process of habituation stimulating the expression of more evolutionary propitious behaviors in mice, such as defensive behaviors. In fact, the increasing in self-protective behaviors as the environment becomes familiar (as indexed by PC6) linked to an increased probability to enter in a potentially dangerous unfamiliar environments (as indexed by PC11) indicates that animals reared in EE reduce their exposition to potentially dangerous environments when there is no longer the need (familiar environment) without stopping exploring the environment when it is still unfamiliar.

Conclusions

In this study we demonstrated that early life transient NMDAR blockade by ketamine administration did not alter Wistar rats' behavior during early adulthood in the three behavioral paradigms evaluated, OF, EPM and inhibitory avoidance task. In contrast, we showed that interferences in the functionality of different NMDAR populations (full NMDAR population x GluN2B-containing NMDAR population) during early development lead to distinct expression of behavior during adulthood. Also, we confirm that animals reared in enriched environments behave different from animals reared in impoverished environments. We postulated that EE reinforces the innate behavioral features of animals, e.g., EE seems to triggers animals wild behavior.

In accordance, EH animals reduced their exposition to potentially unsafe places when the environment was already explored (became familiar), which seems to follow natural conditions, where rodents do not have to expose themselves to risk when it is no longer needed. Importantly, these differences represent subtle alterations in the Wistar rats' behavior and were only recognized by employing the PCA. Therefore, our data provide behavioral support to the concept of EE promoting neuroplastic changes in the brain and reinforce the need of using tools that allow the identification of subtle alterations in behavioral patterns of rodents.

Competing interests

The authors declare that they have no competing interests.

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References

Adams, B. and B. Moghaddam (1998). "Corticolimbic dopamine neurotransmission is temporally dissociated from the cognitive and locomotor effects of phencyclidine." J Neurosci **18**(14): 5545-5554.

Akillioglu, K., E. Babar Melik, et al. (2012). "The investigation of neonatal MK-801 administration and physical environmental enrichment on emotional and cognitive functions in adult Balb/c mice." Pharmacol Biochem Behav **102**(3): 407-414.

Akillioglu, K., S. Binokay, et al. (2012). "The effect of neonatal N-methyl-D-aspartate receptor blockade on exploratory and anxiety-like behaviors in adult BALB/c and C57BL/6 mice." Behav Brain Res **233**(1): 157-161.

Baier, P. C., A. Blume, et al. (2009). "Early postnatal depletion of NMDA receptor development affects behaviour and NMDA receptor expression until later adulthood in rats--a possible model for schizophrenia." Behav Brain Res **205**(1): 96-101.

Baldini, S., L. Restani, et al. (2013). "Enriched early life experiences reduce adult anxiety-like behavior in rats: a role for insulin-like growth factor 1." J Neurosci **33**(28): 11715-11723.

Bondi, C., M. Matthews, et al. (2012). "Glutamatergic animal models of schizophrenia." Curr Pharm Des **18**(12): 1593-1604.

Brim, B. L., R. Haskell, et al. (2013). "Memory in aged mice is rescued by enhanced expression of the GluN2B subunit of the NMDA receptor." Behav Brain Res **238**: 211-226.

Cancedda, L., E. Putignano, et al. (2004). "Acceleration of visual system development by environmental enrichment." J Neurosci **24**(20): 4840-4848.

Cao, L., E. Y. Choi, et al. (2011). "White to brown fat phenotypic switch induced by genetic and environmental activation of a hypothalamic-adipocyte axis." Cell Metab **14**(3): 324-338.

Cao, L., X. Liu, et al. (2010). "Environmental and genetic activation of a brain-adipocyte BDNF/leptin axis causes cancer remission and inhibition." Cell **142**(1): 52-64.

Cao, X., Z. Cui, et al. (2007). "Maintenance of superior learning and memory function in NR2B transgenic mice during ageing." Eur J Neurosci **25**(6): 1815-1822.

Chen, C., Y. Tang, et al. (2012). "Early postnatal benzo(a)pyrene exposure in Sprague-Dawley rats causes persistent neurobehavioral impairments that emerge postnatally and continue into adolescence and adulthood." Toxicol Sci **125**(1): 248-261.

Cull-Candy, S., S. Brickley, et al. (2001). "NMDA receptor subunits: diversity, development and disease." Curr Opin Neurobiol **11**(3): 327-335.

Davies, D. A., Q. Greba, et al. (2013). "GluN2B-containing NMDA receptors and AMPA receptors in medial prefrontal cortex are necessary for odor span in rats." Front Behav Neurosci **7**: 183.

Di Maio, R., P. G. Mastroberardino, et al. (2011). "Pilocapine alters NMDA receptor expression and function in hippocampal neurons: NADPH oxidase and ERK1/2 mechanisms." Neurobiol Dis.

du Bois, T. M. and X. F. Huang (2007). "Early brain development disruption from NMDA receptor hypofunction: relevance to schizophrenia." Brain Res Rev **53**(2): 260-270.

Ehlers, M. D., E. T. Fung, et al. (1998). "Splice variant-specific interaction of the NMDA receptor subunit NR1 with neuronal intermediate filaments." J Neurosci **18**(2): 720-730.

Eilam, D. and I. Golani (1989). "Home base behavior of rats (Rattus norvegicus) exploring a novel environment." Behav Brain Res **34**(3): 199-211.

Eilam, D. and I. Golani (1990). "Home base behavior in amphetamine-treated tame wild rats (Rattus norvegicus)." Behav Brain Res **36**(1-2): 161-170.

Fiala, B., F. M. Snow, et al. (1977). ""Impoverished" rats weigh more than "enriched" rats because they eat more." Dev Psychobiol **10**(6): 537-541.

Gehres, S. W., A. Rocha, et al. (2016). "Cognitive Intervention As an Early Non-pharmacological Strategy in Alzheimer's Disease: A Translational Perspective." Front Aging Neurosci **8**: 280.

Georgiev, D., H. Taniura, et al. (2008). "A critical importance of polyamine site in NMDA receptors for neurite outgrowth and fasciculation at early stages of P19 neuronal differentiation." Exp Cell Res **314**(14): 2603-2617.

Girbovan, C. and H. Plamondon (2013). "Environmental enrichment in female rodents: Considerations in the effects on behavior and biochemical markers." Behav Brain Res.

Hanson, J. E., W. J. Meilandt, et al. (2014). "Chronic GluN2B antagonism disrupts behavior in wild-type mice without protecting against synapse loss or memory impairment in Alzheimer's disease mouse models." J Neurosci **34**(24): 8277-8288.

Hanson, J. E., M. Weber, et al. (2013). "GluN2B antagonism affects interneurons and leads to immediate and persistent changes in synaptic plasticity, oscillations, and behavior." Neuropsychopharmacology **38**(7): 1221-1233.

Hardingham, G. E., Y. Fukunaga, et al. (2002). "Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways." Nat Neurosci **5**(5): 405-414.

Hashimoto, K., T. Fukushima, et al. (2003). "Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia." Arch Gen Psychiatry **60**(6): 572-576.

Hashimoto, K. and M. Iyo (2002). "[Glutamate hypothesis of schizophrenia and targets for new antipsychotic drugs]." Nihon Shinkei Seishin Yakurigaku Zasshi **22**(1): 3-13.

Herkert, M., S. Rottger, et al. (1998). "The NMDA receptor subunit NR2B of neonatal rat brain: complex formation and enrichment in axonal growth cones." Eur J Neurosci **10**(5): 1553-1562.

Hughes, R. N. and M. A. Collins (2010). "Enhanced habituation and decreased anxiety by environmental enrichment and possible attenuation of these effects by chronic alpha-tocopherol (vitamin E) in aging male and female rats." Pharmacol Biochem Behav **94**(4): 534-542.

Javitt, D. C. (2007). "Glutamate and schizophrenia: phencyclidine, N-methyl-D-aspartate receptors, and dopamine-glutamate interactions." Int Rev Neurobiol **78**: 69-108.

Javitt, D. C. and S. R. Zukin (1991). "Recent advances in the phencyclidine model of schizophrenia." Am J Psychiatry **148**(10): 1301-1308.

Juliano, C., S. Sosunov, et al. (2015). "Mild intermittent hypoxemia in neonatal mice causes permanent neurofunctional deficit and white matter hypomyelination." Exp Neurol **264**: 33-42.

Kawabe, K., T. Iwasaki, et al. (2007). "Repeated treatment with N-methyl-d-aspartate antagonists in neonatal, but not adult, rats causes long-term deficits of radial-arm maze learning." Brain Res **1169**: 77-86.

Kim, J. S., H. H. Kornhuber, et al. (1980). "Low cerebrospinal fluid glutamate in schizophrenic patients and a new hypothesis on schizophrenia." Neurosci Lett **20**(3): 379-382.

Li, B., N. Chen, et al. (2002). "Differential regulation of synaptic and extra-synaptic NMDA receptors." Nat Neurosci **5**(9): 833-834.

Loss, C. M., L. B. Binder, et al. (2015). "Influence of environmental enrichment vs. time-of-day on behavioral repertoire of male albino Swiss mice." Neurobiol Learn Mem **125**: 63-72.

Loss, C. M., S. D. Córdova, et al. (2014). "Time-of-day influence on exploratory behaviour of rats exposed to an unfamiliar environment." **151**(14): 1943-1966.

Lu, L., T. Mamiya, et al. (2010). "Prenatal exposure to phencyclidine produces abnormal behaviour and NMDA receptor expression in postpubertal mice." Int J Neuropsychopharmacol **13**(7): 877-889.

Moghaddam, B., B. Adams, et al. (1997). "Activation of glutamatergic neurotransmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex." J Neurosci **17**(8): 2921-2927.

Moncek, F., R. Duncko, et al. (2004). "Effect of environmental enrichment on stress related systems in rats." J Neuroendocrinol **16**(5): 423-431.

Monyer, H., N. Burnashev, et al. (1994). "Developmental and regional expression in the rat brain and functional properties of four NMDA receptors." Neuron **12**(3): 529-540.

Nithianantharajah, J. and A. J. Hannan (2006). "Enriched environments, experience-dependent plasticity and disorders of the nervous system." Nat Rev Neurosci **7**(9): 697-709.

Nithianantharajah, J. and A. J. Hannan (2009). "The neurobiology of brain and cognitive reserve: mental and physical activity as modulators of brain disorders." Prog Neurobiol **89**(4): 369-382.

Nithianantharajah, J. and A. J. Hannan (2011). "Mechanisms mediating brain and cognitive reserve: experience-dependent neuroprotection and functional compensation in animal models of neurodegenerative diseases." Prog Neuropsychopharmacol Biol Psychiatry **35**(2): 331-339.

Novak, C. M., P. R. Burghardt, et al. (2012). "The use of a running wheel to measure activity in rodents: relationship to energy balance, general activity, and reward." Neurosci Biobehav Rev **36**(3): 1001-1014.

Olney, J. W. and N. B. Farber (1995). "Glutamate receptor dysfunction and schizophrenia." Arch Gen Psychiatry **52**(12): 998-1007.

Olney, J. W., J. W. Newcomer, et al. (1999). "NMDA receptor hypofunction model of schizophrenia." J Psychiatr Res **33**(6): 523-533.

Paoletti, P. (2011). "Molecular basis of NMDA receptor functional diversity." Eur J Neurosci **33**(8): 1351-1365.

Pena, Y., M. Prunell, et al. (2006). "Environmental enrichment effects in social investigation in rats are gender dependent." Behav Brain Res **174**(1): 181-187.

Pena, Y., M. Prunell, et al. (2009). "Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats." Psychoneuroendocrinology **34**(9): 1390-1404.

Petralia, R. S. (2012). "Distribution of extrasynaptic NMDA receptors on neurons." ScientificWorldJournal **2012**: 267120.

Petralia, R. S., R. A. Al-Hallaq, et al. (2009). "Trafficking and Targeting of NMDA Receptors."

Petralia, R. S., N. Sans, et al. (2005). "Ontogeny of postsynaptic density proteins at glutamatergic synapses." Mol Cell Neurosci **29**(3): 436-452.

Poels, E. M., L. S. Kegeles, et al. (2014). "Imaging glutamate in schizophrenia: review of findings and implications for drug discovery." Mol Psychiatry **19**(1): 20-29.

Sale, A., M. C. Cenni, et al. (2007). "Maternal enrichment during pregnancy accelerates retinal development of the fetus." PLoS One **2**(11): e1160.

Sale, A., E. Putignano, et al. (2004). "Enriched environment and acceleration of visual system development." Neuropharmacology **47**(5): 649-660.

Sheng, M., J. Cummings, et al. (1994). "Changing subunit composition of heteromeric NMDA receptors during development of rat cortex." Nature **368**(6467): 144-147.

Sircar, R. (2003). "Postnatal phencyclidine-induced deficit in adult water maze performance is associated with N-methyl-D-aspartate receptor upregulation." Int J Dev Neurosci **21**(3): 159-167.

Slater, A. M. and L. Cao (2015). "A Protocol for Housing Mice in an Enriched Environment." J Vis Exp(100): e52874.

Stefani, M. R. and B. Moghaddam (2005). "Transient N-methyl-D-aspartate receptor blockade in early development causes lasting cognitive deficits relevant to schizophrenia." Biol Psychiatry **57**(4): 433-436.

Stern, Y. (2002). "What is cognitive reserve? Theory and research application of the reserve concept." J Int Neuropsychol Soc **8**(3): 448-460.

Tang, Y. P., E. Shimizu, et al. (1999). "Genetic enhancement of learning and memory in mice." Nature **401**(6748): 63-69.

Tsai, P. P., D. Oppermann, et al. (2003). "The effects of different rack systems on the breeding performance of DBA/2 mice." Lab Anim **37**(1): 44-53.

Uehara, T., T. Sumiyoshi, et al. (2010). "Neonatal exposure to MK-801, an N-methyl-D-aspartate receptor antagonist, enhances methamphetamine-induced locomotion and disrupts sensorimotor gating in pre- and postpubertal rats." Brain Res **1352**: 223-230.

Van de Weerd, H. A., P. L. Van Loo, et al. (1997). "Nesting material as environmental enrichment has no adverse effects on behavior and physiology of laboratory mice." Physiol Behav **62**(5): 1019-1028.

van Praag, H., G. Kempermann, et al. (2000). "Neural consequences of environmental enrichment." Nat Rev Neurosci **1**(3): 191-198.

Vianna, M. R., L. A. Izquierdo, et al. (2001). "Pharmacological differences between memory consolidation of habituation to an open field and inhibitory avoidance learning." Braz J Med Biol Res **34**(2): 233-240.

Viola, G. G., P. H. Botton, et al. (2010). "Influence of environmental enrichment on an object recognition task in CF1 mice." Physiol Behav **99**(1): 17-21.

Viola, G. G. and C. M. Loss (2014). "Letter to Editor about: "Physical exercise increases GFAP expression and induces morphological changes in hippocampal astrocytes"." Brain Struct Funct **219**(4): 1509-1510.

Wang, C., J. McInnis, et al. (2001). "Long-term behavioral and neurodegenerative effects of perinatal phencyclidine administration: implications for schizophrenia." Neuroscience **107**(4): 535-550.

Wang, P. Y., R. S. Petralia, et al. (2011). "Functional NMDA receptors at axonal growth cones of young hippocampal neurons." J Neurosci **31**(25): 9289-9297.

FIGURE LEGENDS

Fig. 1. Schematic representation of the Housing Conditions. Enriched Housing is depicted in Top; Standard Housing is depicted in Bottom.

Fig. 2. Effect of housing conditions and transient NMDAR blockade on weight gain of the offspring. Weight gain was calculated as PNDn-PND5, being PNDn the day in which body weight was measured while PND5 was the first day of NMDAR antagonist treatment. (a) Comparison of the body weight variation between Housing Conditions. EH offspring gained weight more slowly along the time than did SH offspring ($P< 0.014$); (b) Comparison of the body weight variation between NMDAR antagonists. Body weight of SAL group is greater than KET ($P= 0.043$) and CI groups ($P< 0.001$). Data were analyzed by Two-way ANOVA with repeated measures.

Fig. 3. Effect of housing conditions and transient NMDAR blockade on neurodevelopment of the offspring. A PCA with the variables measured during development was performed to evaluation of offspring neurodevelopment. The matrix correlation between the original variables and each individual PC (PC1 and PC2) are presented in a and b. Dashed lines in a and b (values 0.5 and -0.5 in the Y-axis) indicate the cutoff points (i.e., the variables which presented correlation values > -0.5 and < 0.5 are not represented in the graphs). Comparison between groups with respect to the PC1 and PC2 values are presented in (c and d, respectively). Data from c and d are expressed as the medians and the interquartile ranges and were analyzed using Two-way ANOVA followed by Sidak's multiple comparisons test. Outliers (represented by filled circles)

are depicted in the graphs but were not included in the analysis. * indicates P< 0.05 when compared with CI groups.

Fig. 4. Adult emotional and cognitive behaviors analyzed by PCA: correlation coefficients between the original behavioral measures and each specific PC. A PCA with the variables from OFT (OF1 and OF2), EPM and from inhibitory avoidance task (testing session) resulted in eleven PCs. The matrix correlation between the original variables and each individual PC (PC1-PC11) are presented. Dashed lines (values 0.5 and -0.5 in the Y-axis) indicate the cutoff points (i.e., the variables which presented correlation values > -0.5 and < 0.5 are not represented in the graphs).

Fig. 5. Adult emotional and cognitive behaviors analyzed by PCA: comparison between groups. Data from (a) PC1, (b) PC6 and (c) PC11 are expressed as the medians and the interquartile ranges and were analyzed using Two-way ANOVA followed by Sidak's multiple comparisons test. Outliers (represented by filled circles) are depicted in the graphs but were not included in the analysis. * indicates P< 0.05 when compared with CI groups. $ indicates P< 0.05 when compared with SH groups. # indicates P< 0.05 when compared with SH+CI group.

Table 1- Battery of behavioral tests

Behavioral and observational tests	PND
Developmental landmarks	
Eye opening	Evaluated every day until each outcome is achieved
Incisor eruption milestone	
Development of fur	
Sensory-motor reflexes	
Surface righting reflex test	PND12
Negative geotaxis test	PND12
Cliff aversion test	PND12
Grip strength test	PND12
Emotionality and cognition	
Elevated plus maze task	PND 60
Open field task	PND 62 and PND 63
Inhibitory avoidance task	PND 65 and PND 66

Developmental landmarks and sensory-motor reflexes were conducted only in female pups;
Emotional and cognitive tasks were conducted only in male rats;

Table 2 - Interpretation of the meaning of each PC generated by PCA using data of 68 rats in the EPM, OF1, OF2 and Inhibitory Avoidance tasks.

Principal component	% of variance explained	Meaning associated
PC1	12.52%	Locomotor activity in OF2 negatively related to habituation in locomotor activity between OF1 and OF2
PC2	12.24%	Anxiogenic-like behaviors in EPM
PC3	11.72%	General locomotor activity in both OF1 and OF2
PC4	10.63%	Central exploration in a familiar environment (anxiolytic-like behaviors in OF2)
PC5	7.33%	Central exploration in unfamiliar environment (anxiolytic-like behaviors in OF1)
PC6	7.32%	Locomotor activity in the EPM related to changes in anxiolytic-like behaviors between unfamiliar and familiar environments
PC7	6.43%	Exploration from home base in an unfamiliar environment
PC8	6.08%	Exploration from home base in a familiar environment
PC9	4.74%	Exploration outside the home base (without immobility)
PC10	4.30%	Average speed in both OF1 and OF2
PC11	3.66%	Neophobic responses to alternate between well-established landmarks dividing contrasting environments
Total variance explained		86.95%

Interpretation of the meaning of each PC was based on the correlation coefficients between behavioral measures and each specific PC; Percentage of variance explained by each PC and total variance explained is depicted; PCs which presented eigenvalues lower than 1 were disregarded.

Figure 1.

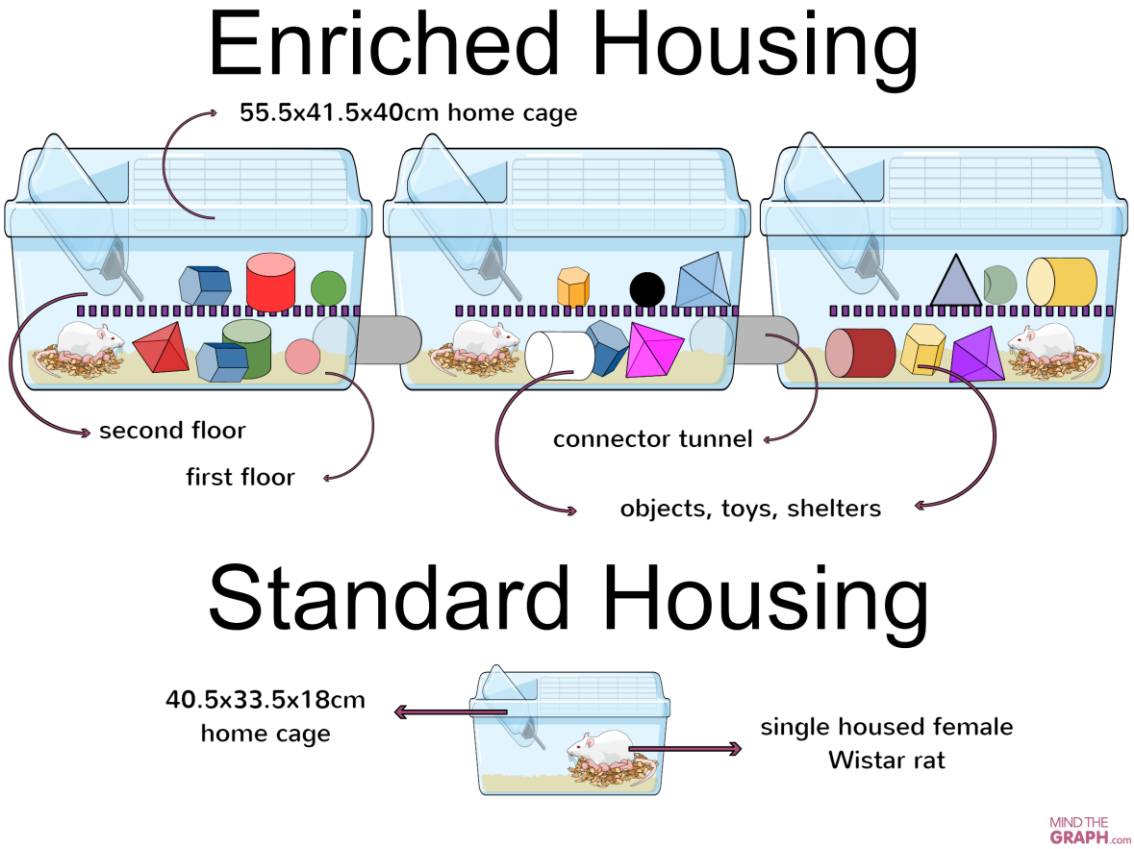

Enriched Housing
55.5x41.5x40cm home cage
second floor
first floor
connector tunnel
objects, toys, shelters
Standard Housing
40.5x33.5x18cm
home cage
single housed female
Wistar rat
MIND THE GRAPH.com

Figure 2.

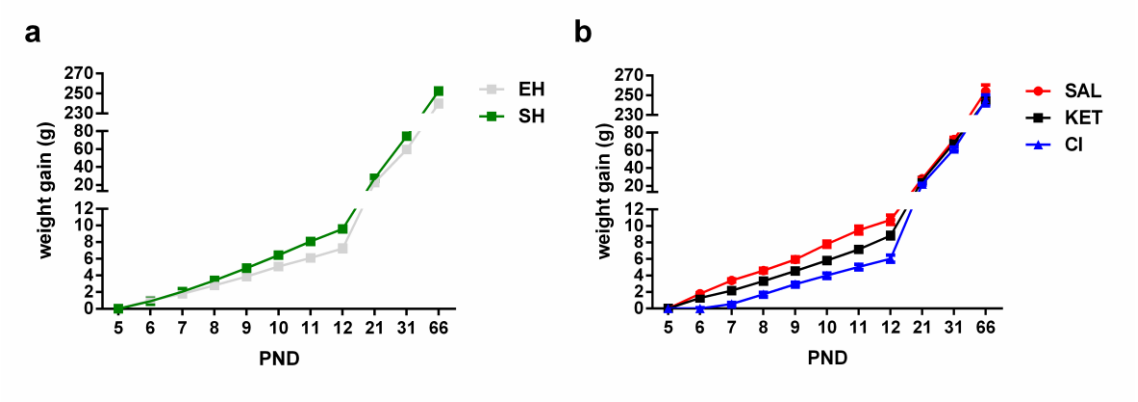


Figure 3.

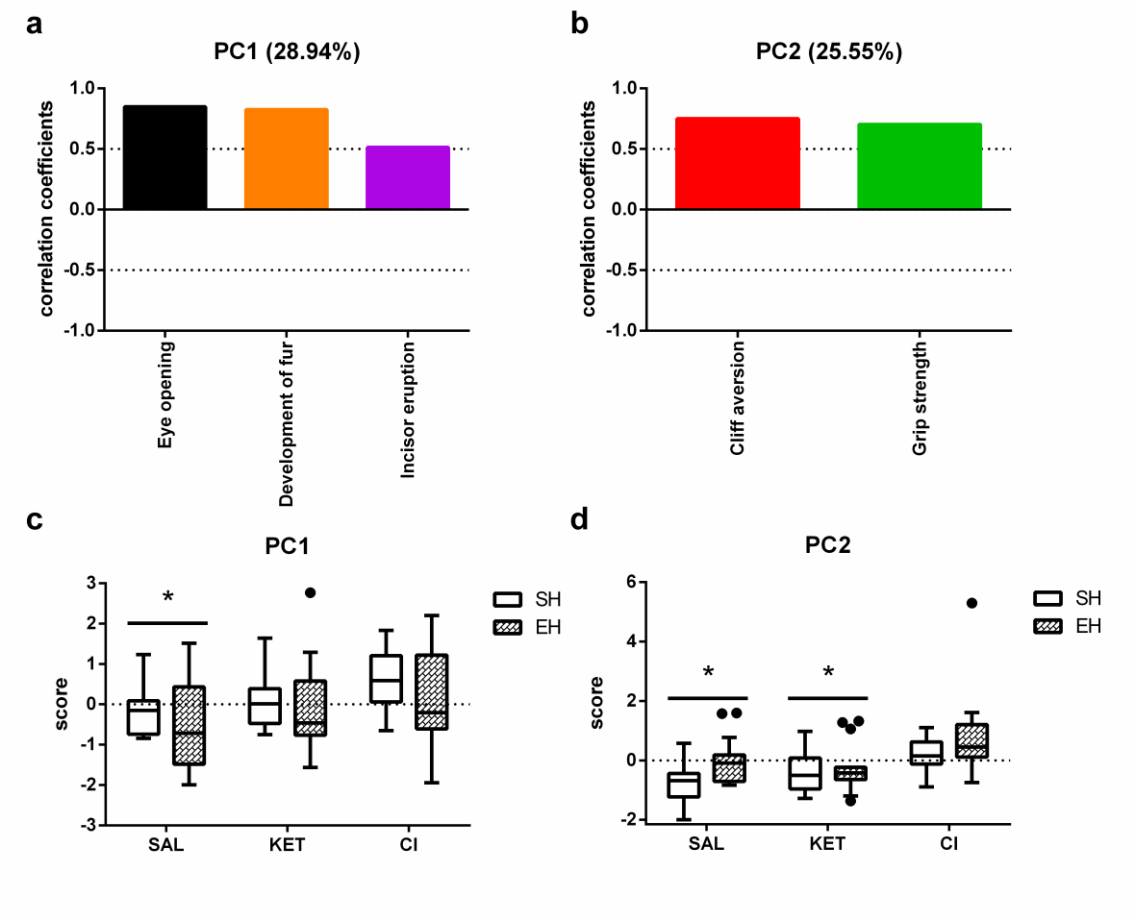

a
PC1 (28.94%)
correlation coefficients
1.0
0.5
0.0
-0.5
-1.0
Eye opening
Development of fur
Incisor eruption
b
PC2 (25.55%)
correlation coefficients
1.0
0.5
0.0
-0.5
-1.0
Cliff aversion
Grip strength
c
PC1
score
3
2
1
0
-1
-2
-3
*
SAL
KET
CI
SH
EH
d
PC2
score
6
4
2
0
-2
*
*
SAL
KET
CI
SH
EH

Figure 4.

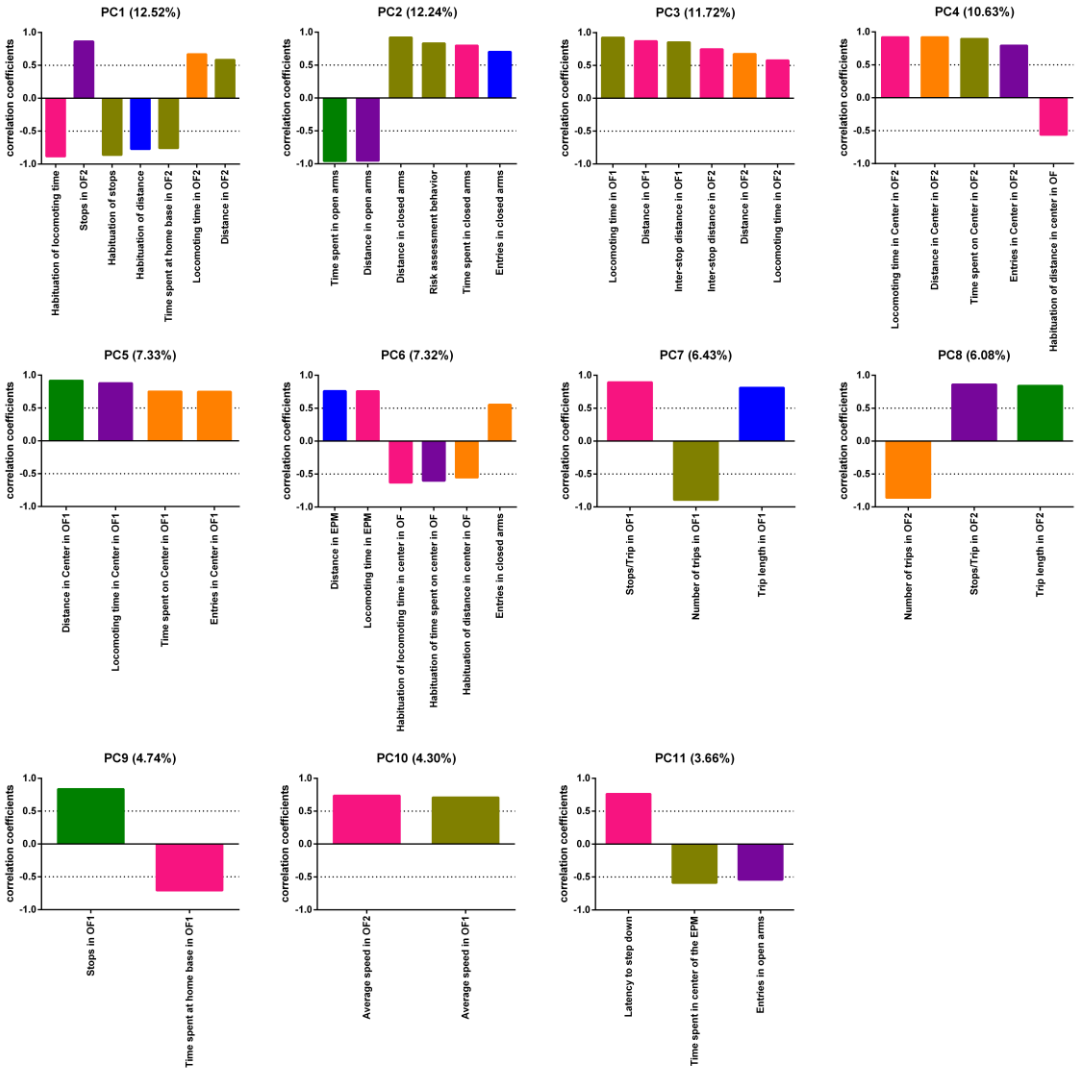
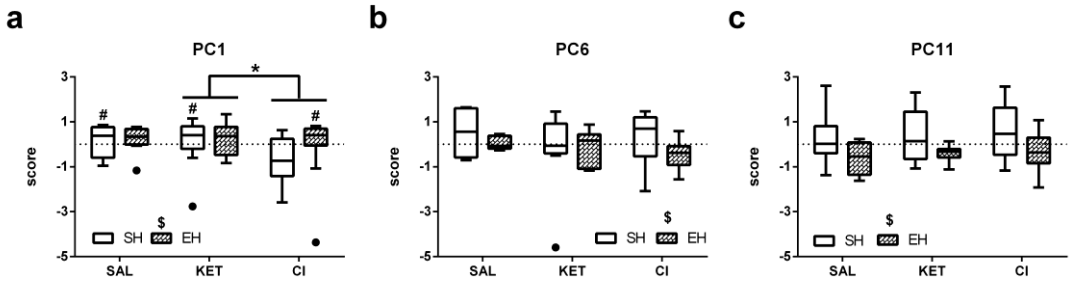

PC1 (12.52%)
correlation coefficients
1.0
0.5
0.0
-0.5
-1.0
Habituation of locomoting time in
Stops in OF2
Habituation of stops
Habituation of distance
Time spent at home base in OF2
Locomoting time in OF2
Distance in OF2
PC2 (12.24%)
correlation coefficients
Time spent in open arms
Distance in open arms
Distance in closed arms
Risk assessment behavior
Time spent in closed arms
Entries in closed arms
PC3 (11.72%)
correlation coefficients
Locomoting time in OF1
Distance in OF1
Inter-stop distance in OF1
Inter-stop distance in OF2
Distance in OF2
Locomoting time in OF2
PC4 (10.63%)
correlation coefficients
Locomoting time in Center in OF2
Distance in Center in OF2
Time spent on Center in OF2
Entries in Center in OF2
Habituation of distance in center in OF
PC5 (7.33%)
correlation coefficients
Distance in Center in OF1
Locomoting time in Center in OF1
Time spent on Center in OF1
Entries in Center in OF1
PC6 (7.32%)
correlation coefficients
Distance in EPM
Locomoting time in EPM
Habituation of locomoting time in center in OF
Habituation of time spent on center in OF
Habituation of distance in center in OF
Entries in closed arms
PC7 (6.43%)
correlation coefficients
Stops/Trip in OF1
Number of trips in OF1
Trip length in OF1
PC8 (6.08%)
correlation coefficients
Number of trips in OF2
Stops/Trip in OF2
Trip length in OF2
PC9 (4.74%)
correlation coefficients
Stops in OF1
Time spent at home base in OF1
PC10 (4.30%)
correlation coefficients
Average speed in OF2
Average speed in OF1
PC11 (3.66%)
correlation coefficients
Latency to step down
Time spent in center of the EPM
Entries in open arms

Figure 5.



Supplementary Table S1- Individual behavioral parameters obtained from Elevated Plus Maze, Open Field (session 1 and 2) and Inhibitory Avoidance Task. Data are presented as Mean±SD.

Parameter	SH			EH		
	SAL	KET	CI	SAL	KET	CI
Elevated Plus Maze						
Distance in EPM	13.72±3.18	12.38±1.67	11.86±2.99	12.80±2.21	11.55±2.50	11.24±1.58
Locomoting time in EPM [a]	66.76±10.39	62.51±7.03	64.28±8.71	60.77±4.48	60.64±7.21	56.79±7.68
Risk assessment behavior	7.55±5.13	7.78±3.58	5.18±2.99	6.67±2.60	7.00±2.93	5.29±3.18
Entries in closed arms	14.00±4.30	14.27±3.90	13.46±3.70	14.78±1.72	11.82±2.09	12.57±3.48
Time spent in closed arms [b]	37.41±13.51	34.36±6.59	36.93±13.15	32.41±9.38	28.43±6.34	29.18±11.06
Distance in closed arms	49.21±14.76	47.89±8.01	48.38±15.82	47.71±6.51	46.97±6.47	45.74±13.61
Entries in open arms	15.27±4.59	13.42±7.00	13.91±4.28	16.44±4.88	14.36±3.50	15.86±5.05
Time spent in open arms	37.38±20.17	30.79±15.78	42.67±18.77	40.58±8.64	40.20±16.22	44.64±14.44
Distance in open arms	31.86±18.75	26.15±14.33	33.47±17.97	29.23±8.56	31.85±15.70	33.64±13.77
Time spent in center of the EPM [c]	25.16±10.30	25.06±9.30	16.04±3.70	27.01±5.93	28.57±9.55	26.19±10.19
Open Field						
Distance in OF1	27.90±10.07	25.17±5.66	22.12±5.02	23.62±5.90	22.88±5.08	24.37±7.42
Locomoting time in OF1	45.10±10.52	42.65±8.89	39.75±8.60	41.31±9.26	38.30±6.77	40.48±9.25
Average speed in OF1	6.08±0.82	5.69±0.15	5.57±0.62	5.60±0.18	5.96±0.65	5.95±0.71

Parameter	SH			EH		
	SAL	KET	CI	SAL	KET	CI
Stops in OF1	115.4±11.00	115.8±14.57	113.8±15.98	118.9±11.12	116.2±10.51	115.1±10.68
Inter-stop distance in OF1	0.22±0.05	0.22±0.06	0.20±0.05	0.22±0.06	0.20±0.03	0.21±0.07
Entries in Center in OF1	30.00±8.33	25.75±7.58	31.64±11.55	29.78±7.81	28.27±12.66	31.36±7.89
Distance in Center in OF1	15.03±2.63	12.65±2.80	16.63±5.11	15.61±2.87	13.93±4.07	15.44±4.76
Locomoting time in Center in OF1	13.03±3.99	11.88±2.85	13.73±3.69	14.09±3.67	12.61±3.89	13.79±4.47
Time spent on Center in OF1 +	7.76±2.71	7.03±2.13	9.24±3.43	7.93±2.84	6.60±2.74	9.39±4.08
Time spent at home base in OF1	55.49±29.17	50.80±20.83	46.27±9.26	57.64±32.57	64.88±20.57	49.22±14.87
Number of trips in OF1	11.60±3.24	11.91±1.87	13.00±4.75	14.22±5.07	14.82±3.55	11.86±4.28
Trip length in OF1	0.79±0.33	0.70±0.30	0.75±0.36	0.64±0.32	0.60±0.22	0.99±0.75
Stops/Trip in OF1	4.50±1.08	4.68±1.21	5.15±2.31	3.91±1.44	4.10±1.24	5.74±3.59
Distance in OF2	22.85±3.69	19.68±6.85	15.36±6.87	18.94±6.23	18.74±5.37	19.83±6.76
Locomoting time in OF2	34.93±7.65	32.98±11.43	26.00±10.39	32.97±8.44	30.19±8.68	32.68±10.08
Average speed in OF2	6.25±0.47	5.97±0.14	5.80±0.63	5.68±0.66	6.23±0.45	6.12±0.96
Stops in OF2 *	114.9±16.91	116.1±13.53	90.09±22.63	104.4±18.84	109.5±23.97	101.8±21.62
Inter-stop distance in OF2	0.19±0.06	0.18±0.06	0.16±0.04	0.18±0.04	0.17±0.02	0.19±0.06
Entries in Center in OF2	22.36±7.97	20.83±13.20	20.82±14.63	22.22±16.98	18.45±8.95	21.71±10.93
Distance in Center in OF2	12.67±3.41	11.12±5.78	13.09±7.12	9.05±4.34	9.56±3.47	12.28±6.68

Parameter	SH			EH		
	SAL	KET	CI	SAL	KET	CI
Locomoting time in Center in OF2 $	11.75±4.08	9.63±5.63	10.90±5.64	7.44±3.47	7.96±3.36	9.49±5.03
Time spent on Center in OF2	6.45±2.88	5.44±4.07	5.86±5.41	3.83±2.38	4.59±2.77	5.88±5.30
Time spent at home base in OF2	67.08±33.74	50.96±13.05	74.48±25.76	68.20±28.59	82.12±35.57	78.86±58.58
Number of trips in OF2	15.27±4.47	13.25±3.62	12.00±7.06	13.89±4.23	12.78±1.56	15.86±10.65
Trip length in OF2	0.57±0.29	0.60±0.17	0.56±0.34	0.62±0.28	0.57±0.18	0.68±0.53
Stops/Trip in OF2	3.94±0.66	4.32±1.13	4.12±1.93	4.10±1.03	4.55±0.33	4.24±2.83
Habituation of distance &	1.17±0.18	1.23±0.17	1.63±0.52	1.29±0.21	1.26±0.22	1.29±0.38
Habituation of locomoting time	1.35±0.45	1.29±0.26	1.70±0.57	1.28±0.21	1.33±0.25	1.35±0.54
Habituation of stops	1.03±0.22	1.09±0.24	1.31±0.23	1.09±0.20	1.10±0.22	1.23±0.51
Habituation of time spent on center in OF	1.45±0.91	1.69±1.02	2.55±2.30	2.42±1.40	1.64±0.71	3.25±3.94
Habituation of distance in center in OF	1.05±0.26	1.24±0.55	1.51±0.97	1.83±0.89	1.31±0.33	1.65±1.11
Habituation of locomoting time in center in OF %	1.04±0.30	1.20±0.40	1.39±0.70	1.91±0.89	1.56±0.42	1.81±1.10
Inhibitory Avoidance task						
Latency to step down in training session @	32.27±34.66	32.00±28.26	24.50±19.55	5.56±5.13	9.67±8.45	12.86±7.80
Latency to step down in testing session	116.18±75.99	140.83±71.16	180.00±0.00	137.11±76.99	102.75±82.79	119.14±77.59

a indicates housing condition effect [F(1, 62) = 7.097; P = 0.0098];

b indicates housing condition effect [F(1, 58) = 5.359; P = 0.0242];

[c] indicates housing condition effect [F(1, 60) = 5.538; P = 0.0219];

[+] indicates treatment effect [F(2, 61) = 3.897; P = 0.0255]; KET groups ≠ CI groups (P = 0.0212).

[*] indicates treatment effect [F(2, 61) = 4.686; P = 0.0128]; KET groups ≠ CI groups (P = 0.023).

[$] indicates housing condition effect [F(1, 61) = 4.442; P = 0.0392];

[&] indicates treatment effect [F(2, 58) = 3.728; P = P = 0.03];

[%] indicates housing condition effect [F(1, 57) = 8.720; P = 0.0046];

[@] indicates housing condition effect [F(1, 62) = 16.25; P = 0.0002];

Supplementary Table S2- Meaning of the parameters used in the PCA.

Parameter	Behavioral category					
	Locomotor activity	Exploratory activity	Spatial distribution	Temporal organization	Anxiety-like behavior	Learning
Elevated Plus Maze						
Distance in EPM	X	X				
Locomoting time in EPM	X	X				
Risk assessment behavior		X			X	
Entries in closed arms	X		X		X	
Time spent in closed arms			X		X	
Distance in closed arms			X		X	
Entries in open arms	X		X		X	
Time spent in open arms			X		X	
Distance in open arms			X		X	
Time spent in center of the EPM			X			
Open Field						
Distance in OF1	X	X				
Locomoting time in OF1	X	X				

Parameter	Behavioral category					
	Locomotor activity	Exploratory activity	Spatial distribution	Temporal organization	Anxiety-like behavior	Learning
Average speed in OF1	X	X				
Stops in OF1	X	X				
Inter-stop distance in OF1	X	X				
Entries in Center in OF1			X		X	
Distance in Center in OF1			X		X	
Locomoting time in Center in OF1			X		X	
Time spent on Center in OF1			X		X	
Time spent at home base in OF1	X		X			
Number of trips in OF1		X		X		
Trip length in OF1	X			X		
Stops/Trip in OF1		X		X		
Distance in OF2	X	X				
Locomoting time in OF2	X	X				
Average speed in OF2	X	X				
Stops in OF2	X	X				

Parameter	Behavioral category					
	Locomotor activity	Exploratory activity	Spatial distribution	Temporal organization	Anxiety-like behavior	Learning
Inter-stop distance in OF2	X	X				
Entries in Center in OF2			X		X	
Distance in Center in OF2			X		X	
Locomoting time in Center in OF2			X		X	
Time spent on Center in OF2			X		X	
Time spent at home base in OF2	X		X			
Number of trips in OF2		X		X		
Trip length in OF2	X			X		
Stops/Trip in OF2		X		X		
Habituation of distance	X	X				X
Habituation of locomoting time	X	X				X
Habituation of stops	X	X				X
Habituation of time spent on center in OF			X		X	X
Habituation of distance in center in OF			X		X	X
Habituation of locomoting time in center in OF			X		X	X

Parameter	Behavioral category					
	Locomotor activity	Exploratory activity	Spatial distribution	Temporal organization	Anxiety-like behavior	Learning
Inhibitory Avoidance task						
Latency to step down		X				

PCA was performed on a set of forty-three behavioral variables (ten from EPM, thirty-two from OF and one from inhibitory avoidance task). Each parameter represents one or more behavioral category.

CAPÍTULO IV

Resultados Parciais

Effect of transient GluN2B-containing NMDAR blockade and brain cognitive reserve stimulation during critical periods of brain development on [18F]FDG uptake in adult rats

Cássio Morais Loss*, Natã Sehn da Rosa, Gianina Teribele Venturin, Samuel Greggio, Eduardo Rigon Zimmer, Giordano Gubert Viola, Diogo Losch de Oliveira.

Justificativa: Evidências crescentes sugerem que uma disfunção do metabolismo encefálico é um aspecto importante da patogênese da esquizofrenia. Além disso, é bem documentado que a estimulação da reserva cognitiva e encefálica (BCR) influencia positivamente funções cognitivas e induz alterações neuroanatômicas, sendo sugerido muitas vezes como uma terapia complementar aos tratamentos farmacológicos convencionais em pacientes com distúrbios neurológicos.

Objetivo geral: Investigar se o bloqueio neonatal de NMDAR é capaz de alterar a captação de glicose encefálica na idade adulta, e se a estimulação da reserva cognitiva e encefálica durante a infância é capaz de prevenir as potenciais alterações na captação de glicose encefálica induzidas pelo bloqueio neonatal de NMDAR.

Objetivo específico: Investigar se o bloqueio não seletivo de NMDAR durante idades precoces do desenvolvimento é capaz de alterar a captação de glicose encefálica na idade adulta; Investigar se o bloqueio não seletivo de NMDAR e o bloqueio seletivo de NMDAR contendo a subunidade GluN2B durante idades precoces do desenvolvimento influenciam de maneira similar a captação de glicose encefálica na idade adulta; Investigar se o enriquecimento ambiental durante a infância é capaz de prevenir as potenciais alterações na captação de glicose encefálica induzidas pelo bloqueio neonatal de NMDAR.

Effect of transient GluN2B-containing NMDAR blockage and brain cognitive reserve stimulation during critical periods of brain development on [18F]FDG uptake in adult rats.

Loss, C. M.*[1]; da Rosa, N. S.[1]; Venturin, G. T.[2]; Greggio, S.[2]; Rocha, A.[1]; Zimmer, E. R[1,2]; Viola, G. G.; de Oliveira, D. L[1].

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rio Grande do Sul – Brazil

[2]Instituto do Cérebro, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul – Brazil

* Corresponding author: Cássio Morais Loss, Departamento de Bioquímica, ICBS, UFRGS. Rua Ramiro Barcelos 2600-Anexo. Zip code: 90035-003. Porto Alegre, RS, BRAZIL. Phone: +55 51 33085555. Fax: +55 51 33085540. E-mail: cassio.m.loss@gmail.com

Rationale

There is a growing body of evidence suggesting that a dysfunction of brain metabolism is an important aspect of the pathogenesis of schizophrenia (Bergman and Ben-Shachar, 2016). An adequate energy supply is essential for neural plasticity, synaptic transmission and brain development, processes that are impaired in schizophrenic patients (Park et al., 2010; Zheng et al., 2016). Studies that evaluated glucose metabolism through [18F]FDG PET scan observed differences in the brain activation profile in both patients and adult animal models of schizophrenia(Ben-Shachar et al., 2007; Bralet et al., 2015; Kosten et al., 2016; Shinto et al., 2014). In this study, we investigate if the transient NMDAR antagonism during early stages of development, a neurodevelopmental model of schizophrenia, is capable of inducing long-term alterations in brain glucose metabolism. Also, we evaluate if the specific GluN2B-containing NMDAR subpopulation antagonism during early development is involved in this process. We also evaluated whether BCR stimulation is able to prevent the putative alterations in brain glucose metabolism induced by transient neonatal NMDAR antagonism.

Material and methods

Animals

Twenty pregnant female Wistar rats (3 months old) were kept in standard housing (SH) or in enriched housing (EH). The lodging occurred immediately after mating (ED0) and the mothers stayed in those environments until weaning. After birth, the puppies were kept with their mothers in the same housing condition until they reached 21-days-old (PND21 – weaning day). After weaning, the offspring stayed in the

same housing condition until they were 30-days-old. From PND30 until the end of the experiment, all the animals were kept in standard housing in groups of 2-3 animals/cage. All animals were maintained under a 12:12 h controlled light/dark photoperiod cycle (lights on at 7:00 AM) and room temperature adjusted to 21±1 ºC, with unlimited access to water and standard rodent food during the entire experiment. Animals in each experimental group always came from different litters, with a minimum of nine litters even for the smallest groups, to prevent litter effects from occurring.

Transient NMDAR blockade

Administration of NMDAR antagonists were based on studies of Akillioglu and colleagues (Akillioglu et al., 2012a; Akillioglu et al., 2012b). Rat pups (male and female) were injected from PND5 to PND10 with (i) ketamine (a non-selective NMDAR antagonist) at a dose of 25 mg/kg; (ii) CI-1041 (a GluN2B-containing NMDAR-selective antagonist) at a dose of 10 mg/kg; or (iii) saline (0.9% NaCl). The volume of injection was 5 ml/kg, and the drugs were administered twice a day (11:00 AM.–6:00 PM.), intraperitoneally.

Housing conditions

Enriched housing apparatus consisted of three Plexiglas box (55.5 x 41.5 x 40 cm each) interconnected by 10 cm diameter plastic tubes (please see Figure 1). Each cage was divided in two floors, except during the second gestational and second postnatal weeks (as described below). In the two boxes of the corners the floor was always covered with sawdust, while in the middle box the floor topping was changed once a week. The different toppings consisted of sawdust, small stones, falcon tubes,

plastic chips, plastic bottle caps, small marbles and paper towels. The base of the second floor was always a metal grid. All cages contained a variety of objects with distinct sizes and shapes, including wood, plastic, metal and glass made objects, nesting material and hiding places (such tunnels for instance), in order to represent eco-ethological expansions for rats including the sense of security and to provide a place where they could avoid open spaces and luminosity (a natural behavior of wild rodents). Running wheels (for adults) were just available during the second gestational and second postnatal weeks. During these periods the second floor of one of the cages was removed to accommodate the wheel. Because not all the rats run when running wheels are available (Novak et al., 2012), we choose not to maintain the running wheels available for the entire housing to minimize the data variability without depriving animals from running. Small running wheels (for pups) were available from PND17 to PND30. Additional cognitive stimulation regarding the formation of spatial mapping was provided by changing half of the objects and by shifting their positions twice a week. In addition, although water and food were available out of the cages (through the top metal grid), food pellets were also available inside the cages to stimulate the natural rodents' behavior of stocking and hoarding food. Based in previous studies (Cancedda et al., 2004; Sale et al., 2004), female rats were housed in groups of four (being 2-3 rats pregnant). The non-pregnant rats provide both social stimulation for the mothers and increased pups' care by acting like "babysitters" in the periods in which the mothers are absent from the nest foraging for water and food. At ED21, the mothers were isolated in one of the EH cages and stayed there with their own offspring until PND4, when they were putted back together with free access to all the EH compartments.

Standard housing consisted of one pregnant rat kept alone with their own offspring during the entire experiment in a Plexiglas box (40.5 x 33.5 x 18 cm) containing just sawdust.

Battery of behavioral tests

Behavioral and observational tests to assess maturation and development of offspring were conducted in female pups during the period leading up to weaning (PND21), which include sensory-motor reflexes, neuromotor behaviors, and physical development stages (Chen et al., 2012; Juliano et al., 2015). Care was taken to minimize the duration of separation from the dam.

Developmental Landmarks

Based in Chen et al. (2012), the emergence of physical maturation landmarks was noted each day, including incisor eruption (the first appearance of upper incisors), eye opening (when both eyelids were completely separated) and development of fur (the entire body appeared to be covered in white fur). All female pups in each litter were assessed every scheduled day even after attaining each milestone.

Neonatal Sensory and Motor Development

Surface righting reflex test, Negative geotaxis test, Cliff aversion test, and grip strength test were measured based in Chen et al. (2012).

In the surface righting reflex test, pups were gently removed from the litter and placed on a warming pad (34 ºC). Pups were timed from the moment of being placed in supine position until it had righted itself and all four feet were in contact with the surface. Pups were tested just once in PND12.

In the negative geotaxis test, pups were timed for completing a 180º turn when placed in a head down position on a 25º inclined surface. Latency to rotate 180º on the inclined surface was measured during a 120-s test. If the pup fell, crawled off the plane, or made no movement, the rat was considered to have failed the task, and a maximum time of 120s was assigned. Pups were tested just once in PND12.

In the cliff aversion test, pups were placed with their heads and forepaws over the edge of a table. The latency to retract their body (to turn the body or to crawl away) 1 cm from the edge was recorded. Pups were tested just once in PND12.

In the grip strength test, pups were allowed to grip on a metal grid which initially was in a vertical position and then was gently moved to a horizontal position. The moving time from vertical to horizontal position was 10s. Once horizontal position was achieved, the grid stayed immobile until the pup drop the grid. The length of time (including the moving grid time) the pup was able to hold on the bar unaided before dropping was recorded. Pups were tested three times on PND12 with a 30s intertrial interval.

[18F]FDG PET uptake

When female mice were 5-months old, they were subjected to evaluation of [18F]FDG uptake. Animals received an intravenous bolus injection (0.2 mL) of [18F]FDG into the tail vein. Mice will be maintained awake for a 40 min uptake period, followed by a 10 min static acquisition under isoflurane anesthesia. Imaging analysis was conducted based in Zimmer et al. (2017). Images were reconstructed by fully-3D ordered subset expectation maximization (3D-OSEM) algorithm, normalized and corrected for scatter, dead time and decay. Imaging analysis was conducted using PMOD and minc-tools (http://www.bic.mni.mcgill.ca/ServicesSoftware/HomePage).

MicroPET images were manually co-registered to a standard rat histological template. Standardized uptake value reference (SUVr) was calculated using pons as the reference region. Mean SUVr values for each brain region analyzed (frontal cortex, temporoparietal cortex, hippocampus, striatum, thalamus, hypothalamus, cerebellum) were compared.

Statistics

Data were first analyzed by Principal Component Analysis (PCA) with Varimax Rotation and Kaizer Normalization. Principal Components (PC) scores were extracted by regression method and analyzed by Two-way ANOVA (between-subjects factor 1: Housing Condition; between-subjects factor 2: NMDAR antagonist) followed by Sidak's multiple comparisons post hoc test. A significance level of 0.05 was set for all analyses.

Results

Partial results from Principal Component Analysis using the fourteen brain regions analyzed (seven from right hemisphere and seven from left hemisphere) resulted in two PC which together explain 83.04% of total data variability. PC1 (which explain 52.23% of data variability) was related to whole brain activation, while PC2 (which explain 30.81% of data variability) was related to cortex activation (Figure 2 a-b). When groups were analyzed by their PC1 scores, Two-way ANOVA revealed no significant NMDAR antagonist effect [$F(2,15)= 0.49$; $P= 0.620$] neither NMDAR antagonist x Housing Condition effects [$F(2,15)= 1.17$; $P= 0.338$] but a marginal Housing Condition effect [$F(1,15)= 3.75$; $P= 0.072$] (Figure 2 c). When groups were analyzed by their PC2 scores, Two-way ANOVA revealed no significant NMDAR

antagonist effect [F(2,15)= 0.61; P= 0.555], neither Housing Condition [F(1,15)= 1.12; P= 0.307] and NMDAR antagonist x Housing Condition effects [F(2,15)= 0.18; P= 0.837] (Figure 2 d). If confirmed, these results suggest that in general, animals reared in EH present an elevated whole brain activity which is not related to greater cortical activity (Figure 3).

References

Akillioglu, K., Babar Melik, E., Melik, E., Kocahan, S., 2012a. The investigation of neonatal MK-801 administration and physical environmental enrichment on emotional and cognitive functions in adult Balb/c mice. Pharmacol Biochem Behav. 102**,** 407-14.

Akillioglu, K., Binokay, S., Kocahan, S., 2012b. The effect of neonatal N-methyl-D-aspartate receptor blockade on exploratory and anxiety-like behaviors in adult BALB/c and C57BL/6 mice. Behav Brain Res. 233**,** 157-61.

Ben-Shachar, D., Bonne, O., Chisin, R., Klein, E., Lester, H., Aharon-Peretz, J., Yona, I., Freedman, N., 2007. Cerebral glucose utilization and platelet mitochondrial complex I activity in schizophrenia: A FDG-PET study. Prog Neuropsychopharmacol Biol Psychiatry. 31**,** 807-13.

Bergman, O., Ben-Shachar, D., 2016. Mitochondrial Oxidative Phosphorylation System (OXPHOS) Deficits in Schizophrenia: Possible Interactions with Cellular Processes. Can J Psychiatry. 61**,** 457-69.

Bralet, M.C., Buchsbaum, M.S., DeCastro, A., Shihabuddin, L., Mitelman, S.A., 2015. FDG-PET scans in patients with Kraepelinian and non-Kraepelinian schizophrenia. Eur Arch Psychiatry Clin Neurosci.

Cancedda, L., Putignano, E., Sale, A., Viegi, A., Berardi, N., Maffei, L., 2004. Acceleration of visual system development by environmental enrichment. J Neurosci. 24**,** 4840-8.

Chen, C., Tang, Y., Jiang, X., Qi, Y., Cheng, S., Qiu, C., Peng, B., Tu, B., 2012. Early postnatal benzo(a)pyrene exposure in Sprague-Dawley rats causes persistent neurobehavioral impairments that emerge postnatally and continue into adolescence and adulthood. Toxicol Sci. 125**,** 248-61.

Juliano, C., Sosunov, S., Niatsetskaya, Z., Isler, J.A., Utkina-Sosunova, I., Jang, I., Ratner, V., Ten, V., 2015. Mild intermittent hypoxemia in neonatal mice causes permanent neurofunctional deficit and white matter hypomyelination. Exp Neurol. 264**,** 33-42.

Kosten, L., Verhaeghe, J., Verkerk, R., Thomae, D., De Picker, L., Wyffels, L., Van Eetveldt, A., Dedeurwaerdere, S., Stroobants, S., Staelens, S., 2016. Multiprobe molecular imaging of an NMDA receptor hypofunction rat model for glutamatergic dysfunction. Psychiatry Res. 248**,** 1-11.

Novak, C.M., Burghardt, P.R., Levine, J.A., 2012. The use of a running wheel to measure activity in rodents: relationship to energy balance, general activity, and reward. Neurosci Biobehav Rev. 36**,** 1001-14.

Park, Y.U., Jeong, J., Lee, H., Mun, J.Y., Kim, J.H., Lee, J.S., Nguyen, M.D., Han, S.S., Suh, P.G., Park, S.K., 2010. Disrupted-in-schizophrenia 1 (DISC1) plays essential roles in mitochondria in collaboration with Mitofilin. Proc Natl Acad Sci U S A. 107**,** 17785-90.

Sale, A., Putignano, E., Cancedda, L., Landi, S., Cirulli, F., Berardi, N., Maffei, L., 2004. Enriched environment and acceleration of visual system development. Neuropharmacology. 47**,** 649-60.

Shinto, A.S., Kamaleshwaran, K.K., Srinivasan, D., Paranthaman, S., Selvaraj, K., Pranesh, M.B., Lakshminarayanan, G.N., Prakash, B., 2014. "Hyperfrontality" as seen on FDG PET in unmedicated schizophrenia patients with positive symptoms. Clin Nucl Med. 39**,** 694-7.

Zheng, X., Boyer, L., Jin, M., Mertens, J., Kim, Y., Ma, L., Hamm, M., Gage, F.H., Hunter, T., 2016. Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation. Elife. 5.

Zimmer, E.R., Parent, M.J., Souza, D.G., Leuzy, A., Lecrux, C., Kim, H.I., Gauthier, S., Pellerin, L., Hamel, E., Rosa-Neto, P., 2017. [18F]FDG PET signal is driven by astroglial glutamate transport. Nat Neurosci.

FIGURE LEGENDS

Fig. 1. Schematic representation of the Housing Conditions. Enriched Housing is depicted in Top; Standard Housing is depicted in Bottom.

Fig. 2. Effect of housing conditions and transient NMDAR blockade on brain [18F]FDG uptake. A PCA with the fourteen regions analyzed was performed to evaluation brain [18F]FDG uptake. The matrix correlation between the original variables and each individual PC (PC1 and PC2) are presented in a and b. Dashed lines in a and b (values 0.5 and -0.5 in the Y-axis) indicate the cutoff points (i.e., the variables which presented covariation coefficients > -0.5 and < 0.5 are not represented in the graphs). Comparison between groups with respect to the PC1 and PC2 values are presented in (c and d, respectively). Data from c and d are expressed as the medians and the interquartile ranges and were analyzed using Two-way ANOVA followed by Sidak's multiple comparisons test. Each filled circle depicted in the graphs represent one individual. For PC1, Housing Condition effect presented marginal significance ($P= 0.072$) when compared with the α level of 0.05.

Fig. 3. Brain [18F]FDG uptake. Representative images showing brain [18F]FDG uptake of the three treated groups (SAL, KET, CI) and created in Enriched Housing or Standard Housing. Columns represent transversal (top), sagittal (middle) and coronal views of the groups' brains. Hot colors represent high levels of [18F]FDG uptake while cold colors represent low levels of [18F]FDG uptake.

Figure 1.

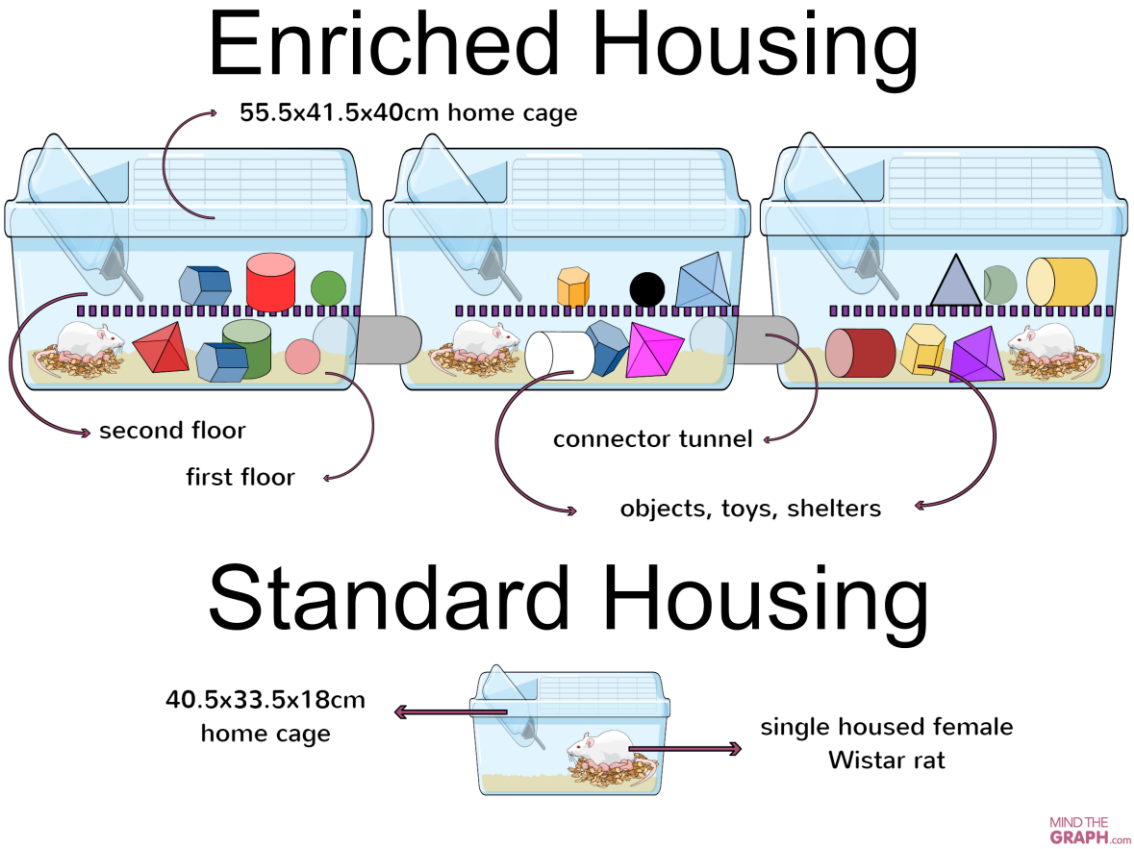

Enriched Housing
55.5x41.5x40cm home cage
second floor
first floor
connector tunnel
objects, toys, shelters
Standard Housing
40.5x33.5x18cm
home cage
single housed female
Wistar rat
MIND THE GRAPH.com

Figure 2.

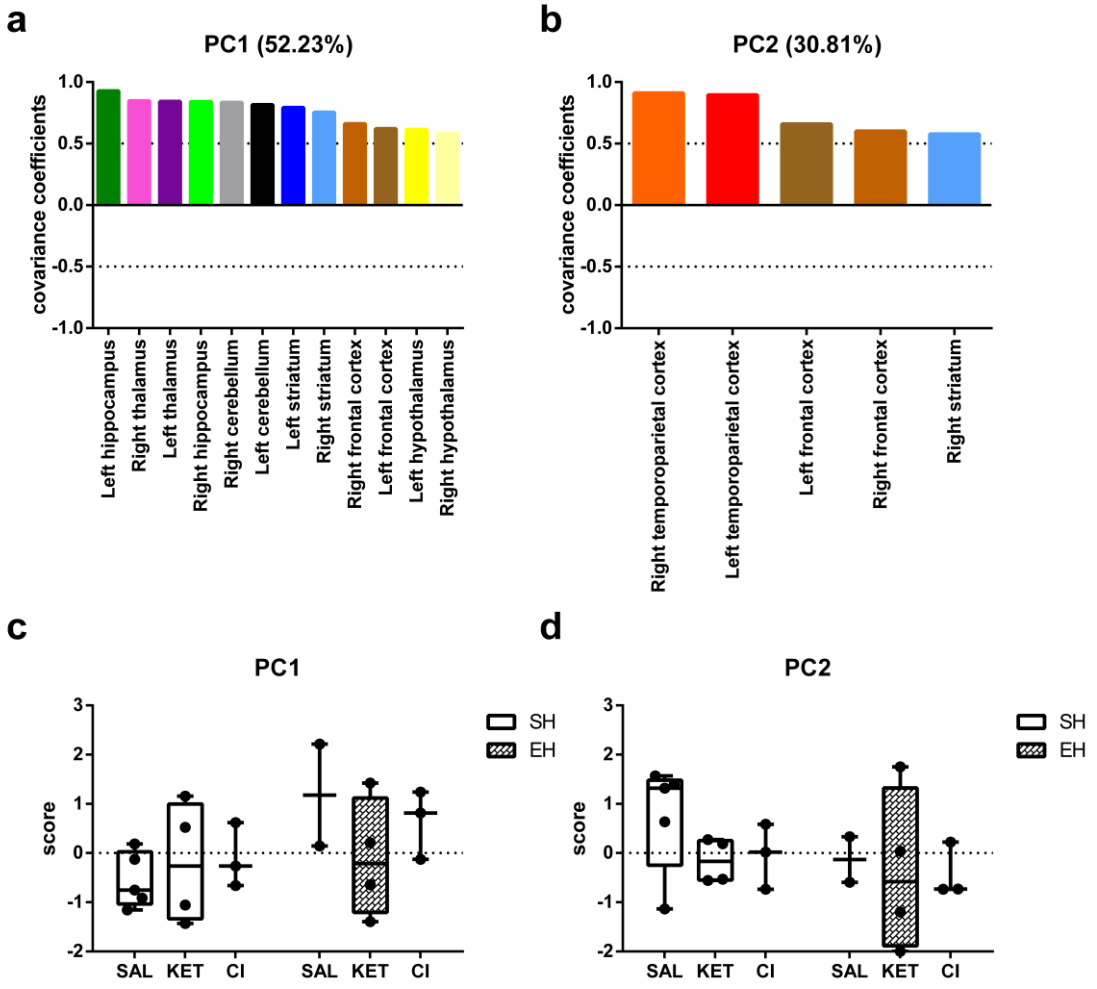
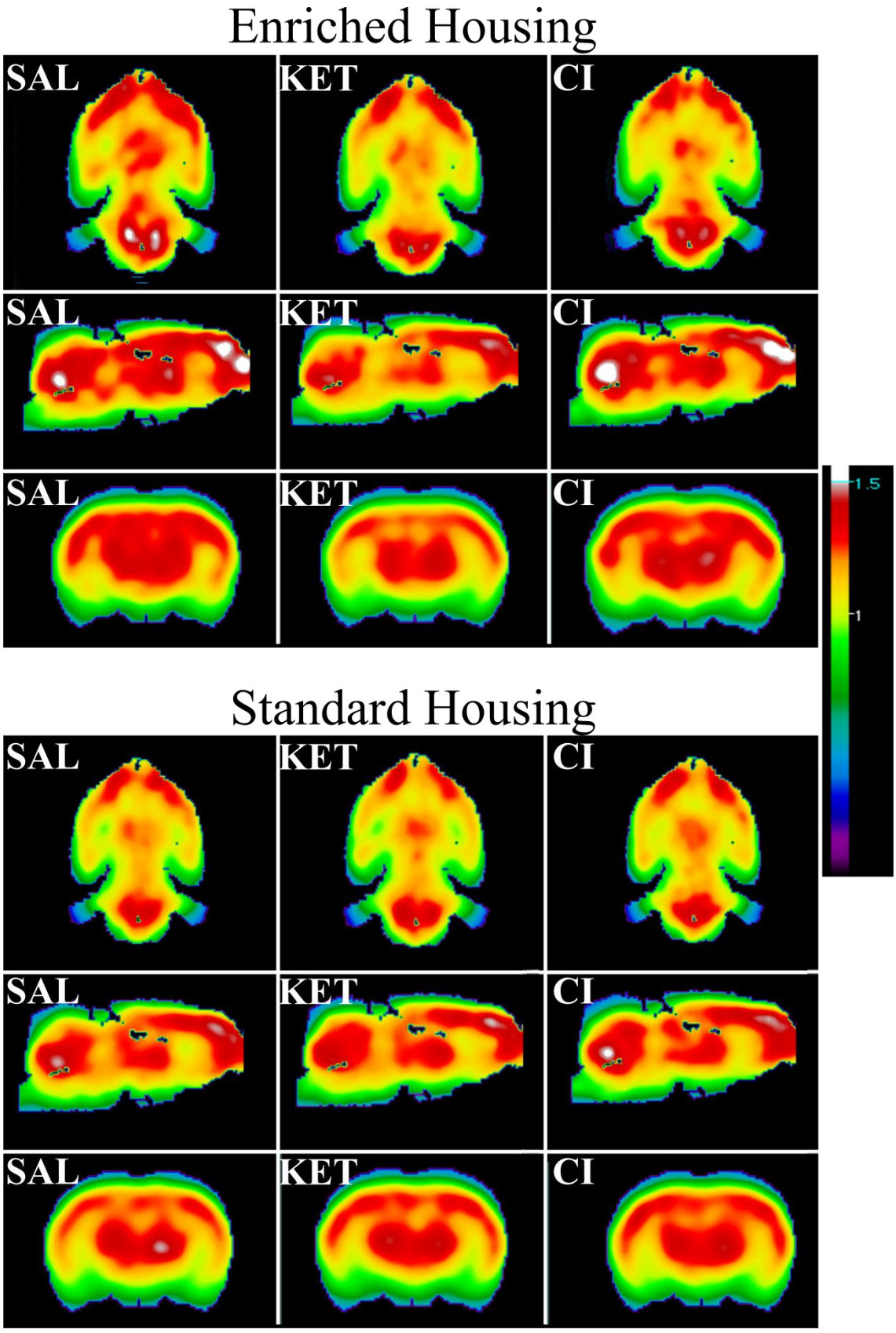

a
PC1 (52.23%)
covariance coefficients
1.0
0.5
0.0
-0.5
-1.0
Left hippocampus
Right thalamus
Left thalamus
Right hippocampus
Right cerebellum
Left cerebellum
Left striatum
Right striatum
Right frontal cortex
Left frontal cortex
Left hypothalamus
Right hypothalamus
b
PC2 (30.81%)
covariance coefficients
Right temporoparietal cortex
Left temporoparietal cortex
Left frontal cortex
Right frontal cortex
Right striatum
c
PC1
score
SH
EH
SAL
KET
CI
d
PC2

Figure 3.


Enriched Housing
SAL
KET
CI
SAL
KET
CI
SAL
KET
CI
1.5
1
Standard Housing
SAL
KET
CI
SAL
KET
CI
SAL
KET
CI

PARTE III. DISCUSSÃO E CONCLUSÃO

DISCUSSÃO

Uma série de alterações fisiológicas como crescimento axonal, maturação dendrítica, estabelecimento de redes neurais, formação de novas sinapses, proliferação de células da glia e mielinização ocorrem durante períodos críticos do desenvolvimento encefálico (Akillioglu *et al.*, 2012). Em roedores, este período começa no nascimento e inclui as três primeiras semanas de vida pós-natal (Ikonomidou *et al.*, 1999). Levando em consideração que disfunções na neurotransmissão glutamatérgica mediada pelos NMDAR têm sido implicadas na patogênese de inúmeros transtornos agudos e crônicos do SNC (Lipton e Rosenberg, 1994; Price, 1999; Chapman, 2000; Anticevic *et al.*, 2012), é lógico pensar que interferências na funcionalidade destes receptores durante a infância podem muitas vezes ser a base dos défices cognitivos e as alterações na emocionalidade observados em transtornos psiquiátricos como esquizofrenia, transtornos de ansiedade e humor.

Estudos têm demonstrado que roedores que foram expostos a episódios repetidos de hipofunção de NMDAR durante idades iniciais do desenvolvimento apresentaram, durante a vida adulta, alterações na atividade exploratória, nos níveis de ansiedade, e em processos cognitivos da aprendizagem espacial (Latysheva e Raevskii, 2003; Stefani e Moghaddam, 2005; Kawabe, Iwasaki e Ichitani, 2007; Akillioglu *et al.*, 2012; Akillioglu, Binokay e Kocahan, 2012). Além disso, em um estudo recente, nós mostramos que um único evento de antagonismo de NMDAR durante a terceira semana pós-natal foi suficiente para induzir alterações de comportamento na idade adulta, tais como níveis elevados de comportamentos relacionados a ansiedade (Loss, Cordova e de Oliveira, 2012). Levando em consideração que os NMDAR são complexos heterotetraméricos que podem ser formados por diferentes combinações das subunidades GluN1, GluN2 e GluN3 (Henson *et al.*, 2010; Paoletti, 2011; Paoletti,

Bellone e Zhou, 2013), e, que tem sido sugerido que as diferentes localizações e composições dos NMDAR refletem diferentes funções fisiológicas desses receptores (Hardingham, Fukunaga e Bading, 2002; Li *et al.*, 2002; Petralia, 2012), torna-se de extrema importância identificar quais são as subunidades dos NMDAR responsáveis pelas alterações comportamentais observadas em animais adultos após sofrerem eventos de hipofunção de NMDAR durante a infância. Uma vez que a subunidade GluN2B é a subunidade predominante durante estágios iniciais do desenvolvimento pós-natal (Ewald e Cline, 2009; Paoletti, 2011; Paoletti, Bellone e Zhou, 2013), nesta Tese investigamos se bloquear seletivamente os NMDAR contendo a subunidade GluN2B durante períodos precoces do desenvolvimento encefálico afetaria as funções cognitivas de maneira similar ao bloqueio não seletivo de NMDAR.

Além disso, a busca por estratégias de neuroproteção e tratamento de distúrbios do SNC é um tema que tem sido amplamente discutido no meio acadêmico. Uma das estratégias que tem ganhado destaque entre os cientistas é a estimulação da BCR (Stern *et al.*, 2005; Nithianantharajah e Hannan, 2009; Stern, 2009; Nithianantharajah e Hannan, 2011) a qual tem se mostrado efetiva não somente no tratamento dos sintomas neurológicos e cognitivos decorrentes de distúrbios do SNC (Scarmeas *et al.*, 2001; Nithianantharajah e Hannan, 2009; Stern, 2009; Gehres *et al.*, 2016), mas também em melhorar o desempenho cognitivo de indivíduos saudáveis (Nithianantharajah e Hannan, 2009; Viola *et al.*, 2009; Loss *et al.*, 2015). Estes benefícios da estimulação da BCR podem estar relacionados tanto a uma prevenção quanto a um atraso na evolução e desenvolvimento de doenças do SNC (Scarmeas *et al.*, 2001; Stern *et al.*, 2005; Nithianantharajah e Hannan, 2009; Stern, 2009; Nithianantharajah e Hannan, 2011; Gehres *et al.*, 2016). Desta forma, um segundo objetivo da presente Tese foi investigar se a estimulação da BCR, por meio do paradigma de EA, em períodos precoces do

desenvolvimento encefálico (desde o desenvolvimento embrionário até o início da adolescência) é capaz de prevenir ou atenuar as alterações cognitivas induzidas pelo bloqueio transiente de NMDAR durante o desenvolvimento encefálico.

Conforme citado anteriormente, episódios repetidos de hipofunção de NMDAR durante idades iniciais do desenvolvimento induzem alterações duradouras em processos cognitivos da aprendizagem espacial (Latysheva e Raevskii, 2003; Stefani e Moghaddam, 2005; Kawabe, Iwasaki e Ichitani, 2007; Akillioglu *et al.*, 2012; Akillioglu, Binokay e Kocahan, 2012). Contudo, ainda não é claro como esse tipo de disfunção da neurotransmissão glutamatérgica afeta a atividade exploratória e os níveis de ansiedade dos indivíduos por elas acometidos. Alguns estudos reportaram ausência de efeitos do bloqueio de NMDAR durante o desenvolvimento sobre a atividade exploratória e os níveis de ansiedade (Stefani e Moghaddam, 2005; Kawabe, Iwasaki e Ichitani, 2007) enquanto outros reportaram alterações destes comportamentos em animais adultos que foram submetidos ao bloqueio de NMDAR na infância (Akillioglu *et al.*, 2012; Akillioglu, Binokay e Kocahan, 2012). Uma vez que o comportamento é o resultado final (ou resposta) do processamento das múltiplas informações (tanto ambientais quanto intrínsecas do organismo) que chegam ao encéfalo, existem diversos fatores que poderiam estar influenciando a interpretação destes dados (Viola e Loss, 2014), tais como as diferentes espécies (e/ou linhagens) utilizadas nos diferentes trabalhos, o período do dia em que os testes foram realizados, e a utilização de parâmetros individuais para avaliação de parâmetros complexos (tal como comportamentos).

Apesar de variáveis individuais serem amplamente utilizadas por décadas e serem ferramentas valiosas para análise comportamental, nós acreditamos que a análise de comportamentos complexos (tais como exploração, locomoção, navegação,

ansiedade, cognição) deva ser feita através da combinação dos múltiplos parâmetros adquiridos durante as tarefas comportamentais. Desta forma, nós propusemos a utilização de uma ferramenta estatística alternativa, a Análise de Componentes Principais (PCA), que integre todos os parâmetros individuais de forma a simplificar a estrutura deste conjunto de dados, e que baseado nas correlações entre as variáveis originais, gere um novo conjunto de dados que serão utilizados na análise comportamental (Jolliffe, 1986; Sanguansat, 2012).

Levando em consideração que os dois objetivos acima citados envolvem a análise de comportamentos complexos, nós primeiramente investigamos se a PCA é capaz de identificar tanto macro quanto micro alterações na estrutura do comportamento. Além disso, uma vez que o ritmo circadiano afeta de maneira significativa o padrão de atividade dos animais (Moore e Eichler, 1972; Stephan e Zucker, 1972; Yao *et al.*, 2006; Panksepp *et al.*, 2008), nós utilizamos a PCA para investigar se o período do dia em que as tarefas eram realizadas influencia o comportamento exploratório e os níveis de ansiedade dos animais.

No primeiro capítulo desta Tese nós testamos a hipótese de que o período do dia em que as tarefas seriam realizadas afetaria a organização espaço-temporal do comportamento exploratório e emocionalidade dos animais. Através da utilização da PCA, nós observamos que além do padrão de atividade dos animais, o período do dia também alterou a micro estrutura do comportamento exploratório. Animais testados durante o período escuro do ciclo claro/escuro apresentaram maior atividade exploratória e aparentemente foram mais meticulosos nas incursões exploratórias, apresentando maior frequência de paradas durante viagens mais longas (e consequentemente ficando menos tempo no *home base*). Apesar disso, o período do dia em que as tarefas foram realizadas não afetou as estratégias empregadas nem a ordem

em que elas foram utilizadas pelos animais para explorar o ambiente (primeiro o patrulhamento do perímetro, seguido pela exploração com incursões acontecendo a partir do *home base*). Além disso, tanto as alterações sutis quanto as mais acentuadas não foram relacionadas aos níveis de ansiedade dos animais. Em conjunto, os resultados obtidos no Capítulo I desta Tese mostraram que a PCA foi capaz de identificar tanto macro quanto micro alterações na estrutura do comportamento, sugerindo que a PCA é uma ferramenta estatística eficaz no estudo do comportamento animal. Além disso, estes resultados nos levaram a sugerir que o período noturno do ciclo claro/escuro era o período mais adequado para investigação da organização espaço-temporal do comportamento exploratório. É importante salientar que nossos resultados foram baseados em testes realizados sempre com as mesmas condições de teste, ou seja, foi utilizada iluminação branca na tarefa de campo aberto e iluminação vermelha na tarefa de labirinto em cruz elevado independentemente do período do dia em que os animais foram testados. Baseado no fato de que tanto o tipo de iluminação quanto a intensidade da iluminação afetam o comportamento de forma significativa (Jones e King, 2001; Garcia, Cardenas e Morato, 2005; Zadicario *et al.*, 2005; Avni, Zadicario e Eilam, 2006), em termos metodológicos, as condições do teste e o cronograma experimental não devem ser ignorados na hora de planejar os experimentos.

No segundo capítulo da presente Tese, nós investigamos se as alterações comportamentais induzidas pelo EA seriam observadas independentemente do período do dia em que os animais fossem testados. Neste trabalho nós utilizamos uma linhagem de camundongo que apresenta resistência aos efeitos do EA (a linhagem de camundongos albinos *Swiss*) (Silva *et al.*, 2011). Desta forma, se através do uso da PCA nós fossemos capazes de identificar alterações comportamentais induzidas pelo EA nesta linhagem, provavelmente estas alterações seriam também detectadas em outros

modelos animais e a PCA se mostraria uma ferramenta importante no estudo do comportamento animal. Nós mostramos que algumas das alterações comportamentais induzidas pelo EA (tais como diminuição na exploração e na auto-exposição a ambientes potencialmente perigosos) são observadas durante o período diurno, mas não durante o período noturno. Contudo, animais submetidos ao EA apresentaram melhor desempenho em uma tarefa que avalia a formação de memórias episódicas independentemente do período em que foram testados. É importante salientar que diferentemente do capítulo I, no segundo capítulo nós utilizamos iluminações diferentes para diferentes períodos do dia. Esta diferença na metodologia ocorreu para evitar o efeito *jet lag*. No primeiro trabalho o intervalo entre tarefas era de sete dias, tempo suficiente para que o padrão de atividade noturna fosse reestabelecido após uma primeira interferência na ritmicidade circadiana (Yan, 2011), enquanto que no segundo trabalho o cronograma experimental exigia que o intervalo entre tarefas fosse de 24 horas. Por estes motivos, no segundo estudo, foi utilizada iluminação branca para testes realizados durante o período claro do ciclo claro/escuro enquanto que iluminação vermelha foi utilizada para o período escuro. Desta forma, nós acreditamos que os dados referentes a ambos os grupos testados no escuro não representam atividade propriamente dita, mas um padrão comportamental que é modulado por uma combinação de iluminação e o período do dia. Esses dados também sugerem que algumas das alterações comportamentais induzidas pelo EA estejam relacionadas a alterações na percepção visual, uma vez que diferenças entre os ambientes de criação não foram observadas na ausência de luz, quando os sentidos visuais estavam indisponíveis. Esta hipótese é perfeitamente plausível uma vez que diversos estudos demonstraram que o EA acelera o desenvolvimento do sistema visual e melhora a

acuidade visual dos animais (Cancedda *et al.*, 2004; Sale *et al.*, 2004; Sale *et al.*, 2007; Sale, Berardi e Maffei, 2009).

Após confirmar que a PCA é uma ferramenta eficaz em identificar tanto alterações comportamentais sutis quanto mudanças acentuadas, e que os efeitos benéficos do EA ocorrem principalmente, mas não exclusivamente, quando os sentidos visuais estão disponíveis, nós investigamos se bloquear seletivamente os NMDAR contendo a subunidade GluN2B durante períodos precoces do desenvolvimento encefálico afetaria as funções cognitivas dos animais, e se a estimulação da BCR, por meio do paradigma de EA, em períodos precoces do desenvolvimento encefálico é capaz de prevenir ou atenuar as alterações cognitivas induzidas pelo bloqueio transiente de NMDAR durante o desenvolvimento encefálico. Neste estudo (Capítulo III) nós utilizamos uma metodologia mista dos dois primeiros capítulos. A tarefa de labirinto em cruz elevado foi realizada conforme descrito no Capítulo I (iluminação vermelha durante o período escuro do ciclo claro/escuro) e, apesar de a tarefa de campo aberto ser realizada com a mesma iluminação utilizada no capítulo I (iluminação branca), os testes ocorreram durante o período claro do ciclo claro/escuro, similar ao Capítulo II. Escolhemos utilizar esta configuração de iluminação/período do dia por quatro motivos: primeiro, optamos por minimizar qualquer possível perturbação no sistema circadiano dos animais para evitar o efeito *jet lag* (Yan, 2011); segundo, os efeitos benéficos do EA se mostraram mais evidentes quando os testes foram realizados durante o período claro na presença de luz (por favor veja o Capítulo II); terceiro, a formação de *home base* no teste de campo aberto acontece mais rápido na presença de luz (Zadicario *et al.*, 2005; Avni, Zadicario e Eilam, 2006); e quarto, parâmetros relacionados a exploração e comportamentos tipo ansiosos seriam coletados tanto durante o período claro (na tarefa de campo aberto) quanto no período escuro (na tarefa de labirinto em cruz elevado).

Os resultados do Capítulo III mostraram que animais criados em ambientes enriquecidos apresentaram ganho de peso corporal reduzido quando comparado a animais criados em AP. Embora a redução no ganho de peso em animais expostos ao EA seja perfeitamente plausível (Fiala, Snow e Greenough, 1977; Moncek *et al.*, 2004; Pena *et al.*, 2006; Pena *et al.*, 2009; Hughes e Collins, 2010) devido ao fato de que o EA permite que os animais aumentem os níveis de atividade física (Moncek *et al.*, 2004) e reduzam a quantidade de comida ingerida (Fiala, Snow e Greenough, 1977), aumentando desta forma a taxa metabólica diária (Moncek *et al.*, 2004; Cao *et al.*, 2010; Cao *et al.*, 2011; Slater e Cao, 2015), muitos estudos observaram um aumento no ganho de peso de animais submetidos ao EA (Van de Weerd *et al.*, 1997; Tsai *et al.*, 2003; Sale *et al.*, 2007) ou não observaram diferenças entre os diferentes ambientes (Mainardi *et al.*, 2010; Beale *et al.*, 2011; Gergerlioglu *et al.*, 2016). Essas diferenças podem ser atribuídas tanto aos diferentes tipos de EA (por exemplo, presença ou ausência de rodas de correr ou complexidade das caixas moradia) quanto aos diferentes *backgrounds* genéticos das espécies (ou linhagens) utilizadas (Simpson e Kelly, 2011; Viola e Loss, 2014).

Além disso, os resultados do Capítulo III também mostraram que o antagonismo transiente de NMDAR durante idades precoces afetou a maturação e o desenvolvimento dos animais uma vez que foi observado um atraso no neurodesenvolvimento (nos filhotes tratados com CI) e ganho de peso reduzido (nos filhotes tratados com KET ou CI). Uma redução do peso corporal induzido por antagonismo transiente de NMDAR durante idades precoces do desenvolvimento foi também observada em outros estudos (Stefani e Moghaddam, 2005; Kawabe, Iwasaki e Ichitani, 2007). Embora os autores não tenham discutido o porquê de o bloqueio de NMDAR causar perda de peso (ou reduzir o ganho de peso), eles descartaram a hipótese de que as alterações

comportamentais duradouras observadas nos mesmos animais sejam efeitos de uma possível desnutrição infantil induzida pelo bloqueio de NMDAR. Esta constatação é baseada no fato de que alterações neuroquímicas e comportamentais induzidas pelo bloqueio neonatal de NMDAR diferem das alterações induzidas por desnutrição (Gorter *et al.*, 1992; Facchinetti *et al.*, 1993; Facchinetti *et al.*, 1994), e pelo fato de que déficits na aprendizagem após bloqueio neonatal de NMDAR foram observados mesmo em estudos que não observaram redução de peso corporal (Griesbach e Amsel, 1998; Akillioglu, Binokay e Kocahan, 2012). Em nosso estudo observamos que mesmo com a redução de peso, animais que foram tratados com KET não apresentaram alterações comportamentais em relação ao grupo SAL. Nós acreditamos que a diminuição do ganho de peso corporal induzida pelo bloqueio neonatal de NMDAR possa ser devido a uma desregulação das redes neurais que controlam a busca por alimento. Essa hipótese é corroborada pelo fato de que o antagonismo de NMDAR diminui o peso corporal e a ingestão de alimentos através de interferências no sistema de neurotransmissão mediado por NMDAR no hipotálamo lateral (Stanley *et al.*, 1996; Schiffelholz, Hinze-Selch e Aldenhoff, 2004).

Embora tanto a administração de KET quanto CI tenham diminuído o ganho de peso dos animais, apenas ratos tratados com CI apresentaram um atraso no neurodesenvolvimento (maturação física e sensório-motora). Esses dados sugerem que diferentes populações de NMDAR estão envolvidas no controle destas funções. Essa hipótese é corroborada pelo fato de que diferentes subunidades (ou subpopulações) de NMDAR estão localizadas em diferentes regiões e refletem diferentes funções fisiológicas destes receptores (Hardingham, Fukunaga e Bading, 2002; Li *et al.*, 2002; Petralia, 2012).

Além de alterar o neurodesenvolvimento dos animais, o bloqueio de NMDAR contendo a subunidade GluN2B durante períodos iniciais do desenvolvimento encefálico também alterou alguns aspectos cognitivos de forma duradoura. Animais injetados com CI e criados em AP apresentaram atividade locomotora reduzida no segundo dia de campo aberto relacionada a uma melhor habituação à tarefa, quando comparados aos animais injetados com SAL ou KET e criados em AP. No entanto, expor os animais ao EA durante a infância reverteu estas alterações. Estes dados sugerem que a estimulação da BCR após um insulto durante idades precoces do desenvolvimento impede que alterações (danosas ou não) sejam instaladas no SNC, provavelmente através de um mecanismo compensatório (Stern *et al.*, 2005; Stern, 2009).

Intrigantemente e em contraste ao encontrado em outros estudos (Stefani e Moghaddam, 2005; Kawabe, Iwasaki e Ichitani, 2007; Akillioglu *et al.*, 2012; Akillioglu, Binokay e Kocahan, 2012), com exceção da melhor habituação induzida pela administração de CI (conforme discutido acima), nenhuma alteração comportamental foi observada entre animais injetados com antagonistas de NMDAR e SAL. Estas diferenças poderiam ser atribuídas aos diferentes mecanismos de ação do antagonista de NMDAR utilizado em nosso estudo (KET), e o antagonista NMDAR utilizado nos demais estudos (MK-801). Embora ambos KET e MK-801 sejam antagonistas não competitivos de NMDAR, o MK-801 bloqueia irreversivelmente o canal do NMDAR impedindo o fluxo de íons de forma permanente (Hardingham, Fukunaga e Bading, 2002) enquanto que a KET bloqueia o NMDAR reduzindo a probabilidade (frequência) de abertura do canal e o tempo de abertura do canal, desta forma, diminuindo o fluxo de íons sem impedir permanentemente o transito de íons (Orser, Pennefather e MacDonald, 1997; Machado-Vieira, Manji e Zarate, 2009).

Entretanto esta hipótese não é corroborada por resultados de Kawabe et al. (2007) os quais mostraram que a administração do antagonista competitivo de NMDAR, CGS 19755, durante idades iniciais do desenvolvimento produziu alterações comportamentais similares aos observados após administração de MK-801. Além disso, estudos anteriores mostraram que a administração de KET em idades precoces do desenvolvimento também foi capaz de induzir alterações neuroquímicas e comportamentais duradouras (Fredriksson *et al.*, 2004; Loss, Cordova e de Oliveira, 2012; Jeevakumar e Kroener, 2016). Desta forma, nós acreditamos que os resultados conflitantes entre os estudos não seja devido ao uso de diferentes antagonistas de NMDAR.

Uma segunda hipótese é a de que o antagonismo de NMDAR durante períodos iniciais do desenvolvimento causa alterações comportamentais que ainda não se manifestaram na idade em que os animais foram testados em nosso estudo (60 a 66 dias pós-natal). Estudos têm sugerido que a esquizofrenia é um transtorno do neurodesenvolvimento causado por alterações da sinalização glutamatérgica durante estágios iniciais do desenvolvimento na qual o indivíduo apresenta sintomas positivos, negativos e cognitivos na idade adulta, após outros sistemas de neurotransmissores atingirem a maturação (Olney, Newcomer e Farber, 1999; Insel, 2010). Contudo, outros estudos reportaram que o antagonismo de NMDAR durante períodos iniciais do desenvolvimento induziu alterações comportamentais em idades similares às avaliadas em nosso estudo (Stefani e Moghaddam, 2005; Kawabe, Iwasaki e Ichitani, 2007; Uehara *et al.*, 2010; Akillioglu *et al.*, 2012; Akillioglu, Binokay e Kocahan, 2012).

Ainda, uma terceira e potencialmente mais provável hipótese que poderia explicar os divergentes achados é a avaliação do comportamento através da utilização de diferentes tarefas comportamentais (que avaliam diferentes tipos de comportamento).

O antagonismo de NMDAR durante períodos iniciais do desenvolvimento já foi demonstrado causar distúrbios sensoriomotores e cognitivos, tais como prejuízos na inibição do pré-pulso (Uehara *et al.*, 2009; Uehara *et al.*, 2010; Uehara *et al.*, 2012), na memória espacial (Stefani e Moghaddam, 2005; Kawabe, Iwasaki e Ichitani, 2007; Akillioglu *et al.*, 2012), e flexibilidade (Stefani e Moghaddam, 2005). Resultados conflitantes foram encontrados com respeito a alterações na atividade locomotora e nos níveis de ansiedade (Stefani e Moghaddam, 2005; Kawabe, Iwasaki e Ichitani, 2007; Uehara *et al.*, 2009; Uehara *et al.*, 2010; Akillioglu *et al.*, 2012; Akillioglu, Binokay e Kocahan, 2012), os quais potencialmente sugerem um efeito espécie-(linhagem)-dependente do antagonismo de NMDAR em idades precoces do desenvolvimento. Em nosso estudo observamos que o antagonismo de NMDAR em idades precoces do desenvolvimento não afetou comportamentos relacionados à locomoção, exploração, ansiedade e memória aversiva associativa. Estes resultados corroboram resultados de alguns estudos (Stefani e Moghaddam, 2005; Kawabe, Iwasaki e Ichitani, 2007; Uehara *et al.*, 2009), mas contradizem os resultados de outros estudos (Akillioglu *et al.*, 2012; Akillioglu, Binokay e Kocahan, 2012), sugerindo que mais estudos são necessários para completa elucidação dos efeitos do antagonismo de NMDAR na infância sobre esses comportamentos.

Os resultados do Capítulo III também apontaram alterações no repertório comportamental de animais submetidos ao EA em relação a animais criados em AP. Nossos dados indicam que conforme o ambiente vai se tornando familiar, os animais criados em ambientes enriquecidos diminuem sua exposição às áreas potencialmente perigosas mais rapidamente do que fazem animais criados em AP. Estudos anteriores (Zambrana *et al.*, 2007; Jule, Leaver e Lea, 2008; Roberts, Taylor e de Leaniz, 2011; Walker e Mason, 2011) juntamente com resultados do Capítulo II, sugerem que o EA

induz uma redução das respostas neofóbicas e da procura por novidade, o que acelera o processo de habituação e estimula a expressão de comportamentos evolutivamente mais favoráveis aos animais, como por exemplo, aumento da expressão de comportamentos defensivos em roedores. De fato, nossos resultados mostraram uma redução das respostas neofóbicas dos animais criados em ambientes enriquecidos, os quais apresentam uma maior probabilidade de entrar em ambientes potencialmente mais perigosos do que animais criados em AP. Juntos, nossos resultados sugerem que o EA induz uma redução da auto-exposição a ambientes inseguros quando não existe mais necessidade de explorá-los (ou seja, quando o ambiente já é familiar) sem afetar, contudo, a exploração do ambiente quando ele ainda não é familiar.

No Capítulo IV da presente Tese, nós investigamos se o bloqueio neonatal de NMDAR é capaz de alterar a captação de glicose encefálica na idade adulta, e se a estimulação da reserva cognitiva e encefálica durante a infância é capaz de prevenir as potenciais alterações na captação de glicose encefálica induzidas pelo bloqueio neonatal de NMDAR. Estudos que utilizaram ferramentas de neuroimagem indicam que existam alterações na utilização de glicose encefálica em pacientes esquizofrênicos (Shinto *et al.*, 2014; Bralet *et al.*, 2015). Uma vez que alterações na utilização de glicose encefálica podem refletir diferenças na ativação encefálica (Sokoloff, 1993; Mosconi *et al.*, 2008; Zimmer *et al.*, 2017), é possível que a organização das redes neurais relacionadas à integração funcional entre os diferentes sistemas de neurotransmissão estejam comprometidas em pacientes esquizofrênicos (Dawson *et al.*, 2012). Através da utilização de uma abordagem de medicina translacional reversa, nós investigamos se o nosso modelo de hipofunção de NMDAR induz alterações no metabolismo encefálico de glicose similar aos observados em pacientes esquizofrênicos. Nossos resultados (embora parciais) de captação de [18F]-fluorodesoxiglicose (18F-FDG), através de

escaneamento por Tomografia por Emissão de Pósitrons, não mostraram haver macro-alterações no metabolismo encefálico de glicose em animais submetidos à hipoativação neonatal de NMDAR. Contudo, alterações no padrão regional de utilização de glicose pelo encéfalo não podem ser descartados. Além disso, nossos resultados sugerem que animais criados em ambientes enriquecidos apresentam uma tendência a aumentarem a captação de glicose encefálica, o que pode indicar um aumento de atividade encefálica (Sokoloff, 1993; Mosconi *et al.*, 2008; Zimmer *et al.*, 2017). Se confirmados, estes resultados aumentarão as evidências que dão suporte às alterações cognitivas duradouras induzidas pela estimulação da BCR durante idades precoces do desenvolvimento encefálico.

CONCLUSÃO

Como considerações finais desta Tese, pode-se concluir que o comportamento animal é fortemente influenciado por fatores ambientais, sendo que os dois fatores ambientais abordados nesta Tese (ritmo circadiano e EA) afetam o comportamento animal tanto de forma individual quanto integrada. Os resultados desta Tese mostraram a importância de se levar em consideração o período do dia em que os animais serão submetidos a tarefas comportamentais no momento de planejamento e desenho experimental, ressaltando a importância da elaboração e definição da pergunta experimental que se pretende responder. Estes dados podem, e devem ser extrapolados para o planejamento de experimentos biológicos que não envolvam análise comportamental, uma vez que alterações comportamentais refletem alterações fisiológicas, e, portanto, experimentos *ex vivo* também devem ser cuidadosamente planejados. De mesmo modo, a Análise de Componentes Principais (PCA) se mostrou uma ferramenta muito útil não somente na análise comportamental (conforme observado nos Capítulos I-III), mas também para a análise do desenvolvimento físico e

sensoriomotor (Capítulo III) além de análise da captação de 18F-FDG encefálico, sugerindo que sua aplicação pode auxiliar a interpretar amplos conjuntos de dados em diversas áreas da ciência e que a análise estatística deve ser melhor explorada pela comunidade científica. A aplicação da PCA permitiu que algumas características sutis do comportamento fossem identificadas sem afetar a identificação de alterações comportamentais mais pronunciadas.

Pode-se concluir também que as diferentes populações de NMDAR estão envolvidas em diferentes processos de maturação encefálica, e que interferências no funcionamento das diferentes populações durante períodos críticos de desenvolvimento encefálico podem alterar o funcionamento do encéfalo gerando adaptações nas comunicações neurais de modo a modificar o fenótipo comportamental de maneira duradoura. Contudo, apesar de o EA ter induzido alterações comportamentais benéficas ao organismo, a hipótese de que a estimulação da BCR durante períodos precoces do desenvolvimento pode prevenir a evolução de um quadro esquizofrênico induzido por períodos de hipofunção de NMDAR durante a infância não pode ser confirmada uma vez que os resultados a respeito desta hipótese se mostraram inconclusivos, e portanto, novos estudos são necessários para elucidação deste tema.

PARTE IV. PERSPECTIVAS

Considerando que o antagonismo de NMDAR durante períodos iniciais do desenvolvimento pode causar neurodegeneração em diversas regiões encefálicas (Ikonomidou *et al.*, 1999; Kaindl e Ikonomidou, 2007; Kaindl *et al.*, 2008) e alterar tanto a neurogênese quanto a sinaptogênese de forma duradoura (Kaindl *et al.*, 2008; Turski e Ikonomidou, 2012), torna-se de extrema importância investigar quais as populações de NMDAR estão envolvidas nestes processos. Nosso grupo já está com experimentos em andamento para investigação dos efeitos que o antagonismo de NMDAR contendo a subunidade GluN2B durante o período neonatal exerce sobre a neurogênese e neurodegeneração cerebral. Além disso, resultados do Capítulo III sugerem que o antagonismo de NMDAR contendo a subunidade GluN2B durante períodos iniciais do desenvolvimento melhora a habituação de animais adultos na tarefa de CA. Essas alterações poderiam estar associadas a uma maior expressão da subunidade GluN2B na idade adulta (Tang *et al.*, 1999; Cao *et al.*, 2007; Brim *et al.*, 2013), induzida pelo antagonismo neonatal de NMDAR contendo a subunidade GluN2B através de um mecanismo de *feedback* negativo. Análises da expressão de diferentes subunidades de NMDAR serão feitas em algumas regiões cerebrais tais como córtex pré-frontal, hipocampo e núcleo accumbens.

Estudos que avaliaram o metabolismo de glicose através de 18-FDG PET *scan* observaram diferenças no perfil de ativação encefálico tanto em pacientes quanto em modelos de esquizofrenia em animais adultos (Shinto *et al.*, 2014; Bralet *et al.*, 2015; Kosten *et al.*, 2016). Contudo, não se sabe se o modelo de repetidos episódios de hipofunção de NMDAR durante estágios iniciais do desenvolvimento é capaz de induzir estas alterações no metabolismo de glicose, se a estimulação da BCR é capaz de prevenir estas alterações, e qual o papel dos NMDAR contendo a subunidade GluN2B neste processo. Análise do perfil de captação de 18-FDG por PET *scan* (conforme

Capítulo IV), além de análise da função mitocondrial para avaliação da atividade dos complexos do sistema de fosforilação oxidativa mitocondrial e ATP sintase através de um método de respirometria de alta-resolução já estão em andamento.

Ainda, propostas para a investigação do efeito do antagonismo neonatal de NMDAR contendo a subunidade GluN2B em tarefas comportamentais que avaliem outros tipos de memória (tais como aprendizado espacial de trabalho, curta e longa duração, além de tarefas que avaliem flexibilidade e atenção), já estão em processo de elaboração para dar continuidade a investigação do papel de NMDAR no desenvolvimento da esquizofrenia.

PARTE V. REFERÊNCIAS BIBLIOGRÁFICAS

Akillioglu, K., Babar Melik, E., Melik, E. and Kocahan, S. "The investigation of neonatal MK-801 administration and physical environmental enrichment on emotional and cognitive functions in adult Balb/c mice." Pharmacol Biochem Behav **102**: 407-414 (2012).

Akillioglu, K., Binokay, S. and Kocahan, S. "The effect of neonatal N-methyl-D-aspartate receptor blockade on exploratory and anxiety-like behaviors in adult BALB/c and C57BL/6 mice." Behav Brain Res **233**: 157-161 (2012).

Akkerman, S., Prickaerts, J., Bruder, A. K., Wolfs, K. H., De Vry, J., Vanmierlo, T. and Blokland, A. "PDE5 inhibition improves object memory in standard housed rats but not in rats housed in an enriched environment: implications for memory models?" PLoS One **9**: e111692 (2014).

Al-Hallaq, R. A., Conrads, T. P., Veenstra, T. D. and Wenthold, R. J. "NMDA di-heteromeric receptor populations and associated proteins in rat hippocampus." J Neurosci **27**: 8334-8343 (2007).

Anderson, C. M. and Swanson, R. A. "Astrocyte glutamate transport: review of properties, regulation, and physiological functions." Glia **32**: 1-14 (2000).

Anticevic, A., Gancsos, M., Murray, J. D., Repovs, G., Driesen, N. R., Ennis, D. J., Niciu, M. J., Morgan, P. T., Surti, T. S., Bloch, M. H., Ramani, R., Smith, M. A., Wang, X. J., Krystal, J. H. and Corlett, P. R. "NMDA receptor function in large-scale anticorrelated neural systems with implications for cognition and schizophrenia." Proc Natl Acad Sci U S A **109**: 16720-16725 (2012).

Auger, C. and Attwell, D. "Fast removal of synaptic glutamate by postsynaptic transporters." Neuron **28**: 547-558 (2000).

Avni, R., Zadicario, P. and Eilam, D. "Exploration in a dark open field: a shift from directional to positional progression and a proposed model of acquiring spatial information." Behav Brain Res **171**: 313-323 (2006).

Bahar-Fuchs, A., Clare, L. and Woods, B. "Cognitive training and cognitive rehabilitation for persons with mild to moderate dementia of the Alzheimer's or vascular type: a review." Alzheimers Res Ther **5**: 35 (2013).

Baldini, S., Restani, L., Baroncelli, L., Coltelli, M., Franco, R., Cenni, M. C., Maffei, L. and Berardi, N. "Enriched early life experiences reduce adult anxiety-like behavior in rats: a role for insulin-like growth factor 1." J Neurosci **33**: 11715-11723 (2013).

Baumans, V. "Environmental enrichment for laboratory rodents and rabbits: requirements of rodents, rabbits, and research." ILAR J **46**: 162-170 (2005).

Baumans, V. and Van Loo, P. L. "How to improve housing conditions of laboratory animals: the possibilities of environmental refinement." Vet J **195**: 24-32 (2013).

Beale, K. E., Murphy, K. G., Harrison, E. K., Kerton, A. J., Ghatei, M. A., Bloom, S. R. and Smith, K. L. "Accurate measurement of body weight and food intake in environmentally enriched male Wistar rats." Obesity (Silver Spring) **19**: 1715-1721 (2011).

Bear, M. F., Connors, B. W. and Paradiso, M. A. (2002). Neurociências: desvendando o sistema nervoso. Porto Alegre, ARTMED**:** 855.

Bear, M. F., Connors, B. W. and Paradiso, M. A. (2009). Neuroscience: exploring the brain. Philadelphia, Lippincott Williams & Wilkins.

Benveniste, H., Drejer, J., Schousboe, A. and Diemer, N. H. "Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis." J Neurochem **43**: 1369-1374 (1984).

Bezzina, C., Verret, L., Halley, H., Dahan, L. and Rampon, C. "Environmental enrichment does not influence hypersynchronous network activity in the Tg2576 mouse model of Alzheimer's disease." Front Aging Neurosci **7**: 178 (2015).

Bigge, C. F. "Structural requirements for the development of potent N-methyl-D-aspartic acid (NMDA) receptor antagonists." Biochem Pharmacol **45**: 1547-1561 (1993).

Blahos, J., 2nd and Wenthold, R. J. "Relationship between N-methyl-D-aspartate receptor NR1 splice variants and NR2 subunits." J Biol Chem **271**: 15669-15674 (1996).

Bondi, C., Matthews, M. and Moghaddam, B. "Glutamatergic animal models of schizophrenia." Curr Pharm Des **18**: 1593-1604 (2012).

Bralet, M. C., Buchsbaum, M. S., DeCastro, A., Shihabuddin, L. and Mitelman, S. A. "FDG-PET scans in patients with Kraepelinian and non-Kraepelinian schizophrenia." Eur Arch Psychiatry Clin Neurosci (2015).

Brim, B. L., Haskell, R., Awedikian, R., Ellinwood, N. M., Jin, L., Kumar, A., Foster, T. C. and Magnusson, K. R. "Memory in aged mice is rescued by enhanced expression of the GluN2B subunit of the NMDA receptor." Behav Brain Res **238**: 211-226 (2013).

Brown, J., Cooper-Kuhn, C. M., Kempermann, G., Van Praag, H., Winkler, J., Gage, F. H. and Kuhn, H. G. "Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis." Eur J Neurosci **17**: 2042-2046 (2003).

Burrows, E. L., McOmish, C. E., Buret, L. S., Van den Buuse, M. and Hannan, A. J. "Environmental Enrichment Ameliorates Behavioral Impairments Modeling Schizophrenia in Mice Lacking Metabotropic Glutamate Receptor 5." Neuropsychopharmacology **40**: 1947-1956 (2015).

Cancedda, L., Putignano, E., Sale, A., Viegi, A., Berardi, N. and Maffei, L. "Acceleration of visual system development by environmental enrichment." J Neurosci **24**: 4840-4848 (2004).

Cao, L., Choi, E. Y., Liu, X., Martin, A., Wang, C., Xu, X. and During, M. J. "White to brown fat phenotypic switch induced by genetic and environmental activation of a hypothalamic-adipocyte axis." Cell Metab **14**: 324-338 (2011).

Cao, L., Liu, X., Lin, E. J., Wang, C., Choi, E. Y., Riban, V., Lin, B. and During, M. J. "Environmental and genetic activation of a brain-adipocyte BDNF/leptin axis causes cancer remission and inhibition." Cell **142**: 52-64 (2010).

Cao, X., Cui, Z., Feng, R., Tang, Y. P., Qin, Z., Mei, B. and Tsien, J. Z. "Maintenance of superior learning and memory function in NR2B transgenic mice during ageing." Eur J Neurosci **25**: 1815-1822 (2007).

Chapman, A. G. "Glutamate and epilepsy." J Nutr **130**: 1043S-1045S (2000).

Chatterton, J. E., Awobuluyi, M., Premkumar, L. S., Takahashi, H., Talantova, M., Shin, Y., Cui, J., Tu, S., Sevarino, K. A., Nakanishi, N., Tong, G., Lipton, S. A. and Zhang, D. "Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits." Nature **415**: 793-798 (2002).

Chazot, P. L. and Stephenson, F. A. "Molecular dissection of native mammalian forebrain NMDA receptors containing the NR1 C2 exon: direct demonstration of NMDA receptors comprising NR1, NR2A, and NR2B subunits within the same complex." J Neurochem **69**: 2138-2144 (1997).

Clare, L., Woods, R. T., Moniz Cook, E. D., Orrell, M. and Spector, A. "Cognitive rehabilitation and cognitive training for early-stage Alzheimer's disease and vascular dementia." Cochrane Database Syst RevCD003260 (2003).

Cotman, C. W., Monaghan, D. T. and Ganong, A. H. "Excitatory amino acid neurotransmission: NMDA receptors and Hebb-type synaptic plasticity." Annu Rev Neurosci **11**: 61-80 (1988).

Cull-Candy, S., Brickley, S. and Farrant, M. "NMDA receptor subunits: diversity, development and disease." Curr Opin Neurobiol **11**: 327-335 (2001).

Cull-Candy, S. G. and Leszkiewicz, D. N. "Role of distinct NMDA receptor subtypes at central synapses." Sci STKE **2004**: re16 (2004).

Dawson, N., Xiao, X., McDonald, M., Higham, D. J., Morris, B. J. and Pratt, J. A. "Sustained NMDA Receptor Hypofunction Induces Compromised Neural Systems Integration and Schizophrenia-Like Alterations in Functional Brain Networks." Cereb Cortex (2012).

Di Maio, R., Mastroberardino, P. G., Hu, X., Montero, L. and Greenamyre, J. T. "Pilocapine alters NMDA receptor expression and function in hippocampal neurons: NADPH oxidase and ERK1/2 mechanisms." Neurobiol Dis (2011).

Dingledine, R., Borges, K., Bowie, D. and Traynelis, S. F. "The glutamate receptor ion channels." Pharmacol Rev **51**: 7-61 (1999).

Diniz, D. G., Foro, C. A., Rego, C. M., Gloria, D. A., de Oliveira, F. R., Paes, J. M., de Sousa, A. A., Tokuhashi, T. P., Trindade, L. S., Turiel, M. C., Vasconcelos, E. G., Torres, J. B., Cunnigham, C., Perry, V. H., Vasconcelos, P. F. and Diniz, C. W. "Environmental impoverishment and aging alter object recognition, spatial learning, and dentate gyrus astrocytes." Eur J Neurosci **32**: 509-519 (2010).

du Bois, T. M. and Huang, X. F. "Early brain development disruption from NMDA receptor hypofunction: relevance to schizophrenia." Brain Res Rev **53**: 260-270 (2007).

EC "Directive 2010/63/EU of the European Parliament and the Council of 22 September on the protection of animals used for scientific purposes." Official Journal of the European Union **276**: 33–79 (2010).

Ernst, T. and Chang, L. "Adaptation of brain glutamate plus glutamine during abstinence from chronic methamphetamine use." J Neuroimmune Pharmacol **3**: 165-172 (2008).

Ewald, R. C. and Cline, H. T. "NMDA Receptors and Brain Development." (2009).

Facchinetti, F., Ciani, E., Dall'Olio, R., Virgili, M., Contestabile, A. and Fonnum, F. "Structural, neurochemical and behavioural consequences of neonatal blockade of NMDA receptor through chronic treatment with CGP 39551 or MK-801." Brain Res Dev Brain Res **74**: 219-224 (1993).

Facchinetti, F., Dall'Olio, R., Ciani, E., Sparapani, M., Virgili, M. and Contestabile, A. "Long-lasting effects of chronic neonatal blockade of N-methyl-D-aspartate receptor through the competitive antagonist CGP 39551 in rats." Neuroscience **60**: 343-353 (1994).

Faherty, C. J., Kerley, D. and Smeyne, R. J. "A Golgi-Cox morphological analysis of neuronal changes induced by environmental enrichment." Brain Res Dev Brain Res **141**: 55-61 (2003).

Fiala, B., Snow, F. M. and Greenough, W. T. ""Impoverished" rats weigh more than "enriched" rats because they eat more." Dev Psychobiol **10**: 537-541 (1977).

Flores-Soto, M. E., Chaparro-Huerta, V., Escoto-Delgadillo, M., Vazquez-Valls, E., Gonzalez-Castaneda, R. E. and Beas-Zarate, C. "[Structure and function of NMDA-type glutamate receptor subunits]." Neurologia **27**: 301-310 (2012).

Fonnum, F. "Glutamate: a neurotransmitter in mammalian brain." J Neurochem **42**: 1-11 (1984).

Fratiglioni, L., Paillard-Borg, S. and Winblad, B. "An active and socially integrated lifestyle in late life might protect against dementia." Lancet Neurol **3**: 343-353 (2004).

Fredriksson, A., Archer, T., Alm, H., Gordh, T. and Eriksson, P. "Neurofunctional deficits and potentiated apoptosis by neonatal NMDA antagonist administration." Behav Brain Res **153**: 367-376 (2004).

Freedman, L. P., Cockburn, I. M. and Simcoe, T. S. "The Economics of Reproducibility in Preclinical Research." PLoS Biol **13**: e1002165 (2015).

Garcia, A. M., Cardenas, F. P. and Morato, S. "Effect of different illumination levels on rat behavior in the elevated plus-maze." Physiol Behav **85**: 265-270 (2005).

Gates, N. J. and Sachdev, P. "Is cognitive training an effective treatment for preclinical and early Alzheimer's disease?" J Alzheimers Dis **42 Suppl 4**: S551-559 (2014).

Gehres, S. W., Rocha, A., Leuzy, A., Loss, C. M., Viola, G. G. and Zimmer, E. R. "Cognitive Intervention As an Early Non-pharmacological Strategy in Alzheimer's Disease: A Translational Perspective." Front Aging Neurosci **8**: 280 (2016).

Gergerlioglu, H. S., Oz, M., Demir, E. A., Nurullahoglu-Atalik, K. E. and Yerlikaya, F. H. "Environmental enrichment reverses cognitive impairments provoked by Western diet in rats: Role of corticosteroid receptors." Life Sci **148**: 279-285 (2016).

Gorter, J. A., Botterblom, M. H., Feenstra, M. G. and Boer, G. J. "Chronic neonatal NMDA receptor blockade with MK-801 alters monoamine metabolism in the adult rat." Neurosci Lett **137**: 97-100 (1992).

Gray, J. A., Shi, Y., Usui, H., During, M. J., Sakimura, K. and Nicoll, R. A. "Distinct modes of AMPA receptor suppression at developing synapses by GluN2A and GluN2B: single-cell NMDA receptor subunit deletion in vivo." Neuron **71**: 1085-1101 (2011).

Griesbach, G. S. and Amsel, A. "Immediate and long-term effects of neonatal MK-801 treatment on nonspatial learning." Proc Natl Acad Sci U S A **95**: 11435-11439 (1998).

Gross, A. N., Richter, S. H., Engel, A. K. and Wurbel, H. "Cage-induced stereotypies, perseveration and the effects of environmental enrichment in laboratory mice." Behav Brain Res **234**: 61-68 (2012).

Hardingham, G. E., Fukunaga, Y. and Bading, H. "Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways." Nat Neurosci **5**: 405-414 (2002).

Henneberger, C., Bard, L., King, C., Jennings, A. and Rusakov, D. A. "NMDA Receptor Activation: Two Targets for Two Co-Agonists." Neurochem Res (2013).

Henson, M. A., Roberts, A. C., Perez-Otano, I. and Philpot, B. D. "Influence of the NR3A subunit on NMDA receptor functions." Prog Neurobiol **91**: 23-37 (2010).

Hertz, L., Dringen, R., Schousboe, A. and Robinson, S. R. "Astrocytes: glutamate producers for neurons." J Neurosci Res **57**: 417-428 (1999).

Hughes, R. N. and Collins, M. A. "Enhanced habituation and decreased anxiety by environmental enrichment and possible attenuation of these effects by chronic alpha-tocopherol (vitamin E) in aging male and female rats." Pharmacol Biochem Behav **94**: 534-542 (2010).

Huttenrauch, M., Salinas, G. and Wirths, O. "Effects of Long-Term Environmental Enrichment on Anxiety, Memory, Hippocampal Plasticity and Overall Brain Gene Expression in C57BL6 Mice." Front Mol Neurosci **9**: 62 (2016).

Ikonomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vockler, J., Dikranian, K., Tenkova, T. I., Stefovska, V., Turski, L. and Olney, J. W. "Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain." Science **283**: 70-74 (1999).

Insel, T. R. "Rethinking schizophrenia." Nature **468**: 187-193 (2010).

Izquierdo, I. and Medina, J. H. "Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures." Neurobiol Learn Mem **68**: 285-316 (1997).

Jansson, L. C. and Akerman, K. E. "The role of glutamate and its receptors in the proliferation, migration, differentiation and survival of neural progenitor cells." J Neural Transm (Vienna) **121**: 819-836 (2014).

Jeevakumar, V. and Kroener, S. "Ketamine Administration During the Second Postnatal Week Alters Synaptic Properties of Fast-Spiking Interneurons in the Medial Prefrontal Cortex of Adult Mice." Cereb Cortex **26**: 1117-1129 (2016).

Johnson, J. W. and Ascher, P. "Glycine potentiates the NMDA response in cultured mouse brain neurons." Nature **325**: 529-531 (1987).

Jolliffe, I. T. (1986). Principal component analysis. New York, Springer New York**:** 271.

Jones, N. and King, S. M. "Influence of circadian phase and test illumination on pre-clinical models of anxiety." Physiol Behav **72**: 99-106 (2001).

Jule, K. R., Leaver, L. A. and Lea, S. E. G. "The effects of captive experience on reintroduction survival in carnivores: A review and analysis." Biological Conservation **141**: 355-363 (2008).

Kaindl, A. M. and Ikonomidou, C. "Glutamate antagonists are neurotoxins for the developing brain." Neurotox Res **11**: 203-218 (2007).

Kaindl, A. M., Koppelstaetter, A., Nebrich, G., Stuwe, J., Sifringer, M., Zabel, C., Klose, J. and Ikonomidou, C. "Brief alteration of NMDA or GABAA receptor-mediated neurotransmission has long term effects on the developing cerebral cortex." Mol Cell Proteomics **7**: 2293-2310 (2008).

Katzman, R., Terry, R., DeTeresa, R., Brown, T., Davies, P., Fuld, P., Renbing, X. and Peck, A. "Clinical, pathological, and neurochemical changes in dementia: a subgroup with preserved mental status and numerous neocortical plaques." Ann Neurol **23**: 138-144 (1988).

Kawabe, K., Iwasaki, T. and Ichitani, Y. "Repeated treatment with N-methyl-d-aspartate antagonists in neonatal, but not adult, rats causes long-term deficits of radial-arm maze learning." Brain Res **1169**: 77-86 (2007).

Kemp, J. A. and Leeson, P. D. "The glycine site of the NMDA receptor--five years on." Trends Pharmacol Sci **14**: 20-25 (1993).

Kobilo, T., Liu, Q. R., Gandhi, K., Mughal, M., Shaham, Y. and van Praag, H. "Running is the neurogenic and neurotrophic stimulus in environmental enrichment." Learn Mem **18**: 605-609 (2011).

Kosten, L., Verhaeghe, J., Verkerk, R., Thomae, D., De Picker, L., Wyffels, L., Van Eetveldt, A., Dedeurwaerdere, S., Stroobants, S. and Staelens, S. "Multiprobe molecular imaging of an NMDA receptor hypofunction rat model for glutamatergic dysfunction." Psychiatry Res **248**: 1-11 (2016).

Latysheva, N. V. and Raevskii, K. S. "Behavioral analysis of the consequences of chronic blockade of NMDA-type glutamate receptors in the early postnatal period in rats." Neurosci Behav Physiol **33**: 123-131 (2003).

Lau, C. G. and Zukin, R. S. "NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders." Nat Rev Neurosci **8**: 413-426 (2007).

Laurie, D. J. and Seeburg, P. H. "Regional and developmental heterogeneity in splicing of the rat brain NMDAR1 mRNA." J Neurosci **14**: 3180-3194 (1994).

Leke, R. and Schousboe, A. "The Glutamine Transporters and Their Role in the Glutamate/GABA-Glutamine Cycle." Adv Neurobiol **13**: 223-257 (2016).

Li, B., Chen, N., Luo, T., Otsu, Y., Murphy, T. H. and Raymond, L. A. "Differential regulation of synaptic and extra-synaptic NMDA receptors." Nat Neurosci **5**: 833-834 (2002).

Limapichat, W., Yu, W. Y., Branigan, E., Lester, H. A. and Dougherty, D. A. "Key binding interactions for memantine in the NMDA receptor." ACS Chem Neurosci **4**: 255-260 (2013).

Lipton, S. A. and Rosenberg, P. A. "Excitatory amino acids as a final common pathway for neurologic disorders." N Engl J Med **330**: 613-622 (1994).

Loss, C. M., Binder, L. B., Muccini, E., Martins, W. C., de Oliveira, P. A., Vandresen-Filho, S., Prediger, R. D., Tasca, C. I., Zimmer, E. R., Costa-Schmidt, L. E., de Oliveira, D. L. and Viola, G. G. "Influence of environmental enrichment vs. time-of-day on behavioral repertoire of male albino Swiss mice." Neurobiol Learn Mem **125**: 63-72 (2015).

Loss, C. M., Cordova, S. D. and de Oliveira, D. L. "Ketamine reduces neuronal degeneration and anxiety levels when administered during early life-induced status epilepticus in rats." Brain Res **1474**: 110-117 (2012).

Low, C. M. and Wee, K. S. "New insights into the not-so-new NR3 subunits of N-methyl-D-aspartate receptor: localization, structure, and function." Mol Pharmacol **78**: 1-11 (2010).

Luhmann, H. J., Fukuda, A. and Kilb, W. "Control of cortical neuronal migration by glutamate and GABA." Front Cell Neurosci **9**: 4 (2015).

Machado-Vieira, R., Manji, H. K. and Zarate, C. A. "The role of the tripartite glutamatergic synapse in the pathophysiology and therapeutics of mood disorders." Neuroscientist **15**: 525-539 (2009).

Mainardi, M., Scabia, G., Vottari, T., Santini, F., Pinchera, A., Maffei, L., Pizzorusso, T. and Maffei, M. "A sensitive period for environmental regulation of eating behavior and leptin sensitivity." Proc Natl Acad Sci U S A **107**: 16673-16678 (2010).

Marquez-Arias, A., Santillan-Doherty, A. M., Arenas-Rosas, R. V., Gasca-Matias, M. P. and Munoz-Delgado, J. "Environmental enrichment for captive stumptail macaques (Macaca arctoides)." J Med Primatol **39**: 32-40 (2010).

Martinez-Rivera, A., Rodriguez-Borrero, E., Matias-Aleman, M., Montalvo-Acevedo, A., Guerrero-Figuereo, K., Febo-Rodriguez, L. J., Morales-Rivera, A. and Maldonado-Vlaar, C. S. "Metabotropic glutamate receptor 5 within nucleus accumbens shell modulates environment-elicited cocaine conditioning expression." Pharmacol Biochem Behav **110**: 154-160 (2013).

Matsuda, K., Kamiya, Y., Matsuda, S. and Yuzaki, M. "Cloning and characterization of a novel NMDA receptor subunit NR3B: a dominant subunit that reduces calcium permeability." Brain Res Mol Brain Res **100**: 43-52 (2002).

McDonald, J. W. and Johnston, M. V. "Physiological and pathophysiological roles of excitatory amino acids during central nervous system development." Brain Res Brain Res Rev **15**: 41-70 (1990).

Meagher, R. K. and Mason, G. J. "Environmental enrichment reduces signs of boredom in caged mink." PLoS One **7**: e49180 (2012).

Menard, C. and Quirion, R. "Group 1 metabotropic glutamate receptor function and its regulation of learning and memory in the aging brain." Front Pharmacol **3**: 182 (2012).

Mohammed, A. H., Zhu, S. W., Darmopil, S., Hjerling-Leffler, J., Ernfors, P., Winblad, B., Diamond, M. C., Eriksson, P. S. and Bogdanovic, N. "Environmental enrichment and the brain." Prog Brain Res **138**: 109-133 (2002).

Monaghan, D. T. and Cotman, C. W. "Distribution of N-methyl-D-aspartate-sensitive L-[3H]glutamate-binding sites in rat brain." J Neurosci **5**: 2909-2919 (1985).

Monaghan, D. T. and Jane, D. E. "Pharmacology of NMDA Receptors." (2009).

Moncek, F., Duncko, R., Johansson, B. B. and Jezova, D. "Effect of environmental enrichment on stress related systems in rats." J Neuroendocrinol **16**: 423-431 (2004).

Monteiro, B. M., Moreira, F. A., Massensini, A. R., Moraes, M. F. and Pereira, G. S. "Enriched environment increases neurogenesis and improves social memory persistence in socially isolated adult mice." Hippocampus **24**: 239-248 (2014).

Mony, L., Kew, J. N., Gunthorpe, M. J. and Paoletti, P. "Allosteric modulators of NR2B-containing NMDA receptors: molecular mechanisms and therapeutic potential." Br J Pharmacol **157**: 1301-1317 (2009).

Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B. and Seeburg, P. H. "Developmental and regional expression in the rat brain and functional properties of four NMDA receptors." Neuron **12**: 529-540 (1994).

Moore, R. Y. and Eichler, V. B. "Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat." Brain Res **42**: 201-206 (1972).

Mosconi, L., Tsui, W. H., Herholz, K., Pupi, A., Drzezga, A., Lucignani, G., Reiman, E. M., Holthoff, V., Kalbe, E., Sorbi, S., Diehl-Schmid, J., Perneczky, R., Clerici, F., Caselli, R., Beuthien-Baumann, B., Kurz, A., Minoshima, S. and de Leon, M. J. "Multicenter standardized 18F-FDG PET diagnosis of mild cognitive impairment, Alzheimer's disease, and other dementias." J Nucl Med **49**: 390-398 (2008).

Mothet, J. P., Parent, A. T., Wolosker, H., Brady, R. O., Jr., Linden, D. J., Ferris, C. D., Rogawski, M. A. and Snyder, S. H. "D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor." Proc Natl Acad Sci U S A **97**: 4926-4931 (2000).

Nedergaard, M., Takano, T. and Hansen, A. J. "Beyond the role of glutamate as a neurotransmitter." Nat Rev Neurosci **3**: 748-755 (2002).

Nicoletti, F., Bruno, V., Copani, A., Casabona, G. and Knopfel, T. "Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders?" Trends Neurosci **19**: 267-271 (1996).

Nishi, M., Hinds, H., Lu, H. P., Kawata, M. and Hayashi, Y. "Motoneuron-specific expression of NR3B, a novel NMDA-type glutamate receptor subunit that works in a dominant-negative manner." J Neurosci **21**: RC185 (2001).

Nithianantharajah, J. and Hannan, A. J. "Enriched environments, experience-dependent plasticity and disorders of the nervous system." Nat Rev Neurosci **7**: 697-709 (2006).

Nithianantharajah, J. and Hannan, A. J. "The neurobiology of brain and cognitive reserve: mental and physical activity as modulators of brain disorders." Prog Neurobiol **89**: 369-382 (2009).

Nithianantharajah, J. and Hannan, A. J. "Mechanisms mediating brain and cognitive reserve: experience-dependent neuroprotection and functional compensation in animal models of neurodegenerative diseases." Prog Neuropsychopharmacol Biol Psychiatry **35**: 331-339 (2011).

Ogden, K. K. and Traynelis, S. F. "New advances in NMDA receptor pharmacology." Trends Pharmacol Sci **32**: 726-733 (2011).

Olney, J. W., Newcomer, J. W. and Farber, N. B. "NMDA receptor hypofunction model of schizophrenia." J Psychiatr Res **33**: 523-533 (1999).

Orser, B. A., Pennefather, P. S. and MacDonald, J. F. "Multiple mechanisms of ketamine blockade of N-methyl-D-aspartate receptors." Anesthesiology **86**: 903-917 (1997).

Ozawa, S., Kamiya, H. and Tsuzuki, K. "Glutamate receptors in the mammalian central nervous system." Prog Neurobiol **54**: 581-618 (1998).

Pachernegg, S., Strutz-Seebohm, N. and Hollmann, M. "GluN3 subunit-containing NMDA receptors: not just one-trick ponies." Trends Neurosci **35**: 240-249 (2012).

Panksepp, J. B., Wong, J. C., Kennedy, B. C. and Lahvis, G. P. "Differential entrainment of a social rhythm in adolescent mice." Behavioural Brain Research **195**: 239-245 (2008).

Paoletti, P. "Molecular basis of NMDA receptor functional diversity." Eur J Neurosci **33**: 1351-1365 (2011).

Paoletti, P., Bellone, C. and Zhou, Q. "NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease." Nat Rev Neurosci **14**: 383-400 (2013).

Paupard, M. C., Friedman, L. K. and Zukin, R. S. "Developmental regulation and cell-specific expression of N-methyl-D-aspartate receptor splice variants in rat hippocampus." Neuroscience **79**: 399-409 (1997).

Pena, Y., Prunell, M., Dimitsantos, V., Nadal, R. and Escorihuela, R. M. "Environmental enrichment effects in social investigation in rats are gender dependent." Behav Brain Res **174**: 181-187 (2006).

Pena, Y., Prunell, M., Rotllant, D., Armario, A. and Escorihuela, R. M. "Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats." Psychoneuroendocrinology **34**: 1390-1404 (2009).

Peng, S., Zhang, Y., Zhang, J., Wang, H. and Ren, B. "Glutamate receptors and signal transduction in learning and memory." Mol Biol Rep **38**: 453-460 (2011).

Pereira, L. O., Nabinger, P. M., Strapasson, A. C., Nardin, P., Goncalves, C. A., Siqueira, I. R. and Netto, C. A. "Long-term effects of environmental stimulation following hypoxia-ischemia on the oxidative state and BDNF levels in rat hippocampus and frontal cortex." Brain Res **1247**: 188-195 (2009).

Petralia, R. S. "Distribution of extrasynaptic NMDA receptors on neurons." ScientificWorldJournal **2012**: 267120 (2012).

Petralia, R. S., Al-Hallaq, R. A. and Wenthold, R. J. "Trafficking and Targeting of NMDA Receptors." (2009).

Price, D. L. "New order from neurological disorders." Nature **399**: A3-5 (1999).

Prybylowski, K. and Wenthold, R. J. "N-Methyl-D-aspartate receptors: subunit assembly and trafficking to the synapse." J Biol Chem **279**: 9673-9676 (2004).

Rampon, C., Tang, Y. P., Goodhouse, J., Shimizu, E., Kyin, M. and Tsien, J. Z. "Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice." Nat Neurosci **3**: 238-244 (2000).

Rauner, C. and Kohr, G. "Triheteromeric NR1/NR2A/NR2B receptors constitute the major N-methyl-D-aspartate receptor population in adult hippocampal synapses." J Biol Chem **286**: 7558-7566 (2011).

Riedel, G., Platt, B. and Micheau, J. "Glutamate receptor function in learning and memory." Behav Brain Res **140**: 1-47 (2003).

Roberts, L. J., Taylor, J. and de Leaniz, C. G. "Environmental enrichment reduces maladaptive risk-taking behavior in salmon reared for conservation." Biological Conservation **144**: 1972-1979 (2011).

Rossi, C., Angelucci, A., Costantin, L., Braschi, C., Mazzantini, M., Babbini, F., Fabbri, M. E., Tessarollo, L., Maffei, L., Berardi, N. and Caleo, M. "Brain-derived neurotrophic

factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment." Eur J Neurosci **24**: 1850-1856 (2006).

Sale, A., Berardi, N. and Maffei, L. "Enrich the environment to empower the brain." Trends Neurosci **32**: 233-239 (2009).

Sale, A., Cenni, M. C., Ciucci, F., Putignano, E., Chierzi, S. and Maffei, L. "Maternal enrichment during pregnancy accelerates retinal development of the fetus." PLoS One **2**: e1160 (2007).

Sale, A., Putignano, E., Cancedda, L., Landi, S., Cirulli, F., Berardi, N. and Maffei, L. "Enriched environment and acceleration of visual system development." Neuropharmacology **47**: 649-660 (2004).

Sampedro-Piquero, P., Castilla-Ortega, E., Zancada-Menendez, C., Santin, L. J. and Begega, A. "Environmental enrichment as a therapeutic avenue for anxiety in aged Wistar rats: Effect on cat odor exposition and GABAergic interneurons." Neuroscience **330**: 17-25 (2016).

Sampedro-Piquero, P., De Bartolo, P., Petrosini, L., Zancada-Menendez, C., Arias, J. L. and Begega, A. "Astrocytic plasticity as a possible mediator of the cognitive improvements after environmental enrichment in aged rats." Neurobiol Learn Mem **114C**: 16-25 (2014).

Sanguansat, P. "Principal Component Analysis." 300 (2012).

Scarmeas, N., Levy, G., Tang, M. X., Manly, J. and Stern, Y. "Influence of leisure activity on the incidence of Alzheimer's disease." Neurology **57**: 2236-2242 (2001).

Schell, M. J., Molliver, M. E. and Snyder, S. H. "D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release." Proc Natl Acad Sci U S A **92**: 3948-3952 (1995).

Schiffelholz, T., Hinze-Selch, D. and Aldenhoff, J. B. "Perinatal MK-801 treatment affects age-related changes in locomotor activity from childhood to later adulthood in rats." Neurosci Lett **360**: 157-160 (2004).

Schousboe, A., Westergaard, N., Waagepetersen, H. S., Larsson, O. M., Bakken, I. J. and Sonnewald, U. "Trafficking between glia and neurons of TCA cycle intermediates and related metabolites." Glia **21**: 99-105 (1997).

Segovia, G., Del Arco, A., De Blas, M., Garrido, P. and Mora, F. "Environmental enrichment increases the in vivo extracellular concentration of dopamine in the nucleus accumbens: a microdialysis study." J Neural Transm **117**: 1123-1130 (2010).

Segovia, G., Porras, A., Del Arco, A. and Mora, F. "Glutamatergic neurotransmission in aging: a critical perspective." Mech Ageing Dev **122**: 1-29 (2001).

Sheng, M., Cummings, J., Roldan, L. A., Jan, Y. N. and Jan, L. Y. "Changing subunit composition of heteromeric NMDA receptors during development of rat cortex." Nature **368**: 144-147 (1994).

Shinto, A. S., Kamaleshwaran, K. K., Srinivasan, D., Paranthaman, S., Selvaraj, K., Pranesh, M. B., Lakshminarayanan, G. N. and Prakash, B. ""Hyperfrontality" as seen on FDG PET in unmedicated schizophrenia patients with positive symptoms." Clin Nucl Med **39**: 694-697 (2014).

Silva, C. F., Duarte, F. S., Lima, T. C. and de Oliveira, C. L. "Effects of social isolation and enriched environment on behavior of adult Swiss mice do not require hippocampal neurogenesis." Behav Brain Res **225**: 85-90 (2011).

Simpson, J. and Kelly, J. P. "The impact of environmental enrichment in laboratory rats--behavioural and neurochemical aspects." Behav Brain Res **222**: 246-264 (2011).

Slater, A. M. and Cao, L. "A Protocol for Housing Mice in an Enriched Environment." J Vis Expe52874 (2015).

Sokoloff, L. "Sites and mechanisms of function-related changes in energy metabolism in the nervous system." Dev Neurosci **15**: 194-206 (1993).

Sonnewald, U. and Schousboe, A. "Introduction to the Glutamate-Glutamine Cycle." Adv Neurobiol **13**: 1-7 (2016).

Stanley, B. G., Willett, V. L., 3rd, Donias, H. W., Dee, M. G., 2nd and Duva, M. A. "Lateral hypothalamic NMDA receptors and glutamate as physiological mediators of eating and weight control." Am J Physiol **270**: R443-449 (1996).

Stefani, M. R. and Moghaddam, B. "Transient N-methyl-D-aspartate receptor blockade in early development causes lasting cognitive deficits relevant to schizophrenia." Biol Psychiatry **57**: 433-436 (2005).

Stephan, F. K. and Zucker, I. "Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions." Proc Natl Acad Sci U S A **69**: 1583-1586 (1972).

Stern, Y. "Cognitive reserve." Neuropsychologia **47**: 2015-2028 (2009).

Stern, Y., Habeck, C., Moeller, J., Scarmeas, N., Anderson, K. E., Hilton, H. J., Flynn, J., Sackeim, H. and van Heertum, R. "Brain networks associated with cognitive reserve in healthy young and old adults." Cereb Cortex **15**: 394-402 (2005).

Tanabe, Y., Masu, M., Ishii, T., Shigemoto, R. and Nakanishi, S. "A family of metabotropic glutamate receptors." Neuron **8**: 169-179 (1992).

Tang, Y. P., Shimizu, E., Dube, G. R., Rampon, C., Kerchner, G. A., Zhuo, M., Liu, G. and Tsien, J. Z. "Genetic enhancement of learning and memory in mice." Nature **401**: 63-69 (1999).

Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., Hansen, K. B., Yuan, H., Myers, S. J. and Dingledine, R. "Glutamate receptor ion channels: structure, regulation, and function." Pharmacol Rev **62**: 405-496 (2010).

Tsai, P. P., Oppermann, D., Stelzer, H. D., Mahler, M. and Hackbarth, H. "The effects of different rack systems on the breeding performance of DBA/2 mice." Lab Anim **37**: 44-53 (2003).

Turski, C. A. and Ikonomidou, C. "Neuropathological sequelae of developmental exposure to antiepileptic and anesthetic drugs." Front Neurol **3**: 120 (2012).

Uehara, T., Sumiyoshi, T., Hattori, H., Itoh, H., Matsuoka, T., Iwakami, N., Suzuki, M. and Kurachi, M. "T-817MA, a novel neurotrophic agent, ameliorates loss of GABAergic parvalbumin-positive neurons and sensorimotor gating deficits in rats transiently exposed to MK-801 in the neonatal period." J Psychiatr Res **46**: 622-629 (2012).

Uehara, T., Sumiyoshi, T., Seo, T., Itoh, H., Matsuoka, T., Suzuki, M. and Kurachi, M. "Long-term effects of neonatal MK-801 treatment on prepulse inhibition in young adult rats." Psychopharmacology (Berl) **206**: 623-630 (2009).

Uehara, T., Sumiyoshi, T., Seo, T., Matsuoka, T., Itoh, H., Suzuki, M. and Kurachi, M. "Neonatal exposure to MK-801, an N-methyl-D-aspartate receptor antagonist, enhances methamphetamine-induced locomotion and disrupts sensorimotor gating in pre- and postpubertal rats." Brain Res **1352**: 223-230 (2010).

Van de Weerd, H. A., Aarsen, E. L., Mulder, A., Kruitwagen, C. L., Hendriksen, C. F. and Baumans, V. "Effects of environmental enrichment for mice: variation in experimental results." J Appl Anim Welf Sci **5**: 87-109 (2002).

Van de Weerd, H. A., Van Loo, P. L., Van Zutphen, L. F., Koolhaas, J. M. and Baumans, V. "Nesting material as environmental enrichment has no adverse effects on behavior and physiology of laboratory mice." Physiol Behav **62**: 1019-1028 (1997).

van Praag, H. "Neurogenesis and exercise: past and future directions." Neuromolecular Med **10**: 128-140 (2008).

van Praag, H., Kempermann, G. and Gage, F. H. "Neural consequences of environmental enrichment." Nat Rev Neurosci **1**: 191-198 (2000).

Vazquez-Sanroman, D., Sanchis-Segura, C., Toledo, R., Hernandez, M. E., Manzo, J. and Miquel, M. "The effects of enriched environment on BDNF expression in the mouse cerebellum depending on the length of exposure." Behav Brain Res **243**: 118-128 (2013).

Viola, G. G., Botton, P. H., Moreira, J. D., Ardais, A. P., Oses, J. P. and Souza, D. O. "Influence of environmental enrichment on an object recognition task in CF1 mice." Physiol Behav **99**: 17-21 (2010).

Viola, G. G. and Loss, C. M. "Letter to Editor about: "Physical exercise increases GFAP expression and induces morphological changes in hippocampal astrocytes"." Brain Struct Funct **219**: 1509-1510 (2014).

Viola, G. G., Rodrigues, L., Americo, J. C., Hansel, G., Vargas, R. S., Biasibetti, R., Swarowsky, A., Goncalves, C. A., Xavier, L. L., Achaval, M., Souza, D. O. and Amaral, O. B. "Morphological changes in hippocampal astrocytes induced by environmental enrichment in mice." Brain Res **1274**: 47-54 (2009).

Vivar, C., Potter, M. C., Choi, J., Lee, J. Y., Stringer, T. P., Callaway, E. M., Gage, F. H., Suh, H. and van Praag, H. "Monosynaptic inputs to new neurons in the dentate gyrus." Nat Commun **3**: 1107 (2012).

Vyklicky, V., Korinek, M., Smejkalova, T., Balik, A., Krausova, B., Kaniakova, M., Lichnerova, K., Cerny, J., Krusek, J., Dittert, I., Horak, M. and Vyklicky, L. "Structure, function, and pharmacology of NMDA receptor channels." Physiol Res **63 Suppl 1**: S191-203 (2014).

Walker, M. D. and Mason, G. "Female C57BL/6 mice show consistent individual differences in spontaneous interaction with environmental enrichment that are predicted by neophobia." Behav Brain Res **224**: 207-212 (2011).

Watanabe, M., Inoue, Y., Sakimura, K. and Mishina, M. "Developmental changes in distribution of NMDA receptor channel subunit mRNAs." Neuroreport **3**: 1138-1140 (1992).

Watkins, J. C. and Jane, D. E. "The glutamate story." Br J Pharmacol **147 Suppl 1**: S100-108 (2006).

Wee, K. S., Zhang, Y., Khanna, S. and Low, C. M. "Immunolocalization of NMDA receptor subunit NR3B in selected structures in the rat forebrain, cerebellum, and lumbar spinal cord." J Comp Neurol **509**: 118-135 (2008).

Williams, K. "Extracellular Modulation of NMDA Receptors." (2009).

Wong, H. K., Liu, X. B., Matos, M. F., Chan, S. F., Perez-Otano, I., Boysen, M., Cui, J., Nakanishi, N., Trimmer, J. S., Jones, E. G., Lipton, S. A. and Sucher, N. J. "Temporal and regional expression of NMDA receptor subunit NR3A in the mammalian brain." J Comp Neurol **450**: 303-317 (2002).

Woods, B., Aguirre, E., Spector, A. E. and Orrell, M. "Cognitive stimulation to improve cognitive functioning in people with dementia." Cochrane Database Syst RevCD005562 (2012).

Yan, L. "Structural and functional changes in the suprachiasmatic nucleus following chronic circadian rhythm perturbation." Neuroscience **183**: 99-107 (2011).

Yao, Z., DuBois, D. C., Almon, R. R. and Jusko, W. J. "Modeling circadian rhythms of glucocorticoid receptor and glutamine synthetase expression in rat skeletal muscle." Pharm Res **23**: 670-679 (2006).

Zadicario, P., Avni, R., Zadicario, E. and Eilam, D. "'Looping'-an exploration mechanism in a dark open field." Behav Brain Res **159**: 27-36 (2005).

Zambrana, C., Marco, E. M., Arranz, L., de Castro, N. M., Viveros, M. P. and de la Fuente, M. "Influence of aging and enriched environment on motor activity and emotional responses in mice." Ann N Y Acad Sci **1100**: 543-552 (2007).

Zhu, S. W., Codita, A., Bogdanovic, N., Hjerling-Leffler, J., Ernfors, P., Winblad, B., Dickins, D. W. and Mohammed, A. H. "Influence of environmental manipulation on exploratory behaviour in male BDNF knockout mice." Behav Brain Res **197**: 339-346 (2009).

Zimmer, E. R., Parent, M. J., Souza, D. G., Leuzy, A., Lecrux, C., Kim, H. I., Gauthier, S., Pellerin, L., Hamel, E. and Rosa-Neto, P. "[18F]FDG PET signal is driven by astroglial glutamate transport." Nat Neurosci (2017).

PARTE VI. ANEXOS

ANEXO I

Artigo publicado

Letter to Editor about: ‘‘Physical exercise increases GFAP expression and induces morphological changes in hippocampal astrocytes’’

Giordano Gubert Viola, Cássio M. Loss.

Revista: *Brain Structure and Function*

Qualis-CAPES-CBII: A1

Fator de Impacto: 5.811

Justificativa: Existe uma crescente necessidade de se discutir a respeito de resultados conflitantes reportados por diferentes trabalhos, afim de aumentar a replicabilidade de resultados entre laboratórios.

Objetivo geral: Discutir sobre a necessidade de considerar a espécie e/ou a linhagem animal a ser utilizada, a idade e o período de tempo de análise durante a aplicação do protocolo e a estrutura encefálica avaliada em estudos científicos, principalmente em estudos envolvendo enriquecimento ambiental, um paradigma que induz benefícios encefálicos de maneira dependente da espécie (linhagem) animal, duração dos protocolos e idades de intervenção.

Brain Struct Funct (2014) 219:1509–1510
DOI 10.1007/s00429-013-0563-1

TECHNICAL NOTE

Letter to Editor about: "Physical exercise increases GFAP expression and induces morphological changes in hippocampal astrocytes"

Giordano Gubert Viola · Cássio Morais Loss

Received: 14 February 2013 / Accepted: 20 April 2013 / Published online: 5 May 2013

Keywords Astrocytes · Species · Exercise · Environmental enrichment · GFAP · Neuroplasticity

Dear Editor,

The article entitled "Physical exercise increases GFAP expression and induces morphological changes in hippocampal astrocytes" recently published in Brain Structure and Functions elegantly demonstrated that a protocol of light intensity exercise induced an increased GFAP expression and number of astrocytes associated with morphological changes in astrocytes of CA1 Stratum Radiatum (Saur et al. 2013).

The authors studied the astrocytic plasticity in adult brain (Gibbs et al. 2008), a very important issue in neuroscience. They discussed different results obtained after the light intensity exercise protocol and after the environmental enrichment (EE) protocol applied by Viola et al. (2009). In fact, despite exercise being one of the components of EE (Nithianantharajah and Hannan 2006), that is these conflicting findings between the two protocols (Faherty et al. 2003; Brown et al. 2003), in our point of view, could be explained by different factors.

First, a critical point to be discussed is the difference between species (rats and mice) that was not mention in this article. In fact, these two species present distinct evolutionary history and consequently present inherent behavioral responses (Branchi and Ricceri 2004), which may influence the response of animals to either physical exercise or EE. Second, there is a possible variation in the mnemonic process between the two species, as has been demonstrated by differences in hippocampal cells immunoreactivity to cGMP between rats and mice (van Staveren et al. 2004). Finally, other important factors worth to be mentioned are the plastic changes of astrocytes to distinct protocols in several species, the time-period of analysis during the protocol application (either exercise or EE), and the brain region analyzed. A recent study demonstrated that prehatch hypoxia in chicken induces a decreased number of neurons, increased GFAP immunoreactivity, and memory impairment in the bead discrimination learning task. Those alterations were dependent of the timing of the insult, the evaluation period, and the damaged brain region (Rodricks et al. 2010).

In view of all the facts, not meaning to retract the elegant article of Saur et al., we want to bring to attention the need to consider the animal species or strain to be used, the age and time-period of analysis during the protocol application, and brain structure evaluated in different time-points of protocols.

We are looking forward to hearing from you.

G. G. Viola (✉)
Programa de Pós-graduação em Neurociências, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina (UFSC), Rua João Pio Duarte Silva, S/N, Córrego Grande, Florianopolis 88040-900, Brazil
e-mail: giorgviola@gmail.com

G. G. Viola
UFSC Campus Curitibanos, Rodovia Municipal Ulysses Gaboardi, km 3, Curitibanos 89520-000, Brazil

C. M. Loss
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul (UFRGS) – Rua Ramiro Barcelos, 2600, Anexo, Porto Alegre 90035-000, Rio Grande do Sul, Brazil

References

Branchi I, Ricceri L (2004) Refining learning and memory assessment in laboratory rodents. An ethological perspective. Ann Ist Super Sanita 40(2):231–236

Brown J, Cooper-Kuhn CM, Kempermann G, Van Praag H, Winkler J, Gage FH, Kuhn HG (2003) Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. Eur J Neurosci 17(10):2042–2046. ([pii] 2647)

Faherty CJ, Kerley D, Smeyne RJ (2003) A Golgi-Cox morphological analysis of neuronal changes induced by environmental enrichment. Brain Res Dev Brain Res 141(1–2):55–61. doi:S0165380602006429

Gibbs ME, Hutchinson D, Hertz L (2008) Astrocytic involvement in learning and memory consolidation. Neurosci Biobehav Rev 32(5):927–944. doi:10.1016/j.neubiorev.2008.02.001S0149-7634(08)00039-0

Nithianantharajah J, Hannan AJ (2006) Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat Rev Neurosci 7(9):697–709. doi:nrn197010.1038/nrn1970

Rodricks CL, Gibbs ME, Castillo-Melendez M, Miller SL (2010) The effect of hypoxia on the functional and structural development of the chick brain. Int J Dev Neurosci 28(4):343–350. doi:10.1016/j.ijdevneu.2010.02.004S0736-5748(10)00022-5

Saur L, Baptista PP, de Senna PN, Paim MF, Nascimento PD, Ilha J, Bagatini PB, Achaval M, Xavier LL (2013) Physical exercise increases GFAP expression and induces morphological changes in hippocampal astrocytes. Brain Struct Funct. doi:10.1007/s00429-012-0500-8

van Staveren WC, Steinbusch HW, Markerink-van Ittersum M, Behrends S, de Vente J (2004) Species differences in the localization of cGMP-producing and NO-responsive elements in the mouse and rat hippocampus using cGMP immunocytochemistry. Eur J Neurosci 19(8):2155–2168. doi:10.1111/j.0953-816X.2004.03327.xEJN3327

Viola GG, Rodrigues L, Americo JC, Hansel G, Vargas RS, Biasibetti R, Swarowsky A, Goncalves CA, Xavier LL, Achaval M, Souza DO, Amaral OB (2009) Morphological changes in hippocampal astrocytes induced by environmental enrichment in mice. Brain Res 1274:47–54. doi:10.1016/j.brainres.2009.04.007

ANEXO II

Cartas de aprovação emitidas pela Comissão de Ética no Uso de Animais (CEUA)

Resultado de Solicitação de Protocolo

Protocolo
PP00795
Título
Metodologias utilizadas no laboratório de Neurobiologia da Depressão para ensaios in vivo.
Data de Entrada
17/05/2012

Resultado:
Aprovado
Data/Prazo
26/07/2012

Considerações
Oficio nº 62/CEUA/PRPE/2012
Do: Presidente da Comissão de Ética no Uso de Animais-CEUA
Ao(à): Prof(a) Dr(a) Ana Lúcia Severo Rodrigues, Departamento de Bioquímica - CCB

Prezado(a) Professor(a),
Em relação ao protocolo de pesquisa sob sua responsabilidade o presidente da CEUA-UFSC deliberou o seguinte:

Os procedimentos elencados no protocolo e corrigidos na carta anexa estão credenciados para uso no seu laboratório pelo período de quatro anos. Qualquer alteração destes, ou inclusão de novos, deverão ser apreciados pela CEUA-UFSC novamente.

Este credenciamento é válido para a utilização das espécies animais: sete mil camundongos (Mus musculus) e duzentos e cinquenta ratos (Rattus Norvegicus).

Procedência do animal: Biotério Central da UFSC.

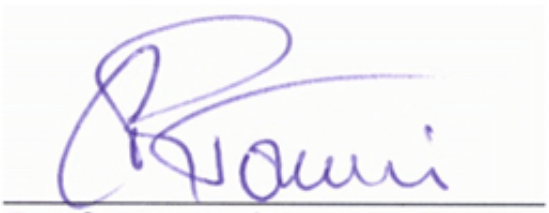
Por ocasião do término deste período de credenciamento, DEVERÁ SER APRESENTADO RELATÓRIO detalhado relacionando o uso de animais com estes procedimentos aos resultados obtidos, conforme formulário ON LINE CEUA.

Atenciosamente,

Relatório Final previsto para (90 dias após término da vigência do protocolo ou no momento da apresentação de um novo protocolo)
Data 26/10/2016

Data 26/07/2012

Parecer(es):



Prof. Assoc. Carlos Rogério Tonussi, D.Sc.
COMISSÃO DE ÉTICA NO USO DE ANIMAIS – PRPE – UFSC
PRESIDENTE

Abrir Solicitação | Criar Relatório

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CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 27071
Título: Participação de receptores NMDA na capacidade cognitiva de ratos expostos a ambientes enriquecidos durante períodos críticos do desenvolvimento cerebral

Pesquisadores:

Equipe UFRGS:

DIOGO LOSCH DE OLIVEIRA - coordenador desde 01/06/2014
Cássio Morais Loss - Aluno de Doutorado desde 01/06/2014

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 01/09/2014 - Sala 330 - Anexo I do Prédio da Reitoria - Campus Centro/UFRGS- Porto Alegre, em seus aspectos éticos e metodológicos, para a utilização de ratos Wistar (108 fêmeas, 22 machos e 432 filhotes), de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Segunda-Feira, 3 de Novembro de 2014

STELA MARIS KUZE RATES
Coordenador da comissão de ética

ÍNDICE DE ILUSTRAÇÕES

ÍNDICE REMISSIVO

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