UNIVERSIDADE FEDERAL DE ALFENAS

KAROLYNE CORDEIRO DE OLIVEIRA

ESTUDO DOS EFEITOS DO ETANOL SOBRE O ENCÉFALO DE RATOS WISTAR

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Ciências Ambientais, pela Universidade Federal de Alfenas. Área de concentração: Ciências Ambientais. Orientador: Prof. Dr. Tales Alexandre Aversi-Ferreira
Coorientador: Prof. Dr. Breno Régis Santos

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A Banca examinadora abaixo-assinada aprova a Dissertação apresentada como parte dos requisitos para a obtenção do título de Mestre em Ciências Ambientais pela Universidade Federal de Alfenas. Área de concentração: Ciências Ambientais.

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RESUMO

A ingestão de álcool por mulheres em idade reprodutiva associado à uma gravidez não planejada submete a gravida à um grande risco de consumir álcool durante os estágios iniciais da gravidez, quando os sintomas ainda não são evidentes, o que pode resultar em uma variedade de problemas morfológicos, cognitivos e comportamentais na criança que virá a nascer, sendo um grave problema de saúde pública. Por isso, examinamos se apenas uma exposição ao etanol durante os estágios iniciais da migração neuronal foi capaz de afetar a citoarquitetura do neocórtex e a massa encefálica da prole. A injeção de etanol foi realizada intraperitonealmente em três doses (3g de etanol/kg peso corporal) com intervalos de 8 horas em fêmeas grávidas de ratos Wistar (180-230 g) doze dias após o coito (E12) para modelar a exposição ao álcool entre as 5-6 semanas de gravidez em humanos. Para a visualização das células que migraram, foi realizada a marcação com Bromodesoxiuridina (BrDU) 24 horas após a primeira injeção de etanol. A exposição aguda causou a perturbação da migração celular produzindo ectopia, heterotopia e despovoamento neuronal, o que pode caracterizar a Síndrome Alcoólica Fetal, mas não teve impacto substancial no volume cerebral total. Em resumo, a exposição aguda restrita ao início da gestação teve efeitos sutis na massa encefálica da prole, mas trouxe evidências histológicas do comprometimento da migração neuronal, o que contribui para danos cognitivos característicos antes apenas descritos na exposição pré-natal crônica ao etanol.

Palavras-chaves: neocórtex; efeito de drogas; migração neuronal; síndrome alcoólica fetal; toxicidade do etanol.

ABSTRACT

The consumption of alcohol by women of reproductive age associated with an unplanned pregnancy puts the pregnant woman at a greater risk of consuming alcohol during the early stages of pregnancy, when the symptoms are not yet evident, which can result in a variety of morphological, cognitive, and behavioral problems in the unborn child, being a serious public health problem. Therefore, we examined whether just one prenatal exposure to ethanol during the early stages of neuronal migration was able to affect the cytoarchitecture of the neocortex and the brain mass of the offspring. Ethanol injection was performed intraperitoneally in three doses (3g ethanol/kg body weight) at 8-hour intervals in pregnant female Wistar rats (180-230 g) twelve days after conception (E12) to model the alcohol exposure between 5-6 weeks of pregnancy in humans. To visualize the cells that migrated in this period, labeling with Bromodeoxyuridine (BrDU) was performed 24 hours after the first ethanol injection. Acute exposure caused the disruption of cell migration producing ectopia, heterotopia, and neuronal depopulation, which may characterize Fetal Alcohol Syndrome, but had no substantial impact on total brain volume. In summary, acute exposure restricted to early pregnancy had subtle effects on the brain mass of the offspring, but brought histological evidence of impaired neuronal migration, which contributes to cognitive impairments previously only described in chronic prenatal exposure to ethanol.

Keywords: neocortex; drug effects; neuronal migration; fetal alcohol syndrome; ethanol toxicity.

SUMÁRIO

1 INTRODUÇÃO GERAL

O consumo de drogas de abuso durante a gestação constitui um importante problema de saúde pública mundial que é evitável. A gravidez é um período extremamente delicado, pois existem interações estreitas entre o sistema materno e o sistema fetal através da placenta, que resultam em uma interligação entre a mãe e o feto, que fica vulnerável às exposições medicamentosas, infeciosas, de produtos químicos ou até mesmo à agentes físicos que uma mulher pode sofrer durante a gravidez (ALWAN; CHAMBERS, 2015).

O álcool, também denominado etanol, é uma droga psicoativa que é capaz de alterar o comportamento, percepção e a consciência do usuário que o consome (STAFFORD; FERNANDES; AGOBIANI, 2012; SALLING *et al.*, 2018). O etanol age como depressor do sistema nervoso, ao retardar o funcionamento do cérebro e das atividades neurais, resultando em fala arrastada, movimentação instável, perturbação da percepção e diminuição de inibições (SALLING *et al.*, 2018). Apesar disso, o álcool está dentro do grupo de drogas lícitas e é amplamente consumido no mundo inteiro (BEYDOUN *et al*., 2014) por diversos fatores, como celebrações culturais, eventos sociais, entre outros.

A ingestão de álcool durante a gravidez pode resultar em uma variedade de problemas morfológicos, cognitivos e comportamentais. Dependendo do tempo de exposição ao álcool e do padrão de consumo da mãe, o álcool pode ter efeitos diferentes em uma criança (FOLTRAN *et al.*, 2011). Não há janela de segurança para o uso de etanol durante a gestação, pois ele pode causar sérios problemas para o feto ao longo da gravidez, incluindo antes da mulher saber que está grávida.

A exposição fetal ao álcool durante o período de desenvolvimento do cérebro tem sido associada à déficits no aprendizado, memória e funcionamento executivo tanto em humanos quanto em modelos animais (WARREN, 2011; MARQUARDT *et al.*, 2014; WANG *et al.*, 2020). Estes déficits muito provavelmente resultam de Distúrbios da Migração Neuronal (DMN), que é um grupo de más formações congênitas causadas pela migração anormal de neurônios no cérebro em desenvolvimento e no sistema nervoso (BUCHSBAUM; CAPPELLO, 2019; SUBRAMANIAN, CALCAGNOTTO; PAREDES, 2020).

A migração neuronal, que ocorre durante o segundo mês de gestação em humanos, é controlada por uma variedade complexa de guias e sinais químicos. Quando esses sinais estão ausentes ou incorretos, os neurônios não vão para o lugar a que pertencem (RAKIC, 1990;

HWANG, KU HASHIMOTO-TORII, 2019), resultando em áreas do cérebro estruturalmente anormais ou ausentes nos hemisférios cerebrais, cerebelo, tronco cerebral ou hipocampo. As anormalidades estruturais encontradas em DNMs incluem esquizencefalia, porencefalia, lissencefalia, heterotopias, agenesia do corpo caloso e agenesia do crânio (SCHILLER *et al.*, 2020; SUBRAMANIAN; CALCAGNOTTO; PAREDES, 2020).

Estudos sobre os efeitos crônicos da exposição pré-natal ao etanol demonstraram que houve dessincronização do desenvolvimento cortical (BARTH, 1987; MILLER, 1993; GUERRI, 1998; CAMARILLO; MIRANDA, 2007; SHENODA. 2017), e consequentemente, danos ao estabelecimento de circuitos neuronais, que geram uma reorganização do neocórtex caracterizada por grupos heterotópicos de neurônios (MILLER, 1993; FIUZA; MORAIS, 2017). Além da desestabilização da matriz celular, dos componentes moleculares e da glia (IKONOMIDOU; SERGIADIS, 2000; GUERRI *et al.,* 2009; ARONNE *et al.,* 2011) e dos próprios neurônios (GIL-MOHAPEL *et al.,* 2010), prejuízos na expressão gênica (CAMARILLO; MIRANDA, 2007; EHRHART *et al.*, 2019) e apoptose das células neurais (IKONOMIDOU; SERGIADIS, 2000).

Em relação aos efeitos da exposição aguda ao etanol, não existem muitos relatos na literatura (AVERSI-FERREIRA; NASCIMENTO, 2008). Estudos gerais e mais completos sobre os efeitos agudos do etanol no cérebro no desenvolvimento são necessários, considerando aspectos moleculares e gênicos; de fato, a maioria dos poucos estudos agudos está focada em aspectos histológicos (FERREIRA *et al.*, 2004; AVERSI-FERREIRA *et al.,* 2006; AVERSI-FERREIRA; NASCIMENTO, 2008).

Nossa hipótese é que apenas um evento de exposição aguda já seria suficiente para causar resultados, como o distúrbio da migração neuronal e redução da massa encefálica, relatados nos estudos crônicos e encontrados na Síndrome Alcóolica Fetal.

2 REVISÃO DA LITERATURA

2.1 A GÊNESE DO CÓRTEX CEREBRAL

O córtex cerebral de mamíferos é composto por uma diversidade de tipos celulares - neurônios excitatórios de projeção, neurônios inibitórios, oligodendrócitos e astrócitos - distribuídos através de camadas e regiões. Para que as funções normais do cérebro sejam executadas é de extrema importância que o processo da estratificação horizontal do córtex durante o desenvolvimento ocorra sem prejuízos, pois problemas na formação destas camadas podem resultar em distúrbios anatômicos e funcionais (THOMPSON; LEVITT; STANWOOD; 2009).

Os processos de geração, migração e diferenciação do córtex ocorrem principalmente no período pré-natal, mas em algumas áreas do Sistema Nervoso Central (SNC), estes fenômenos podem ocorrer após o nascimento, como no cerebelo (FIUZA *et al.*, 2017; RAHIMI-BALAEI *et al.*, 2018), no hipocampo (NOGUCHI; LI; PLEASURE, 2020) e na zona subventricular (GUERRERO-CAZARES *et al.*, 2020).

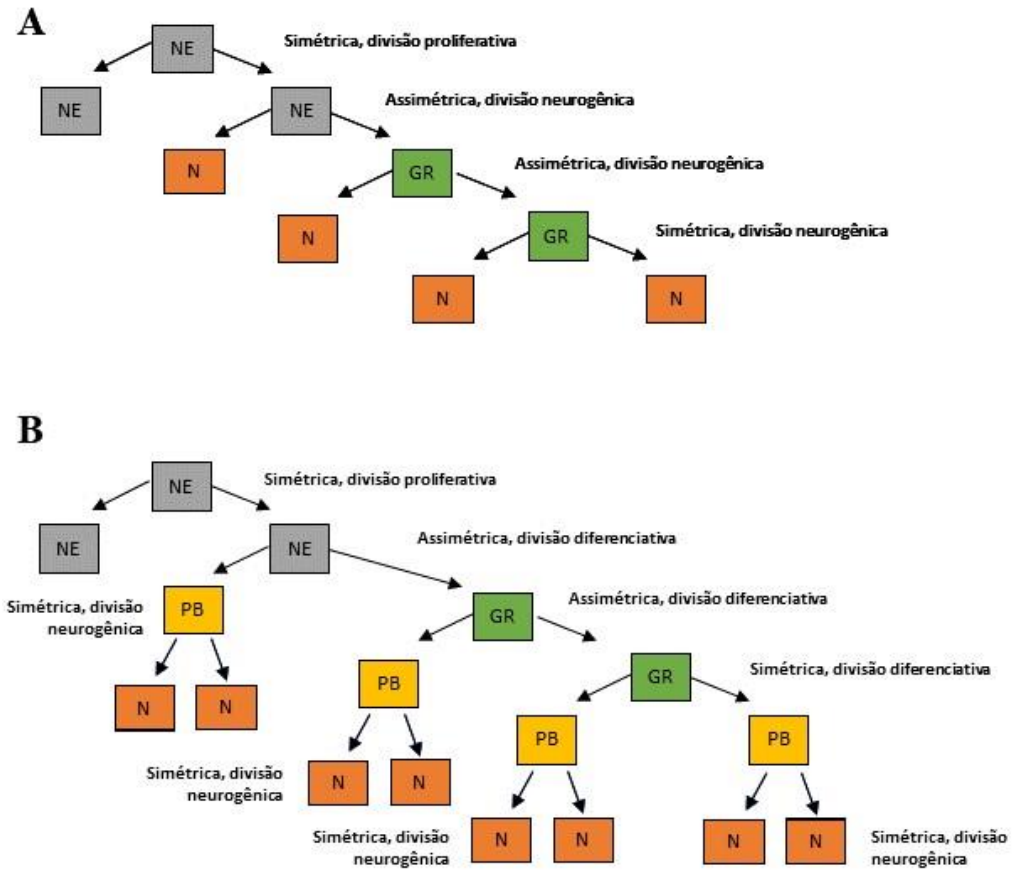
Por volta do nono dia embrionário (E9) em ratos, o tubo neural é composto por células neuroepiteliais, que se dividem simetricamente para ampliar sua população. A divisão de cada célula é sincronizada com o movimento basal-apical do núcleo em um processo conhecido como migração intercinética (GÖTZ; HUTTNER, 2005). Como as divisões celulares de cada célula não é sincronizada com as outras ao seu redor, o neuroepitélio aparenta ser pseudoestratificado (KRIEGSTEIN; ALVAREZ-BUYLLA, 2009).

Até este momento, as células neuroepiteliais são o único tipo celular que compõem a placa e o tubo neural, e serão responsáveis por dar origem aos neurônios e células da glia. A neurogênese é o primeiro processo do desenvolvimento do SNC, em que por volta de E9.5/E10.5, as células neuroepiteliais começam a transformar-se em células gliais radiais (MILLER, 1993; TAKAHASHI; NOWAKOWSKI; CAVINESS JR, 1996).

Estas células possuem propriedades neuroepiteliais residuais, como a polaridade apical-basal e a migração intercinética em seu ciclo celular, além da ancoragem no ventrículo lateral. Seu núcleo fica no lado apical voltado para o ventrículo, formando uma camada celular muito densa chamada de Zona Ventricular (ZV) (GÖTZ; HUTTNER, 2005; TAVERNA; GÖTZ; HUTTNER, 2014).

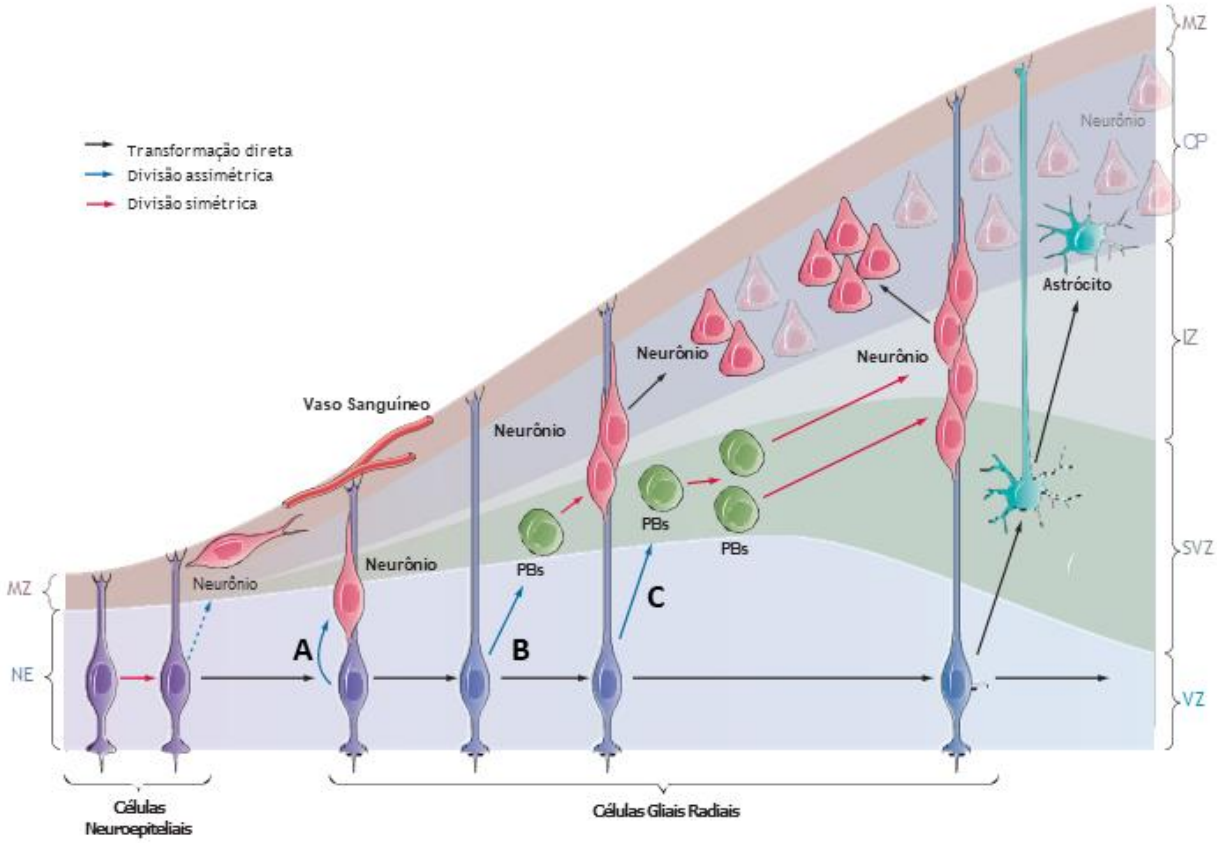
As células gliais radiais dão origem à maior parte dos neurônios, mesmo que de forma indireta, através da geração de progenitores chamados de Progenitores Basais (PB) que possuem maior capacidade de proliferação (Figura 1), o que permite a posterior ampliação exacerbada do número de neurônios no córtex (IMAYOSHI *et al.*, 2013; KICHEVA *et al.*, 2014), pois eles passam por mais ciclos celulares em comparação com as células da glia, aumentando o número de neurônios que são gerados a partir de um determinado número de progenitores, permitindo que ocorra uma nova rodada de divisão celular em camadas distantes da superfície apical. O corpo celular dos PB está localizado fora da ZV, e eles formam outra camada celular, a Zona Subventricular (ZSV) (Figura 2) (IMAYOSHI *et al.*, 2013; KICHEVA *et al.*, 2014).

Figura 1 - Relação simplificada entre as células neuroepiteliais (NE), células gliais radiais (GR) e neurônios (N).



Fonte: Adaptado de GÖTZ e HUTTNER (2005).
Legenda: A) Linhagem sem progenitores;
B) Linhagem com progenitores basais (PB).

Figura 2 - Três formas de neurogênese durante o desenvolvimento cortical.



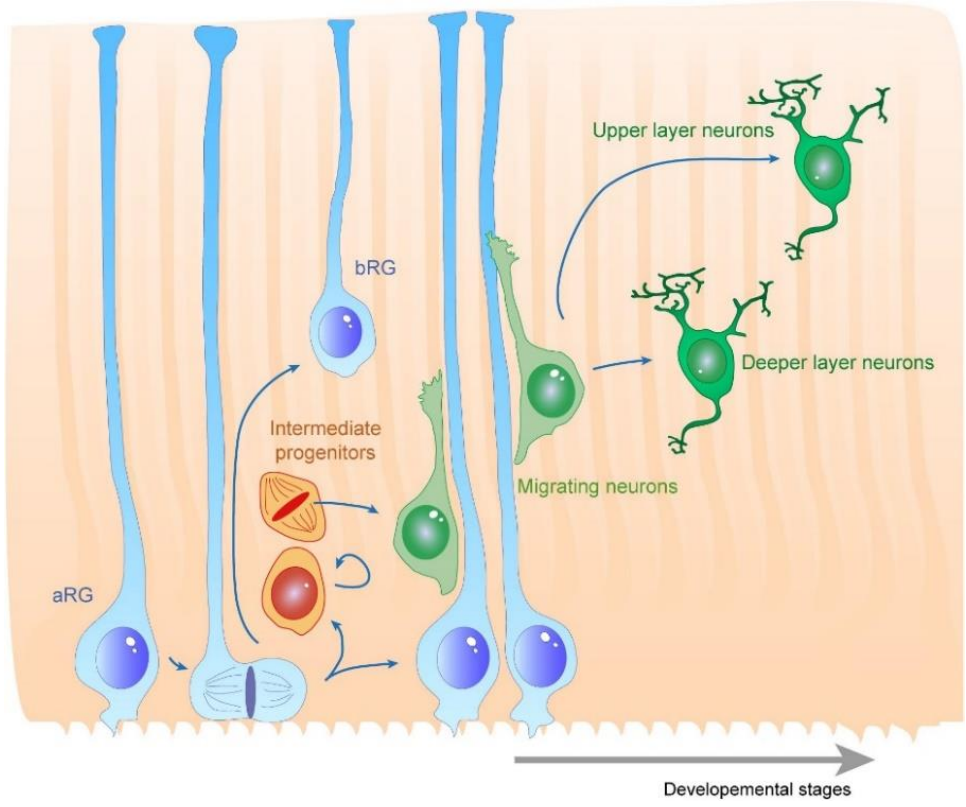
Fonte: Adaptado de KRIEGSTEIN e ALVAREZ-BUYLLA (2009).
Legenda: A) Células Gliais Radiais geram neurônios diretamente através de divisão assimétrica;
B) Indiretamente através da geração de Progenitores Basais (PBs) e uma rodada de amplificação;
C) Indiretamente através dos PBs com duas rodadas de divisão simétrica e amplificação.

Em ratos, esta sequência de acontecimentos ocorre entre E11 até E17, que é equivalente ao segundo trimestre de gravidez em humanos (TAKAHASHI; NOWAKOWSKI; CAVINESS, 1996).

Durante o desenvolvimento, os neurônios excitatórios são gerados na zona ventricular paliativa voltada para o ventrículo lateral ou para a zona subventricular adjacente. Em seguida, eles migram radialmente em direção à superfície pial do cérebro, passando por neurônios predecessores gerados durante os estágios anteriores e interrompendo sua migração no topo da placa cortical em desenvolvimento. Como os neurônios excitatórios do neocórtex cerebral são gerados e migram sequencialmente durante o período de desenvolvimento, os neurônios nascidos no início estão localizados nas camadas profundas e os neurônios nas últimas fases

estão localizados nas camadas superficiais, nascendo "de dentro para fora" (Figura 3) (FERENT, 2020).

Figura 3 - As glias radiais funcionam como fonte e suporte de neurônios recém-nascidos no córtex em desenvolvimento. A glia radial apical (aRG) estende um processo apical atingindo a superfície ventricular, onde expõe seus cílios primários, bem como um processo basal atingindo a superfície cortical. A glia radial basal (bRG) tem seus corpos celulares localizados em áreas mais basais da parede cortical. Os processos apicais e basais dessas células (azul) estabelecem o arcabouço em toda a parede cortical. As GRs sofrem divisão celular, dando origem a uma célula filha que pode ser outra GR (divisão simétrica) ou um progenitor basal (divisão assimétrica, os progenitores intermediários são representados em laranja). Essas células dão origem a neuroblastos migratórios (verdes) que se movem ao longo dos processos basais da RG para alcançar sua posição final dentro das camadas corticais. Os primeiros neurônios da camada profunda são gerados, então os neurônios da camada superior nascem.



Fonte: FERENT (2020).

2.2 MIGRAÇÃO NEURONAL

O Sistema Nervoso Central (SNC) se desenvolve a partir de diversos eventos celulares e moleculares extremamente organizados, tais como a criação de padrões neuronais, a ocorrência da migração de neurônios e o crescimento axonal (LEE *et al*., 2020).

A partir das regiões proliferativas, as células migram para os seus sítios destino para que sejam diferenciadas em neurônios. Em vista disso, observa-se que a maioria dos neurônios no sistema nervoso vertebrado em desenvolvimento tem sua origem em locais significativamente diferentes daqueles onde residirão no cérebro adulto já formado (KANEKO; SAWADA; SAWAMOTO, 2017; RAKIC, 1990).

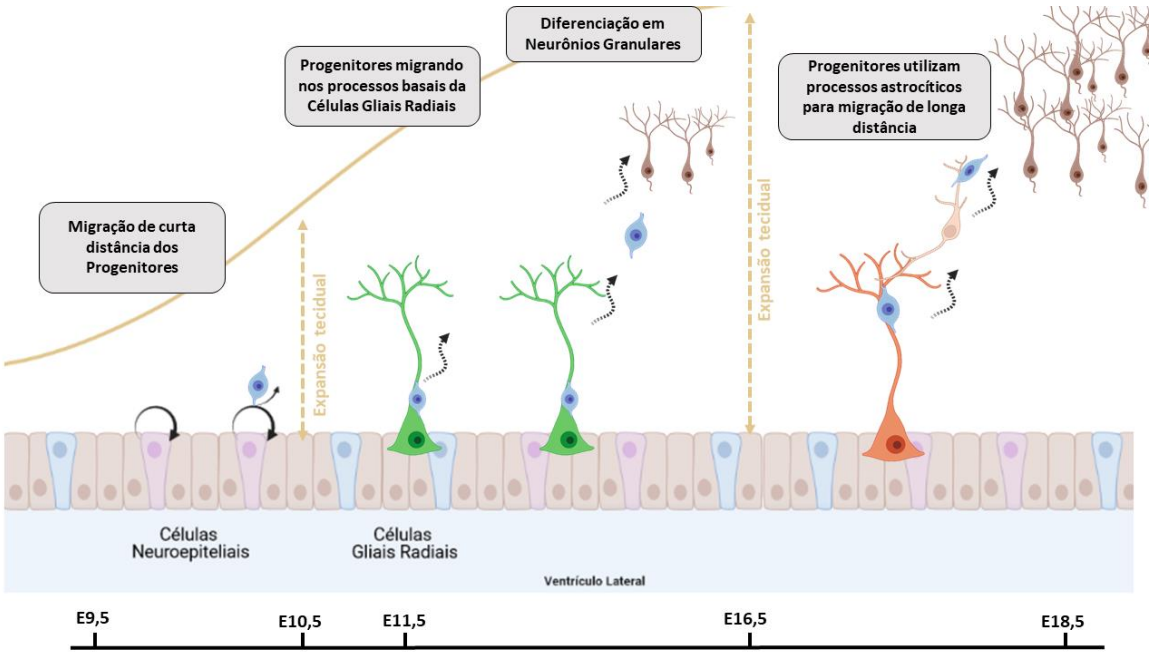
A migração de neurônios começa no período pré-natal e continua na infância. Este processo de desenvolvimento é crucial para a formação de uma rede neuronal adequada, e a perturbação deste processo resulta em prejuízos cognitivos e comportamentais para o resto da vida do indivíduo (BUCHSBAUM; CAPPELLO, 2019). Durante o desenvolvimento do cérebro, os neurônios recém-gerados sofrem mudanças morfológicas seguidas pela migração da camada germinativa através da intrincada rede da matriz extracelular para estabelecer conexões com outras células de uma forma altamente ordenada (RAKIC, 1972).

Há dois modos principais de migração no córtex cerebral conhecidos (GUERRINI; PARRINI, 2010).

2.2.1 Migração Radial

A migração radial direcionada à pia, em que os neurônios excitatórios gerados nas zonas proliferativas do primórdio migram radialmente em direção à superfície pial do córtex ao longo do eixo radial. Esses neurônios usam os processos celulares que são alongados das células gliais radiais como a estrutura de sua migração (AYALA, SHU; TSAI, 2007; RAKIC, 1972). Ao chegar à camada superficial, os neurônios se desprendem dos processos gliais radiais para se estabelecerem na placa cortical (Figura 4).

Figura 4 - Ilustração esquemática da migração radial direcionada à pia de neurônios em ratos entre E9.5 e E18.5.

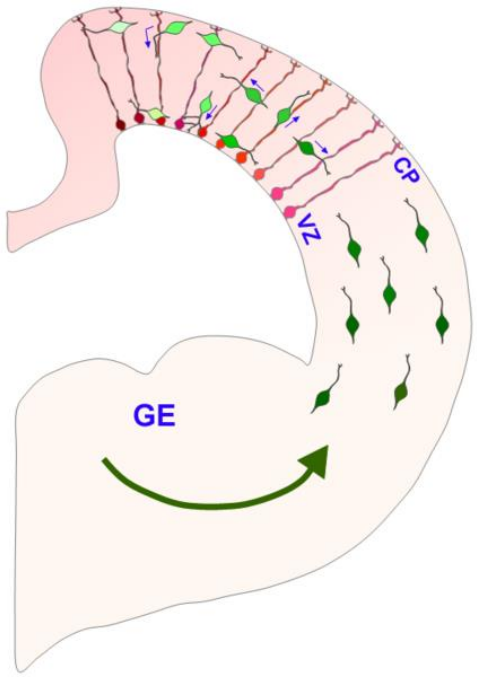


FONTE: Adaptado de CARAMELLO (2019).

2.2.2 Migração Tangencial

Já o outro modo de migração neuronal, conhecido como migração tangencial, é empregado por interneurônios inibitórios. Esses neurônios migram tangencialmente a partir das eminências ganglionares (GE) para o córtex cerebral (Figura 5) (AYALA; SHU; TSAI, 2007; MARÍN; RUBENSTEIN, 2001).

Figura 5- Interações entre interneurônios e células radiais da glia durante o desenvolvimento do córtex cerebral. Interneurônios (verde) migram para a parede cerebral a partir da eminência ganglionar (GE), interagem com células radiais da glia (vermelho) e podem exibir mudanças na direção da migração após este contato.



Fonte: YOKOTA *et al*. (2007).

2.2.3 Regulação da migração

Com essas informações, entende-se, então que a migração neuronal implica no movimento de um corpo celular a partir da zona proliferativa até o seu destino final no cérebro já maduro (RAKIC, 1972). Esses eventos de deslocamento são regulados de forma que a densidade neuronal produzida é aproximadamente a mesma de uma área para outra, tanto no cérebro humano quanto no cérebro de outras várias espécies de mamíferos (TAKAHASHI; NOWAKOWSKI; CAVINESS JR, 1996).

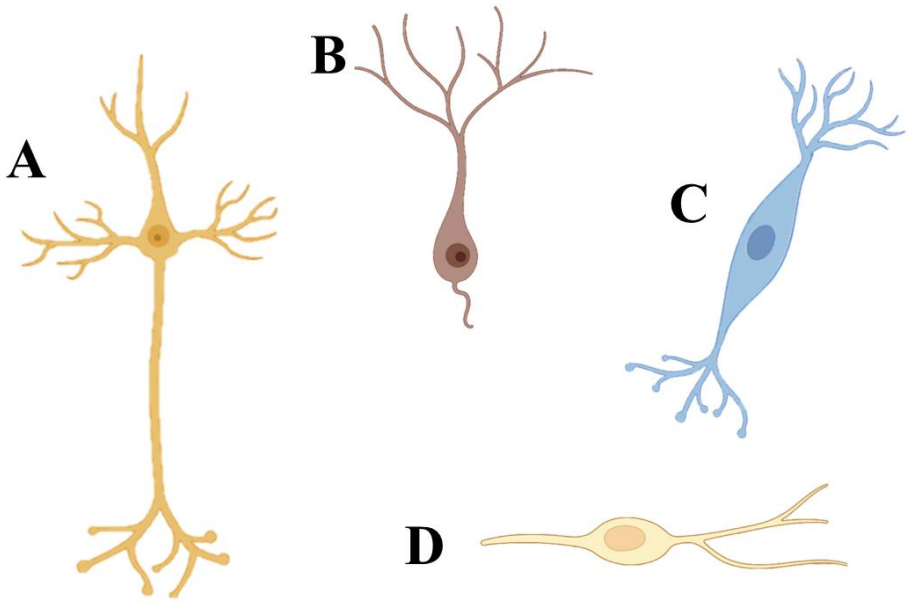
Diferentes populações de células estão envolvidas na regulação da migração neuronial, como por exemplo neurônios de Cajal-Retzius, neurônios da subplaca, neurônios precursores ou glia radial, além de implicar múltiplos mecanismos moleculares, como controle do ciclo celular, interações célula-célula - estas, geralmente mediadas por células de adesão - liberação

de neurotransmissores, disponibilidade de fatores de crescimento, vias de transdução de sinal, entre outros (EDELMAN *et al*., 2003; GRESSENS, 2000).

2.2.4 Camadas corticais

As camadas corticais são formadas principalmente por quatro tipos neuroniais: as células piramidais, as células granulares, células fusiformes e as células horizontais ou de Cajal (Figura 6).

Figura 6 - Ilustração do formato típico de neurônios.



Fonte: Do autor (2022).
Legenda: A) Piramidal;
B) Granular;
C) Fusiforme;
D) Horizontal ou de Cajal.

Os diferentes tipos celulares organizam-se no córtex em seis camadas, que são enumeradas de I a VI, da porção externa para a interna (Figura 7) (CAVINESS JR, 1975).

A camada I, a camada molecular, contém poucos corpos celulares de neurônios e consiste principalmente em axônios paralelos (horizontais) à superfície do córtex. Os dendritos apicais das células localizadas nas camadas mais profundas também se ramificam na camada I.

A camada II, a camada granular externa, é composta por uma mistura de pequenos neurônios chamados células granulares e neurônios ligeiramente maiores que são chamados de células piramidais. Os dendritos apicais destas células piramidais se estendem para a camada I e seus axônios descem para dentro e através das camadas corticais mais profundas.

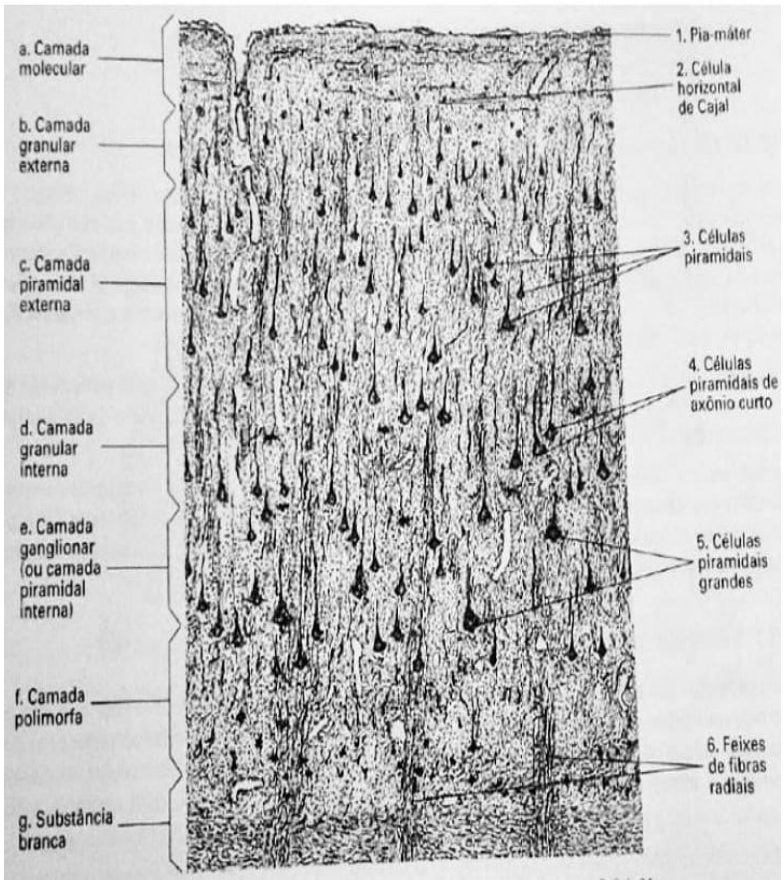
A camada III, a camada piramidal externa, contém principalmente células piramidais de tamanho pequeno a médio, juntamente com alguns neurônios de outros tipos. Em geral, as células piramidais menores ficam localizadas na porção externa ou superficial da camada III, enquanto as células piramidais maiores estão localizadas na porção interna ou mais profunda dessa camada. Seus dendritos apicais ascendem para a camada I e seus axônios também descem para as camadas mais profundas.

A camada IV, a camada granular interna, consiste quase exclusivamente de neurônios granulares lisos e espinhosos, ambos às vezes categorizados apenas como células granulares. Esta camada é livre de células em forma de pirâmide. Pode ser dividido em porções externa (IVa) e interna (IVb) em muitas áreas neocorticais e em três porções (IVa, IVb, IVc) no córtex visual primário.

A camada V, a camada piramidal interna, consiste predominantemente de células piramidais médias a grandes. Os dendritos apicais das células piramidais médias podem se estender para cima em uma ou duas camadas, enquanto os das células piramidais grandes se estendem para fora até a camada I. Alguns axônios corticais também se originam na camada V.

A camada VI, chamada camada multiforme, contém uma variedade de tipos de neurônios, incluindo alguns com corpos celulares piramidais e fusiformes. Os dendritos das células maiores se estendem para a camada I, sendo que aqueles que surgem das células menores geralmente não se estendem além da camada IV.

Figura 7 - Microfotografia do Córtex cerebral. Coloração nitrato de prata reduzido de Cajal.



Fonte: PIEZZI e FORNÉS (2008).

2.3 DISTÚRBIOS NA MIGRAÇÃO NEURONAL

Havendo problemas de migração de neurônios no córtex cerebral, as camadas podem ter sua ordem natural invertida, com despovoamento neuronial na camada cortical profunda (REDECKER *et al.*, 1998). Muitas doenças, como a epilepsia (CHEVASSUS *et al.*, 1998; EVSYUKOVA; PLESTANT; ANTON, 2013), estão associadas a desordens na migração neuronial.

Anormalidades genéticas ou fatores epigenéticos induzem distúrbios em células gliais ou interação neurônio-glial durante o período crítico de desenvolvimento (KRAVITZ *et al.*, 2019), o que pode conduzir a várias anormalidades moleculares, estruturais e funcionais no

cérebro (LIU *et al.*, 2011; SUPER; SORIANO; UYLINGS, 1998; TABATA; NAGATA, 2016).

As desordens na migração neuronial representam um grupo de malformações congênitas que afetam a migração de milhões de neurônios ectodérmicos para a camada germinal, o que produz mudanças na citoarquitetura, laminação e na fisiologia neural, particularmente no córtex cerebral. Elas são determinadas geneticamente ou causadas por infecções, intoxicações ou radiações (GUERRINI; PARRINI., 2010; LIU *et al.*, 2011; VALIENTE; MARÍN, 2010; VELEZ-DOMINGUES, 1998).

Os problemas na migração neuronal podem ser provocados por desarranjos da glia radial. A glia radial conduz os neurônios para a posição que devem ocupar no adulto, a fim de gerar circuitos normais pela formação das sinapses (BATOOL *et al.*, 2019; BEATTIE; HIPPENMEYER, 2017; MILLER, 1993; SUPÉR *et al.*, 1998). Um atraso na migração pode promover dessincronização do desenvolvimento cortical, o que prejudica a formação de circuitos normais nos neurônios do adulto (FALKNER *et al.*, 2016; MILLER, 1993).

Quaisquer distúrbios desses dois modos de migração causam Distúrbios de Migração Neuronal (DMN), e as consequentes malformações são detectáveis por imagens do cérebro em pacientes com DNM (ROBERTS, 2018). Casos profundos de DNMs incluem lisencefalia, heterotopia e displasia focal (GUERRINI; PARRINI, 2010).

2.4 TERATOGENICIDADE DO ÁLCOOL

Agentes teratógenos são aqueles responsáveis por causarem anormalidades durante o desenvolvimento pré-natal (FRIEDMAN, 2011). Os efeitos teratogênicos não estão restritos apenas a alterações físicas, mas também funcionais e comportamentais, que só são evidenciadas quando a criança atinja a idade na qual estas funções e comportamentos se desenvolvem (HOYME, *et al*., 2016; MATTSON; SCHOENFELD; RILEY, 2001). O etanol é um teratógeno capaz de induzir diversas anormalidades no desenvolvimento tanto em animais quanto em humanos.

A gama de déficits decorrentes da teratogenicidade do álcool depende do momento e da quantidade de álcool que a mãe consumiu, causando uma variedade de distúrbios categorizados na Síndrome Alcoólica Fetal (SAF) (WARREN; HEWITT; THOMAS, 2011). Indivíduos com

SAF normalmente apresentam deficiências no neurodesenvolvimento e fenótipo facial distinto, com lábio superior fino e comprimento curto da fissura palpebral (BLACKBURN; CARPENTER; EGERTON, 2010; CHUDLEY *et al.,* 2005; FOLTRAN *et al.,* 2011; SCHNEIDER; MOORE; ADKINS, 2011). Embora se trate de uma exposição evitável, a incidência da SAF ainda permanece elevada (MAY *et al*., 2018), com custos econômicos para a criança, família e sociedade em geral (POPOVA *et al.,* 2017).

2.5 O RATO COMO MODELO EXPERIMENTAL

As pesquisas de estudos neurais utilizam animais de laboratório (CLANCY; DARLINGTON; FINLAY, 2001; CLANCY *et al*., 2007), que tem sido usado sucessivamente nos estudos de defeitos induzidos pelo etanol na estrutura cortical (MILLER, 1993).

Em ratos, a migração para a formação do córtex começa entre o décimo segundo (E12) e décimo-terceiro (E13) dia de vida intrauterina (KANEKO; SAWADA; SAWAMOTO, 2017; TAKAHASHI; NOWAKOWSKI; CAVINESS JR, 1996). A exposição de fêmeas grávidas ao etanol afeta os embriões e conduz a alterações no citoesqueleto dos neuroblastos (MILLER, 1993), causando danos na arquitetura cortical (FERREIRA *et al*., 2004).

A praticidade da utilização desse animal é fundamental para trabalhos com o sistema neural em desenvolvimento, pois permite acompanhar estas etapas no embrião a partir do dia em que em a fêmea foi fecundada. As etapas de migração dos neurônios corticais no rato já foram caracterizadas morfologicamente por marcação com bromodesoxiuridina (BrDU) (TAKAHASHI; NOWAKOWSKI; CAVINESS JR, 1996).

3 JUSTIFICATIVA

O desenvolvimento do sistema nervoso é um período extremamente delicado durante a gestação, e a perturbação deste processo pode gerar problemas físicos e comportamentais na criança que virá a nascer. Devido ao etanol ser uma substância lícita amplamente consumida ao redor do mundo, diversas mulheres em idade reprodutiva estão entre estes consumidores, e podem vir a expor seus filhos durante o desenvolvimento fetal sem nem ao menos detectar os primeiros sintomas de gravidez.

A utilização do rato Wistar como modelo experimental neste estudo é justificada devido ao fato do seu desenvolvimento neuronal ter sido extensivamente descrito na literatura e ser similar ao de humanos. Além disso, permite uma acurácia na marcação dos dias gestacionais, e uma maior confiança nos resultados apresentados.

Embora sejam conhecidos os efeitos da exposição crônica ao álcool na formação do néocortex em modelos animais em diferentes estágios da gestação, poucos estudos observaram os efeitos que apenas um evento de exposição pode gerar durante o evento da migração neuronal. Portanto, é necessário saber a severidade dos efeitos que a exposição aguda pode causar no desenvolvimento do sistema neural, discutir as consequências desta exposição e trazer mais informações sobre um problema de saúde pública totalmente evitável, como a Síndrome Alcóolica Fetal (SAF).

4 OBJETIVOS

Esse estudo teve como objetivo avaliar os efeitos da exposição pré-natal aguda sobre a massa do encéfalo e a arquitetura do neocórtex em filhotes de ratos Wistar submetidos à injeção de etanol.

5 ARTIGO

Postnatal effects of the ethanol on the encephalon mass and neocortex architecture in Wistar rat offspring submitted to acute prenatal injection and *in vitro* ethanol effects on Wistar rat and human erythrocytes.

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Abstract

Background: A population of proliferative cells forms the ventricular and subventricular shells, where the neuroblasts begins its migration to generate the six typical shells of the adult neocortex. In rats, this migration begins by the 12th day of pregnancy (E_{12}).

Methods: In this work, we studied the effects of the acute exposition to ethanol on E_{12} on the morphology of the neural tissue. Ethanol was injected intraperitoneally (3 doses of 3 g of ethanol/kg body weight, at 8 hours intervals) in pregnant females of Wistar rats (180-230 g) with their embryos on E_{12}.

Results: Under these conditions, the acute treatment with ethanol perturbed the cell migration, producing ectopia, heterotopia, and neuronal depopulation.

Conclusions: Acute ethanol treatment of rats on the twelfth day of intrauterine life determines a degree of severity on the neural system that is very similar to the chronic treatment.

Keywords: drug effects, acute exposure, neocortex, development, erythrocytes, *in vitro*

Background

The chronic effects of ethanol on the brain in development have been associated, *inter alia*, to damages in cortical migration and generation of neurons [1-3] causing heterotopy and ectopia [4], neuronal depopulation and neuroapoptosis [5,6] as well as disruption in callosal projection neurons [7], glial alterations [8,9], disruption on the second messengers and protein phosphorylation [10], alterations of the genetic expression [11-13], alterations of the growth factors [14] and neurotoxicity of homocysteine [15] as well as alterations in *substantia nigra* [16], in the neural crest [17], in the hypothalamic-pituitary-adrenal axis [18], in the cholinergic development [19,20] along with decreases in Purkinje cell density [21] and hippocampus neurons [22,23].

Different regions of the encephalon are not uniformly vulnerable to ethanol and other drug effects during development [17,24], mainly because of the absence or inefficiency of a protective fetal blood-brain barrier [25,26].

Furthermore, the deleterious effects of ethanol on the organism in general and specifically on the development of the encephalon are scarcely understood [27,28] and the *modus* of action behind the ethanol effects on biological structures is uncertain, putatively because ethanol effects are ubiquitous on cells constituent as well as receptors, growth cones, nucleic acids, membranes what could affect the migration routes, synaptogenesis and the structure of tissues and organs as cited above.

Most studies about the effects of ethanol on the neural system are focused on chronic effects, and just a few study acute effects from a single day of exposure to this drug on prenatal development [29]. The acute assays can be made on a specific day in the gestation while prioritizing crucial periods as gastrulation, migration of cells, for instance, neural and glial migration or synaptogenesis period, hypothetically destroying the basis of the cortical structure and consequently decreasing the cerebral mass [30].

In this way, ethanol effects in the birth of neurons in the neocortex that occurs from gestational day 11 to 13 in rats [31,32] could be studied, because that is a crucial time to the construction of neocortex layers and corresponds to first trimester-equivalent gestational days in humans [33,34] for the cerebral mass. Thus, the first purpose of this work was to acutely inject ethanol (single day) in the development neocortex when the neuronal migration is starting (neuron birthday) and to measure the evolution of the brain mass in some days after birth, using rats as a model.

On the other hand, ethanol is a known chaotropic that acts by denaturing proteins [35,36] and modifying the membrane bilayer [28]. It has been verified that chronic ethanol exposure in rats changes membrane fluidity [37] and increases the osmotic fragility of erythrocytes [38], producing hemolysis [39,40].

In vitro effects on erythrocytes indicate that ethanol destabilises the membranes [28,41,42] generating pores [43] and modifying erythrocyte shapes [42]. Nevertheless, membrane stability is amply dependent on the environment that cells live in [44] and the entropy of the aqueous solution-lipids system [45].

Indeed, before the ethanol acts on internal cellular structures, it must pass the extracellular environment and penetrate the cells by going through the plasma membrane and disrupting it [41,42]. Thus, it is reasonable to think, hypothetically, that the membrane rupture should generate apoptosis and migration errors in the cells because the membrane stability is essential to maintain cell integrity.

Water and saline solutions containing ethanol were tested separately to verify the direction and under what physiological conditions the effect of these solutions on plasma membranes would occur by the ethanol concentration injected in the rats in this experiment. To reach these objectives, the main model used was the erythrocytes because of their particular constitution, i.e. a cytoplasm without nucleus and organelles surrounded by a plasma membrane, such that the plasma membrane is most of the membrane in this structure. Rat and human erythrocytes were used.

In summary, the effects of the ethanol on the neocortex histology and the brain mass were verified. Then, the acute injection of ethanol (single day) on the offspring´s neurons (E_{12}) in Wistar rats was studied and effects in offspring were observed on the eighth postnatal day (P_8). Additionally, measures of the brain and corporal masses were made after birth until the 50th postnatal day to verify the effect of ethanol on brain mass. Then, *in vitro* tests were performed to verify the stability of erythrocyte membranes under aqueous and saline ethanol solutions. Finally, the results and other data from the literature were used to understand the general effect of ethanol on brain tissues.

Methods

Immunohistochemistry and encephalon/body masses

Female Wistar rats (180–230 g) were housed from 3:00 pm to 6:00 am the next day with male rats. The presence of a vaginal plug and sperm in the vagina confirmed successful mating and indicated the first day of gestation (E_0). Eighteen pregnant female rats were housed in cages at 22 ± 0.4°C on a 12 h light/dark cycle, with free access to food and water. On the 11th day of pregnancy (E_{12}), 12 rats received three intraperitoneal (i.p.) injections of a 20% ethanol solution (3 g of ethanol/kg of body weight), at 8 h intervals. Ethanol was administered intraperitoneally because this route ensured that all rats received an equal volume and amount of drug and there was almost 100% absorption. In addition, at E_{12}, six control pregnant rats received saline injections of 0.9% at the same intervals as the ethanol-treated rats. Proliferating cells were labelled as described elsewhere [32]. All rats received a single i.p. injection of BrdU (5 mg/mL in 0.9% NaCl, containing 70 mM NaOH) at a dose of 60 mg/kg 2 h after the last injection of ethanol or saline solution.

On the eighth day of postnatal life (P_8), 18 litters were chosen randomly and anesthetised with sodium pentobarbital (50 $mg \cdot kg^{-1}$, i.p.) and perfused with saline followed by 70% ethanol. The brains were removed and processed as described elsewhere (Miller 1993). Briefly, the brains were embedded in paraffin, sectioned sagittally (7 μm thick sections) and mounted on gelatine-coated slides. The sections were deparaffinised, hydrated in a graded ethanol series and treated with 1 molar (M) NaOH

followed by 1 M sodium borate buffer (20 min each), before washing with phosphate buffer. After blocking non-specific sites with serum, the sections were incubated for 2 h with a monoclonal anti-BrdU antibody (diluted 1:500; Sigma, St. Louis, MO, USA) followed by incubation for 1 h with a goat anti-rat secondary antibody (diluted 1:200; Vector, Burlingame, CA, USA) and then treated with an avidin-biotin complex (Vector) and incubated with 3,3'-diaminobenzidine (Sigma). Sections were cover slipped with Entellan. Some sections were not incubated with monoclonal antibodies to verify the possible background. Finally, the slides were contrasted with safranin.

The presence of anomalous neuron clusters in the neocortex areas was indicative of heterotopia, and the presence of unusual neurons in the neocortex layers was indicative of ectopia.

The lobes were identified via anatomical region and type of neurons for each layer according to Caviness Jr [46]. The number of cells immunohistochemically labelled in cortices was obtained from a trinocular microscope Olympus BX40-F4, coupled to an Oly-200 (Olympus American) camera and IBM computer via an Olympus U-SPT coupling accessory. The images were processed with the use of the HL image ++97 software. Six areas of 15,000 μm^2 from each lobe for each encephalon were captured and analysed.

The same proceedings used in the immunohistochemistry assays were done with eight female rats prior to mating, ethanol and BrdU injections. The encephalon and body masses of offspring were obtained on postnatal days 1, 8, 17, 26, 33, 45 and 50 after the perfusion (as the perfusion described before). Between four to six weight measures were obtained from each litter, depending on the number of individuals born to the nest. An effort was made to cut the head between the atlas vertebrae and the occipital using a fine scissor and the encephalon was removed carefully. All materials were weighed on an analytical balance (Mettler Toledo mod. AG245).

Osmotic fragility

Blood samples (3 mL) were collected, with a syringe containing heparin, from the antecubital vein of male human volunteers (14) after overnight (8–12 h) fasting and from 2 adult Wistar rats (198 and 230g) via the left ventricle.

The stability of erythrocytes was tested in solutions with different compositions: 1) ethanol from 0.1 to 20 $g \cdot dL^{-1}$ in 0.9% NaCl, 2) NaCl from 0.1 to 0.9 $g \cdot dL^{-1}$ and 3) ethanol from 0 to 19.8 $g \cdot dL^{-1}$ in deionised water to humans and ethanol from 3.9 to 74.1 $g \cdot L^{-1}$ in 0.9% NaCl. The general conditions of the assays were adapted from the literature [47]. To each unit of a duplicate set of Eppendorf flasks, 1 mL of the testing solution and 10 µL of blood were added. After homogenisation and incubation at 37°C, the flasks were centrifuged for 10 minutes at 2000 rpm, and the supernatant fractions were analysed by visible spectrophotometry at 540 nm with a Micronal B442 spectrophotometer.

Aliquots of 10 µL of blood were added to concentrations of 1.56, 3.9, 7.8, 5.6 and 11.7 $mg \cdot dL^{-1}$ ethanol (equivalent to 2, 5, 10, 15 and 20% of ethanol) solutions prepared in 0.9% NaCl. After homogenisation and incubation at 37°C for 10 minutes, the mixtures were added to histological slides and labelled with May-Grünwald-Giemsa stain and Sudan black. The slides were analysed in a trinocular microscope Olympus BX40-F4, coupled to an Oly-200 (Olympus American) camera and IBM computer via an Olympus U-SPT coupling accessory. The images were processed with the use of the HL image ++97 software.

Statistics

For the cell count, the normality test of data was performed first. Data submitted and approved by Kolmogorov-Smirnov/Liliefor and Shapiro-Wilk W tests were considered normal. After the acceptance, the normality was applied to a T-test to comparing means between the control and treated groups to each lobe and afterwards to both complete groups. Differences were considered significant at $p < 0.001$.

Data from encephalon and body weight were submitted to curve fit to verify the type of regression that had a better fit for the data. Logarithmic and linear regressions were used in the

regressions. Logarithmic regression was used to compare the brain and body weight for both treated and control rats, and linear regressions were used to compare control and treated brain and body weights. The comparison between the relationship between brain and body weight for both treated and control groups was performed using linear regression. For all cases, the ANOVA was calculated for $p < 0.05$.

For both experiments' cell counts and encephalon-body weights, the statistical analyses and graphical plots were performed via the StatPlus program (AnalystSoft Inc. v.7.3.3).

Data obtained for ethanol/saline concentrations and only saline concentration were adjusted to a sigmoidal curve according to the Boltzmann equation and the D_{50} was obtained by 50% of erythrocyte lysis.

Results

Immunohistochemistry of acute ethanol effects on the neocortex

In quantitative terms, the number of cells was lower in rats treated with ethanol than to controls to each lobe and total count in all lobes (frontal, parietal, temporal and occipital) considering absolute numeric data and after statistical analysis of means comparison with a t-test to $p < 0.001$ (Fig 1).

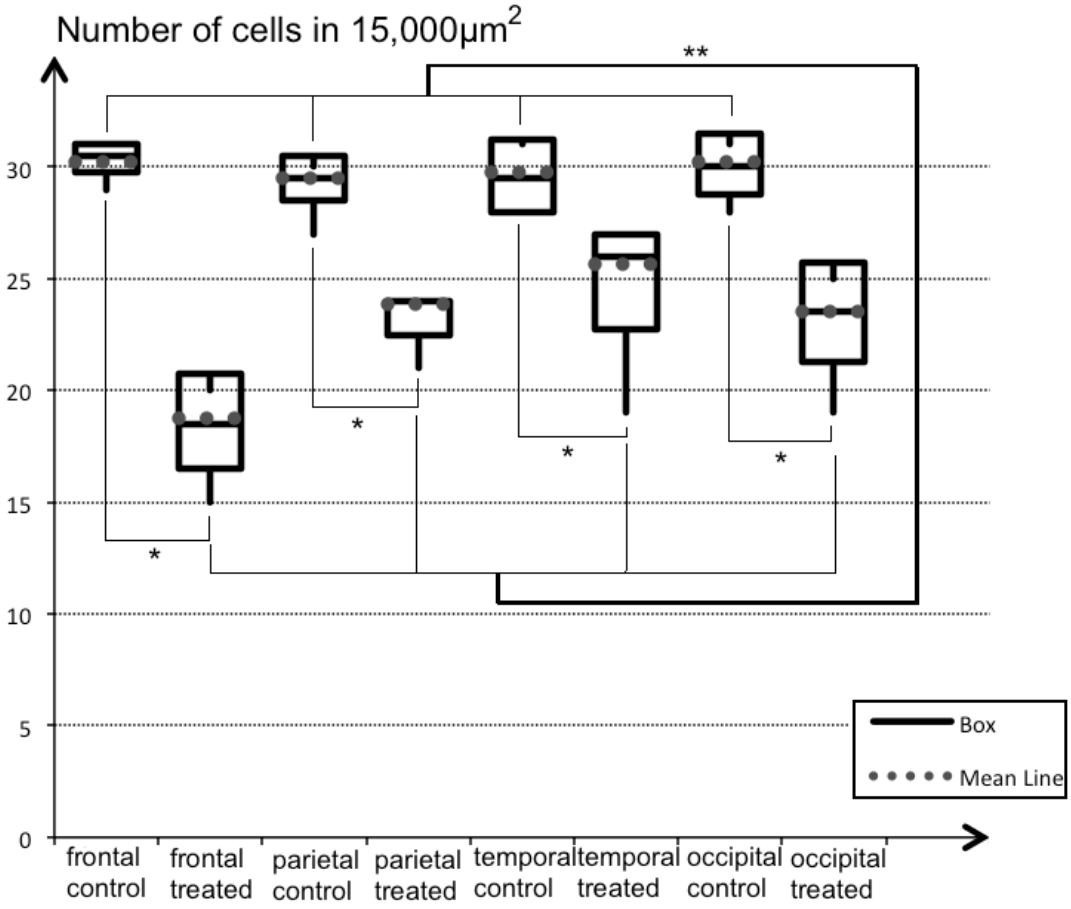

Fig 1. Box graphic of the number of cells counted in the neocortex (15,000 µm^2). The asterisk (*) indicates a significant difference between control and treated groups for each lobe, and two asterisks (**) indicate a significant difference between all controls and treatments by comparing means (T-test) to $p < 0.001$.

In qualitative terms, significant alterations of neocortical tissue in ethanol-treated rats relative to control ones were observed. The characteristic pyramidal neurons were used as a marker to define brain layers in Wistar rats (Fig 2A). The BrdU stained nuclei; however, many times, the content of the nucleus may leak during the proceedings, and the cytoplasm shape is visualised in black or shades of grey. On the other hand, the safranin stains permit a pale visualisation of the cytoplasm.

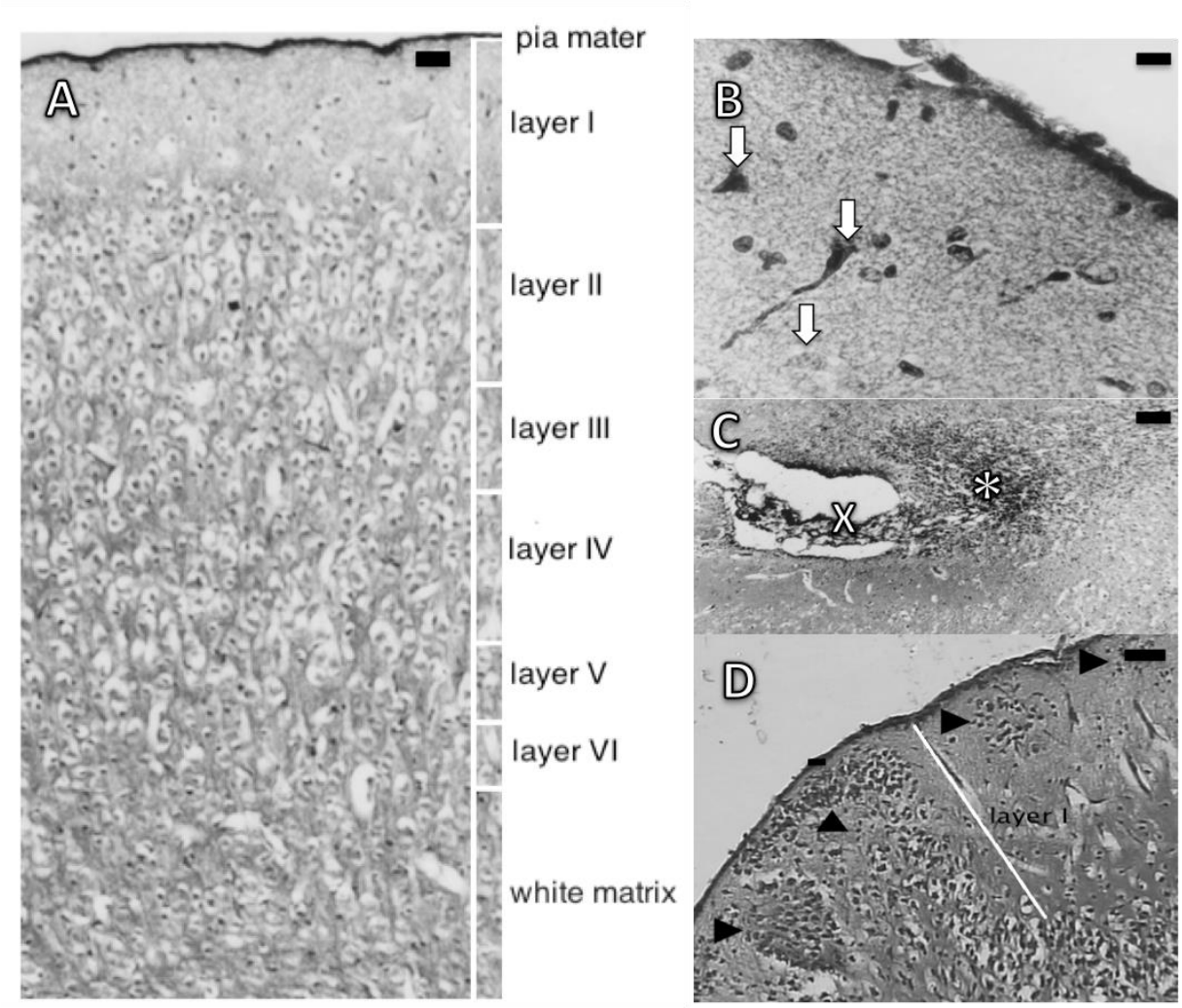

Fig 2. Photomicrographs of the neocortex (prefrontal lobe) of Wistar rats with cells labelled with BrDU. **A** Photomicrography of the histology of layers in the parietal lobe of a control (Bar = 200 µm); **B** Photomicrography of layers I and II of a frontal lobe of a treated animal indicating (white arrows) the unusual large cells (ectopia) with pyramidal shape in layer I (Bar = 20 µm); **C** Photomicrograph of the tangential route from the ventricular zone to the olfactory bulb generating a heterotopic group of cells (*) under fissure between the frontal lobe and olfactory bulb (X) (Bar = 240 µm); **D** Photomicrograph of superficial layers of the temporal lobe of a treated animal indicating (arrowheads) anomalous group of cells (heterotopy) in layer I close to the pia mater (Bar = 200 µm).

The main alterations observed in ethanol-treated animals were ectopic neurons with pyramidal shapes found in layer I (molecular layer) (Fig 2B) and heterotopic groups into the molecular (Figs 2C, 2D, 3A) and external granular (layer II) layers (Fig 3C).

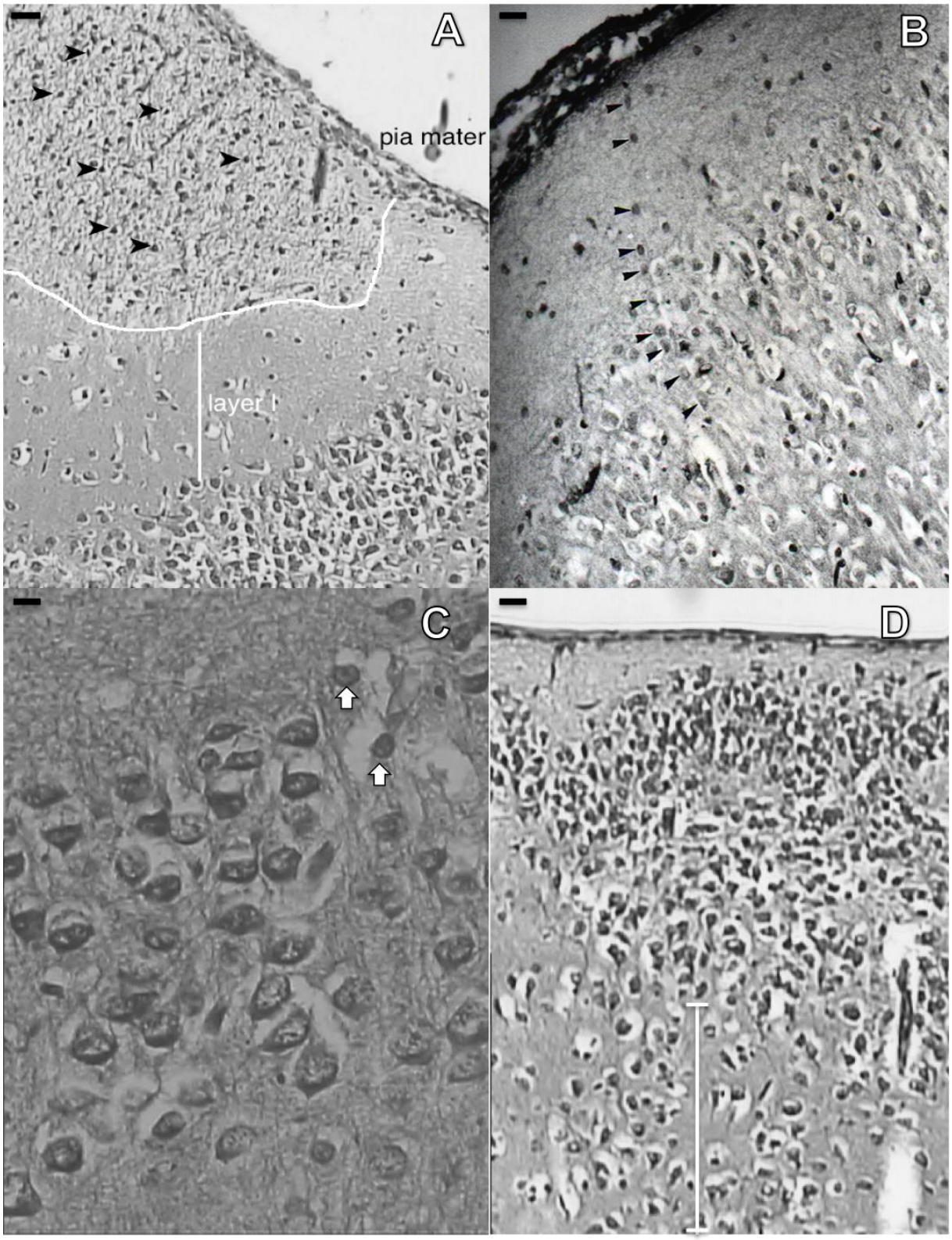

Fig 3. Photomicrographs of the neocortex of treated Wistar rats with cells labelled with BrDU. A Photomicrography of the histology of superficial layers in the parietal lobe with a large heterotopic group of cells presenting pyknotic nuclei (arrowheads) (Bar = 90 µm); **B** Photomicrography of layers of a frontal indicating (arrowheads) a group of cells in line seeming to follow a pathway to the pia mater (Bar = 70 µm); **C**

Photomicrography of superficial layers I, II and part of III of the parietal lobes indicating an anomalous group of cells (heterotopy) in layer II; the normal nuclei expected to this layer are shown by head arrows (Bar = 25 µm); **4** Photomicrography of layers of an occipital lobe showing (white line) a few populations of cells in deep layers (V and VI) relative to other more superficial ones (Bar = 50 µm).

Cell depopulation in deep layers, manly V and VI (pyramidal internal and fusiform layers, respectively) was observed qualitatively (Fig 3D) and quantitatively (Fig 1). Apoptotic cells characterised by pyknotic nuclei were observed in heterotopic clusters (Fig 3A) and a putative radial pathway to the pia mater was found by the observation of a line of nuclei (Fig 3B).

Acute ethanol effects on encephalon mass

The relation of the encephalon weight to body weight demonstrated a statistical difference according to the ANOVA from the 1st day forward until the 50th post-natal day to $p < 0.05$ (Fig 4). The statistical logarithmic regression was calculated to compare the brain and body weight for both treated ($R^2 = 0.95504$) and control ($R^2 = 0.9407$) rats. The linear regressions were calculated to compare control and treated brain weights ($R^2 = 0.7166$) and body weights ($R^2 = 0.8212$), as well as the comparison between the relation between brain and body weights for both treated and control groups ($R^2 = 0.0052$), at $p < 0.05$ (Fig 4).

The ANOVA shows the acceptance of H_0 for the comparisons between the brain and body weight for both treated and control groups as well as the control and treated brain and body weights; however, for the comparison between the relation between brain and body weight for both treated and control groups, the H_0 was rejected at $p < 0.05$.

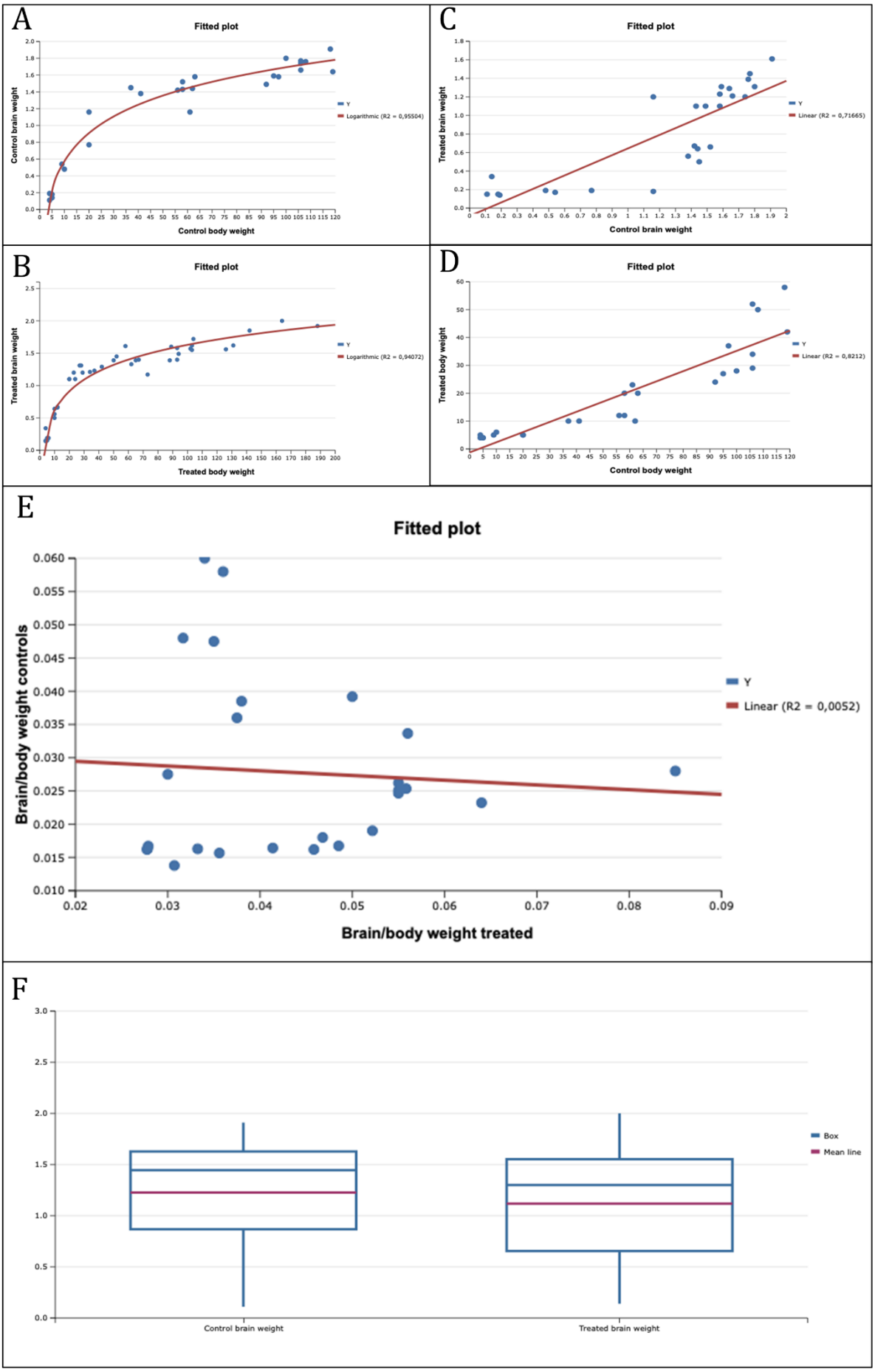
A
Fitted plot
Control brain weight
Control body weight
Y
Logarithmic (R2 = 0,95504)
C
Fitted plot
Treated brain weight
Control brain weight
Y
Linear (R2 = 0,71665)
B
Fitted plot
Treated brain weight
Treated body weight
Y
Logarithmic (R2 = 0,94072)
D
Fitted plot
Treated body weight
Control body weight
Y
Linear (R2 = 0,8212)
E
Fitted plot
Brain/body weight controls
Brain/body weight treated
Y
Linear (R2 = 0,0052)
F
Box
Mean line
Control brain weight
Treated brain weight

Fig 4. Graph showing the acute ethanol effects on encephalon mass. A Showing the logarithmic regression to compare the brain and body weight for both treated (R^2 = 0.95504) and **B** control (R^2 = 0.9407) rats; **C** Showing the linear regressions to compare control and treated brains weight (R^2 = 0.7166) and **D** body weights (R^2 = 0.8212); **E** Also the comparison between the relation between brain and body weights for both treated and control groups was performed using linear regression (R^2 = 0.0052) in all cases, to $p<0.05$; **F** Box plot indicating the brain mass difference between the control and treated rats.

Ethanol effects on osmotic fragility (membrane stability) of human erythrocytes

The stability of erythrocyte membranes was verified under different concentrations of NaCl from 0.1 to 0.9 g·dL^{-1} (Fig 5A) with D_{50} equal to 0.46 g·dL^{-1} (± 0.05). It was performed by two motifs, at least, 1) to verify the device accuracy and 2) to represent an osmotic parameter to study the ethanol osmotic effects on membranes of erythrocytes. Crescent ethanol concentrations from 0 to 19.8 g·dL^{-1} were used to verify the membrane stability in erythrocytes (Fig 5B), demonstrating membrane rupture for all analysed concentrations.

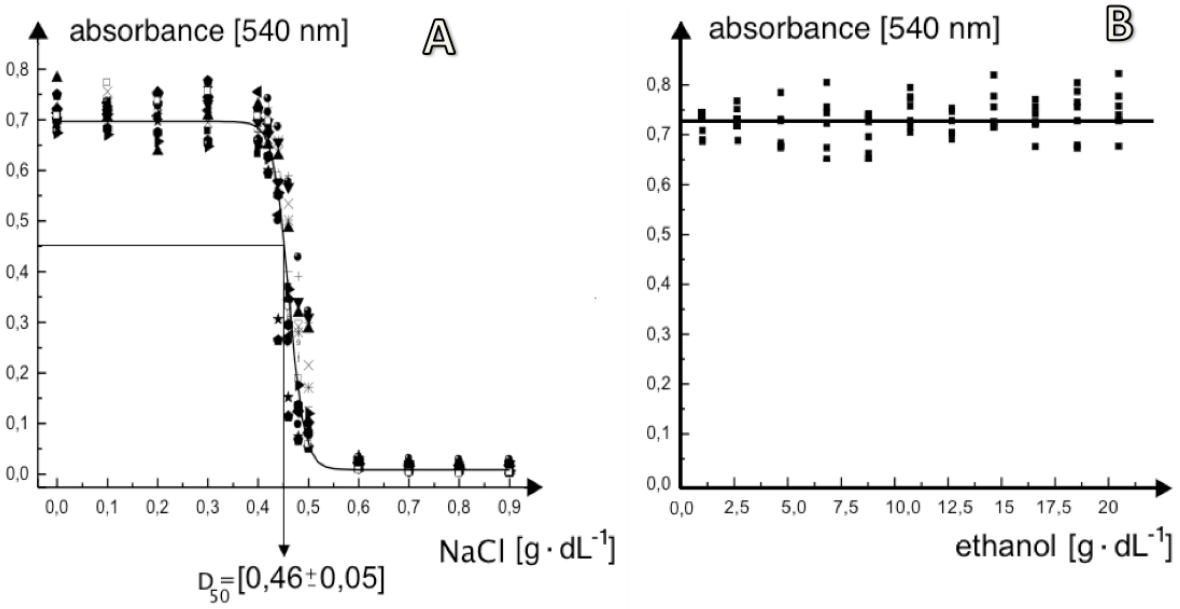

Fig 5. Data from the crescent concentration of NaCl and ethanol on human erythrocyte membranes. A Data from the crescent concentration of NaCl on human erythrocyte membranes; observe that the membrane stability of erythrocytes starts at D_{50} (0.46 ± 0.05 g·dL^{-1}); **B** Data from the crescent concentration of ethanol on erythrocyte membrane stability; observe that for these concentrations membrane stability for erythrocytes is not present.

The membrane stability of Wistar rats and human erythrocytes was tested under the effect of association among crescent ethanol concentrations (0.1 to 20.0 g·dL^{-1} in humans and 3.9 to 74.1 g·dL^{-1} in Wistar rats) diluted in a constant concentration of NaCl (0.9%) with a D_{50} equal to 11.05 ± 0.25 g dL^{-1} for humans (Fig 6A) and a D_{50} equal to 11.31 ± 0.04 g·dL^{-1} for Wistar rats (not shown graphically). According to these data, the 0.9% NaCl in solution with ethanol stabilises erythrocyte membranes until 11.05 g·dL^{-1} for humans and 11.31 g·dL^{-1} for Wistar rats. The average concentration of the ethanol in the blood of Wistar rats for the histological study was approximately 24.4 g·dL^{-1}; therefore, into the destruction of erythrocytes by ethanol in rats, in considering the rats' average mass of 205 mg and the blood volume of approximately 13 mL [48].

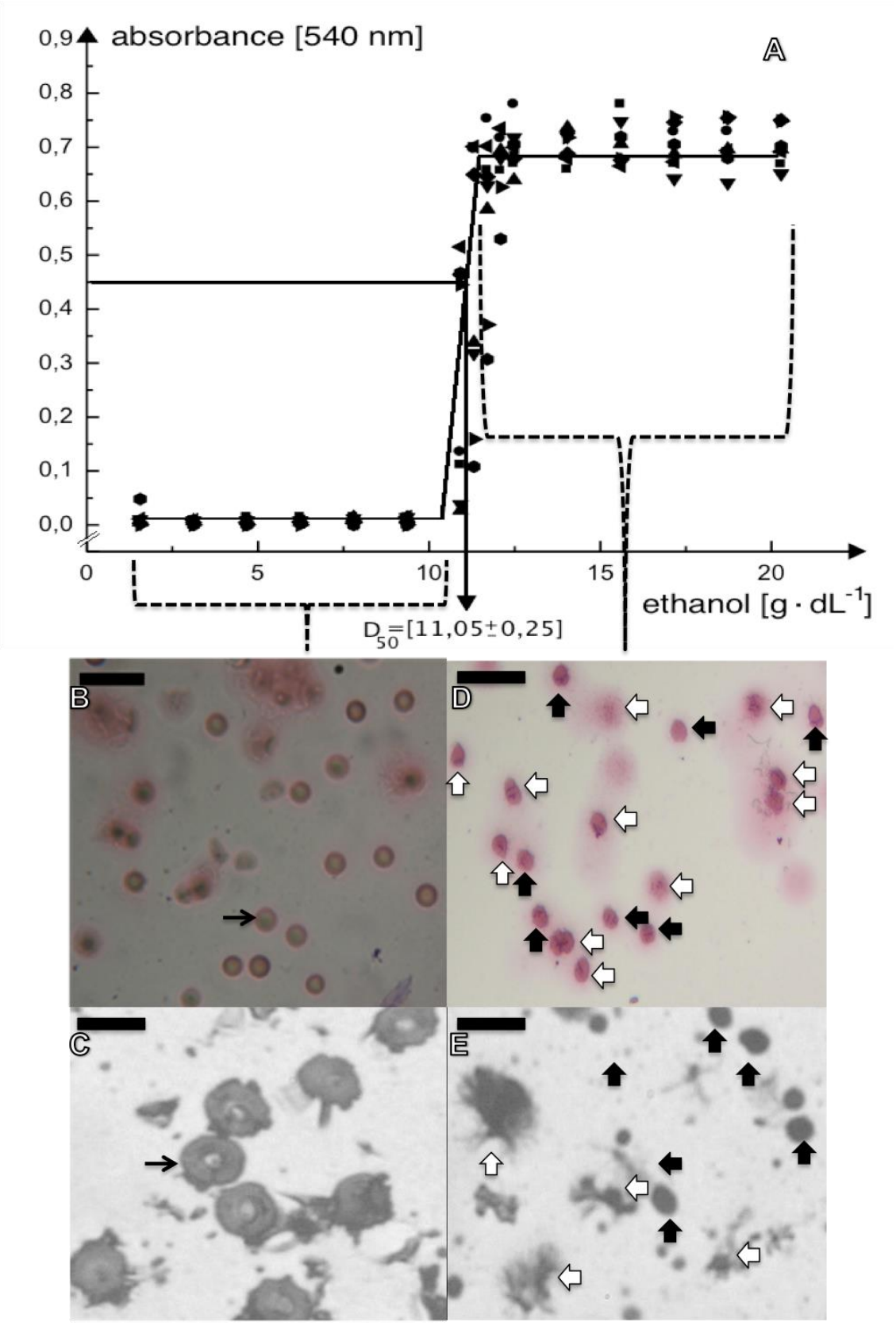

Fig 6. Ethanol effects on membrane stability of human erythrocytes. A Plotted data of the crescent ethanol concentration effect on the erythrocyte's membrane keeping a constant concentration of the NaCl (0.9%)

generating a sigmoidal curve adjusted to the Boltzmann equation. The pre-transition region must contain most of the erythrocytes intact and the post-transition region must contain most of the destroyed erythrocytes. The calculated D_{50} (a point that represents 50% of erythrocytes intact) was 11.05 ± 0.25 $g \cdot dL^{-1}$. **B** Photomicrography of erythrocytes labelled by May-Grünwald-Giemsa obtained from samples that generate pre-transition curve (2% or 1.56·$g \cdot dL^{-1}$) indicating the intact or normal shape of erythrocytes (the thin black arrow indicates an example of intact erythrocyte) (Bar = 35 μm). **C** Photomicrography of erythrocytes labelled by Sudan black obtained from samples that generate pre-transition curve (2% or 1.56 $g \cdot dL^{-1}$) indicating the intact or normal shape of erythrocytes in detail (the thin black arrow indicates an example of intact erythrocyte) (Bar = 10 μm). **D** Photomicrography of erythrocytes labelled by May-Grünwald-Giemsa obtained from samples that generate post-transition curve (20% or 15.6 $g \cdot dL^{-1}$) indicating small (black arrows) and destroyed (white arrows) erythrocytes (Bar = 38 μm). **E** Photomicrography of in detail erythrocytes labelled by Sudan black obtained from samples that generate post-transition curve (20% or 15.6 $g \cdot dL^{-1}$) indicating small (black arrows) and destroyed (white arrows) erythrocytes (Bar = 10 μm).

To verify the shape of the erythrocytes, histological assays were performed using May-Grünwald-Giemsa (Figs 6B, 6D) and Sudan black (Figs 6C, 6E) stains. Most erythrocytes shown in Fig 6B and with more detail in Fig 6C at a concentration of 2% ethanol (1.56 $g \cdot dL^{-1}$) presented intact membranes. Meanwhile, at a concentration higher than 11.05 $g \cdot dL^{-1}$, specifically 20% ethanol (15.6 $g \cdot dL^{-1}$) in Fig 6D and with more detail in Fig 6E, the erythrocyte membranes ruptured and/or the erythrocytes presented small sizes in comparison with the erythrocytes in Figs 6B and 6C.

Discussion

Immunohistochemistry of acute ethanol effects on the neocortex and loss of the cerebral mass

In the development neocortex, post-mitotic neurons migrate from ventricular pseudostratified epithelium from days E_{11} to E_{13} [32] to generate, in order, the deepest to superficial layers in the adult neocortex [31,32,47]. This migration phenomenon obeys spatial and temporal patterns dependent on the

formation and differentiation of the radial glia stimulated via chemical signs [49,50] for which molecular mechanisms are scarcely known [51-53].

The horizontal and vertical layers of organization in the adult neocortex are essential to its normal function and histological/molecular alterations during the development can affect the learning, attention, behavior, motor abilities [11,54] and memory [55,56] in animals and humans.

Chronic ethanol effects are associated with the desynchronization of cortical development and consequently the establishment of neuronal circuits generating a neocortex reorganization characterized by heterotopic groups of neurons [7] as well as destabilization of the matrix, molecules and other components in glia [53] and neurons [58,59], including processes such as gene expressions [11,60] and apoptosis [53,61-63].

General and more complete studies about acute effects of ethanol on the brain in development are necessary, considering molecular and genic aspects; indeed, most of the few acute studies are focused on histological aspects [64-67].

The acute studies indicated similar effects in the neocortex of Wistar rats in relation those verified to chronic ones, mainly in the presence of heterotopic groups of cells and depopulation in deep layers [66] and apoptosis in primate brains [67]. In rats, however, episodic prenatal exposure to ethanol in macaques has recently been shown to affect neurogenesis [68].

The literature refers mainly to chronic ethanol treatment problems, with some exceptions, as in the studies of the lobes for acute effects of ethanol specifically in P_8 with pregnant rats exposed in E_{12} [65-67].

These causes of the brain disorders could be caused by several factors, such as disorganization of the cytoskeleton [49,69], problems in the expression of molecular clues (GGF, RF60, laminin, proteoglycans) or even some metabolic alterations, generating heterotopia, which is one of the most important derangements of the migration in the neural system [70-74].

The presence of evident heterotopic groups forming cell mass of a nodular type, determined by chronic exposure to ethanol [72], was also verified in this work as the deviation of the migratory route of neurons, i.e. putatively because the cells lost the ability to locate in the correct space-time place, perhaps they did not obtain the necessary connections to avoid apoptosis.

The neuroblast, when it leaves the germinal lamina to start its migration, has already specified its type, location and shape [50] and ethanol could disrupt these organizations. Indeed, in this work, the presence of ectopic neurons was observed in all rats acutely treated with/exposed to ethanol.

When neurons lose chemical contact with glial fibres, they continue migrating following the radial pathway until the *pia mater* where they undergo apoptosis [74,75]. This certainly justifies the neuronal depopulation mainly in the deep layers observed in animals treated with ethanol (Fig 4). In this sense, putatively, the ethanol inhibits the chemical relation between glia and neuroblasts necessary for correct neuron localisation in the correct cortex layer.

As ethanol is chaotropic, it can affect the chemical environment of the cell matrix or the receptors and disrupt cellular components, in addition promoting changes in some or several of the basic activities of cells such as gene expression [60,76]

Molecular, cellular and histological changes in the neural system in initial development may affect general functions such as learning, inhibition, attention, regular behaviour and motor skills [54,77,78] in the adult brain, and ethanol promotes these kinds of disturbances both in chronic and acute exposure, as shown in this work in the last case.

Accordingly, histophysiology modifications from ethanol administration may be responsible for problems such as memory loss reported in adults who had Foetal Alcohol Syndrome (FAS) [29,56,79]; however, this kind of analysis were not performed here.

Indeed, our data, i.e. the effects of the acute exposure to ethanol, are in agreement with reports of chronic effects studies [4,7], including migration problems in the cortex, with the layers having their natural order reversed and neuronal depopulation in the deep cortical layer, inter alia.

Hypothetically, the deep neuronal depopulation can be justified by the fact that the cells that form these layers are the first to initiate the migration [48,50,57,74] and, therefore, were subjected to a higher concentration of ethanol on the twelfth day of intrauterine life when the neurons started their migrations.

Another explanation is linked to horizontal or Cajal-Retzius cells that are responsible for the way the direction and stimulation of neuronal migration guides the astrocytes fibres, until their anchorage in the *pia mater* [50]. Therefore, the disorganisation of the structure of the cytoskeleton of those cells by ethanol's chaotropic effects [53] could alter, at least secondarily, the time of fibre formations or fibre competence and, consequently, compromise neuron migration.

In summary, most works in the literature report the chronic effects of ethanol with few studies about the acute effects on specific days of embryonic or foetal development [27]. However, this work shows the severity of acute ethanol intoxication at the beginning of neuronal migration in rats, around the twelfth day of rat intrauterine life.

It seems that the earlier the biological structure of the tissue is affected, the larger the consequences are [29], since the complex structures derived from the movement of cells during the development and differentiation into many cell subtypes depend directly on the initial basic structure of the tissue or organ, mainly the matrix integrity.

As the chronic studies about the ethanol effects are performed in rats [9,48,75,80], and the consequences are projected for humans, the same might be done here, i.e. the acute effects of the ethanol on the cortex are valid for humans, since the toxic concentrations of ethanol in rats and humans are equivalent, and the neuronal densities existing between different regions of the same brain and between brains of different species are also equal for all mammals.

Brain mass alterations

Neural apoptosis is a normal way for cerebral depopulation after birth in animals [51,81]. The measures of the brain mass diminish across time; however, the corporal mass increases simultaneously [82]. Thus, the relative measure of brain mass/body mass was performed to verify the ethanol effects on the brain mass, because when the numbers of neurons decrease, the brain mass must also decrease. However, the brain mass is not only dependent on neurons, the glia can divide and substitute for the dead neurons [83].

The chronic studies about ethanol effects, including the FAS, indicates a cerebral mass decrease [30] relative to controls; however, this was not verified here except for the relation brain/body mass for both control and treated groups with linear regression, with R^2 indicating no correlation and ANOVA indicating the H_0 rejection. Some correlation was expected for this last case and a deeper study in trying to fit the curve for other parameters as logarithmic, exponential, square, or cubic regressions also indicate a null correlation. The expected regression for other parameters indicated a good precision in the data obtention, i.e., the body and brain growth for both control and treated groups. Thus, some alterations seem to occur with a decrease of the brain mass of the treated according to the box graph (Fig 4), following the information from the FAS, but weakly.

Disruption of the erythrocyte membrane by ethanol

The literature cites the disruption of the cytoskeleton as well as DNA and protein disarrangement to explain the ethanol injuries in the brain [36]; however, logically, the ethanol first acts on the environment or cell matrix and the membranes.

In this work, the study of erythrocyte fragility was to verify ethanol effects on the membranes. The concentration used for brain histology was 20% (24.4 $g \times dL^{-1}$), which was in the zone of the ethanol destruction of erythrocytes. Accordingly, it is plausible to think that membrane destruction of the neurons and glia could generate the histological problems in the migrated and dead cells. A study using neurons and glia under the effect of the ethanol in comparison with histological data could be performed to clarify this hypothesis.

Other important information from the experimentation is that NaCl can act as a protector of erythrocyte membranes. It behaves in a proportion relative to the concentration of solutions, i.e. a doubling of ethanol concentration by the number of particles is equivalent to half the NaCl concentration, because this salt ionises into Na^+ and Cl^- in an aqueous solution. Therefore, the concentration of particles in the system cannot explain the membrane destruction by ethanol, and the interactions among the molecules in the environment must be considered.

Indeed, the bipolarity of the ethanol should generate a disruption of the cell membranes [35]. If one considers that the ethanol hydrocarbon could dissolve the membrane, at least, partially, the general membrane structure should be disarranged, and all cell metabolism could suffer these effects, with the addition that the polar head of the ethanol can link with the membrane lipid heads, but it could have a lesser effect on the membrane stability that is generated by the molecular Van der Waals interactions. Despite the Van der Waals force being a weak one, in proximity, it becomes very strong because this interaction is proportional to the inverse of the distance raised to the power of 14. As the arrangement of the cell membranes is linked to the solvent properties, ethanol, because of its chaotropic effects, could change the solvent features and interfere with the Van der Waals forces.

Accordingly, the ethanol effects occur, on all cell membranes by the alteration of the cell environment (cell matrix) and disruption of the membrane stability, according to the interaction force proportions. In this way, partial disruption of the blood-brain barrier could occur as verified before [25,26] in neurons.

Conclusions

According to this work, the ethanol injection in the rats shows marked brain tissue destruction and decrease of brain mass, relative to the control, and ethanol's effects on erythrocytes indicated that membrane destruction could be one of the causes of brain cell disruption in neural migration.

On the other hand, the chaotropic ethanol properties could destroy the matrix clues for neuron migration and glia organisation, thus altering the cell environment and membrane stability.

The histology data linked to theoretical concepts indicate that neuron migration involves cellular structures such as the cytoskeleton, cell membranes and biomolecules of the extracellular matrix, which are together responsible for the space-time relationship that determines the specific location of neurons in the layers of the neocortex.

Chronic exposure to ethanol affects neuronal migration, producing severe changes in the structure of the neocortex, which comprise 1) decreased neuronal density in all lobes studied in animals treated with ethanol, 2) change in the location of neuronal cells, such as the occurrence of pyramidal neurons in the molecular layer and 3) alteration in the specific migration path of cells in the formation of the cortex.

Acute ethanol treatment of rats on the twelfth day of intrauterine life determines a degree of severity on the neural system that is very similar to the chronic treatment with this drug throughout pregnancy in rats, including brain mass loss.

These results are an indication for controlled ethanol ingestion or no use of ethanol during known or suspected gestation in human females.

Declarations

Ethical approval and consent to participate

The institutional ethics committee approved the experimental protocols described here, i.e. the Federal University of Tocantins – Brazil for immunohistochemistry and the University of Toyama – Japan for experiments on brain and body masses and the stability of erythrocyte membranes.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Author's Contributions

KCO, RAGMFAV, TA, SFS, EVS, RPA, DSA, MPFM, RBB, RBV, RCRD, BRS, TAAF: Investigation and methodology; KCO & TAAF: Validation; KCO, TAAF: Writing draft; TAAF: Conceptualization and formal analysis; KCO, TA, SFS, EVS, RPA, DSA, BRS, TAAF: Writing review and editing; TAAF: Supervision. All of the authors have read and approved the manuscript.

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