PERINATAL HIPOXIC ISCHEMIC INJURY IN THE AUDITORY PATHWAY AND THE EFFECT OF SEVERAL NEUROPROTECTIVE AGENTS

ABSTRACT

Despite improvements in neonatology, perinatal hypoxic-ischemic (HI) encephalopathy remains one of the main causes of disabilities in term-born infants. This specific pathology underlies many neurological disorders such as learning difficulties, language and attention deficit, hyperactivity disorders and cerebral palsy. Moreover, it is also a notable risk factor for hearing impairments which affect neonates.

Insult from hypoxia-ischemia causes immediate neuronal injury and exhaustion of cellular energy stores, as the main cause of HI brain injury is the deprivation of glucose and oxygen supply, which initiates a multi-faceted cascade of biochemical events. The combined effects of cellular energy failure, acidosis, glutamate release, intracellular calcium accumulation, lipid peroxidation, and nitric oxide neurotoxicity provoke, in many cases, the death of the cells, either by necrosis or apoptosis, a divergence that will depend on the severity of the insult, the maturational state of the cell or the brain region affected, among others. After a thorough understanding of the mechanism underlying neural plasticity following hypoxic-ischemic brain injury, various neuroprotective therapies have been developed for alleviating brain injury.

The aim of this work is to evaluate with morphofunctional, molecular and cellular methods the effect of a panel of antioxidants on HI-induced auditory deficits. To this end, we studied the effects of Nicotine, Melatonin, Resveratrol and DHA on the neonatal auditory system via measurement of auditory evoked potentials and characterization of the morphological, molecular and cellular integrity of the IC.

Miren Revuelta Aramberri, Leioa 2015

DOCTORAL THESIS

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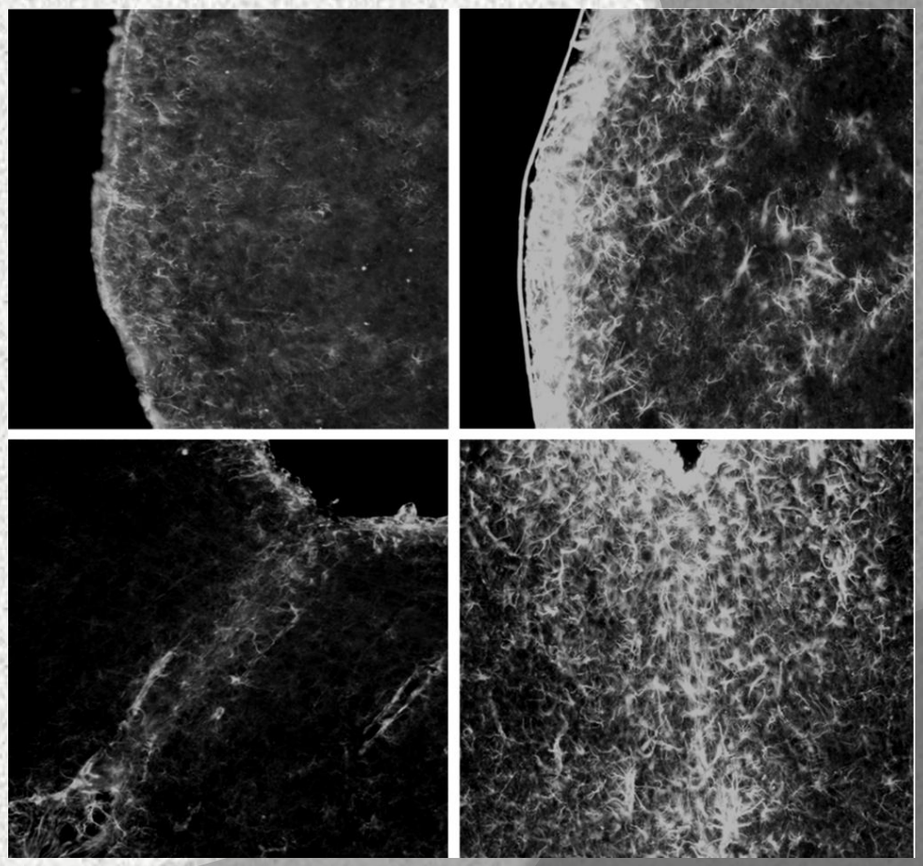

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LABURPENA

Despite improvements in neonatology, perinatal hypoxic-ischemic (HI) encephalopathy remains one of the main causes of disabilities in term-born infants. This specific pathology underlies many neurological disorders such as learning difficulties, language and attention deficit, hyperactivity disorders and cerebral palsy. Moreover, it is also a notable risk factor for hearing impairments which affect neonates.

Insult from hypoxia-ischemia causes immediate neuronal injury and exhaustion of cellular energy stores, as the main cause of HI brain injury is the deprivation of glucose and oxygen supply, which initiates a multi-faceted cascade of biochemical events. The combined effects of cellular energy failure, acidosis, glutamate release, intracellular calcium accumulation, lipid peroxidation, and nitric oxide neurotoxicity provoke, in many cases, the death of the cells, either by necrosis or apoptosis, a divergence that will depend on the severity of the insult, the maturational state of the cell or the brain region affected, among others. After a thorough understanding of the mechanism underlying neural plasticity following hypoxic-ischemic brain injury, various neuroprotective therapies have been developed for alleviating brain injury.

The aim of this work is to evaluate morphofunctionally, molecularly and cellularly the effect of a panel of antioxidants on HI-induced auditory deficits. To this end, we studied the effects of nicotine, melatonin, resveratrol and DHA on the neonatal auditory system via measurement of auditory evoked potentials and characterization of the morphological, molecular and cellular integrity of the IC.

AURKIBIDEA

Orrialdea

LABURDURAK

ABR	Auditory Brainstem Response
ACPD	1-amino-1,3-cyclopentrane dicarboxylic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Varinace analysis
APE	Auditory Evocated Potential
ATP	Adenosine tri phosphate
BAX	bcl-2-like protein 4
BSA	Bobin serum albumin
Bcl-2	B-cell lymphoma 2
Ca^{2+}	Calcium ion
$[Ca^{2+}]_I$	Intracellular calcium concentration
CBF	Cerebral blood flow
cGMP	Cyclic guanosine monophosphate
CIC	Central part of the inferior colluculus
CNS	Central nervous system
CPA-4	Lysophsphatidic acid-4
DAB	Diaminobencidin
dB	Decibelium
DCFH-DA	2,7-dichlorodihydrofluorescein diacetate
DHA	Docosahexaenoic acid
DMSO	Dimetilsulfoxid
DNA	Deoxyribonucleic acid
ECIC	External cortex of the inferior colliculus
ECochG	Electrocochleogram
EHI	Hypoxic Ischemic Encephalopathy
FALS	Forward Angel Light Scatter
GFAP	Glial fibrilary acidic protein
GLUT1	Glucose transporter 1
GST	glutation S-transferasa
H^+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
HI	Hypoxia ischemia
HIF1	Hypoxia inducible factor 1
HSA	Human serum albumin
HSF1	Heat shock factor1
HSP70	Heat shock protein 70
Hz	Herz
IC	Inferior colliculus
IP3	Inositol tri phosphate
ISS	Integrated Side Scatter
K^+	Potassium ion
K^+-ATPase	Potassium ATPase
LPS	Lypopolisacarid
MBP	Myelin basic protein
MET	Mechanoelectric channels
MPTP	Mitochondrial Permeability Transition Pore
ms	milliseconds
N_2	Nitrogen ion

Na^+	Sodium ion
Na^+/K^+	Sodium/potassium channel
nAChR	Nicotinic acetylcholine receptor
NADH	Nicotinamide Adenosine Dinucleotide
NAO	10-N-nonyl-acridin orange
NEUN	Neuronal specific protein
NMDA	N-Metyl-D-aspartate
NO	Oxide nitric
nNOS	Oxide nitric sintase
O^-	Superoxide ion
O_2	Oxygen
OH^-	Hydroxide ion
$ONOO^-$	Peroxinitrite
PaO2	Arterial Oxygen partial presure
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PVL	Periventricular leukomalacia
ROS	Reactive oxygen species
RT	Retrotranscriptase
SOD	Superoxide dismutase
SOD1	ZN-SOD
SOD2	Mn-SOD
TNF	Tumor necrosis factor
TTC	Tetrazolium chloride
VEGF	Vascular endothelial growth factor
ZO	Zonula ocludens
µV	Microvolt
µl	Micro liter
ΔO2Hb	Oxygenated hemoglobin
ΔHHb	Desoxygenated hemoglobin
ΔcHb	Total hemoglobin change

IKERKETAREN JUSTIFIKAZIOA

Nahiz eta gaur egun neonatologian hainbeste aurrerapen izan, entzefalopatia hipoxiko iskemikoak (HI) hainbat patologia eragiten ditu (garun-paralisia, lengoaia atzerapena eta atzerapen mentala, ikasketetan ezgaitasunak eta epilepsia edo kasu batzuetan heriotza ere sor dezake) eta jaio berrien ohiko ezgaitasuna izaten jarraitzen du. 1000 jaio berrien artetik bik pairatzen duten ezgaitasuna da baina jaiotza goiztiarretan eragin hau %60ra igotzen da.

Komunikatu ahal izateko entzumena beharrezkoa da. Komunikaziorako gaitasuna bizitzako lehenengo urteetan eskuratzen da eta garatu ahal izateko entzumen egokia beharrezkoa da. Honegatik, entzumenaren gaixotasun goiztiarrak lengoaian atzerapena eragin dezakete, izan ere, lengoaia eskuratzearen erraztasuna galdu egiten da hipoakusia bat gertatzen den momentuan, eguneroko bizitzan ondorio anitzak eraginez.

Hipoxia iskemiak entzumen-sisteman eragiten dituen ondorio gehienak Kortiren organoan eta gongoil-espiralean dauden zeluletan aztertu dira, eta ondorioz, entzunbidean duen efektuaren azterketak oso eskasak eta zatikatuak dira.

Gure ikerketa honetan jaio berrien bi modelo esperimental erabiliko ditugu, arratoia eta txerria. Izan ere, 7 eguneko arratoi baten eta txerri jaio berri baten (< 5 egun) garunaren garapena, gizaki jaio berri goiztiar baten antzekoa da. Honegatik, 7 eguneko arratoiak eta txerri jaio berrien garunak gizakia jaio berriaren garunarekin konparatu daitezke garapen neurologikoa aztertzeko.

Azken hamarkadan, aurrerapen ugari egon dira hipoxia iskemiak eragiten dituen aldaketa biokimiko eta molekularren azterketan eta helburua, kaltearen lehen orduetan gertatzen den eraso sakona zehaztea izan da. Aurrerapen hauen eraginez, aukera terapeutiko zehatzen erabilera begiztatu dute. Gainera, azken urteetan ikerketa basiko, esperimental eta klinikoak aurrerapen ikaragarria izan du eta baita hipoxia iskemia baten ostean aukera terapeutiko zehatzak erabiltzea kaltea aurreikusi edo murrizteko. Ikerketa hauek antioxidatzaileek hipoxia iskemia baten ondoren kaltea murrizten dutela baieztatu dute, baina ikerketa hauek kortex edo

hipokanpoan soilik oinarritu dira. Honegatik, hipoxia iskemia baten ondorioz entzumen-sisteman gertatzen diren kalteak aztertzeaz gain, antioxidatzaileen eragina ikertuko dugu eta garuneko eremu honetan ere efektu neurobabestzailea duten edo ez.

SARRERA

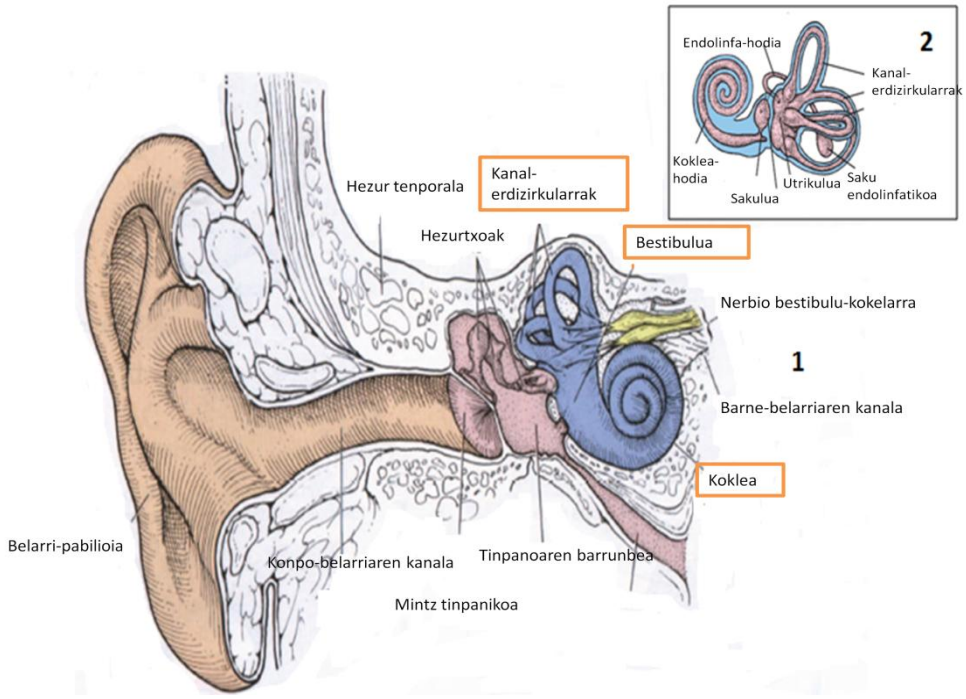
Gizakiaren kalte neurosentsorial ohikoena entzumen gaitasunaren galera da. Helduen artean, entzumenaren ezgaitasunak ohiko diren 10 gaixotasunen artean aurkitzen da eta gizon zein emakumeetan eragina duten gaixotasunen artetik bosgarren lekuan dago. Gainera, mundu osoan zehar 70 milioi pertsonengan eragina duela uste da.

Entzumenaren gaitz hauen artean % 18a kalte perinatalen ondorioz gertatzen da (Mazurek et al., 2006) eta 1000 jaio berrien artetik batek pairatzen duela uste da. Baina kaltearen gogortasunaren edo goiztiartasunaren arabera, ume hauek arreta neonatal intentsibo bat behar izan ezkero, proportzio hau % 2-4ra igotzen da. Honegatik entzumen galeraren detekzio goiztiarra beharrezkoa da eta hau, diagnosi teknika espezifikoen bitartez soilik egin daiteke. Gainera, hobe da, jaio eta lehen hilabeteetan “screening” programa bereziak erabiltzen badira (Moreno-Aguirre et al., 2012).

2.1 BELARRIAREN MORFOLOGIA

Belarria organo sentsorial konplexua da, hiru zatitan banatua dagoena, kanpo-belarria, erdiko belarria eta barne-belarria eta entzumen-sistema (soinuaren pertzepzioaz arduratzen dena) eta bestibulu-sistema (oreka mantentzeaz arduratzen dena) partekatzen ditu (1.irudia).

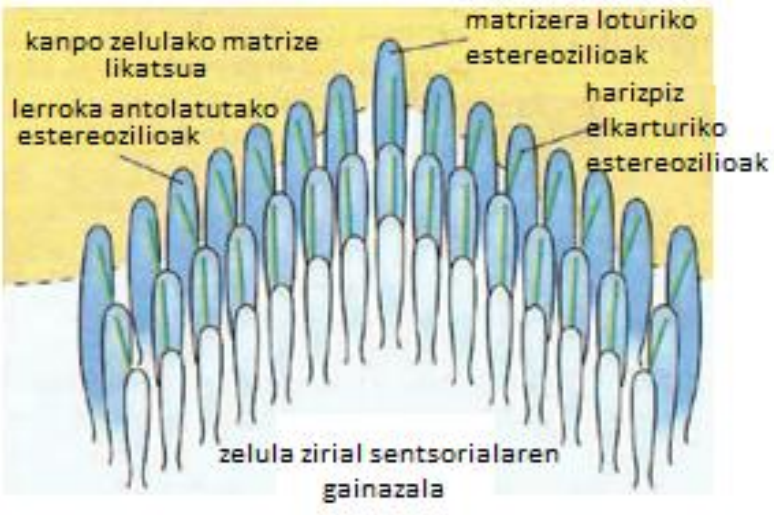
Kanpo-belarriko hodia (haragi kolerez 1.irudian) soinua tinpanoko mintzera garraiatzeaz arduratzen da, non erdiko belarria kokatua dagoen. Honek, soinu uhinak jasotzen ditu eta bibrazio mekaniko bihurtzen ditu, ondoren belarriko hezurtxoek anplifikatu, arroza kolorez 1.irudian (mailu, ingude eta estriboa), eta barne-belarrira (urdin kolorez) transmititzen dituelarik. Azken hau bi konpartimentu labirintikoz osatua dago, bata bestearen barnean: **hezur-labirintoa** eta **mintz-labirintoa** (hezur-labirintoaren barnean dagoena).



1.irudia: 1. Belarriaren zatien eskema: Kanpo-belarria (haragi kolorea), tinpanoko mintza eta hezurtxoak (arrosa) eta barne-belarria bere hezur-labirinto eta mintz-labirintoz osatua (urdin kolorez). 2.Barne-belarriko mintz-labirintoaren irudi zehaztuagoa (Ross & Pawlina, 2011).

a) **Hezur labirintoa**, hezur tenporalaren barnean konektatuak dauden hiru espazioz osatua dago (1.irudia, lauki laranja):

- Bestibulua, erdiko eremua, mintz-labirintoko sakulu eta utrikulua dituena.
- Kanal-erdizirkularrak, tutu formako eremuak beraien artean perpendikularki kokatuak eta bestibuluari elkartzen direnak.
- Koklea edo barraskiloa, bestibuluaren jarraipena dena kanal-erdizirkularren kontrako aldean.



2.irudia: Zelula sentsorial ziliatuen banaketa (Stevens & Lowe, 2006).

b) **<u>Mintz-labirintoa</u>** (2.irudia), koklea-labirintoz eta bestibulu-labirintoz banatua dago.

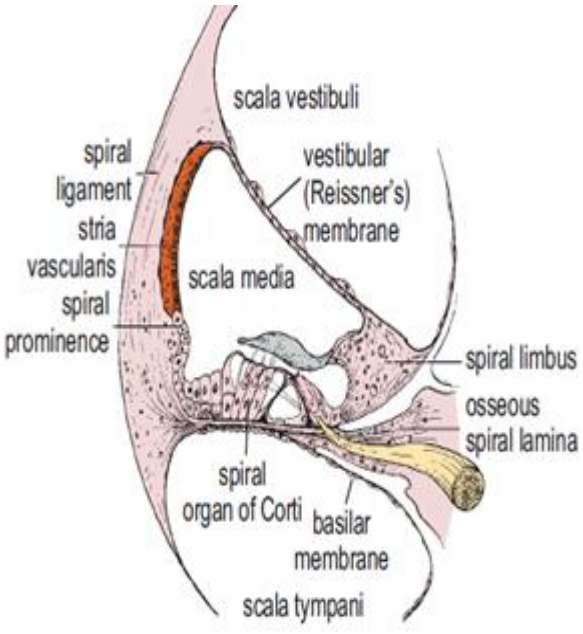
Mintz-labirintoaren eremu batzuetan 2 jatorri ezberdineko zelulak aurkitzen dira, entzumen-funtzioan eragina dutenak, zelula sentsoriala ziliatuak eta euskarri zelulak.

Zelula sentsorial ziliatuak (2.irudia) bestibulu eta koklea-labirintoko mekano-hartzaile epitelialak dira, energia mekanikoa energia elektriko bihurtzen dutenak gero nerbio bestibulo-koklearraren bitartez entzefalora garraiatua izateko. Zelula hauek, beraien gainazalean banatuta daude zilio-bala deritzonaren bitartez, estereo-zilio ilara (edo zilio sentsorial) batez osatua, atzeko eremuan kokatzen diren zilioak altuagoak izanik, 2.irudian ikus dezakegun bezala.

2.1.1 Soinuaren detekzioa barne-belarrian

3. irudian ikus dezakegun bezala, koklea-hodiak paralelo diren 3 konpartimentutan banatzen du koklea "eskala-media" deritzona, endolinfaz betea dagoena eta "bestibulu-eskala" eta "tinpano-eskala" perilinfaz beteak daudenak.
Bitarteko aldatsean (bestibulu-eskala), Kortiren organoa aurkitzen da. Bertan 3 zelula mota bereizi dira: sentitze-zelulak, euste-zelulak eta kanpo falange-zelulak (Young & Heath, 2000). Kokleako behe-pareta mintz basilarra da eta Kortiren organoa hemen eta mintz tektorioan finkatzen da (3.irudia).

Horrela, frekuentzia jakin bateko soinu batek mintz basilarraren (hodi koklearraren beheko pareta) desplazamendua eragiten du, desplazamendu maximoa zonalde oso estu batetan gertatzen delarik. Desplazamendu hau espezifikoa da soinu frekuentzia bakoitzarentzako, honela, frekuentzia altuko soinu batek bibrazio maximo bat eragiten du

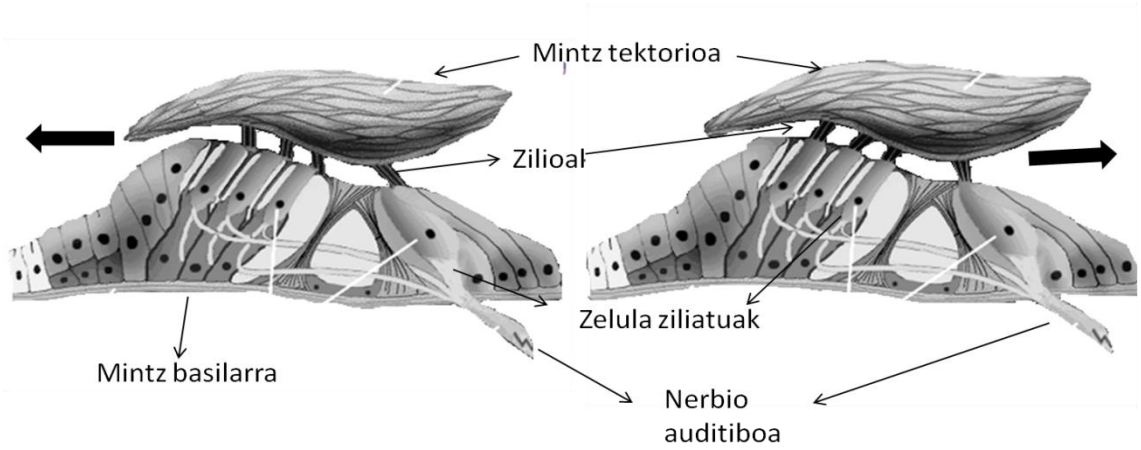


3.irudia: Koklearen irudi eskematikoa (Ross & Pawlina, 2011).

mintz basilarreko koklearen oinarrian eta alderantziz, frekuentzia baxuko soinuek desplazamendu maximoa koklearen erpinean eragiten dute (3.irudia). Fenomeno honi kokleako banaketa tonotopikoa deritzo.

2.1.2 Transdukzio neuronala

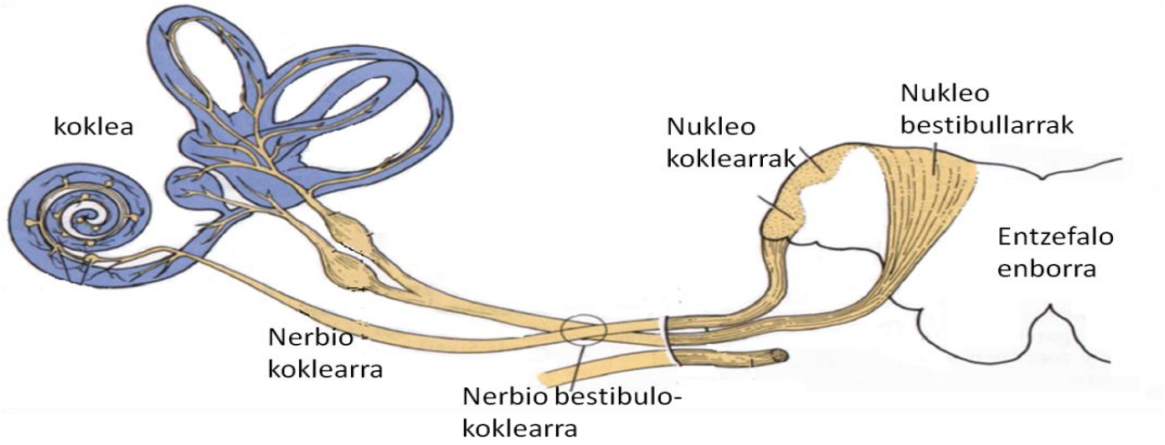
Transdukzio neuronala kokleako zelula ziliatuen esterozilioen (ile itxurazko luzapenak) mugimenduen bitartez hasten da (4.irudia). Zelula ziliatuak mintz basilarrari lotuta daude eta soinua heltzean dar-dar egiten dute. Mintz basilarrak heltze puntu ezberdinak dituenez, zitzailadura efektu bat gertatzen da bibrazioak barne belarriarekin talka egiten dutenean. Bibrazio hauek, transdukzio mekanoelektrikozko kanalak aktibatzen dituzte (MET), esterozilioen ertzetan kokatzen direnak eta honela mintz plasmatikoan potentzial elektrikoak sortzen dira, estimulu hau nerbio koklearraren bitartez entzefalora iristen delarik.



4. irudia: Transdukzio neuronalaren ilustrazioa zilioen mugimendua

Nerbio koklearra osatzen duten neuronak bipolarrak dira, hau da, 2 luzapen dituzten neuronak dira, axoia eta dendrita (giza neurona gehienak luzapen ugariz osatuak daude, axoi bakarra izanik baina dendrita ugari). Neurona bipolarrek gehienetan funtzio sentsoriala dute eta zentzuen bitartez gertatzen diren nerbioen seinalearen garraioan espezializaturik daude.

Kokleako neuronen soma Kortiren gongoilean kokatzen da eta axoiak entzefalo enborrean sartzen dira erraboileko nukleo koklearretan amaituz (5.irudia). Hemendik irteten diren nerbio zuntzak talamora arte heltzen dira eta hemendik lobulu tenporaleko entzumen kortexera.

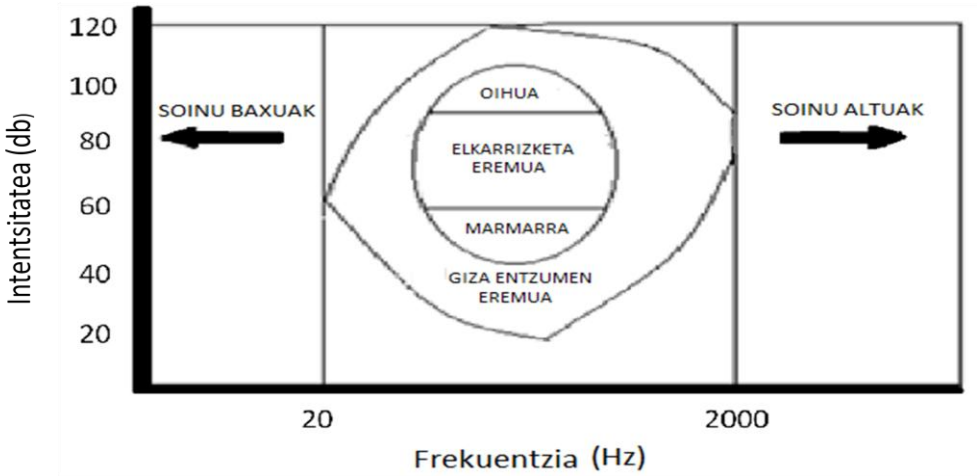


6.irudia: Giza entzumen-atariaren irudia. 16 y 18.000 Hz-ko soinu frekuentzien artean sentsibilitate-ataria ezberdina da. Pujol & Puelen (1999) aldatutako irudia.

2.2 SOINUAREN MONITORIZAZIOA

Soinu uhinen propietate fisikoak, **frekuentzia** edo tonua, ziklo segundoko neurtzen dena edo Hertz (HZ) eta **intentsitatea** dira. Azken honen neurketa unitatea anplitudea edo bolumena da eta dezibelioetan neurtzen da (dB).

Giza belarria 16 y 18.000 Hz-ko soinu frekuentziak hautemateko gai da eta ardatz itsurako forma dauka non beheko frekuentzien arabera giza entzumen-ataria neur daiteke eta goiko frekuentzien arabera minaren atariak zeintzuk diren azter daitezkeen (Payehuanca, 2004) (6.irudia).



5.irudia: Nerbio koklearrean dauden neuronen kokapen puntuen irudia. Soma Kortiren gongoilean dute kokatua eta axoiak entzefalo enborreko nukleo koklearretara iristen dira (Ross & Pawlina,, 2007).

Jaio berrietan entzumen gaitasuna neurtzeko metodo objektiboak beharrezkoak dira, sentsibilitate eta espezifikotasun altua dutenak. Gehien erabiltzen direnak entzumeneko potentzial ebokatuak (AEP) eta igorle otoakustikoa dira (Bogacz et al., 1985).

Entzumeneko potentzial ebokatuak aztertzeko garunean elektrodoak kokatzen dira. Baina animalien bitarteko azterketetan elektrodoak entzumen-bidearen zeharreko nukleoetan koka daitezke eta era honetan sentsibilitate handiago lor daiteke, distortsioak eta interferentziak gutxituz.

2.3 ENTZUMENEKO POTENTZIAL EBOKATUAK (AEP)

Estimulu akustiko baten ostean entzumen bidean zehar gertatzen diren boltaje aldaketak neurtzen dituzte. Potentzial hauek ondorengoen bitartez sortzen diren aktibitatea jasotzen dute:

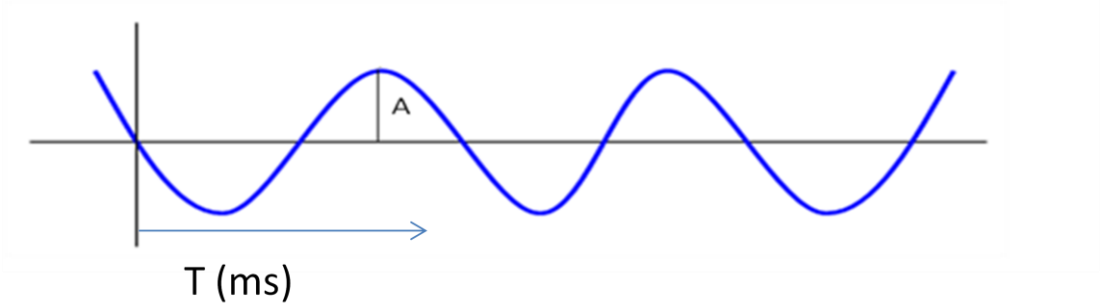
a) Neuronaren despolarizazioa, akzio potentzialen bitartez.

b) Kitzikatzaile edo inhibitzaile izan daitezkeen potentzial postsinaptikoengatik.

AEPk jasotzeko gaitasuna lau faktoreren araberakoa izango da (Plourde, 2006):

1- Estimuluko aktibatzen diren zelula kopurua
2- Aktibazioa sinkronizatzeko gaitasuna
3- Aktibazioaren geometria
4- Inguruko ehunek duten eroapen elektrikoaren araberakoa (hezur, muskulu, glia…)

Estimuluaren intentsitatearen aldaketak, anplitudearen aldaketak (oreka puntutik uhinaren banatze puntu maximoa (μV) eta latentziaren aldaketak dakartzate eta baita beraien morfologiaren aldaketa (7.irudia). 2 aldagai hauek intentsitatearekin duten erlazioa kontrakoa da, honela, anplitudea intentsitatearekiko zuzenki proportzionala da eta latentzia alderantzizko proportzionaltasuna dauka.



7.irudia: Uhin baten irudikapena non latentzia eta anplitudea irudikatzen diren.

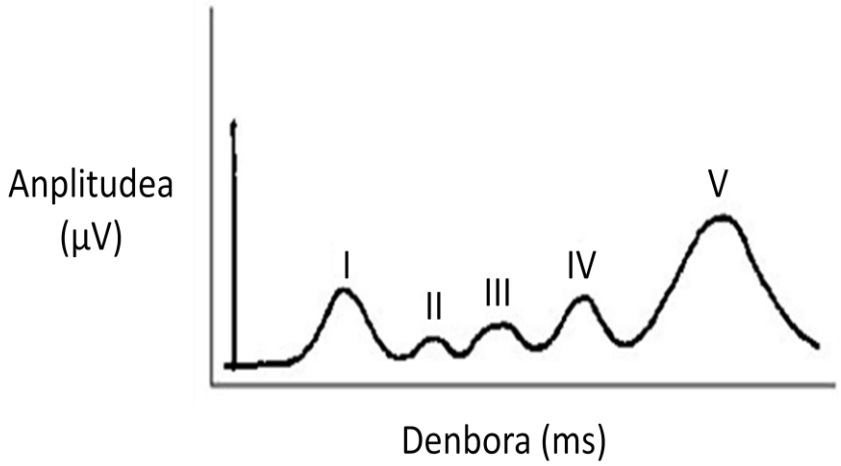
AEPen sailkapena latentziaren araberakoa da, hau da, estimulua sortzen den momentutik beraren erregistrora arte igaro den denbora. Lehen 10 ms-tan gertatzen direnak potentzial azkarrak dira, entzefalo enborrekoak hain zuzen ere, denbora honetan hona iristen direlako eta hemen kokatzen dira nukleo koklearrak. Nukleo hauek neurona ezberdinez osatutako taldeak dira ugaztunen burmuineko enborrean kokatzen direnak eta nerbio koklearraren soinuen informazioa jasotzen duten lehenak dira.

Grason-Staler konpainiak teknologia berri bat garatu du GSI Audera bitartez entzumen diagnostikoa egiteko. Programa honek funtzio koklear zein erretrokoklearra neurtzea baimentzen du AEP bitartez, test ezberdinak burutuz latentziaren araberakoak direnak:

- **Elektrokokleograma** (ECochG): Entzumen estimulu baten ondoren, koklearen erregistro elektrofisiologikoak egitea ahalbidetzen du.
- **Entzefalo enborreko entzumenaren erantzuna (ABR) eta ABR elektrikoa**: “clic” bati erantzuteko sortzen diren garuneko uhinak neurtzeko proba bat da.
- **Erdiko latentziako erantzuna (AMLR**): 10 eta 100 msko latentziak dituzten uhinak dira, talamoan eta kortexean sortzen direnak.
- **Latentzia luzeko erantzuna:** Erantzun kortikalak dira, 100 eta 500 ms bitartean neurtzen direnak.

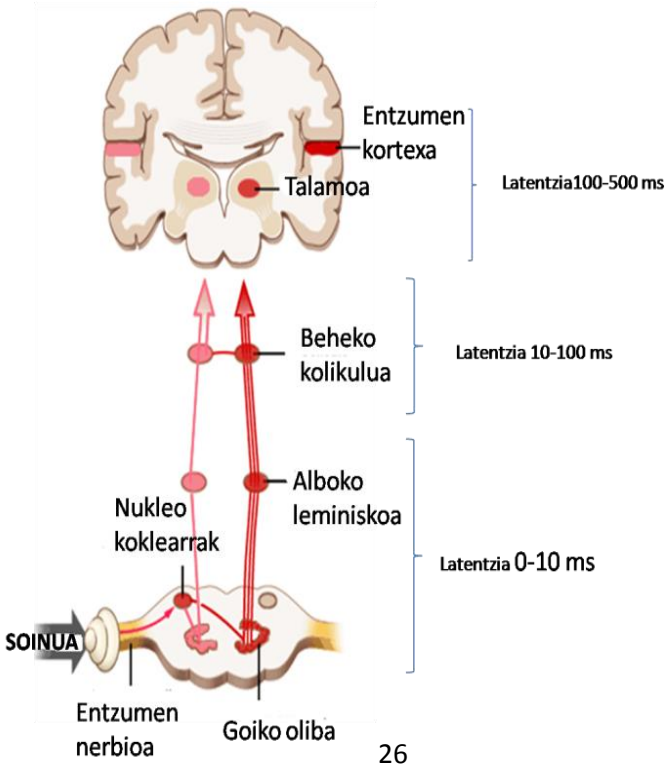
2.4 SOINUAREN INTERPRETAZIO ZEIN IRUDIKAPENA

AEPk neurtzerako orduan 5 tontor aurkitzen ditugu estimulu baten ondorioz egitura anatomiko ezberdinek ematen dituzten erantzunen tontorrak direnak, hain zuzen ere (8.irudia). Estimuluaren intentsitatea aldatu egin daiteke eta honela pertsonen entzumen-ataria zein den ezagutu dezakegu. Gortasunarekin erlazionaturiko arazoren bat diagnostikatu daiteke entzumen-atari hau 40dB-tik gora dagoenean.



8.irudia:Entzumeneko potentzial ebokatuen irudikapen grafikoa (Torrente et al., 2007).

AEP goiztiarrak (latentzia motzekoak (<10ms)) azterketa klinikoetan erabiltzen dira entzun-bideren aktibitatea neurtzeko:



9. irudia: Estimulu baten ondorioz sortzen diren zelulen erantzunen kokapen anatomikoa entzefalo enborretik kortexera arte (Pujol, 1999).

- Entzumen nerbioa = I tontorra
- Nukleo koklearrak = II tontorra
- Goi oliba =III tontorra
- Alboko lemniskoa = IV tontorra
- Behe kolikulua = V tontorra

9. irudian talamo eta entzumen kortexa (tenporala) irudikatzen dira, hemen hasten dira AEP erdiko uhinak eta uhin berantiarrak.
Potentzialak anplitude baxukoak dira (<uV) eta batezbesteko adierazgarria behar dute (1000 eta 2000 errepikapen artekoa) hondoko zaratetatik banatzeko.

2.5 ENTZEFALOPATIA HIPOXIKO-ISKEMIKOAREN KONTZEPTUA

Entzefalopatia hipoxiko iskemiko (EHI) oxigenoaren (O_2) ekarpenaren murriztea (hipoxia) eta entzefalora doan garuneko odol fluxuaren (CBF) murriztea (iskemia) gertatzen denean sortzen den sindromea da. Hipoxemia sistemiko baten ostean sor daiteke (asfixia, arnaste eskasa), O_2-aren garraioan aldaketaren bat gertatu delako (anemia zorrotz bat, karbono monoxidoaren bidezko intoxikazioa) edo garuneko odol fluxuaren murrizte orokor batengatik (gelditze kardiakoa) (Volpe, 1995; Caplan L., 2000).

Asfixia perinatalak aldaketa neurologikoak sortzen ditu, justu jaiotze ostean agertzen direnak, esnatzeko gaitasuna galtzen dutelarik, tonu muskularrean eta erantzun motorretan aldaketak gertatzen direlarik eta erreflexu aldaketak konbultsioekin edo gabe. Asfixiak sortzen duen kaltea larritasun, iraupen denbora eta sentikortasun iskemiko selektiboaren araberakoa da (Johnston, 1998; Hilario et al., 2006, Álvarez-Díaz et al., 2007). Azken honen arabera, zelula batzuk heriotza pairatuko dute eta beste batzuk ilunantz iskemiko batean geldituko dira. Hauetariko neurona asko hil egingo dira, nahiz eta garuneko fluxua berreskuratu. Fenomeno honi atzeratutako heriotza zelularra deritzo eta bere adierazpen zelularra nekrosi selektiboa eta apoptosia dira.

EHI larrian adi egote eta aldizkako arnasketan arazo larriak egoten dira, hipotonia orokor eta hipoaktibitate garrantzitsu batekin. %50ak krisi konbultsiboak izaten ditu lehen 6-12 orduetan. Hurrengo 12-24 orduetan adi egote honen hobekuntza nabaria izaten dute, baina krisiak aurrera jarraitzen du eta baita apneak eta hipotoniak ere. Jaio osteko 2 eta 3 egunean kontzientzia mailan (koma) arazo larrienak izaten dituzte eta heriotza pairatzeko arrisku gehiena ere. Denbora hau igaro ostean, bizirik irauten dutenak, hobekuntza izaten dute normalean (Arenillas et al., 2009).

Lesio hipoxiko iskemiko baten ostean gertatzen diren fenomeno biokimiko eta molekularrei buruz gaur egun ditugun ezagutzengatik, geroz eta aukera terapeutiko gehiago aztertu izan dira asfixia perinatalaren mina arintzeko nerbio sistema zentralean. Honegatik ikerketa basiko eta esperimental honen bitartez kalte honek eragiten dituen heriotza zelularrak murriztea eta ikerketa klinikoei laguntzea espero da.

2.6 ENTZEFALOPATIA HIPOXIKO ISKEMIKOAREN AZKEN BERRIAK

Hipoxia giza gorputzeko oxigenoaren murrizketa da, honen eragilea, anemia, asfixia, apnea… izan daitekeelarik (James & Cherian, 2010). Garai perinatalean, garunak oxigeno gabezia izan dezake bi mekanismo patogenikoren bitartez: hipoxemia, odoleko oxigeno kontzentrazioaren murrizketa, eta iskemia, garunera doan odolaren kontzentrazioaren gutxitzea, eta ondorioz, bi kasuetan zeluletara iristen den oxigeno kontzentrazioa baxuagoa da. Honegatik zelulak beharrekoa duen energia sortzeko arazoak sortzen dira.

Giza nerbio-sistema zentralaren %80ko garapena ernaltze garaiko azken bi hiruhilekotan eta jaiotze osteko lehen hilabeteetan gertatzen da eta momentu honetan odol emariaren murrizketarekiko oso sentikorra da (du-Plessis & Volpe, 2002; Soehle et al., 2003). Honela, garai perinatalean gertatzen den hipoxia iskemiak sorturiko garunaren kaltea defizit neurologiko larrien eragile nagusienetakoa da (Volpe, 2001; Perlman, 2004). Bizirik jaiotzen diren umeen %0,5-

1ak hipoxia iskemia pairatzen du eta nahiz eta gaur egun intzidentzia asko jaitsi den, portzentaje honen heriotza tasa %27-50 ingurukoa da.

Asfixia (hipoxia) eta odol-emariaren murriztearen (iskemia) arteko konbinazioak, agerikoak diren kalte fisiologikoez gain, zelula mailan aldaketak sortzen ditu, batez ere neuronetan, izan ere, oxigeno gabeziarekiko oso sentikorrak dira eta hipoxia oso larria badin bada, beste zelula talde batzuk ere kaltetuak ager daitezke, hala nola, gliako zelulak besteak beste (Hilario et al., 2005; Goñi-de-Cerio et al., 2007). Egoera honetan, garatzen ari diren oligodendrozito zein astrozitoak sentikortasun maila altuagoa dute gertatzen den exitotoxizitatera eta sortzen diren erradikal askeei, honegatik heriotza pairatzeko joera handiagoa dute (Inder & Volpe, 2000; Alvarez et al., 2007).

2.7 ENTZUMENA ETA KALTE HIPOXIKO ISKEMIKOAREN ARTEKO AZKEN BERRIAK

Ikerketa esperimental eta klinikoek baieztatu dute hipoxia iskemiak entzumen gaitasunaren galera dakarrela. Lehenago esan dugun bezala, lengoaiaren gaitasuna lortzeko entzumena beharrezkoa da gizakian eta komunikatzeko gaitasun hau garatzeko jaiotzetik entzun-bide egoki eta osoa izatea beharrezkoa da. Hau honela ez bada, entzumeneko gaitzak lengoaian atzerapen garrantzitsuak izan ditzakete pertsonen garapen psikikoetan eragin handia izanez.

Entzun-bidearen heltzea 18 hilabeterekin bukatzen da eta nerbio-sistema zentralaren mielinizazioarekin erlazionaturik dago (Hepper & Shadidullah, 1994). Entzun-bidearen garapen eta mielinizazio normala kaltetua gerta daiteke hainbat gaitzengatik, hala nola, birus bidezko infekzio, hiperbilirrubinemia, meningitisa, fetuaren sufritzea eta entzefalopatia hipoxiko iskemikoa. Adibide gisa, kernikterusean, entzun-bidea kaltetua dago nukleo koklearretan eta hipoxia larria denean, atzerapen metal eta desordena muskularrarekin lotzen da (Lefebvre, et al., 2002; Cao et al., 2010). Gaitzaren zergatia geroz eta lehenago aurkitu orduan eta

errazago berreskuratu ahalko da lengoaiaren gaitasuna, emaitza terapeutiko hobeagoekin (Lefebvre et al., 2002; Gallardo & Vera, 2003).

2.7.1 Entzumen atariaren aldaketak

Entzefalo enborreko entzumenaren erantzunen neurketa (ABR) oso diagnosi método garrantzitsua da ezgaitasunak aztertzeko eta oso erabilia da entzumen ezgaitasunak aztertzeko asfixia perinatal baten ostean. Aurretik egindako hainbat ikerketetan ikusi da ABRak odoleko oxigeno kontzentrazioaren murrizketaren ostean sortzen diren lesio koklear eta neurologikoekiko oso sentikorrak direla. Izan ere, murrizketa hauek Kortiren organoko zelula neurosentsorialetan kalteak sortzen dituzte edo neuronen galera entzefalo enborreko nukleoetan, hau da, nukleo koklearretan edo behe kolikuloetan (IC) (Jiang et al., 2000; Wilkinson & Jiang, 2006). Neuronen kalte honek nerbioaren garraioan eragina izan dezake, entzumeneko ezgaitasunak sortuz (Misra et al., 1997).

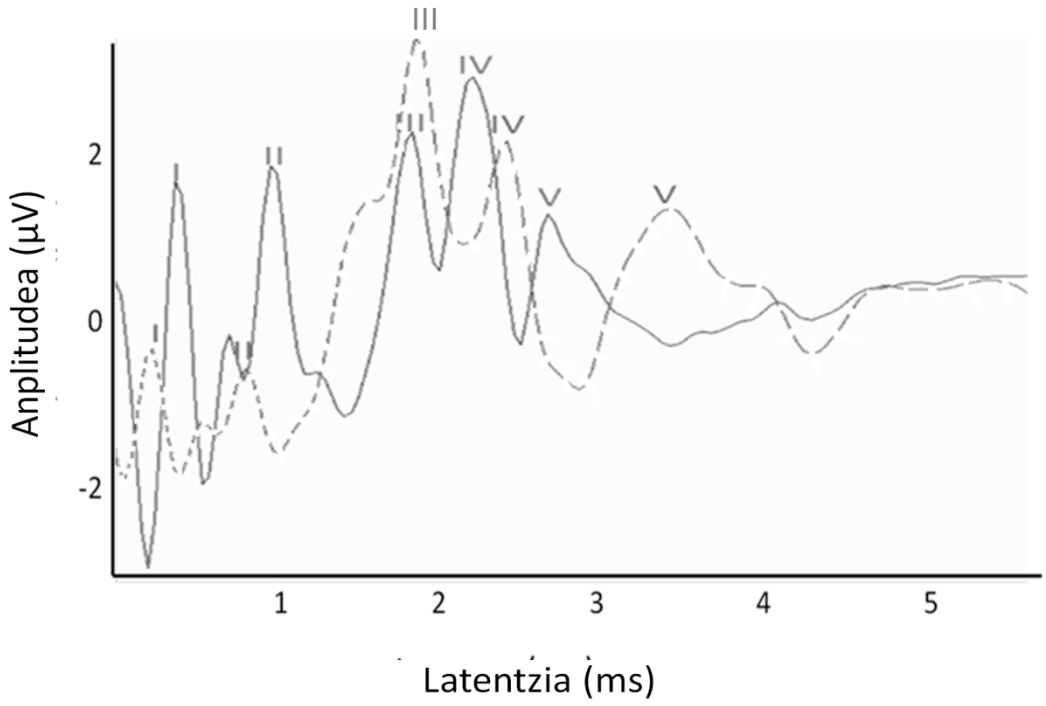
ABRen neurketa metodo sentikor eta fidagarrienetako bat da entzumeneko ezgaitasunak neurtzeko (Tomimatsu et al., 2003; Smit et l., 2013). Espezifikoki, gehien erabiltzen diren aldagaiak I eta V uhinen latentziak dira eta baita I eta V uhinen bitartea, entzefalo enborreko eroapen denbora bezala ezagutzen dena eta neuronen eroapen, funtzio sinaptiko eta mielinizazioarekin zuzenki erlazionatuta dagoena (Jiang et al., 2008).

Entzefalo enborrean gertatzen diren entzumenaren erantzunaren aldaketak eta neuronen heltzea urteetan zehar ikertua izan da. Animaliekin egindako ikerketan, entzefalo enborreko entzumen erantzun egokiak izateko anatomia egokia izatea beharrezkoa dela ikusi da, hala nola, neurona taldea, bere axoiarekin, sinapsi egokiarekin eta amaierako hartzailearekin non ABR bakoitza bertara iristen den. Hauetariko zatiren batetan geldiuneren bat gertatu ezkero, anplitude eta latentzietan aldaketak gerta daitezke (Jiang et al., 2006).

I uhinaren latentzia handitzeak, entzumen-sistema periferikoko egituraren batean aldaketak daudela esan nahi du, bitartean, entzefalo enborreko barne-sisteman kalteak egoten direnean, erantzun hauen desagerpena gertatzen da edo latentzien luzatzea, hau da uhinen latentziak atzeratuak egotea (Simpson et al., 1985). I eta V uhinen bitarteko latentzia handitua egoteak berriz, gaitz neurologiko baten adierazle da (Wilkinson & Jiang, 2006).

Asfixia perinatal baten ostean, ABRak entzumen atarian aldaketak daudela erakusten dute, uhinen latentziak eta uhinen bitarteko latentziak handituz. Honen arabera, entzefalo enborreko eroapen denboran aldaketa esanguratsuak daude, uhinen anplitudearen murrizketarekin eta I eta V uhinen anplitude arteko ratioaren aldaketarekin (Hecox & Cone, 1981; Wilinson & Jiang, 2006; Jiang et al., 2009) (Fig. 10).

Entzumeneko estimulu baten ostean entzefalo enborreko egituren erantzunen ikerketan, I-III eta III-V uhinen bitarteko latentzien handitzea ikusi da (Tomimatsu et al., 2003; Jiang et al., 2009). HI baten ostean, batez ere latentzia eta anplitudearekin erlazionaturiko aldaketak ikusi dira eta entzumen-sistemako erdiko eremua (III-V uhinen tartea) kaltetuagoa agertzen da periferiako eremua baino (I-III uhinen tartea) (Jiang et al., 2004).

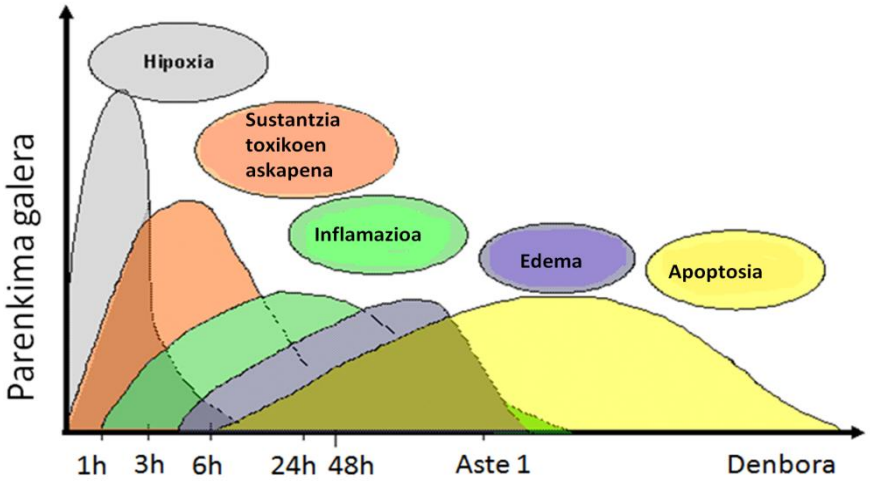


10.irudia: 7 eguneko Sprague-Dawley arrazako entzun-bidearen estimulu baten osteko egituren erantzunen irudi adierazgarria. Lerro zuzenak kaltetu gabeko arratoi baten entzun-bidea adierazten du eta marrazko lerroak HI pairatu duen arratoi batena.

2.8 LESIO HIPOXIKO ISKEMIKOAREN MEKANISMO FISIOPATOLOGIKOA

Odol fluxu eta oxigenoaren gutxitzeak metabolismoko eta nerbio ehunen funtziorako beharrezkoak diren 2 substratu energetiko nagusienak sortzea galarazten du: oxigenoa eta glukosa. Honengatik 2 aldaketa mota gertatzen dira, bat nerbio inpultsuaren transmisioaren aldaketa dakarrena eta bestea neuronen eta glia zelulen aldaketa metabolikoa dakarrena (Pascual et al., 2000).

Kalte hipoxiko iskemikoaren ondoren sortzen diren gertaerak kapitulu komun batzuetan banatu daitezke (11. Irudia): kalte energetikoa, homeostasi ionikoaren aldaketa, exzitotoxikotasuna eta erradikal aske eta oxido nitrikoak eragindako kaltea (Edwards et al., 1997; Biagas, 1999; Dammann & Leviton, 1999; Edwards & Azzopardi, 2000), garuneko kortexeko eremu parasagital eta garun basalak eremu kaltetuenak izanik.



11. irudia: Garun iskemia baten seinale kliniko eta patologikoen garapena denboran zehar (Castillo & Rodríguez, 2004).

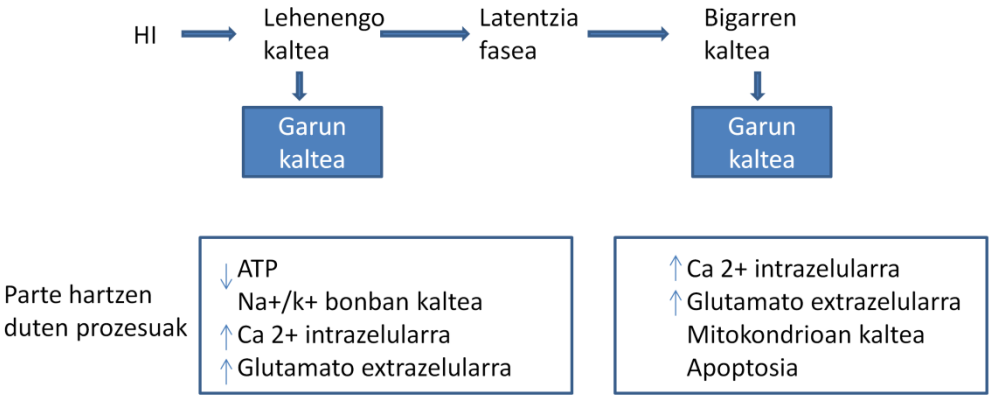
2.8.1 Kalte energetikoa

Mintzen integritate eta potentzia mantentzeko beharrezkoa da homeostasi egokia edukitzea eta honetarako ATP gisako energia beharrezkoa da. Energia hau glukosaren metabolismo aerobioren bitartez lortzen da, baina oxigenoaren kontzentrazioa gutxitzen denean, energia altuko fosfatoen desagerpen azkarra gertatzen da eta ehun mailan laktato kantitatea handitzen da (Cater et al., 2003). Honetaz gain, kalte energetiko honengatik, hipoxia iskemiak aminoazido exitotoxikoen kantitatea handitzen du, glutamatoaren adibidez. Na^+ aren gutxitzearen eraginez, K-ATPasa aktibitatea eta homeostasi honen galeraren eraginez gradiente ionikoa mantentzeko ezgaitasun zelularraren eraginez, lehen heriotza gerta daiteke. Gainera, N-methyl-D-aspartate (NMDA)ren gehiegizko estimuluak, glutamatoaren hartzaile bat, neuronen kalteak eta zelulen degenerazioa eragin dezake.

Aurrerago esan dugun bezala, garuneko energia iturri nagusiena glukosa da, hau garun kapilaretatik neuronen zitoplasmararte garraiatzen da. Hemen, glukosa molekulak bide glukolitikoan sartzen dira pirubatoaren, acetil-CoA eta azkenik NADH sortzeko, elektroien iturri nagusiena. Elektroiak, zitokromoaren entzimaren

bitartez, mitokondriaren mintz barruan sartzen dira ATPa sortuz (Volpe, 2012; McLean & Ferriero, 2004).

Asfixia perinatal bat gertatzen denean, bi motako kalte energetiko gertatzen dira: lehenengoa eta bigarrena (12.irudia)



12.irudia: Hipoxia iskemia baten osteko kalte energetikoaren azalpena. Flores-Compadre et al., (2013) aldatutako irudia.

Lehen kasuan, metabolismoa anaerobioki gertatzen da eta pirubato eta laktatoa metabolizatzen da, neuronen zitoplasman pilatuz eta pHa jaitsiz. Momentu honetan kortexeko aktibitate elektrikoa galtzen da eta garun (talloko) funtzioak mantentzen dira, baina ATP menpeko Na^+/K^+ ponpa kaltetua dagoenez, edemak agertzen hasten dira. Galera hau denboraren eta larritasunaren menpekoa da (James & Cherian, 2010; Ferriero, 2004; Fatemi et al., 2009).

Bigarren kalte energetikoa lesioa gertatu eta 24 ordutara gertatzen da. ATP eta fosfokreatina kantitateak murrizten dira mitokondrio mailako fosforilazio oxidatzailearen aldaketaren adierazle izanik. Elektroiak zitokromo entzimako katean biltzen dira eta laktatoaren kontzentrazio intrazelularrak handitzen dira, pilatze hau handiagoa izanik talamoan eta nukleo basaletan. Bigarren kalte energetiko hau aktibitate parosistikoaren paraleloa da eta baita arrail presinaptikoko glutamato eta aspartatoaren gehiegizko askapenaren ondorioz gertatzen diren konbultsioen paraleloa. Aktibitate parosistiko hau elementu zitotoxiko batzuen ondorio da (Esteve et al., 1999):

→ **Zelula barneko kaltzioaren handiagotzea**: Zelula barnera kaltzioaren sarrera, neuronako mintzeko glutamato bidezko boltaje menpeko kanalen bitartez. Kaltzioa erretikulu endoplasmatiko eta mitokondrioetatik askatzen da eta fosfolipasa, proteasa eta nukleasak aktibatzen ditu kalte nuklear eta zelularra eraginez. Kaltzioak gainera hainbat entzimen aktibitate pizten du, hala nola, xantina oxidasa eta oxido nitiko sintetasena, erradikal askeen askapena dakartenak.

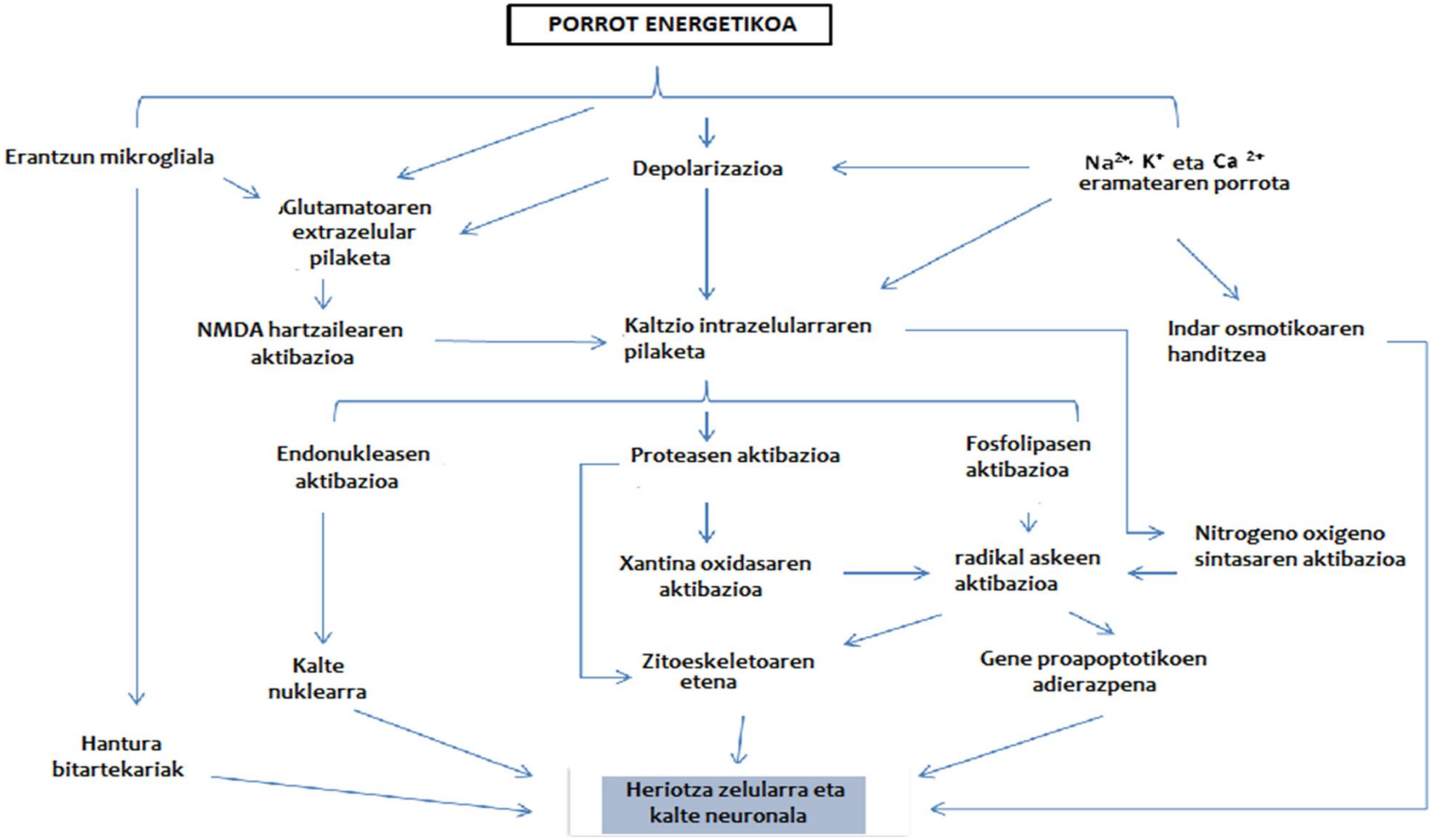
→**Zelula kanpoko gehiegizko glutamato kontzentrazioa**: Zelula kanpoko glutamatoaren igotzeak NMDA eta AMPA hartzaileak aktibatzen ditu eta hauek era berean Na^+ y $Ca2^+$ barneratzen ditu zelula barnera. Azken hauek hanturaren sortzaileak dira eta zelularen ondorengo apurketarena (Flores-Compadre et al., 2013).

→**Erradikal askeen askatzea**: Erradikal askeen pilatzeak zitokromoaren katean, superoxido ioien sortzea eragiten du. Oxido nitrikoa askatzen da (NO) mikrogliaren, astrozitoen eta neutrofiloen aktibazioaren eraginez eta NO gehitze honek garuneko basodilatazioa eragiten du.

→**Hanturaren mekanismoak**: Interleukina 1β, 6 eta TNF zitokina hanturaren askatzearen eraginez gertatzen da (13.irudia).

Kaltea sortzen duten mekanismo zelularren ostean, hipoxia iskemiak bi heriotza zelular mota bultzatzen ditu, morfologikoki oso ezberdinak direnak: nekrosia, edema zelular bat, mintz zelularraren haustura eta hantura erreakzio baten ondorioz sortzen dena eta apoptosia edo heriotza zelular programatua, uzkurtutako zelula, kondentsatutako kromatina eta zatitutako DNA (Inder & Volpe, 2000; Ohyu et al., 2000). Heriotza zelularra bat-batean hasten da eta egun edo asteetan zehar luzatzen da. Berriki egindako hainbat ikerketek uste dute apoptosiak paper garrantzitsua betetzen duela kalte hipoxiko iskemiko baten ostean jaioberrien garunean (Hu et al., 2000; Yakovlev & Faden, 2004). Umetoki barneko kaltearen ondoren hiltzen diren jaioberriek apoptosi morfologia duten zelula ugari aurkezten dituzte.

13.irudia: Hipoxia iskemia baten ostean gertatzen den porrot energetikoaren eraginak.



2.8.2 Kaltzioaren homeostasiaren galera

Aurrerago esan dugun bezala, gertakizun hipoxiko iskemiko baten ostean kaltzio askearen eta eskualde sinaptosomikoaren sarrera, NOren bidez, gertatzen da nukleoaren barnealdera (Mishra et al., 2002). $[Ca^{2+}]_i$-ren handiagotzea boltaje edo agonista bitartez erregulaturiko hartzaileei, hala nola, NMDA glutamato hartzaileak, loturiko kanalen bitartezko kaltzioaren sarreraren bidez gertatzen da (Mills, 1996; Peinado et al., 2000).

Kaltzioaren aldaketa eta honen mugimendua kanpo zonaldetik zelularen barne zonaldera, hiru aldagairen menpekoa da; Zelularen mintzera lotuta dauden kanale kopurua, mintzaren bitartezko kaltzioaren garraioa eta bere bahitzea mitokondrio eta erretikulu endoplasmatikoarengatik (Irvin, 1986; Denton & McCormack, 1990; Paschen, 1996). Baina kaltzioaren sarrera gelditzen ez bada, aipaturiko organoen bitarteko bere hartzea eta gordetzea gutxi izatera hel daiteke. Gainera glutamato eta NMDA hartzaileen arteko loturak kaltzio kanala irekitzeaz gain, mintzeko fosfolipasa A_2 eta C aktibatzen ditu, fosfoinositol bifosfatoa fosfoinositol trifosfato (IP3) eta diazilglizerol bihurtzen duena. IP3ak, erretikulu endoplasmatikoko kaltzio kanalak zabaltzen ditu eta kaltzioaren kontzentrazio handitzeak, kaltzioa gordetzeko kaltzio kanalak zabaltzen ditu, honela sorgin-gurpil bat gertatzen da non ATP menpeko Ca^{2+} ponpa ez eraginkor gertatzen den (Paschen & Doutheil, 1999).

Mitokondrioak ere paper garrantzitsua betetzen du zitosoleko Ca^{2+} altua mantentzen (Rustin et al., 2000). Egoera normalean, zitosoleko kaltzioa mitokondrioan gordeta aurkitzen dugu eta azido trikarboxilikoaren zikloko hainbat deshidrogenasa aktibatzeko erabiltzen da. Gainera, Kaltzioa mitokondriotik irteteak, Na^+ sartzea baimentzen du zitoplasmatik, mitokondrio barnera, ondoren berriz ere zitoplasmara askatzean H^+ aren sarrera baimenduz mitokondria barnera. Era honetan, H^+ gradiente bat sortzen da fosforilazio oxidatzailea gertatzeko beharrezkoa dena. Baina sistema guzti honen desoreka gerta daiteke mitokondrioaren mintzaren potentzialaren galeraren ondorioz (Sugrue et al., 1999). Zelula kanpoko Ca^{2+}-ren pilatzeak mitokondrioaren iragazkortasuna eta mintzaren potentziala kaltetu dezake, izan ere, honen eraginez,

mitokondrioko mintzean poroak (MPTP: Mitochondrial Permeability Transition Pore) zabaltzen dira gradiente elektromekanikoaren galera (Cassarino & Bennen, 1999; Puka-Sundvall et al, 2000).

2.8.3 Exzitotoxikotasuna

Exitotoxikotasunak, aminoazido hartzaileen aktibazioaren ondorioz gertatzen den neuronen heriotzari egiten dio erreferentzia. Garuneko eta muineko neurogarraiatzaile kitzikagarri nagusiena azido glutamikoa da (McGeer, 1989; Thomas, 1995). Glutamato kontzentrazio altuak, minutu bat baino denbora gehiagoz mantentzen bada, heriotza zelularra dakar (Sattler et al., 2000).

Nerbio sistema zentralaren barruan, glutamatoak funtzio ugari ditu, neurogarraiatzaile kitzikagarria izateaz gain (Michaelis, 1998):

1. Ekintza azkarreko neurogarraiatzaile kitzikatzatzailea izatea.
2. Neuronen kitzitagarritasunaren (plastikotasun sinaptikoan) epe luze eta erdiko aldaketen eragile eta mantentzean parte hartzen du.
3. Neuronen migrazioaren eragile diren prozesuetan parte hartzen du
4. Axoien heltze eta sinapsi formazioa modulatzen du
5. Talde neuronal ezberdinen biziraupena sustatzen du.
6. Proteinen aktibitate metaboliko, adierazpen geniko eta sintesian aldaketak eragiten ditu.
7. Endekapeneko prozesuetan paper garrantzitsua dauka.

Glutamatoak bere hartzaile ezberdinen bitartez eragiten du. Hauek molekularki eta ezaugarri elektrofisiologiko zein farmakologikoz desberdindu daitezke (Michaelis, 1998; Ozawa, 1998). Orokorki, glutamato hartzaileak ondorengo sailkapena daukate: hartzaile ionotropikoak, AMPA, kainatoa edo N-Metil-D-Aspartatoa (NMDA) bezalakoak, kanal ionikoz osatuak daudenak eta hartzaile metabotropikoak, LPA-4 eta ACPD bezalakoak, G proteinei loturik aurkitzen direnak (Farooqui & Horrocks, 1991). Bi kasuetan, aminoazidoak amaiera sinaptikotik kentzen dira berriz hartze mekanismo

baten bitartez. Mekanismo hau neurona zelulen %80k eta glia zelulen %20ak burutzen dute (Storm-Mathisen e Iversen, 1979).

Egoera ezberdinak daude hartzaile kitzikatzaileak gehiegi estimulatzen, neurogarraiatzaileka askatu eta ondorengo exzitotoxizitatea eragiten dutenak. Aminoazidoen despolarizazio eta askatzea eragiten duten kausen artean, erreserba energetioen gutxitzea, homeostasi ionikoaren galera, lesio traumatikoak, zahartzea, endekapeneko gaixotasunak eta prozesu iskemikoak aurki ditzakegu (Olney, 1994; Peinado et al., 2000).

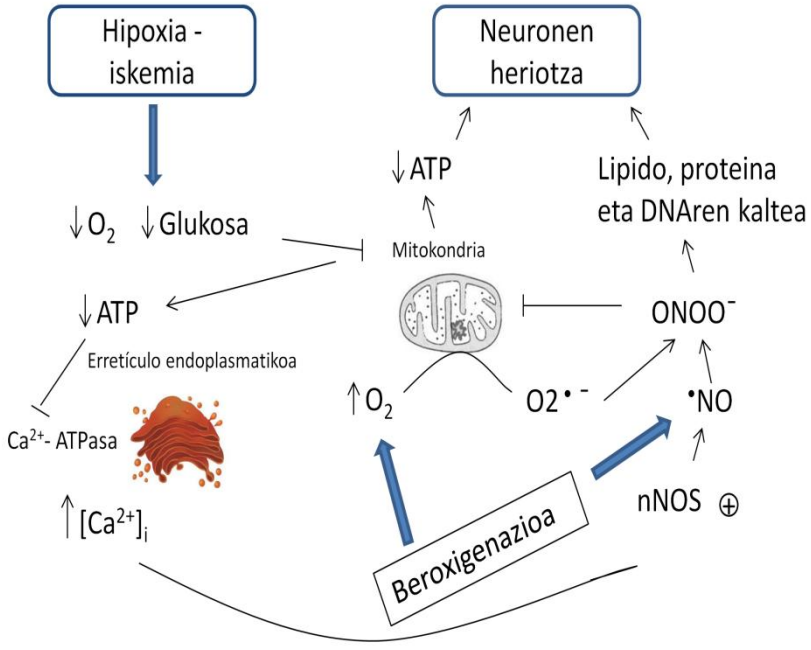
Glutamato hartzaileen gehiegizko aktibitateak, glutamatoaren gehiegizko askatzea eragiten du eta ondorioz mintz postsinaptikoko hartzaileen aktibitatea handitzea. Hauen aktibazioak kanale ionikoak zabaltzea eragiten du eta sarrera zein irteera fluxuak zabaltzen dira. Honek, barne zelulako kontzentrazio ionikoaren aldaketa Dakar: gehien ezagutzen diren fluxuak, sodio, kloro zein kaltzioarenak dira. Gehiegizko kaltzio ioiak, barne zelulako seinalizazioaren menpeko den kaltzioa aktibatzen du eta honek era berean bigarren mezulariak aktibatzen ditu, normalean heriotza zelularra dakartenak (Sattler & Tymianski., 2000).

2.8.4 Estres oxidatzailea

NMDA eta exitotoxikotasun bidezko heriotza zelularreko hartzaileen arteko konexioak oxido nitrico sintasa (nNOS) aktibatzen du eta ondorioz oxido nitrikoa eratzea. Honek, mitokondrioak erreaktibo diren espezie gehiago (ROS) sortzea eragiten du , kalte oxidatzailea eraginez eta zelulen heriotza (Dugan & Choi, 1994; Vannucci & Hagberg, 2004).Erradikal askeak, toxikotasun maila altuagoa eragiten dute ioi superoxido (O-), hidroxilo (OH^-), ur oxigenatua (H_2O_2), oxido nitrikoa (NO) eta peroxinitritoaren ($ONOO^-$) bitartez (Herce-Pagliai et al., 1998; Hogg, 1998; Suzuki et al., 2002).Oxigeno espezie erreaktibo hauek zelulak kaltetzen dituzte, lipidoak (mintzetako azido poli asegabeak), proteinak (entzimak batez ere) edo azido nukleikoak oxidatuz (Floyd & Carney, 1992; Wei, 1998).Aminoazido kitzikatzaileak (Pérez-Velázquez et al., 1997) berriz ere hartzea

galarazi dezake eta kaspasa edo endonukleasa bezalako entzima proteolitikoak aktibatzea (Sapolsky 1992; Puka-Sundvall et al., 2000; Fukuda et al., 2003).

Oxido nitrikoa adibidez, nerbio sistema zentraleko mezulari fisiologikoa da eta oxido nitriko sintasaren (NOS) erreakzio katalitikoaren bidez sintetizatzen da. Entzima honen aktibazioak NO, L-citrulina eta L-arginina askatzen ditu transdukzio bidean parte hartzen dutenak, barne zelulako cGMP kontzentrazioa handituz. Hipokanpoko neuronen zirkuitu maila batzuetan, NOaren askapenak epe luzeko potentziazioa eragiten du eta zerebeloan berriz, epe luzeko depresioa (Fedele & Raiteri, 1999).Baina gehiegizko NOak galera energetikoa, aminoazido kitzikatzaileen askatzea eta beste erradikal askeekin erreakzionatzeko gaitasuna handitzea eragiten du (Gross & Wolin, 1995; Bolaños et al., 1998; Bolaños & Almeida, 1999). Honela, NO eta superoxido ioiaren sarrerak, nitrogenoaren espezie erreaktiboak sortzen dituzte, hala nola, peroxinitritoa ($ONOO^-$), azken hau erreakzio oso toxiko izanik (Pryor et al., 2002) (14.irudia).



14. irudia: Oxido nitrikoaren toxikotasuna hipoxia iskemia baten ostean (Bolaños, 1999).

Honegatik estres oxidatzailea apoptosiaren kausa nagusiena da eraso hipoxia iskemiko batean (Lin et al., 2003).

Antioxidatzaile gaitasun baxua eta burmuin kontzentrazio altua dela eta, garatu gabeko burmuina oso sentikorra da kate oxidatzailea. Burmuineko antioxidatzaile endogenoak, superoxido dismutasa (SOD), Cu bezala aurkitzen dena, ZN-SOD (SOD1) zitoplasman dagoena eta Mn-SOD (SOD2) mitokondrioan dagoena dira. Entzima hauek erradikal askeak H_2O_2 bilakatzen dituzte eta hau katalasa edo glutation peroxidasa bitartez, detoxifikatu daiteke H_2O bezala ezabatua izateko (Yu et al., 1994). Karbonil, peroxido edo epoxido taldeak dituzten metabolitoen aktibazio eza eragiten duen beste entzima bat glutation S-transferasa (GST) da, hauek erradikal askeen oxidazioaren eraginez sortzen direlarik (Hayes & Pulford, 1995). Erregenerazio sistema hauek, babespen antioxidatzailea manten dezaten, zelularen energiaren menpekoak dira, honegatik, hauek kaltetuak gertatzen badira edo hauen gabezia baldin badago, eraso hipoxiko iskemikoan gertatzen den bezala, garunak kalte oxidatzailea pairatzen du makromolekuletan eta ondorioz heriotza zelularra.

2.8.5 Geneen eta transkripzio faktoreen aktibazioa

Azken urteetan zehar adierazpen goiztiarreko geneak asko aztertu dira kodifikatzen dituzten proteinak transkripzio faktoreak direlako. Hipoxia iskemiak estresarekin erlazionaturiko hainbat geneen sintesia handitzen du, “heat shock proteins” bezala ere ezagutzen direnak. Proteina hauek, nahiz eta bere funtzioa zein den ez den oso ongi ezagutzen, kaltearekiko erresistentziaren handitzearekin erlazionatzen dira zelula mota askotan (Tang et al., 1997).

Hauetariko proteina batzuk kaltearen ostean adierazpen maila altua dute, adibidez, c-fos, c-jun eta proteo-onkogeneak neuronetan. Gene hauek kodifikatzen dituzten proteinak (FOS eta Jun) erantzun biologikoetan beharrezkoak diren gene batzuk erregulatzen dituztela uste da (Chen & Liu, 1996).

Transkripzio faktore ezagunetariko bat hipoxiak eragiten duen 1 faktorea da (HIF1), oxigeno eta erredox potentzialaren homeostasia erregulatzen dutena eta oxigeno

faltak eta oxigeno erreaktiboen bidezko estresarekiko adaptazioa errazten dutelarik (Kanngiesser et al., 2014). Hipoxia iskemia ondorengo HIF1aren aktibazioak inguruneko aldaketei egokitzea erraztea ahalbidetzen du, hala nola, intsulina menpekoa ez den glukosa garraiatzailearen absortzioaren handitzea (GLUT1) eta entzima glikolitiko, eritropoietina eta garapen endotelial baskular (VEGF) faktoreen adierazpena handitzea (Huang et al., 2000; Matsuda et al., 2005).

Kaltearen ostean berehala adierazten den beste gene bat heat shock factor 1 (HSF1) da, heat shock eragileren adierazpena (HSP70) positiboki erregulatzen duena. Azken honen aktibazioa garun kaltearen babesle bezala erlazionatu da (Kim et al., 2001).

Aipaturikoez gain, hainbat gene gehiago daude zeintzuen aktibazioa hendekatze prozesuekin lotu diren. Honegatik, garrantzitsua da kaltearen ostean berehala adierazten diren geneak zeintzuk diren ezagutzea eta beraien funtzioa zein de, honela, tratamendu neurobabesle bezala ikertu ahal izango dira.

2.8.6 Apoptosi eta nekrosi bidezko neuronen heriotzaren base molekular eta zelularra

Heriotza zelularra lesio zelularraren azken emaitza da, anatomia patologikoko gertaera garrantzitsuena, zelula mota guztiak kaltetzen dituenak eta hipoxia iskemiaren ondorio nagusiena dena. 2 era ezberdinetan gerta daiteke: ohikoena, nekrosi bidezko heriotza da, porrot energetiko zorrotz baten eraginez gertatzen dena, morfología zelularraren galerarekin eta azkenik hantura prozesu eta lisiarekin. Beste alde batetik, apoptosi bidezko heriotza ere gerta daiteke, kasu honetan energia bidezko zelula barneko mekanismoak aktibatzen dira eta zelularen degradazio erregulatua gertatzen da, beranduago zelula fagozitikoen bitartez ezabatuko dena hantura erreakziorik gertatu gabe (Arango-Davila et al., 2004).

Apoptosi bidezko heriotza zelularra ezagutzeko mekanismo bat gertaera goiztiarrak dira, gorpu apoptotikoak adierazle bezala dituztenak eta mintzaren asimetriarekin erlazionaturik daudenak. Mintz plasmatikoan zeharreko fosfolipidoen banaketa asimetrikoa da eta asimetría honen mantentzea hainbat aktibitate entzimatikorekin

erlazionaturik dago. Fosfatidilserinaren kanporatzea (normalean fosfolipidoak mintz plasmatikoaren barne aldean aurkitzen dira) aminofosfolipido translokasa eta eskamblasa entzimen aktibitatearen orekaren araberakoa da eta zelula barneko kaltzio mailak erregulatzen du. Zelula apoptotikoetan aminofosfolipido translokaza inhibitzen da eta eskrambasa aktibatu, honegatik, mintz plasmatikoaren asimetriaren galera gertatzen delarik. Honek, fosfatidilserina kanpo mintzean ipintzea eragiten du, hau makrofago hartzaile espezializatuen bidez ezagutua dena eta fagozitatu egiten du zelula (Lizarbe, 2007).

Apoptosiak kaspasen mekanismoa aktibatzen du. Kaspasak, zistein-proteasen familiako kideak dira, estrukturalki erlazionatuak daudenak eta denboran oso mantenduak, gainera oso beharrezkoak dira apoptosi bidezko zelulen heriotzaren seinalizaziorako (Holtsberg et al., 1998).

Gainera, mitokondrioak apoptosi eta nekrosi bideetan paper garrantzitsua jokatzen duela ezaguna da, zeinen eragina kaltearen intentsitatearen, kaltetutako eremuaren eta seinalizazio bideen eratorriaren araberakoa den (Ankarcrona, 1998; Green & Reed, 1998).Kanpo mintzaren permeabilizazioa Bcl-2 proteina familien menpekoa da. Mitokondrioaren zitosoleko Bax proteinaen translokazioak kanpo mintzean poroak sortzen ditu eta ondorioz proteina proapoptotikoak askatzen dira. Gainera, garatu gabeko garunena BAX proteinen adierazpena handiagoa da (Thornton & Hagberg, 2015). Honekin batera, apoptosi bidezko garapen faktore batzuen eskasia edo gabezia ezaguna da. Gainera, neuronen kasuan, garapen enbrionarioan zehar, beraien helmugara iristen ez direnak edo beharrezko loturak egiten ez dituztenak, bere buruaz beste egiten dute apoptosi bitartez (Clarke et al., 1998). Garapen faktore batzuen modulazio faltagatik gertatzen da apoptosi hau. Gainera, antiestres entzima (Taglialatela et al., 1998) batzuen indukzioak eragiten duten geneen gabeziak, edo faktore hauek kaltzioa bahitzen duten proteinetan duten akzioaren arabera, kalbindina adibidez, apoptosi hau garatzea eragin dezakete ere (Lipton & Kalil, 1995). Hainbat faktore trofikoren bidezko heriotza zelularraren makinariaren errepresio faltagatik ere heriotza prozesua eragin daiteke, izan ere, endonukleasak zelula guztietan aurki ditzakegu (Boonman & Isacson, 1999; Puka-Sundvall et al., 2000).

Bcl-2 proteinen erregulatzaile bat, p53 tumore supresore bezala proposatu den, mitokondrioaren iragazkortasuna eta apoptosia estimulatzen duela uste da, era honetan heriotza zelularra erregulatuz. Gainera, estres proteinen aktibazioa, bero talka sortzen duten proteinak adibidez, apoptosi bidezko heriotza gelditu edo galaraziko dute bai neurona zein zelula glialetan ere. Aktibitate hau burutzen duten geneen gehiegizko adierazpenak hipoxia iskemiak eragindako kalte zelularrarekiko tolerantzia bat eratzea baimenduko du (Ko et al., 2002).

2.8.7 Heriotza zelularraren ezaugarri morfologikoak

Zelula hiltzen denen, bere buruaz beste egiteko duen barne programa baten bidez, apoptosi bidezko heriotza gertatzen da. Apoptosian zehar, tamaina zelularraren murrizketa gertatzen da eta gorpu apoptotikoen sorrera (besikulak), ondoan aurkitzen diren makrofagoek fagozitatzen dituztenak.Zelulak bere tamaina murrizten du uraren galerarengatik (kloro kanalak) eta zitoeskeletoan aldaketak gertatzen dira. Fagozitatua izateko, hartzaile edo seinaleak izan behar ditu makrofagoek ezagutu dezaten. Gainera, ADNaren zatiketa gertatzen da, beti leku berdinetan.

Nekrosi izenak berriz, aldaketa morfologiko ugariri egiten dio erreferentzia, bizirik dauden ehunen heriotza zelularra eragiten duena, kalteturik dauden zeluletan entzimen degradazio akzioak erreakzionatuz. Zelula nekrotikoak eosinofilia handipena dute, zitoplasmako ARNak baimentzen duen basofilia normalaren eraginez eta desnaturalizaturik dauden barneko zelulako proteinak eta eosinen arteko loturak handitzen direlako.

Zelulak itsura homogeneoagoa izan dezake zelula normalekin konparatuz, glukogeno molekularen galeraren eraginez (Lizarbe, 2007).

Beste alde batetik, geroz eta hipotesi gehiago daude nekrosiak ere hipoxia iskemian eragina duela esaten dutenak, izan ere, programaturiko nekrosia, oso erregulaturik dago eta aurrerago azaldutako nekrosiaren ezaugarri morfologikoak ditu. Kaspasa menpeko apoptosia inhibitua dagoenean aktibatzen da, adibidez, birus edo ATP gabezi baten eraginez eta heriotza zelularreko hartzaile dituzten TNFalfa..en bidez eragiten da. Garatua dagoen garun batean iskemia baten ostean edo garatu gabeko garun

batean hipoxia iskemia baten ostean gerta daiteke. Momentu honetan, nekrosoma deituriko egitura eratzen da nahiz eta oraindik ez den ezaguna hau nola gertatzen den (Thornton, 2015).

Azken urteetan, nekrosi bidezko heriotza edo nekroptosia (kasu gehienetan) eta apoptosi bidezko heriotzan parte hartzen duten sustantzien ikerketan aurrerapen asko egon dira eta neuronen bizitzan eragina duten faktoreak zeintzuk diren (Arango-Davila, 2004).

2.9 ZELULEN AHULTASUN SELEKTIBOA

Garatu gabeko garun baten lesioen mekanismoak ezberdinak dira garatutako garunekiko (Volpe, 1995; Biagas, 1999; Edwards & Azzopardi, 2000; Inder & Volpe, 2000). Animalietan burututako ikerketak, jaio berrietan, hipoxia iskemia baten ostean eta efektu exitotoxikoen ostean zelula multzo ahulagoak daudela uste da (Ferriero & Miller, 2010).

Garatzen hari de garun sistemaren eta zelula moten ahultasun intrintsekoak kaltearen amaierako emaitza eta funtzioen aldaketak zehazten ditu. Garapenarekiko sentikor diren prozesuak garun organizazioa, haurdunaldiko 5.hilabetean hasten dena, jaio osteko hainbat urte beranduagora arte eta mielinizazioa, axoien inguruko mielinaren espezializazio altuak dira, azken hau jaiotze ostean azkartzen dena (Plessis & Volpe, 2002; Volpe , 1991).

Asfixiak aldaketa fisiologiko eta biokimikoak sortzen ditu, jaio berriaren nerbio sistema zentraleko aldaketetan eragina dutenak, hauen artean: odol fluxuaren alterazioa eta neurotransmisore kitzikagarri funtzioa duten aminoazidoen askatzea, hala nola, azido glutamikoa eta aspartikoa. Eszitotoxizitaea ere beste eragile bat da, garun metabolismoaren azkartzea eta hipokapnia (karbono bioxidoaren handiagotzea) eragiten duena, eta arnas arazo larriak, hipertentsio pulmonarra eta hipoxemi larria eragin dezakeena (Volpe, 1997).

1.taula: Perinatal garaiena garunaren garapenean eta giza mielinizazioan gertatzen diren gertaera nagusiak eta hauei gertatzen ahal zaien aldaketak.

GERTAERA NAGUSIAK	GARAIA	LOTUTAKO ALDAKETAK
ORGANIZAZIOA ➢ **Kortexeko neuronen geruzen orientazioa eta ijezketa** ➢ **Neuriten garapena, axoien eta dendriten adarkatzea** ➢ **Sinaptogenesia** ➢ **Heriotza zelular selektiboa** ➢ **Ugaritze glial eta desberdintzea**	Garai perinatala	• Azterapen mentala • Haur autismoa • Down sindromea • Duchene muskular distrofia • Axonal garapenaren alterazio familiarrak • Loturiko alterazioak…
MIELINIZAZIOA ➢ **Oligodendrozitoen ugaritzea** ➢ **Alineación de la cubierta de mielina**	Giza mielinizazioa: Bizitza osorako gestazioa bigarren hiruhilabetetik aurrera.	• Materia zuriaren hipoplasia • Leukomalazia parabentrikularra • Hipotiroidismo kognitiboa • Jaiotze ondorengo desnutrizioa…

Garuneko eremu batzuk beste batzuk baino sentikorragoak dira iskemia baten ostean, izan ere, egoera normal baten aurrean ere bere perfusioa txikiagoa da ondoko eremuekin konparatuz (1.taula). Eremu hauei baskularizazio eremu terminala deritzo eta garun arteria nagusienek irrigatzen dituzten eremuekin bat datoz. Oxigeno murrizpenarekiko sentikortasun handia duten neuronak hipokanpoko CA1 eremuko neuronak dira, Garun kortex hondoko kapak eta zerebeloko purkingel zelulak. Garun enborreko eremuan berriz, kaltetuenak agertzen diren eremuak behe kolikulua, nukleo okulomotorrak eta troklearrak, nerbio faziala eta trigeminoa eta bagoko nukleo motor dortsala dira (Romero-Esquiliano et al., 2004).

2.10 LESIO HIPOXIKO-ISKEMIKOAN PARTE HARTZEN DUTEN ELEMENTUAK

2.10.1 Odol fluxu eta sare baskularra

2.10.1.1 Garapen baskularra, hipoxia iskemia eta garun lesioak

Nerbio sistema zentralaren (CNS) garapenean, garunaren baskularizazioa angiogenesi bitartez garatzen da, odol hodi berrien haztearen bidez. Baskularizazio egoki baten gabeziaren dagoenean, neuroepitelioak apoptosi prozesu bat pairatzen du, ehunak suntsituz eta ondorio gisa garai enbrionarioko heriotza eraginez. Elikagai emate eta gasen hartu-emanaz gain, nerbio sistema zentralaren baskularizazioa espezializatua dago astrozito, zelula endotelial eta perizioen arteko interakzioen bitartez, barrera hematoentzefalikoa eratzen dutelarik eta sustantzien pasatzea mugatu (Brea et al., 2009).

2.10.1.2 Barrera hematoentzefalikoa

Barrera hematoentzefalikoa endotelio baskularreko zelulez osaturiko egitura morfologiko eta funtzionala da eta nerbio sistema eta inguruko odol fluxuaren arteko hartu-emana baimentzen du, nerbio sistema zentralaren funtzionamendu egokirako, beharrezkoa izanik difusio sistema hau (Escobar & González, 2008). Garuneko sare baskularreko funtzioen edozein motako aldaketak garunera elikadura ematean arazoak sortzeaz gain, tonu baskularra ere kaltetzen du, barrera honetan zeharreko sustantzien garraioan arazoak sortuz (de Vries et al., 1997; Fischer et al., 2002).

Barreraren hematoentzefalikoaren beste funtzio batzuen artean, nerbio sistema zentralaren babespena dago, odol sisteman ohikoak diren sustantzia neurotoxikoen efektuetatik eta baita periferiatik hedatzen eta garraiatzen diren sustantzietatik babesten dituena (Fishman, 1991; Sharma, 2003)

Laburbilduz, barrera hematoentzefalikoak hiru funtzio garrantzitsu betetzen ditu:

1. Garuna babesten du odol korrontean zirkulatzen dauden molekula eta konposatuez, endotelio eta garun kapilarren arteko lotura zorrotzen bitartez, izan ere era honetara oxigeno, glukosa, aminoazido eta beharrezko diren elikagaiak soilik pasa dezakete barrera.

2. Sare kapilarretik garun parenkimara garraio zehatza selektiboa, glukosa bidez eratzen den garraio erraztu baten bitartez edo ATP menpekoa den difusio aktiboz.
3. Odolean aurki ditzakegun elementuen metabolismoan edo aldaketa nerbio ehunera edo alderantziz.

Neuronako inguruneak paper garrantzitsua betetzen du kapilarretako zelula endotialen barrera hemantoentzefalikoaren funtzioan, nahiz eta gertatzen diren mekanismoak oraindik ezaguna ez diren (Bauer & Bauer, 2000). Zentzu honetan, barrera hemantoentzefalikoaren eratzea neurona arteko neurobaskularizazioaren ostean gertatzen da (Ballabh et al., 2004). Haurdunaldiaren 12.astean, barrera hematoentzefalikoan aurkitzen diren okludina eta klauduna-5 proteinak, telentzefaloko lehen basoetan adierazten dira (Virgintino et al., 2004). 12 eta 14 asteen artean, gizakian, teleentzefaloko baso txikiak, barreraren funtzioak betetzen hasten dira (Virgintino et al., 1998; 2004) eta giza helduetan agertzen den lotura endotelial estuak dituzte (Coomber & Stewart, 1985; Stewart et al., 1988)

Endotelioko lotura estuen egitura molekularra begiratzen badugu, mintzeko hiru proteina aurki ditzakegu: klaudina, okludina eta loturen itsaspeneko molekulak eta baita zitoplasmako proteina osagarriak diren Zonula ocludens (ZO), hala nola, ZO1, ZO2, ZO3 eta zingulina (Krause, 2002). Zitoplasmako proteinak aktinari loturik daude, aktina, zitoeskeletoko proteina nagusia da, endotelioaren estruktura eta funtzioa mantentzen duena. Aktina, proteina hauen amaierako zatiarekin lotzen da eta honela zelula endotelialen zitoplasmako lotura estuei elkartzen (Papadopoulos et al., 2001, Daniel Alonso-Alconada et al., 2011).

Astrozitoes gain, horma baskularra ordenatzeaz arduratzen direla, perizito eta zelula adbentitzioak estrukturan laguntzen dute eta hormaren egonkortasunean laguntzen du (Lai et al., 2005).

Hipoxiko edo iskemiko den edozein gertaera ostean, lotura hauetan aldaketak sortzen dira eta baita barrera hematoentzefalikoan. Aldaketa hauek zitokinen bitartez gertatzen dira eta baita endotelio baskularraren hazkuntza faktorea (VEGF) eta NO

bitartez ere. Batez ere hantura zitokinak handitzen dira, hala nola, IL1 eta TNF. Hipoxia iskemiak zitokinak, VEGF eta NOen aldaketak eratzean kalteak sortzen ditu baina dirudienez, astrozitoak babesle bezala jokatzen dute (Escobar, 2008; Fisher, 2002; Zhang et al., 2000).

2.10.2 Astrozitoak

Astrozitoak nerbio sistema zentraleko zelula ugarienak dira eta garapen eta barrera hematoentzefalikoaren mantentze funtzioa dute. Gainera, sinaptogenesia eta homeostasi metabolikoa erregulatzen dute (Barres 2008; Barnett & Linington, 2011).

Astrozitoak garuneko kaltearen modulazioan garrantzi handia daukate entzefaopatia hipoxiko iskemikoan (Takuma et al., 2004). Glukogenoa pilatzen dute, neuronentzako energia iturria baimenduz; faktore neurotrofikoak sintetizatu eta askatzen dituzte eta azkenean, NO, beste oxidatzaileen eta zitokinen pilaketa murrizten laguntzen dute, gainera glutamatoaren pilaketa galarazten duten arduradun nagusienak dira (Desagher et al., 1996; Chen et al., 2001; Volpe, 2001; Chen & Swanson, 2003).

Hipoxia iskemia baten ostean, astrozitoak berehalako heriotza pairatzen dute edo apoptosi prozesua hasten da. Zelularitate astrozitikoaren murrizteak garun kalte larriagoa izatea dakar, beraz, astrozitoentzat babesle den estrategiak erabiliz, neurobabesle ere izango litzateke (Martínez-Orgado, 2006). Garun iskemiaren ostean, astrozitoak neuronen biziraupenean eragina daukate, in vitro egindako ikerketen arabera, hipoxiak Faktore-1aren aktibitatea eragiten du, astrozitoetako eritropoietinaren adierazpenaren gehiegizko erregulazioa eraginez. Ondorioz, eritropoiesia gertatzen da eta neurobabespenean bitartekari bezala jokatzen du. Zentzu honetan, astrozito eta neurona arteko barneko berezko seinalizazio bideak hipoxia iskemia baten osteko babesean paper garrantzitsua izan dezake (Liu et al., 2006).

Glial fibrilary acidic protein (GFAP) delakoa, nerbio sistema zentraleko astrozitoetako zitoeskeletoan agertzen den bitarteko filamentua da. Proteina honen kantitatea

handitu egiten da hipoxia iskemia baten ostean eta honegatik astrozitoen bereizle den markatzaile espezifiko bezala erabiltzen da (Li et al., 1995).

Neuronak galdu direnean eta garun ehuna kaltetua dagoenean, astrozitoak ugaritu egiten dira eta konponketa histologikoko prozesuetan parte hartzen dute. Astrozitoen ugaritzeak ehunaren hutsuneak betetzen dituzte eta honi gliosia deritzo. Gliosi hau, ugaztun helduen nerbio sistema zentraleko kalte energetikoaren eragile nagusiena dela uste da. Baina, astrozitoak erradikal askeak eta hantura bitartekariak sortzen dituzte (Deguchi et al., 1997; Maslinska et al., 2002), eta hauek potentzialki onuragarriak dira nerbio sistema zentraleko zelulentzat.

2.10.3 Oligodendrozitoak

Hipoxia iskemia perinatala leukomalazia peribentrikularraren (PVL) eragile handiena da, aldeetako bentrikuluen sustantzia zuriaren kaltearen ondorioz sortzen dena (Noetzel & Brunstrom, 2001). PVL foku nekrosi bezala definitzen da, kaltetua dagoen sustantzia zuriaz inguratua dagoena eta selektiboki pro-oligodendrozitoak kaltetzen dituena (Nesse et al., 2005). Pro-oligodendrozito hauek berantiar epeko oligodendrozitoen gurasoak dira, sustantzia zuriko zelula nagusienak eta beraien murrizketak mielina gutxitzea dakar. PVL garun kaltearen eragile nagusiena da eta baita desordena kognitibo eta garun kaltearen portaerarena.

Aurreago esan dugun bezala, hainbat faktore fisiologikok garuneko sustantzia zuria kalte hipoxiko iskemikoarekiko aldez aurretik prestatzen duela uste da. Garunaren baskularizazio jaio berrietan garatu gabea da eta ondorioz odol presio aldaketa bortitzei moldatzeko arazoak dituzte. Hau honela, sustantzia zuriko zelulak, odol fluxu eta oxigeno murrizketari oso sentikorrak dira. Gainera, pro-oligodendrozitoak glutation kantitate murriztuak dituzte, eta honegatik bereziki sentikorrak dira estresaren ondorioz gertatzen den oxigeno murrizketari.

leukomalazia peribentrikularra gertatzeko arrisku une handiena oligodendrozitoen aintzindariak sustantzia zurian nagusitzen diren momentuarekin bat dator, hau da,

ugaritu eta desberdintzen direnean. Epe hau, mielina zorroen sintesi aktiboa gertatu aurretik gertatzen da (Back et al., 2001), gizakian haurdunaldiaren 23.astetik 32ra gertatzen delarik.

Mielina eratzen duten zelulak oligodendrozitoak dira, eta mielina hau garrantzitsua da nerbio inpultsuak nerbio sistema zentralera garraiatzeko. Hipoxia iskemiaren ondorioz sorturiko glutamatoaren exitotoxikotasuna, estres oxidatzailea eta sorturiko hanturak aldaketa morfologikoak sortzen ditu, nukleoen hedapena, piknosia eta mielina zakuen bakuolizazioa, oligodendrozitoen heriotza edo funtzionamendu okerra eraginez (Montaner, 2007). Glutamatoarekiko oso sentikor izateaz gain, (Ness et al., 2001; Park et al., 2004; Simonishvili, 2013), oligodendrozitoak estres oxidatzailearekiko oso sentikorrak dira, lipido, burdin kontzentrazio altu eta entzima antioxidatzaile gutxi dutelako.

Oligodendrozitoak kaltetu ditzaketen mekanismoak:

1. Neurotransmisore bitarteko toxikotasuna
2. Glutamato bitarteko toxikotasuna
3. ATP bitarteko toxikotasuna
4. Mitokondrioaren egoera eta estres oxidatzailea

Hantura kaltearen bidez eragindako mielinizazio ezan egindako ikerketek, oligodendrozitoak hantura eragiten duten zitokinekiko oso sentikorrak direla baieztatu dute (Hartung et al., 1992). Zitokinaren efektua, TNFα eta interferoiaren efektu zitotoxioaren eraginez gertatzen da eta efektu hau handiagoa da garatu gabeko oligoendrozitoetan garatutakoetan baino (Skof & Ghandour, 1995; Andrews et al., 1998).

Honegatik oso garrantzitsua da kaltearen lehenengo momentuetan ematen diren aldaketak identifikatzea, izan ere, kaltearen ostean oligodendrozitoetan gertatzen diren aldaketa hauek neuronetan gertatzen diren aldaketekin erlazionatu daitezke.

2.10.4 Mikroglia

Mikroglia jatorri mesenkimaleko zelulen multzoa da, odoleko monozitoetatik eratorriak direnak eta garapen enbrionarioko garai goiztiarretan nerbio sistemara Joan zirenak. Zelula hauek nerbio sistema zentrala betetzen dute garai fetalean, batez ere fetuaren garapenaren bigarren hiruhilabetekoan, ondoren, garapenaren zehar desberdinduak izateko (Gehrmann et al., 1995; Rezaie et al., 2004). Odoleko makrofagoen antzekoak dira eta honegatik mikrogliari *garuneko makrofago* deritzo.

Atsedenean dagoen mikroglia edo inaktibo dagoena, adarkapen txikiez osatua dago, baina aktibo dagoenean (lesio baten ostean) ameboide itsura edo borobilduago baten itsura hartzen du, makrofagoen antzekoa dena (Streit et al., 1999). Lehenengo fase batean, mikroglia fagozitiko ez diren irizpideen bitartez aktibatzen da (mikroglia hipertrofikoa) eta bigarren fasean, batez ere terminal edo neuronen degenerazioa gertatzen bada, mikroglia zelula fagozitiko bilakatzen da (mikroglia fagozitikoa) (Gehrmann et al., 1995).

Hipoxia iskemia zehar, mikrogliako zelulak oso azkar aktibatzen dira eta hantura eta konponketa prozesuetan parte hartzen dute. Garuneko odol fluxua handitu egiten da eta ondorioz, neurona eta gliaren funtzioa aldatzen da, garun kaltea eraginez eta edema zitotoxikoa (De Vries et al., 1997; Patel et al., 2003). Gaitasun fagozitikoa dute eta sustantzia ezberdinak askatzen dituzte aktibo direnean, hala nola, elastasa entzima, oxidatzaile diren hainbat erradikal aske eta hantura eragin edo deusezten duten zitokinak, interleukina 1, 3, 5, 6, TNFa, neurona garatzen duen faktorea eta garapen eta transformazio faktorea. Hantura bitartekari hauetariko batzuen eraginez (TNFa, IL-1b y LPS), mikrogliak astrozito eta oligodendrozitoengan eragiten duen kaltea handitzen du (Tahraoui et al., 2001).

Mikrogliaren aktibazioak duen eragin onuragarri edo kaltegarrietan eztabaida ugari dauden arren, hainbat ikerketek nerbio sistema zentraleko hainbat patologiatan neurobabesle bezala joka dezaketela uste dute. Honegatik, mikrogliak ere hainbat funtzio onuragarri izan ditzake hipoxia iskemia baten ostean, fagozitosi bidezko

hainbat sustantzien kentzea eragiten duelako, neuronei elikagaia ematen laguntzen du eta kanpoko zelulako mintza birmoldatzen (Imai et al., 2007; Lai & Todd, 2006; Hanisch, 2002).

2.11 PROBA TERAPEUTIKOAK

Asfixia baten osteko larritasun eta garrantzia, iraupenaren, sentikortasun iskemiko selektibo eta sakontasunaren araberakoa da. Sentikortasun iskemiko selektibo honek erabakiko du zelula batzuk hiltzea eta beste batzuk iluntasun iskemiko batean mantentzea.

Zehaztutako leiho terapeutiko bat dago non tratamendu batek garun kalte hau murriztu dezakeen, baina denbora hau igaro ostean, kaltea ezin da berreskuratu. Jaiotza normala duten umeetan, garun reperfusioa geroz eta lehenago hasi eta tratamendu bidezko erreskatatzea, orduan eta handiagoa izango da histologian eta klinikan duen ohiartzuna (Vannucci & Perlman, 1997; Tan & Parks, 1999). Garun kalte neurrizkoa edo txikia duten gaixoetan, onura hobeagoak izango dituzte parte-hartze terapeutiko honen ostean, baina hauek epe goiztiarrean identifikatzeko zailenak dira.

Orain arte, asfixia perinatala pairatzen duten jaio berriari, arreta intentsiboa egitera mugatu dira, hau da, oinarri hemodinamiko, arnasketa eta metabolikoak aztertu, eta konbultsioen tratamendu eta organo anitzeko disfuntzioen monitorizaziora. Estrategia terapeutiko honek ez du kalte hipoxiko iskemikoan eraginkorki jokatzen, izan ere, ez du kalte fisiopatologikoan eragiten. Kontrolpeko hipotermia izan da neuronen kalte sekundarioa murrizteko proposatu den parte-hartze terapeutiko bakarra. Animalietan egindako ikerketa esperimentalek, gorpu tenperatura 2-5ºC bitartean jaistean, kalte histologikoa eta kalte energetikoa gutxitzen dela erakutsi dute eta gizakian, neurrizko hipotermiak (33-34ºC) hipoxia iskemikoak eragiten duen ondorio neurologiakoak gutxitu ditzake, ondorio larririk gabe. Kontrolpean eginiko hipotermiak heriotza zelular eta neuronen garapeneko aldaketak murrizten ditu eta haurren heriotza tasa gutxitzen du (Silvera, 2011).

Hipotermiaz gain beste hainbat aukera terapeutiko proposatu dira, hipotermiarekin konbinatu daitezkeenak, bere eragin neurobabeslea handitzeko. Hipoxia iskemia baten ostean gertatzen den egoera nagusienetariko bat, oxigenoaren espezie erreaktiboen (ROS) garapen goiztiarra da (Li et al., 2009). ROSen produkzioaren handitzea, lipido eta proteina endogenoen antioxidatzaileak galtzea dakar eta ondorioz, neurona zelulen heriotza (Kelly, 1993; Margaill et al., 2005). Honegatik, geroz eta ikerketa gehiago egiten ari dira asfixia perinatalaren osteko antioxidatzaileen bitartezko tratamendua (Moosmann & Behl, 2002; McMahon et al., 2006).

Eragile asko ikertu dira terapia antioxidatzaile gisa, baina lan honetan 4 antioxidatzaileetan oinarritu gara, aurrerago egindako ikerketetan eragin neurobabesle bat dutela frogatuak daudenak eta efektu onuragarriak dituztela baieztatu direnak asfixia perinatal baten osteko garun kaltean. Hala ere, antioxidatzaile hauek eragina, hipokanpo eta kortexean aztertu da, beraz, gure lan honek, eragile hauen efektua aztertu nahi du entzefalo enborrean eta hauen eragina hipoxia iskemiaren ondoriozko entzumen ezgaitasunean.

Resberatrola (3,5,4-trihydroxystilbene) konposatu polifenol ez flabonoidea da, bi eraztun aromatikoz osatua dago, metileno bidez lotuak eta 72 landare espezie ezberdinek sortzen dute, hala nola, ardoaren mahatsak, pinuak, leguminosoek, kakahueteak eta sojaren eta granada landareak, baina Resberatrolaren iturri ezagunena ardo beltzean dago (Liu et al., 2007). Resberatrolaren efektu neurobabestzaileak beraren eragin antioxidatzailearengatik dator, beraren polifenolez osaturiko 2 eraztun metilenozko egiturarengatik daukana (Lopez-Miranda et al., 2012).

Melatonina (N-acetyl-5-methoxytryptamine) indolamina endogenoa da batez ere guruin pinealak eratzen duena eta odol zirkulaziora eta garun eta bizkarrezuz fluidora askatzen dena. Funtzio asko iradokitu zaizkio, hauen artean, erradikal askeen berriztapena (Tan et al., 2007) eta biomolekula oxidatzaileen oxidazioa (Reiter, 1998).

DHA (Docosahexaenoic acid) (22:6n-3) omega 3 kate luzeko gantz azidoa da, arrainean, izokin eta atunean aurki dezakeguna. Gizakian DHA kontzentrazio baxuak aurki

ditzakegu odolean, baina kontzentrazio altuak garun, erretina eta espermatozoideetan. DHAk plasmaren jariakortasuna baimentzen du, mintzetatik eremu sinaptikoetara eta ondorioz kitzikadura eta neuronen funtzio sinaptikoa ahalbidetuz (Wurtmar, 2008; Davis-Bruno & Tassinari, 2011). Haurdunaldian zeharreko DHA dieta bat izatea, babesa ematen dio jaio aurretiko haurrari, estres oxidatzailearen inhibizioaren bidez (Suganuma et al., 2013).

Nicotinaren hartzaile azetilkolinikoa (nAChR) aurrekoekin konparatuz, ikerketa gutxien dituen sustantzia da, azpitalde ugari dituelako bakoitzaren funtzio eta propietateen bidez sailkatzen direnak, baina gehienak funtzio antioxidatzailea dute. Gainera, glutamato bidezko neuronen toxikotasuna inhibitzen dute eta Bcl-2 eta beste proteina antiapoptotikoren adierazpena handitu (Hejmadi, 2003).

Estrategia terapeutiko hauek garatu gabeko garuneko kalteak murrizteko ditugun gaur egungo aukerak dira eta hainbat aukera ezberdin ditugu, izan ere hipoxia iskemia baten osteko heriotza zelularra gelditzeko aukera asko egon daiteke.

HIPOTESIA

Hipoxia iskemiak entzumen-sisteman aldaketak eragiten ditu. Kaltearen larritasuna gertaeraren graduaren arabera, iraupenaren arabera eta sentikortasun iskemiko selektiboaren araberakoa da (mekanismo fisiopatologikoa). Faktore guzti hauek, neurona multzo ezberdinen sentikortasuna zehartuko dute, batzuen heriotza eraginez eta beste batzuk iluntasun iskemikoan sartuz (leiho terapeutikoa). Jaiotzera iristen diren jaio berritan, garunaren erreperfusioa geroz eta lehenago hasi, orduan eta eragin handiagoa izango dute tratamenduak eta ondorio histologiko eta klinikoak txikiagoak izango dira. Hipoxia iskemia ostean erabiltzen diren estrategiak dira garrantzi handiena dutenak klinikan. Antioxidatzaile bidezko esku-hartzeak leiho terapeutikoa handitu dezake eta bere efektuak modelo esperimentalak erabiliz aztertuak izan daitezke.

Gure lanerako hipotesia, hipoxia iskemiak entzumen-sisteman, mesentzefaloan eta zehazki behe kolikuluan eragina duela da eta antioxidatzaile bidezko tratamenduak babes efektua izan dezakeela, kalte zelularra murriztuz eta entzumen defizita leunduz.

HELBURUAK

Gure hipotesia egiaztatzeko, gure lana ondorengo helburuekin antolatu da:

Helburu nagusia

Eraso hipoxiko-iskemikoak mesetzefaloan eta zehazki behe kolikuluan eragiten duen kaltea aztertzea eta antioxidatzaileek (Nikotina, Melatonina, Resveratrol eta Azido Dekosahenoikoa (DHA)) berarekiko duten babes-efektua aztertzea.

Helburu zehatzak:

1. ABR bitartez hipoxia iskemiak eragiten dituen barne belarriaren eta entzumen-bidearen aldaketa elektrofisiologikoak ikustea eta beraien berreskuratzea tratamendu neurobabestzaileen bitartez.
2. Talde ezberdinen azterketa histologikoa egitea, kaltearen gradua, hedapena, kokapen eta larritasuna ikusteko.
3. Mesentzefalo eta zehazki behe kolikuluko neurona eta zelula glialen heriotza zelularra aztertzea.
4. Mitokondrioaren egoera mitokondrioko mintzaren potentzialitate eta osotasuna aztertuz eta baita erradikal superoxidoen produkzioa eta eremu honetako kalte oxidatzailea.
5. Hipoxia iskemia ostean eta antioxidatzaileen osteko tratamenduan geneen adierazgarritasuna aztertzea
6. Aurreko ikerketen bitartez, tratamendu neurobabesleen efektua ikustea entzumen-bidean eta mesentzefaloan.

MATERIAL ETA METODOAK

ANIMALIAK

Lan honetan *Sprague Dawley* espezieko arratoiak erabili ziren, Euskal Herriko Unibertsitateko SGIKER animaliategiak emandakoak eta txerriak, Sus Scrofa espeziekoak, Galarretako Arri-Turri abeletxeak Gurutzetako Ospitale Unibertsitarioko Ikerketa eta esperimentazio zailera emandakoak.

Egindako prozedura esperimental guztiak Europar Batasunak ikerketa eta animaliekin esperimentazioa egiteko ezarrita dituen arauen arabera egin da (2010/63/EU). Gainera arratoiekin egindako prozesu guztiak Euskal Herriko Unibertsitateko animaliekin esperimentazioa egiteko etika batzordeak baimendu zituen (CEBA) (CEEA/341-344/2014/ALVAREZ DIAZ) eta txerriekin egindako prozesu guztiak Gurutzeta ospitale Unibertsitateko animalien ongizateko etika batzordeak onartu zituen (SP#009_09; SP#012_11).

A) ARRATOIAK

Animaliak Euskal Herriko (UPV/EHU) animaltegian mantendu ziren argi eta iluntasuneko 12 orduko zikloekin, janaria eta ura edozein momentutan eskuragarri zutelarik.

5.1 PROZESU KIRURGIKOA

Kalte hipoxiko-iskemikoa 7 eguneko arratoiei eragin zitzaien eta prozesua 2 fasetan banatua zegoen:

- Gertaera iskemikoa: Animaliek isofluorano bidezko anestesia pairatu zuten, %3,5 indukziorako eta %1,5 mantentzeko. Lehenengo ebakidura bat egin zitzaien arratoiei lepoan, ezkerreko arteria karotida isolatu eta bi korapiloz lotu zen 6/0 setak erabiliz. Ondoren karotida moztu eta kauterizatu zen eta intsizioa 3/0 seta bitartez itxi zen. Klorhexidina %1 bidez desinfektatu zen zauria eta animaliek beroa gal ez zezaten, prozesu guztia manta termiko bidez egin zen.

Gertaera iskemikoaren ostean, kumeak amarengana itzuli ziren prozesutik errekuperatu ahal izateko. Anestesiatik errekuperatzeko 2 ordu inguru behar zituzten.

Operazio bakoitzean igarotako denbora ez zen 15minutu baino luzeagoa izan inongo kasuan.

- Gertaera hipoxiako: 4 kumeko taldeak 1000mlko beirazko ontzietan sartu ziren eta hermetikoki itxi ziren. Ontziak 36,5ºC tako bainuan ipini ziren eta %8ko 0_2-%92ko N_2-a ontzi barnera zirkulatzea egin zen 135 minutuz.

Gertaera hipoxikoaren ostean, manta elektrikoan kokaturiko animaliak berpiztu ziren kanpo estimulu fisikoz lagunduz. Sakrifikatu ziren egunera arte bere amarekin ipini ziren.

5.2 TALDE ESPERIMENTALAK

Hauek dira talde esperimental bakoitzean erabilitako animali kopurua azterketa bakoitzerako:

-Azterketa elektrofisiologikoa; n=8

-Azterketa histologikoa: n=8

-Azterketa inmunohistologikoa: n=8

-Finkaturik ez dauden zelulen zitometria bidezko azterketa: n=5

-Finkaturik dauden zelulen zitometria bidezko azterketa: n=5

-Adierazpen genikoaren azterketa: n=5, Hiru kopiaz

Talde esperimentalak zoriz hautatu ziren eta tratamenduen dosia intraperitonealki eman zitzaien. Ondorengo hauek ziren talde esperimentalak:

<u>Kontrol taldea</u>

Talde honetan sartutako animaliei ez zitzaien inongo prozesu kirurgikorik egin, izan ere, aurrerago ikertua zegoen SHAM (anestesiaturiko animaliak eta ebaketa txiki bat zutenak, ondoren josi egin zena, honela animali hauek anestesia pairatu eta orbaindu beharreko zauria zuten) eta kontrol animaliek (inolako operazio kirurgiko gabe) emaitza berdinak emate zituztela.

Hipoxia iskemia taldea

Talde hauek gertaera hipoxiko iskemiakoa pairatu zuten, aurrerago zehazki deskribatu dena, ezkerreko arteria karotida moztu eta ondoren, oxigenoa %8ra murriztuz 2 ordu eta laurdenez.

Tratamendu zuten taldeak

Talde honetako animaliek Nikotina, Melatonina, Resveratrol eta DHA tratamenduak jaso zituzten, dosi, administrazio denbora eta disoluzio zehatz batzuetan, Aldagai hauek aurrerago egindako ikerketetan oinarritu ziren.

Tratamendua	Dosia	Disoluzioa	Administrazio denborak
Nikotina (N)	1.2 mg/kg (Chen et al., 2013)	Serum gazia	Hipoxia baino 2h lehenago
Melatonina (M)	15 mg/kg (Carloni et al., 2008)	Serum gazia eta DMSO 5%	Hipoxiatik 10 minutura
Resberatrola (RV)	20 mg/kg (West et al., 2007)	DMSO	Hipoxia baino 10 min lehenago
Docosahexaenoic acid (DHA)	1 mg/kg (Berman et al., 2009)	HSA %25 Serum gazian disolbatuta	Hipoxia baino 10 min lehenago

5.3 KALTEAREN IKERTZEN DENBORAK ETA ERABILITAKO METODOLOGIA

Ikerketa denbora ezberdinetan egin zen kalte hipoxiko iskemikoaren efektua eta tratamenduen eraginkortasuna zehazteko

Hipoxia iskemia baten ondoren egindako ikerketa eta erabilitako denborak ondorengoak izan ziren:

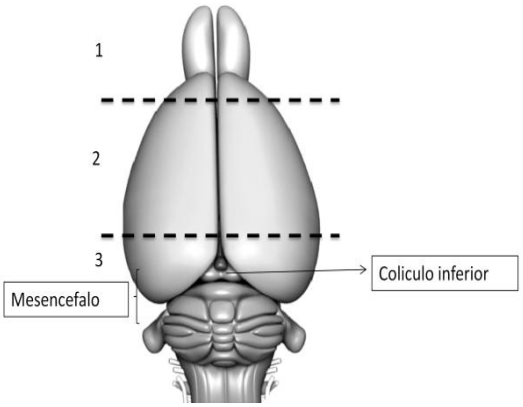
- Epe laburreko efektuak:

→ Bat bateko erantzuna: 0, 3, 12 ordutan (inmunohistokimika bitartez, hipoxiaren markatzailea erabiliz, qPCR bidezko geneen adierazpena eta mitokondriaren *"in vivo"* markatzaileak zitometria bidez).

→ Erantzun berantiarra: 24ordutara (TTC bidezko teknika infartua zein izan den ikusteko eta inmunomarkatzaileak, GFAP, NEUN eta Syp fluxuzko zitometria bitartez).

- Erdi epeko efektuak: 7egun (Elektrofisiologia, histología Nissl eta hematoxilina eosina tindatzaileen bitartez eta inmunohistokimika GFAP eta MBP bitartez).

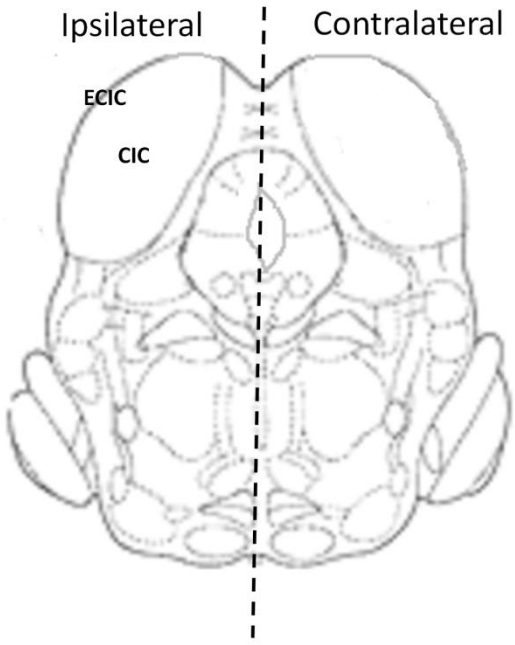
5.4 IKERTUTAKO ZONALDEA



15. irudia: Arratoi garunaren ikusmen dortsala. 3.zatia da guri interesatzen zaigun eskualdea,non mesentzefaloa aurkitzen den.

Animalien burmuinak 3 zatitan banatu ziren eta guk ikertutakoa azken zonaldea zen (15.irudia). Hemen mesentzefaloa aurkitzen interauraletik 0,30mmko distantziara eta bregmatik 9,30mmko distantziara (Paxinos, 1986). Gainera, entzefalo enborra aztertzeaz gain behe kolikulua aztertu genuen zehazki.

Azterketa morfologiko eta inmunohistologikoetarako 5µmtan moztutako mesentzefaloko zatiak erabili ziren, behe kolikulua aurkitzen dugun tokia (16.irudia). Infartuaren zonaldea, zitometria bidezko parametro zelularraren eta adierazpen genikoaren analisirako berriz, mesentzefalo osoa erabili zen.

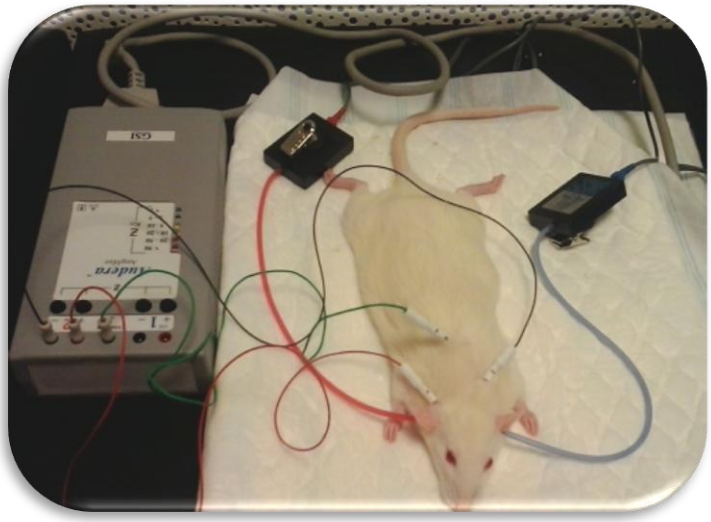


16.irudia: Mesentzefaloaren irudia (Bregmatik 9,30mmtara eta interauraletik 0,3mmra) non ikerketaren ipsilateral hemisferioa irudikatua dagoen. Laburdura: Behe kolikuloko erdiko nukleoak (CIC) eta behe kolikuloko kanpoko kortexa (ECIC).

5.5 AZTERKETA ELEKTROFISIOLOGIKOA

Animaliak pisatu ostean Ketolar/Xilazinarekin anestesiatu ziren 40-90/5-10 mg/kg dosia emanez intraperitonealki eta ondoren entzumeneko potentzial ebokatuak neurtu ziren GSI Audea programa erabiliz, softwaren 1.0.3.4 bertsioa erabiliz.

Honetarako garunean 3 hiltze sartu zitzaizkien, bat garunaren erdian (polo positiboa) beste bat ipsilateral edo kaltetutako aldeko belarrian (polo negatiboa) eta hirugarrena gorputzean (lurra) (17.irudia). Neurketa hau soinurik gabeko gela batean egin zen, kanpoko soinuak gure emaitzetan eraginiz izan ez zezaten.



17.irudia: Elektrodoen kokapen zehatzaren irudi adierazgarria arratoi heldu batean (guk 7eguneko arratoietan egin dugu analisia).

Aparatuak potentzial elektrikoaren aldaketak jasotzen zituen, estimulua egitura anatomiko ezberdinetara iristen zenean sortzen ziren aldaketak hain zuzen ere eta emaitzak, erantzuna jaso arte igarotako denbora (ms) eta erantzunaren intentsitate (µV) bezala jasotzen ziren.

Frekuentzien jasotzearen ezaugarri teknikoak ondorengoak ziren: 10msko eskaneatzea, 150Hzko iragazkiarekin frekuentzia baxuetarako eta 3000HZko iragazkiarekin altuetarako; sentsibilitatea 10mcv/div. Estimulua click bitartez egin zen eta 1024 eta 2048 bitarteko erantzunen media egin zen. Azterketa 100db HLko intentsitatedun click bitartez egin zen.

Neurketa 3 aldiz errepikatu zen belarri bakoitzean eta ondoren animaliak perfunditu ziren nahi genuen lagina hartuz.

Analizaturiko parametroak: pasatako denbora (ms) erantzuna jaso arte, intentsitate (µV) eta tontorren arteko denbora tartea (ms) izan ziren.

5.6 AZTERKETA MORFOLOGIKORAKO ANIMALIEN NEKROPSIA ETA LAGINEN JASOTZEA

5.6.1 Fizatzea

Animaliak 50µl Dolethal (20g/100ml-ko kontzentrazioa) intraperitonealki emandako injekzio baten bitartez sakrifikatu ziren: Bere printzipio aktiboa pentobarbital sodikoa da, azido barbiturikotik datorren konposatua, akzio motzekoa, bere efektu nagusiena nerbio sistema zentrala deprimitzea izanik. Animalia garbitzeko eskuineko bentrikulutik serum fisiologikoa eta 1%era zegoen heparina injektatu ziren, ponpa peristaltiko bidez administratu zirenak, presio eta fluxu konstantean. Ondoren, ezker aurikula moztu zen odolaren drainatze askea izateko. Gorputz-adarra begiratuz, (poliki-poliki zuritzen doazenak), animaliak %4ko paraformaldehido perfusioarekin fizatu zen eta animaliaren zurruntasuna baieztatu ondoren, interesatzen zitzaigun mesentzefalo lagina atera genuen. Ondorengo fizatzea laginak %4ko paraformaldehidoan 24 ordu mantenduz egin zen.

5.6.2 Parafinan inklusioa

Lagina fizatua zegoenenan izendutako saskitxoetan sartu ziren parafinan inklusioa egiteko, ondorengo protokoloaren bitartez:

Laginak, geroz eta handiagoko alkohol kontzentrazioetan sartu ziren: 50ºko alkoholean (ordu 1 eta 30 minutuz), 70ºko alkoholean (ordu 1 eta 30 minutuz), 96ºko alkoholean 2 pausu (bakoitza 30 minutukoa) eta 100ºko alkoholean 3 pausu (bakoitza ordu 1 eta 30 minutukoa). Ondoren, metilozko benzoatoan bi pausu egin ziren eta amaitzeko parafina likidoan inklusioa egin zen 60ºko estufan. Parafina likidoan dauden laginak parafina moldetan ipini ziren. Prozesua bukatu ostean, egindako moldeak mikrotomo (1150/ Acounto Reichert Jung) bidez moztu ziren. Jarraikako 5 zati moztu ziren, bakoitza 5 µmtakoa, eta hematoxilina-eosinaz tindatu ziren. Aukeratutako laginak Nissl bidez ere tindatu ziren eta inmunohistokimikako erreakzioak ere lagin hauetan egin ziren, GFAP eta MBP proteinentzako.

5.6.3 Hematoxilina-eosina tindaketa

Tindaketa hau 2 koloratzaile bitartez osatua dago, Harrisen hematoxilina, izaera basikoko koloratzaile naturala da. Elkarketak ditu intentsitate baxuko eta ahulki azidoa diren kromogenoak, gogorki basikoak diren auxokromo kationikoekin, azken hauek koloratzailearen karga globalaren arduradunak direnak eta azido nukleiko formako zelulen nukleoak koloreztatzen ditu batez ere, egitura basikoak direlako. Eosinak berriz, intentsitate baxuko eta ahulki basikoak diren kromogenoak gogorki azidoak diren auxokromo kationikoekin egiten dituzte loturak. Eosina hidroxixanteniko halogenatuetan dauka jatorria eta koloratzaile zitoplasmatikoak espezifikotasun gutxiago duenez nukleoko koloratzaileekin konparatuz, kanpoko zelulako ehunen egiturarekin elkartzen da, kolorazioa eraginez.

Parafinan inklusioa duten laginen tindaketa egiteko, lehenengo 2 orduz 60ºko estufan mantendu ziren eta ondoren zitrosolean desparafinatu ziren (10 minutuko 2 pausutan). Laginak alkoholen bidezko murgiltzearen bitartez hidratatu ziren (minutu bat alkohol bakoitzean), kasu honetan, geroz eta kontzentrazio baxuagoko alkoholetako pausuak eginez: 100ºko alkohola, 96ºko alkohola (2 pausu), 70ºko alkohola eta azkenik uretan. Laginak hematoxilina sartu ziren 3 minutu eta erdiz eta urez garbitu ziren. Ondoren eosinan sartu ziren 20 segundoz eta urarekin garbitu ziren berriz. Hau amaitzean laginak berriz ere deshidratatu ziren (10segunduz pausu bakoitzean): 70ºko alkohola lehenengo, ondoren 96ºko alkohola eta azkenik 100ºko alkoholena sartuz (2 aldiz), fizatzeko zitrosolean mantendu ziren 10 minutuko bi pausu eginez eta DPX (Fluka, Sigma-Aldrich, St Louis, Mo, DPX EEUU) bidez muntai histologikoa egin zen.

5.6.4 Kresilo violeta bidezko tindaketa (Nissl)

Teknika hau Kresilo violeta koloratzailearen bitartez egin zen, nerbio ehunetarako argentikoa ez den koloratzaile bat. Kresilo bioleta azido nukleikoari lotzen da erakarpen elektrostatiko bidezko mekanismo fisiko kimiko bitartez, Nissl granuluak eta nukleoloak tindatzen, izan ere, RNAz osatuak daude. Granuluak erretikulu

endoplasmatiko bikortsuaren pilaketak dira neuronen eta polirribosomen zitoplasman aurkitzen direnak.

Lagin histologikoen tindaketarako hurrengo pausuak jarraitu ziren: xilolarekin desparafinatu zen 10 minutuzko 2 pausuz eta laginak geroz eta kontzentrazio baxuko alkoholetan sartu ziren, aurrerago azaldu dugun bezala, 100ºko alkoholeko minutu bateko pausu bat, minutu bateko 2 pausu 96ºko alkoholean eta minutu bateko pausu bat 70ºko alkoholean, azkenik ur destilaturekin garbitu ziren.

Jarraian Kresilo bioletazko tanpoi batean sartu ziren (1g kresilo bioleta 500ml ur destilatutan eta azido azetikoaren 4 tantanekin) 10 minutuz eta ondoren ur destilatuarekin garbitu ziren, gehiegizko koloratzailea kentzeko. Laginak kontzentrazio baxutik alturako alkohol kontzentrazioetan sartu ziren deshidratatzeko aurrerago azaldu dugun bezala eta xilolean fizatu ostean muntaia DPXaren bidez egin zen.

5.7 ZELULA HIPOXIKO ETA INFARTU ZELULARRAREN AZTERKETA

5.7.1 Hipoxia markatzaile baten bidezko tindaketa

Arratoien garunean hipoxia gertatu den edo ez ikusteko hipoxia markatzaile berezi bat erabili zen, hypoxyprobe-1 (Hypoxyprobe Inc. Burlington MA, USA). Intraperitonealki hypoxiprobe (60mg/kg) dosia injektatu zitzaien gertaera hipoxia gertatu aurretik. Hipoxia ostean animalia hil eta mesentzefalo lagina hartu genuen, 6.1 eta 6.2 puntuetan azaldu dugun bezala.

Hypoxyprobe-1 ehun guztietan zehar banatzen da, baita garunean ere, baina loturak thiol duten proteinei soilik egiten die: zelula hauek 14 µM baino gutxiagoko oxigeno kontzentrazioaren gutxitzea pairatu dutenak dira. Barne peroxidasaren H2O2arekin (%3) blokeatu ostean eta parafinan fizaturiko laginaren hidratazioaren ostean Hypoxyprobe - 1 MAb1 antigorputz primarioarekin (1/5) inkubatu ziren ordu batez.

Serumarekin garbiketa ugari egin ostean, laginak peroxidasarekin elkartuta dagoen serum anti-mouse-arekin (1/5000) inkubatu ziren 10 minutuz. Antigorputz primarioaren presentzia laginak 7 minutuz diaminobenzidinarekin (DAB) inkubatuz

lortu zen. Honen ostean, laginak hematoxilinarekin tindatu ziren 10 segunduz eta urarekin garbiketak egin ostean, laginak deshidratatu eta muntaia egin zen aurrerago azaldutako era berean.

5.7.2 TTC Tindaketa

Hipoxia iskemiak, arratoien garunean sortzen duen infartuaren zonaldea balioztatzeko, tetrazolio kloruro (TTC) tindaketa erabili genuen (Syp, Sigma-Aldrich, St Louis, Mo, EEUU). Kalte hipoxiko-iskemikoa egin eta 24 ordu ostean, animalia hil eta mesentzefaloa zatikatu zen 2mmko lodierako zatiak eginez. Ehuna TTC %2ko soluzioan inkubatu zen 37ºCtan 30 minutuz eta ondoren 24 orduz %4ko paraformaldehidoan fizatu zen

TTC bidez tindaturiko ehunen argazkiak kamera digital bidez (Canon PC1059, Canon U.S.A., Inc.One Canon Park, Melville, NY) atera ziren, kamara hau lupa (Edmund Optics Inc, East Gloucester Pike, Barrington, USA) bati akoplatua egonik

5.8 INMUNOHISTOKIMIKA BIDEZKO KALTEAREN BALORAZIOA

5.8.1 Aktibitate glialaren azterketa

Garun kaltearen ezaugarrietako bat aktibitate glialaren agerpena da eta bere ikerketarako GFAP (Dako, Denmark) bidezko markapen bat egin zen.

Markatzea parafinan fizaturiko laginetan egin zen 7.1 eta 7.2 puntuetan azaldu dugun bezala. Laginak hidratatu egin ziren, hematoxilina-eosina eta Nissl kasuetan bezala eta ondoren barne peroxidasa blokeatu zen %1eko H2O2aren bidez. Ondoren mintza iragazkortu zen %1eko PBS eta %0,1eko Tween bidez 10 minutuz eta behi serum (BSA) bidez blokeatu zen %0,5 PBSan 20 minutuz. Laginak rabbit anti-GFAP antigorputz primarioarekin (1/500) inkubatu ziren PBS-BSA-TWEEN %1eko kontzentrazioan, ganbara heze (4ºC) batean gau guztia mantenduz.

Hurrengo egunean, %1eko PBS arekin 3 garbiketa egin ostean, peroxidasadun antigorputz sekundarioarekin inkubatu ziren (HRP anti-rabbit 1:100, Santa Cruz

Biotechnology, CA, USA) ordu batez. Inkubazioaren ostean, PBSarekin garbitu ziren antigorputzaren hondarrak kentzeko eta DABkin tindatu ziren 7 minutuz. Ondoren hematoxilinarekin tindatu ziren 10 segundoz eta aurreko prozesuetan bezala, deshidratatu ostean xilol bidez fizatu eta DPXarekin muntaia egin zen.

5.8.2 Sustantzia zuriaren azterketa

Sustantzia zuriaren kaltea ikusteko MBP bidezko markatzailea erabili zen, laginak 9.1 puntuan bezala tratatu ziren antigorputz primarioarekin inkubazioa egin arte, hau mouse anti-MBP (1:200, Santa Cruz Biotechnology, CA, USA) bidez egin zen. 4ºCz inkubatu zen gau osoan zehar eta hurrengo egunean, peroxidasarekin konjugaturiko antigorputz sekundarioarekin inkubatu zen (HRP anti-mouse 1:100, Santa Cruz Biotechnology, CA, USA) ordu batez. GFAP bidezko inmunohistokimikan egin genuen bezala, laginak DABrekin tindatu ziren eta ondoren hematoxilinarekin deshidratatu aurretik, fizatu eta muntaia egin aurretik.

5.9 KALTEAREN BALORAZIO MORFOLOGIKOA

1) Kaltearen balorazio morfologikoa egiteko, hematoxilina-eosinaz zein Nissl bidez tindatutako laginak mikroskopio bidez begiztatu ziren eta zelulen analisi kuantitatibo bat egin genuen.Hau da, neuronak bere morfologia naturala mantentzen zuten edo ez begiratu zen ondoko puntuak kontuan hartuta:
    a. Zelulen tamaina
    b. Luzapenak
    c. Zitoplasmaren kondentsazio maila
    d. Nukleoko kromatina maila
    e. Neuropiloaren dentsitatea

2) Zeluletan gertatu den hipoxiari dagokionez, mikrokopio optiko bitartez aztertu ziren hipoxia iskemia pairatu zuten animaliek hypoxyprobe-1 markatzailea erakusten zuten edo ez.

3) Aktibazio glialaren azterketan, mikroskopio optikoz begiratu zen ia astrozitoen erreaktibitate maila handiagoa zen edo ez kalteturik zeuden animalien kasuan eta trataturiko animalien arteko ezberdintasunak. Analisia kualitatiboa izan zen.

4) Inmunomarkatzeile bidezko dentsitometria bideo kamera erabiliz aztertu ziren, irudiaren analisian oinarriturik dagoena (National Institutes of Health Image software, public domain) (http://rsb.info.nih.gov/nih-image/), lehenago Liu et al., (2002) deskribatu zuen bezala. TIFF formatoa zuten irudiak digitalizatu, moztu eta binarizatu ziren (zuria vs beltza). Pixel beltzak kontatu ziren hemisferioko eta mediak kalkulatu ziren garun bakoitzeko. Gutxienez 8 lagin erabili genituen azterketa egiteko. Dentsitometriako emaitzak, esker hemisferioa eskumarekiko MBP pixel ratio bezala azaltzen dira (ezkerra/eskuina).

5.10 FLUXUZKO ZITOMETRIA BIDEZKO ZELULEN FUNTZIONALTASUNAREN AZTERKETA

Fluxuzko zitometria erreminta garrantzitsua izan zen zelulen analisirako. Zelulen ezaugarri fisikoen azterketarako ondorengo parametroak kontuan hartu ziren.

FALS (Forward Angel Light Scatter): Laserraren argia da zelulak beraiek islatzen dutenak. Tamaina, zelulen tamainarekin bat dator, izan ere, zelularen diametroarekin zuzenki erlazionatua dago eta banakako zelulak, zelular multzoetatik eta zelula hondakinetatik desberdintzea baimentzen du, gainera hildako eta bizirik dauden zelulak desberdintzea ahalbidetzen du.

ISS (Integrated Side Scatter):Laserraren argiarekiko 90ºko angelura zelulak islatzen duen argia da. Zitoplasmaren azterketa egitea baimentzen du, zitoplasma konplexua denean, islatzea handiagoa da desbideratzen den laserraren argiaren proportzioa handiagoa delako. Gainera hildako eta bizirik dauden zelulak desberdintzen ditu ere, hildako zelulak zitoplasma oso kondentsatuta eta pikortsuagoa dutelako eta seinale gehiago ematen dutelako.

5.10.1 In vivo suspentsio zelularraren lortzea

Zelularen funtzionaltasuna aztertzeko erabili diren animalien garunak, glukosalina daukan serum bitartez perfunditu ziren hodietan dagoen odola ateratzeko. Interesatzen zitzaigun zonaldea aukeratu eta zati txikitan moztu zen, izotzean eta glukosalinarekin. 70 µmtako poroak zituen iragazkietan ipini zen lortutako suspentsio zelularra eta 6 putzutako plaketan jarri ziren. Plaka hau 4ml kolagenasarekin 5 mg/ml Hanksen (HBSS; Sigma-Aldrich, St Louis, Mo, EEUU) diluitua inkubatu zen, mugimenduan, 37ºCko estufa baten 20 minutuz.

Iragazkiak zeuden plaka hauetatik suspentsio zelularra hartu zen eta iragazkiak bota egin ziren. Falcon hodietan ipini zen suspentsioa eta 5mlko Hanks soluzioa gehitu zitzaien eta 1640gtan zentrifugatu zen 5 minutuz. Zentrifugazioaren ostean, pelleta berriz 5mlko Hanks soluzioan suspenditu zen.

5.10.1.1 Mitokondrio mintzaren potentzialtasunaren azterketa

Azterketa hauek zitometria bidez egin ziren garunaren ehunaren suspentsio zelularretan. Mitokondrio mintzaren potentzialtasuna bizirik zeuden zeluletan ikertu zen Rhodamina 123 (Rh-123, Invitrogen, The Netherlands) fluorokromo lipofiliko kationikoaren barneraketa kuantifikatuz, hau mitokondrio barnean pilatzen da mintz potentzialaren intentsitatearekiko proportzionalki.

Mitokondrioaren aktibitatea barne eta kanpo mintzaren arteko potentzial diferentziaren ($\Delta\Psi m$) mantentzean islatzen da, ondorioz, mintzen aldeetako kargen distribuzio asimetrikoa beharrezkoa da ATParen sintesirako. Mintzeko $\Delta\Psi$ neurtzeak, mitokondrioaren aktibitate metabolikoa neurtzea baimentzen digu, izan ere, honen aldaketan, hau da, mitokondrio mintzaren potentzial galera mitokondrio bideko apoptosi prozesuan bereizgarria da.

Suspentsio zelularra (650µl, $1x10^6$ zelula/ml) 37ºCko estufan inkubatu ziren 30 minutuz Rho123 (4 µl 100ml Hanksen) soluzioan. Denbora honen ostean zelulak bi aldiz garbitu ziren 5 minutuko 1640g bidezko zentrifugazio bidez eta ondoren berriz inkubatu ziren 30 minutuz 37ºCko estufan. Zelulak berriz garbitu ondoren (5min 1640g) zitometrora eraman ziren analisia egiteko.

5.10.1.2 Erradikal superoxidoen produkzioa

Erradikal askeen analisirako oxidazioari sentikorra den fluorokromo bat erabili genuen, flureszeinako diazetatoa (2,7-dichlorodihydrofluorescein diacetate, DCFH-DA, Molecular Probes, Herbehereak). Erradikal-DArekin konfigurazioak zitoplasmara pasiboki sartzea ahalbidetzen du. Barnean egonik, diazetato taldeak barne zelulako esterasa bitartez askatzen dira, DCFH sortuz, konposatu polarra zelula barnean bahituta gelditzen dena. DCFH molekula fluorescente bilakatu daiteke, 2,7 diklorofluoreszeina (DCF) hidrogenioiak galtzen dituenean zelulan dagoen oxigeno peroxidoaren (H_2O_2) akzioaren eraginez.

Suspentsio zelularra (650 µl, 1 x10^6 zelula/ml gutxi gorabehera) 30 minutuz inkubatu zen Hanksen zegoen 10 µM DCFH-DArekin. Ondoren, bi garbiketa egin ziren (bakoitza 5minutukoa eta zentrifugazioa 1640g), 350µl Hanksen berriz suspenditu zen eta fluxuzko zitometriara eraman zen analisirako.

5.10.2 Suspentsio zelular fizatuen lortzea

In vivo egindako analisien ostean, soberan gelditu zitzaigun suspentsio zelularra, Hanksen suspendituta zegoena 70ºko alkoholarekin fizatu zen, 2ml alkohol botaz eta 4ºCtan gordez.

5.10.2.1 Mitokondrio mintzaren integritatearen kuantifikazioa

10-N-nonyl-acridin orange (NAO) (NAO, Invitrogen, The Netherlands) fluorokromoa kardiolipinari selektiboki lotzen zaio, mitokondrioaren barne mintzean espezifikoki aurkitzen den fosfolipidoa, era honetan zelularen mitokondrioaren egoera zein den azter daitekeelarik.

Honetarako suspentsio zelularreko (650 µl, 1 x10^6 zelula/ml gutxi gorabehera) bi aldiz garbitu ziren (zentrifugazioz, 1640g 5minutuz bakoitza) eta pelleta NAO (10^{-2} M)ekin markatu zen 30 minutuz Hanks medioan eta 37ºCko estufan. Gehiegizko NAO kantitatea kendu zen aurrerago bezala egindako garbiketen bitartez eta igortzen duen fluoreszentzia neurtu genuen fluxuzko zitometro bidez.

5.10.3 Funtzionaltasunaren azterketan erlazionaturik dauden jatorri ezberdinen zelulen identifikazioa

Azterketa honetarako 10.2 puntuan azaldu dugun eran fizaturiko zelulen suspentsioak erabili ziren. Astrozito eta neuronen identifikazioa kaltea gertatu eta 24 ordutara, fluxuzko zitetria bidez egin zen eta baita sinaptofisinarekiko positibo ziren zelulen kuantifikazioa.

Honetarako inmunofluoreszentzia protokolo komun bat erabili zen. Neuronen azterketarako, markatzaile espezifiko bat erabili zen, NeuN (Millipore, Massachussetts, EEUU), astrozitoak aztertzeko, GFAP (DAKO, Glostru, Denmark) markatzailea eta sinapsi bidezko loturak aztertzeko Synaptophisyn (Syp, Sigma-Aldrich, St Louis, Mo, EEUU) proteina markatzaileren bidez.

Antigorputz primarioa	kontzentrazioa primarioarena	Antigorputz sekundarioa	kontzentrazioa sekundarioarena
Mouse Anti-NeuN	1:100	Anti-mouse IgG Alexa fluor 488	1:500
Rabbit Anti-GFAP	1:500	Anti-rabbit IgG Alexa fluor 488	1:500
Mouse Anti -Syp	1:500	Anti-mouse IgG Alexa fluor 488	1:500

Zelulak (10^6 zelula/ml) bi aldiz garbitu ziren PBSarekin eta %1ean zegoen tween20an inkubatu ziren 10 minutuz, mintza iragazkortzeko. Ondoren, 5 minutuko zentrifugazio 16450g bidezko garbiketaren ostean, %0,5ean zegoen PBS-BSA bidez blokeatu zen bi aldiz bakoitza bost minutuz. Pelletak berriro 200 µl %0,5ean zegoen PBS-BSA Tween20an suspenditu ziren antigorputz primarioarekin eta 4ºCtan mantendu ziren gau osoan zehar.

Hurrengo egunean, antigorputz primarioarekin egindako inkubazioaren ostean, bi garbiketa egin ziren %0,5ean zegoen PBS-BSAn 5 minutuz bakoitza eta antigorputz sekundarioan inkubatu ziren %0,5 PBS-BSArekin konjugaturik giroko tenperaturan ordu

batez. Ondoren suspentsioa PBSarekin garbitu zen eta 400µl PBS bota zitzaion fluxuzko zitometriara eraman aurretik.

Lotura ez espezifikoa, zelulak fluorokromoari lotuta zegoen antigorputz sekundarioarekin soilik inkubatuz neurtu zen. Eta lagin zuria zelulak soilik izanik.

5.11 MAILA MOLEKULARREAN EGINDAKO AZTERKETA: qPCR

Analisi molekularra egiteko, animalia hil zen kaltea egin eta denbora ezberdinetan, 0,3 eta 12 ordutara, gene ezberdinen adierazpenaren aldaketa ikusteko.

5.11.1 RNA erauzketa

Animalia glukosadun serumarekin perfunditu ostean eta garuna atera ostean, mesentzefaloa eppendorf hodi baten jaso zen eta RNA erauzi genuen PureLink® RNA Mini Kit (life technologies, 5791 Van Allen Way | Carlsbad, CA 92008 USA) kitaren bitartez.

Laginari 600 µl lisis bufferra bota zitzaion 2-merkaptoetanolarekin eta “rotor-stator homogenizador” homogeneizatu ondoren 45 segundoz abiadura maximora, laginak 5 minutuz zentrifugatu ziren 2600gtara giroko tenperaturan.Ondoren lagina fizatu, garbitu eta eluzitu egin zen, fizatu %70eko alkoholaren bitartez, garbiketa kitean zetorren Wash Buffer I eta II bitartez eta eluzioa RNasa gabeko 90 µl urez minutu bat inkubatuz giroko tenperaturan. Prozesu guzti hau erauzketa zutabe bidez egin zen. Erauzketa egin ondoren, RNAren kantitate eta kalitatea neurtu zen Elisa plakak irakurtzen dituen aparatu baten bidez, zeinek Nanodrop (Multi-Mode Microplate Reader, Biotek Instruments, INC. Winooski,Vermont, USA) sistema gehituta duen.

5.11.2 RT (Reverse transcription)

RTa Fluidigm ® Reverse Transcription Master Mix (Fluidigm Europe B.V. Herikerbergweg, 238 1101 CM Amsterdam Zuidoost, The Netherlands) cDNA preparazio baten bidez egin zen.

Lehen primer mixa prestatu zen RTrako Master mixarekin ondorengo erara:

Primer Mix

	OSAGAIAK	BOLUMENA/ERREAKZIOA (µL)
Pre-Mix	RT Master Mix	1
	RNasa free water	3
	RNA (2,5 ng/µl-250 ng/µl)	1
	BOLUMEN TOTALA	5

Zentrifugatu ostean termozikladorean ipini ziren laginak erreakzioa gerta zedin:

Egoera	**Mantentzea**	**Mantentzea**	**Mantentzea**	**Mantentzea**
Tenperatura	25 ºC	42 ºC	85 ºC	4 ºC
Denbora	5 min	30 min	5 min	

Erreakzioaren ostean laginak -20ºCtara izoztu ziren ondoren preamplifikatuak izateko.

5.11.3 Anplifikazioa

Primer mix (10x)aren 500 nM prestatu ziren ondorengo erara:

	Osagaiak	Bolumena / Erreakzioa (µl)
Pre-Mix	2 x TaqMan PreAmp Master Mix	2,5
	500nM (10x) primer mixture	0,5
	Ura	0,75
	cDNA	1,25
	Totala	5

Zentrifugatu ostean termozikladorean anplifikatu ziren laginak ondorengo zikloak eraginez:

Egoera	Mantentzea	14 ziklo		Mantentzea
Tenperatura (°C)	95	95	60	4
Denbora (min)	10	15	4	∞

Laginak -20ºCtan mantendu ziren geneen analisira arte.

5.11.4 Interesatzen zaizkigun geneen azterketarako txiparen prestakuntza

Laginen Pre-Mixa prestatzea

	OSAGAIAK	Bolumen/erreakzioa (µl)
Laginaren Pre-Mix	2x SsoFast Eva Green Supermix with low ROS	3
	20x DNA Binding dye sample loading reagent	0,3
	STA	2,7
	Totala	6

Laginaren Pre-Mixa prestatu ondoren, zentrifugatu egin ziren eta 4ºCtan gorde plakan kargatu aurretik.

Entseguaren Mix soluzioa

Osagaiak	Bolumen /Erreakzioa (µl)
2x entsegu eragilea	2,5
1x DNA suspentsio bufferra	2,25
100µM del forward and reverse primer	0,25
Totala	5

Entseguaren Mixa zentrifugatu genuen eta txipa kargatu genuen bere analisirako.

Dynamic Array IFC optimizatu eta kargatu

Txiparen pilaketa gune bakoitzean kontrol izango den likidoa ipini genuen, babespen film urdina kendu genuen txiparen hondotik eta txipa IFCaren MX kontrolatzaile gunean ipini genuen, 96.96rentzat eta Prime (136x) script aktibatu 96.96 Dynamic Array IFCarentzat.

Ondoren, 5 µl pipeteatu genituen entsegu bakoitzetik eta lagin bakoitzetik beste 5 µl txiparen putzu bakoitzean eta txipa IFC kontrolatzailean ipini genuen berriz. IFC kontrolatzailearen softwarea erabiliz, Load Mix (136x) scripta martxan ipini genuen 96.96entzako, laginak eta entseguak kargatzeko eta Load Mix scripta amaitzerakoan, txipa kendu genuen IFC kontrolatzailetik eta ondoren laginen analisia egiteko txipa jarri genuen martxan.

B) TXERRIAK

5.12 PROZESU KIRURGIKOA

Animaliak (1-3 egunekoak) Ketaminarekin, atropinarekin eta diazepanarekin (15mg/kg, 0,05mg/kg y 2mg/kg, hurrenez hurren) lokartu ziren. Gero, elektrokardiograma, pulsioximetria transkutaneoa eta gorputzeko tenperatura monitorizatu zen (Philips IntelliVue MP70, Philips Healthcare, Andover, MA, EEUU) eta zain barneko kateter bat ipini zitzaion de 24G (Introcan Safety®, B-Braun, Melsungen, Alemania) belarriko zain periferiko batean fentanilo eta propofol (0,005 mg/kg y 1,2 mg/kg hurrenez hurren) anestesikoa ipintzeko. Ondoren animaliak trakeotomizatu edo tutu endotrakeal bidez intubatu ziren 4 I.D eta presio positibozko aireztamen mekanikoz mantendu ziren (Bird Vip Gold Ventilator, Bird Products Corporation, Palm Springs, CA, EEUU) normoxia egoera mantenduz (Arteriako oxigenoaren presio partziala (PaO2): 80 - 100 mmHg; Arteriako karbono dioxidoaren presio partziala (PaCO2): 35 - 45 mmHg; pH: 7,35 - 7,45).

Aireztapen mekanikoa hasi eta minaren sintomarik ez zegoela baieztatu ondoren (hanketako erreflexua, erreflexu palpebrala, frekuentzia kardiakoaren igotzea…) muskulu paralisiari ekin genion bekuronio bromuroaren bidez (1,5 mg/Kg). Anestesia, analgesia eta muskulu paralisia mantendu egin ziren porpofol, midozolan, fentanilo eta bekuroni bromuroaren jarraikako difuzioz (3 mg/Kg/h, 0,5 mg/Kg/h, 0,004 mg/Kg/h y 3 mg/Kg/h hurrenez hurren).Berriz emate fluidoen bolumena 10 mL/Kg/hkoa izan zen.

5.12.1 Monitorizazioa

Animali guztietan ondorengo aldagaiak monitorizatu ziren:

Garuneko oxigenazioa

Garuneko oxigenazioa infragorriaren hurbileko espectroskopia bidez neurtu zen (NIRS) (NIRO 200, Hamamatsu Photonics, Hamamatsu, Japon). Teknika honek, karbonoari, nitrogenoari eta oxigenoari loturiko hidrogenoen taldekatze funtzionalak dituzten

konposatuen analisi kuantitatiboa egitea ahalbidetzen du, era honetan, ehunetako oxigeno portzentajea zein den kalkulatzen baimenduz.

Tresnak infragorriaren hurbileko argia era seguruan erabiltzen du ehuneko oxigenazio indizea neurtzeko (TOI), oxigenoaren saturazio mailatik adierazgarri dena. Gainera, hemoglobina ehunaren indizea normalizatzen du nTHI eta era honetan hemoglobina totalaren aldaketa ikusi ahal dugu esperimentu osoan zehar, eta baita hemoglobina oxigenatu (ΔO2Hb), hemoglobina desoxigenatu (ΔHHb) eta hemoglobina totalaren (ΔcHb) aldaketak, denbora errealean.

Elektroentzefalografia anplifikatua

Elektroentzefalografia teknika neurofisiologiko bat da garunak garun kortexeko zelulen bidez espontaneoki eratzen dituen aktibitate elektrikoa neurtzen duena eta buru-azalean kokatutako elektrodoen bitartez neurtzen dena. Neonatologian erabiltzen diren tekniketatik objektibo den gutxiengo bat da eta garatu gabe dagoen garun kortex eta bere konexioen integritate funtzionala aztertzen du. Honela, herraminta garrantzitsu bat bilakatu da garunaren garapeneko ezagutza klinikorako eta baita garunek ezgaitasun, garuneko malformazio eta hemorragien, adibidez entzefalopatia epileptikoak, ezjakintasunak garbitzeko. Gure kasuan, bi kanaletako elektroentzefalografoa erabiltzeak (Brainz BRM2, BrainZ Instruments. Auckland, Nueva Zelanda), garunareko bi hemisferioen aktibitatea denbora errealean neurtzea baimendu digu.

5.12.2 Kalte hipoxiko iskemikoaren indukzioa

Kalte hipoxiko iskemikoaren indukzioa bi karotiden itzulgarria den oklusio bidez egin zen eta oxigenoaren arnas-hartze frakzioaren murrizpenaren bitartez, 20 minutuz %8-10era. Bi karotiden oklusioa traumatiko ez diren oklusore baskularren bidez egin zen (Vasseloops™, Medica Europa BV, Holanda), odol fluxuaren desagertzea baieztatuz karotidan kokatutako sunda doppler bidez. Arnas-hartze frakzioa gutxitzeko, arnaste sistema aldatu zen, medizinako aire ematea nitrogeno bidez aldatuz eta oximetro bidez oxigeno kontzentrazioak berriz doituz (Ohmeda 5120, Datex Ohmeda, General

Electric Company, NY, EEUU). Denbora hau igaro ostean, karotiden fluxua eta arnas-hartze oxigenazio frakzioak berriz ere egoera normalera itzuli ziren eta arnas-hartzea analgesia eta anestesia bidez kaltearen ostean 6 orduz mantendu zen.

5.13 AZTERKETA ELEKTROFISIOLOGIKOA

Arratoiekin egin genuen bezala, GSI Audera aparatua erabili genuen entzumeneko potentzial ebokatuak neurtzeko. Orratzak arratoien azalpenean 5. Puntuan azaldu genuen bezala ipini ziren eta eskuratzearen ezaugarri teknikoak ere berdinak izan ziren: 10msko eskaneatzea, 150Hzko iragazkiarekin frekuentzia baxuetarako eta 3000HZko iragazkiarekin altuetarako; sentsibilitatea 10mcv/div. Estimulua click bitartez egin zen eta 1024 eta 2048 bitarteko erantzunen media egin zen. Azterketa 75dB HLko intentsitatedun click bitartez egin zen. Baina kasu honetan dB ak hogeinaka gutxitzen joan ginen (55,35 eta 15 dB) entzumen ataria identifikatu ahal izateko.
Neurketa 3 aldiz errepikatu zen belarri bakoitzean ondorengo egoeretan: 1) kontrol (HI aurretik), 2) animaliaren egonkortze fasea (karotiden oklusio aurretik), 3) 30minutuko HI ostean, 4) 30 minuturo kaltearen hurrengo 6 orduetan zehar.
Analizaturiko parametroak: pasatako denbora (ms) erantzuna jaso arte, intentsitate (μV) eta tontorren arteko denbora tartea (ms) izan ziren.

C) ANALISI ESTATISTIKOA

Estatistika GraphPad prism 5 programa bidez egin zen sorftwararen 5.01 bertsioarekin (GraphPad Software, Inc. CA, USA). Emaitzak media ± desbideratze estandar bezala aurkezten dira kasu guztietan eta taldeen arteko diferentziak Studenten T bitartez egin genituen edo bariantza faktore bakarreko analisia talde guztien artean ANOVA bitartez eta Bonferroni-Dunn bitartez zuzenketak.

EMAITZAK ETA EZTABAIDA

6.1 LIBURU KAPITULUA

MICROSCOPY: ADVANCES IN SCIENTIFIC RESEARCH AND EDUCATION
A. MÉNDEZ-VILAS

REVISION BOOK CHAPTER

Correlation between auditory threshold and the auditory brainstem response (ABR) in rats. A possibility for the experimental study of the auditory impairments.

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Keywords

Auditory impairments, rat, auditory system myelination, auditory brainstem response, perinatal asphyxia and audition.

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Abstract

Auditory impairments are considered to be one of the most frequent neurosensorial problems in human. The normal development and myelination of the auditory system can be impaired by certain pathological conditions such as viral infections, hyperbilirubinemia, meningitis, fetal distress and perinatal asphyxia. The early detection of this hearing loss can be diagnostic with sensitive techniques, such as the auditory brainstem response (ABR). These techniques are the relatively early component of the auditory evoked potential that reflects the electrophysiological activity of a large number of neurons in the brainstem auditory pathway after an acoustic stimulation. Normal ABR requires the integrity of the anatomical system, composed by a set of neurons, their axons, the synapses between them and the neurons on which each ABR terminate. Disruption of any portion of the system will alter the amplitude or the latency.

INTRODUCTION

Morphology of the Auditory System

The ear is a sensorial organ divided in three parts; the external, the middle and the inner part, and is shared with the auditory system (in charge of sound perception) and the vestibular system (in charge of the sense of balance and equilibrium) (Ross, 2007).

General considerations

The function of the external auditory system is to convey the sound to the tympanic membrane which is the external part of the middle ear. There, the sound waves are turned into mechanic vibrations by the small bones (malleus, incus and stapes) and transmitted to the inner ear (Young & Heath, 2000). The inner ear is composed of two labyrinth cavities, **the bony labyrinth**, an irregular, hollowed out cavity located within the petrous portion of the temporal bone and **the membranous labyrinth** which is inside the bony labyrinth.

The bony labyrinth is composed of three communicated spaces inside the temporal bone;

c) The vestibule, central portion of the bony labyrinth that contains the saccule and the utricle of the membranous labyrinth.
d) The semicircular canals, tubular spaces arranged perpendicularly and in touch with the vestibule.
e) The cochlea, next to the vestibule in the other side of the semicircular canals (Stevens & Lowe, 2006).

The membranous labyrinth is divided in the cochlear labyrinth and the vestibular labyrinth.

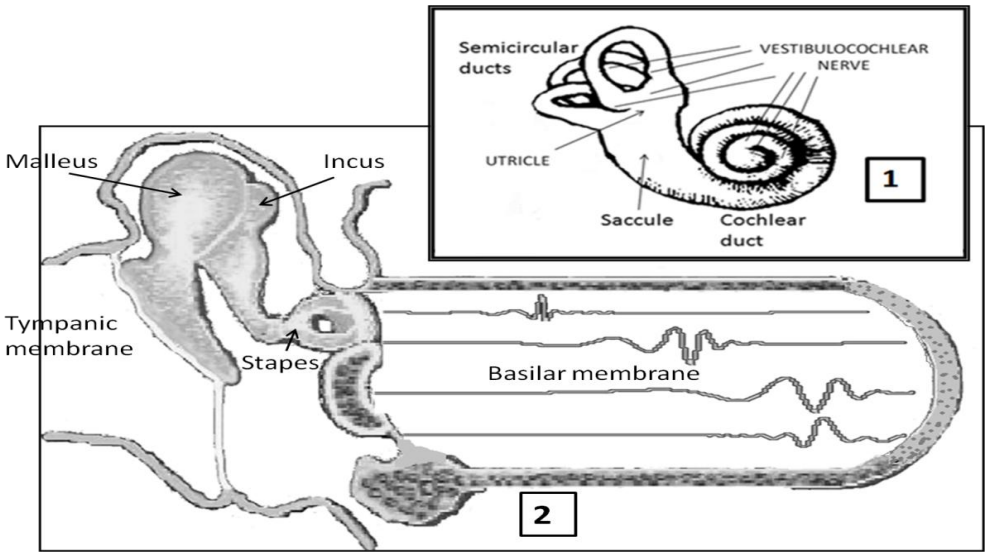

Figure1: 1. Illustration of the bony labyrinth of the inner ear. The stapes is located in the central portion of this labyrinth. **2.** Illustration with exaggerated amplitudes of the basilar membrane response to a tone. The high frequency sounds selectively vibrate the basilar membrane of the inner ear near the entrance port (the oval window) and lower frequencies travel further along the membrane before causing appreciable excitation of the membrane.

These regions have specialized areas with ciliated neurosensorial cells and supporting cells, implicated in the hearing or in the equilibrium functionality.

The sensorial vestibular or cochlear labyrinth so they transform the mechanic energy into neurology impulse that is transmitted by the vestibulocochlear pathway nerve to the brain. The hair cells derive their name from the organized bundle of rigid projections at their apical surface. This surface holds a hair bundle that is formed of rows of stereocilia called sensory hairs that increase in height in a particular direction across the bundle (Ross & Pawlina, 2007).

The cochlea

The cochlea is the auditory part of the ear and is divided into three parallel compartments: Scala Media (endolymph-containing space) and Scala Vestibuli and Scala Tympani (perilymph-containing spaces).

The organ of Corti is located in the Scala Media and it is composed of different cell types; hair cells (sensitive cells), phalangeal cells and pillar cells (both of them supporting cells) (Ross & Pawlina, 2011).

The sound pressure produces the tympani vibration and these vibrations are transmitted to the oval window by the small bones. When the sound waves are transmitted from the tympani to the oval window, the middle ear amplify the sound before entering to the inner ear. The vibration pass into the fluid inside the cochlear and here they shake thousands of tiny hair cells. The hair cells are attached to the basilar membrane and to the tectorial membrane that vibrate when they receive the sound. These movements produce the activation of mechanoelectric transducer channels that are located in the extreme of the stereocilia producing cell depolarization and generating membrane potentials that are transmitted to the spiral ganglion (band of auditory nerve cell bodies) from the auditory nerve fibbers.

The frequency processing occurs in the cochlea that has a tonotopic distribution, so the base of the cochlea processes high frequencies and the apex processes low frequencies (Young & Heath, 2000).

The Statoacoustic nerve (Cranial nerve VIII)

The statoacoustic nerve is divided in a vestibular portion that carries the sensorial receptors associated to the vestibular labyrinth (vestibular nerve) and a cochlear or auditory portion (cochlear nerve) that carries the sensorial receptors associated to the cochlear labyrinth.

Vestibular nerve is formed by bipolar neurons where their somas synapse with the base of the vestibular sensorial cells and their axons go into the brain stem ending in the vestibular nuclei.

Cochlear nerve meanwhile is also formed by bipolar neurons, but their somas synapse with the ganglion of Corti, and their axons go into the

brain stem and terminate in the cochlear nuclei of the medulla oblongata. Nerve fibbers that come from these nuclei reach the thalamus and from there continue to the auditory cortex of the temporal lobe.

The Cochlear Nuclei

In the mammalian brain, the sound impulse coming from the fibers of the cochlear nerve arrives to the brainstem, where we find an interconnected chain of nuclei dedicated to process the acoustic signal (Heimer, 1968).

There are three major nuclei:

f) The cochlear nuclei located in the brainstem where fibers come from the cochlear nerve.
g) The superior olivary complex located in the ventrolateral portion of the bulge.
h) Inferior colliculus (IC).

The first ones are divided in two groups; the dorsal (DCN) and the ventral (VCN) **Cochlear Nuclei** (Fekete et al., 1984; Arnesen & Osen, 1968). In the VCN it has been described 5 types of neurons: spherical, globular, starry, octopus and granular cells (Osen, 1969). The most important neurons of the DCN are the pyramidal (Blackstad et al., 1984) and the giant neurons [Zhang & Oertel, 1993; Kane et al., 1981].

The **Superior Olivary Complex (SOC)** is a group of neurons located in the lower brainstem. The nuclei of the superior olive are important for comparing sound at the two ears, in order to localize the sources of sounds in the environment. The human SOC includes two principal nuclei: the medial superior olive and the lateral superior olives (Kulesza, 2008).

The **Inferior Colliculus** is an anatomical structure of the midbrain that receives direct input from the cochlear nuclei as well as from the nuclei of the superior olive and from the lateral lemniscus. The inferior colliculus is very important for selecting specific features of sound and for activating pathways that provide output to motor systems.

Auditory Physiology

Most of the sounds produced in the nature, including the human voice, are a combination of waves of different frequency and amplitude. The hearing field in human is between 16 and 20000 Hz below this range the auditory system cannot identify the sounds, showed in Fig.2.

The conversational zone is the one frequently investigated because is the most damage in acoustic injuries. To identify these injuries it is necessary to determine the auditory threshold with a complete clinic diagnose that include the audiology probe.

The child audiology probes are divided in two groups: the subjective and the objective probes. The first ones need the child cooperation to observe a behavior change or to respond to sound stimuli. The objective probe doesn't need the child cooperation and between these kinds

of probes we can find, the otoacoustic emissions (OAE) and the brainstem auditory evocated potentials (ABR) (Bogacz et al., 1984).

Auditory brainstem response

The auditory brainstem responses (ABR) are the relatively early component of the auditory evoked potential that reflects the electrophysiological activity of a large number of neurons in the brainstem auditory pathway after an acoustic stimulation (Wilkinson & Jiang, 2006; Tomimatsu et al., 2002). The submicrovolt deflections in ABR occur in the first 10 ms after the stimuli and are identified as waves I to V (Jewett & Williston, 1971).

The ABR waves I and II are generated in the extracranial and intracranial portions of the VIIIth nerve, respectively. Subsequent waves III and V are generated in auditory centers at gradually higher levels of the pathway, with partially overlapping contributions to individual waves.

Wave III is derived from the cochlear nucleus; wave IV is generated in the superior olivary complex; and wave V is generated in the region of the lateral lemniscus and inferior colliculus. The most reproducible and easily definable components are waves I, III and V, showed in Fig. 3.

Auditory Threshold alteration

It has been well documented that the ABR changes with neurological maturation and to vary with functional integrity of the brainstem.

Animal experiments indicate that a normal ABR requires the integrity of the anatomical system composed by a set of neurons, their axons, the synapses between them, and the ending effectors on which each of the ABR components terminate. Disruption of any portion of the system will alter the amplitude and/or the latency of that component (Jiang et al., 2006).

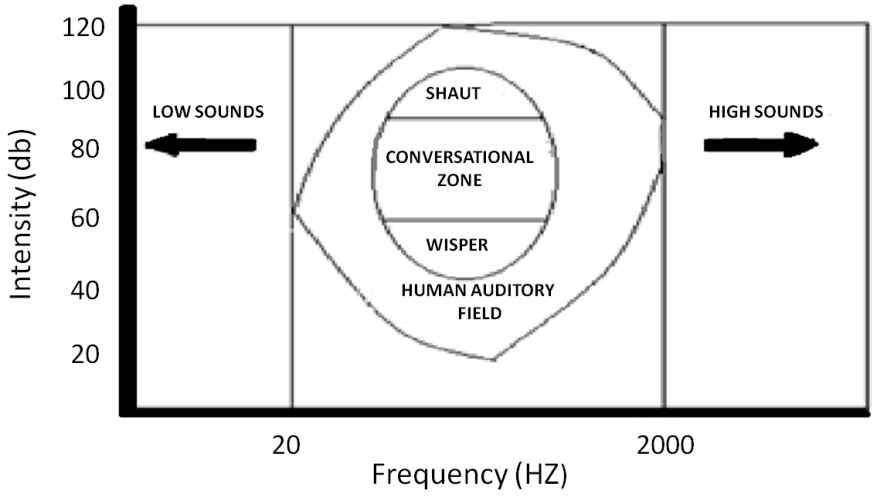

Figure. 2Illustration of the human auditory field. Between 20 Hz and 20 kHz, the threshold of our sensitivity is different and the conversation area demonstrates the range of sounds most commonly used in human voice perception. If a hearing loss affects this area, conversation is not possible. Modified figure of Rémy Pujol (Pujol & Puel, 1999).

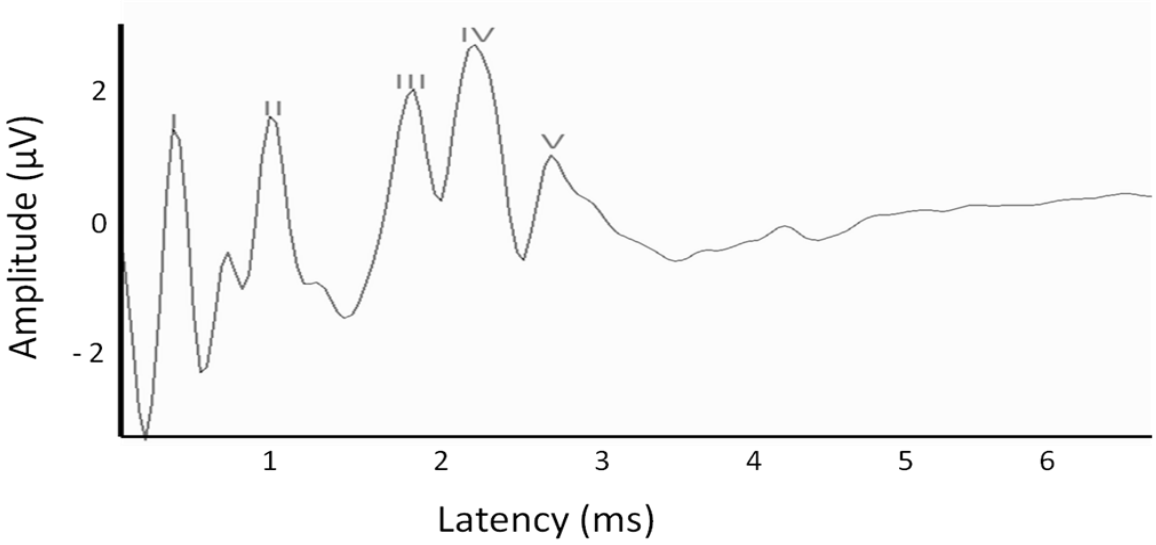

Figure. 3 A representative figure of the auditory threshold of a common 7-day-Sprague-Dawley rat. We can distinguish five waves with different amplitude and latency that correspond to the response of the brainstem anatomical structures to a stimulus**.**

A prolonged latency of wave I is indicative of an abnormality in the peripheral auditory structures, whereas intrinsic brainstem dysfunction is indexed by the absence of components occurring after wave I or by prolongation of the latencies between these components (Simpson et al., 1985). An increased I e V interval is the most frequent abnormal finding in neurological diseases (Wilkinson & Jiang, 2006).

Intensity

Waves intensity and latency are inversely proportional, the more intensity the less latency and backward. However, it is different in the case of the intensity and potential that are proportional, the more intensity the more potential.

Age

The ABR can be register in the first moments of life and there are morphological differences

between the infants and the adult brainstem responses. In the new born child the wave I has mayor amplitude and latency comparing to the adult wave. This mayor latency is because of the incomplete maturation, menor mielinization, of the low frequencies in the cochlea and/or the transmission of the ciliated cells and the auditory nerve fibers (Hecox & Galambos, 1974).

Sex

Women present a lower latency in their evocated responses comparing to men (Jacobson et al., 1980), and the amplitude of I and V waves are increased (Kjaer, 1979). Some studies suggest that these results may be because women have a lower cranial perimeter than men, so that they can have a shorter auditory pathway.

Temperature

Stockar et al., in 1978 studied the influence of the temperature in the brainstem potentials and they observed that the latency of the waves were decreased when increase the temperature; and backwards.

Drugs

It is proved that ingest of alcohol and marijuana disturb the cortical potentials but not the brainstem ones as well as using barbiturates and sedative ones (Lewis et al., 1978). Brainstem response is disturbed with cholinergic drugs that alter the neuronal transmission.

1. Auditory Brainstem Response (ABR) and Perinatal Asphyxia

Perinatal asphyxia is a major problem often leading to neurodevelopment deficits or disabilities, such as learning difficulties, language and attention deficit, hyperactivity disorders and cerebral palsy in newborn infants (Anand et al., 1991). It is also a notable risk factor for hearing impairment that affected neonates and can also affect the brainstem (Misra et al., 1997; Chayasirisobhon et al., 1996).

The brainstem auditory pathway has been shown to be very sensitive to low blood oxygen concentrations with the consequent damage in the Organ of Corti or loss of brainstem neurons, such as cochlear nuclei or inferior colliculi neurons (Jiang et al., 2004; Freeman, 1991). This neuronal damage may interfere with nerve conduction and synaptic transmission of the brain producing an acute impairment (Jiang et al., 2000).

After hypoxic-ischemic (HI) injury, diverse mechanisms produce cell damage (Goñi-de-Cerio et al., 2007), perinatal brain is especially susceptible to energy failure (decrease of ATP levels), cellular excitotoxicity and oxidative stress which can in turn promote cellular death (Alvarez-Diaz et al., 2007; Vexler & Ferriero, 2001), as we can see in Fig 4. Neurons are the

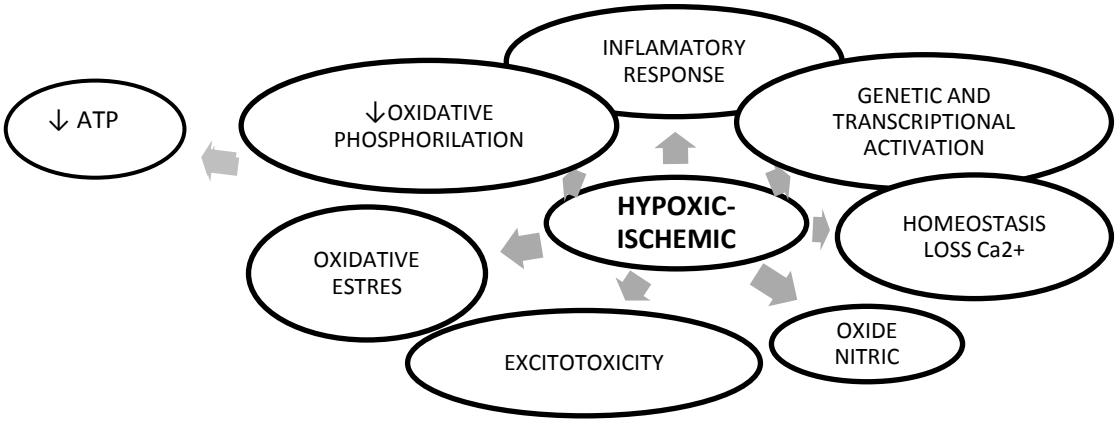

Fig.4: Effect of hypoxic ischemic injury in the metabolism of cells. Decrease of ATP lead to a cascade mechanism including membrane depolarization, increases in intracellular Ca 2+, accumulation of excitotoxic aminoacid, inflammation, generation of free radicals, activation of caspases and of cytokines, and activation of phospholipases, proteases and nucleases.

most sensitive cells to the lack of oxygen, which also show a selective vulnerability (Hilario et al., 2005; Johnston et al., 1998). The HI event disturbs the metabolism of neurons and depresses the synapse between cells (Jiang, 2008), being this function important for the synthesis, release and uptake of neurotransmitters during the neural development. Glutamate concentration increases in the presynaptic site resulting in an irreversible neuronal injury (Jiang et al., 2004; Johnston et al., 2001; Bemveniste et al., 1984).

The ABR is an important diagnosis method to evaluate the brainstem functionality and is used to detect HI auditory impairments. In the ABR, latency of the wave V and of the I–V interval are the two most widely used parameters that reflect neuronal conduction, brainstem´s conduction time, related to myelination and synaptic function; both in the brainstem or in the central auditory pathway (Jiang et al., 2008). In the study of these brainstem responses after a perinatal asphyxia, some authors found abnormalities in their ABR comparing to healthy infants. These differences included: elevated response threshold, increase in the wave latencies, brainstem´s conduction time, and interpeak intervals, a reduction in the wave amplitudes and a decrease in the V/I amplitude ratio [Jiang et al., 2009; Wilkinson & Jiang, 2006; Hecox & Cone, 1981).

The studies concerning to the response from different anatomic structures to a specific sound have showed a significant increase both in the I–III and in the III–V intervals; although the increase in the III–V interval was slightly more significant (Jiang et al., 2010; Tomimatsu et al., 2003). It appears that the generators of wave V following perinatal HI may be particularly

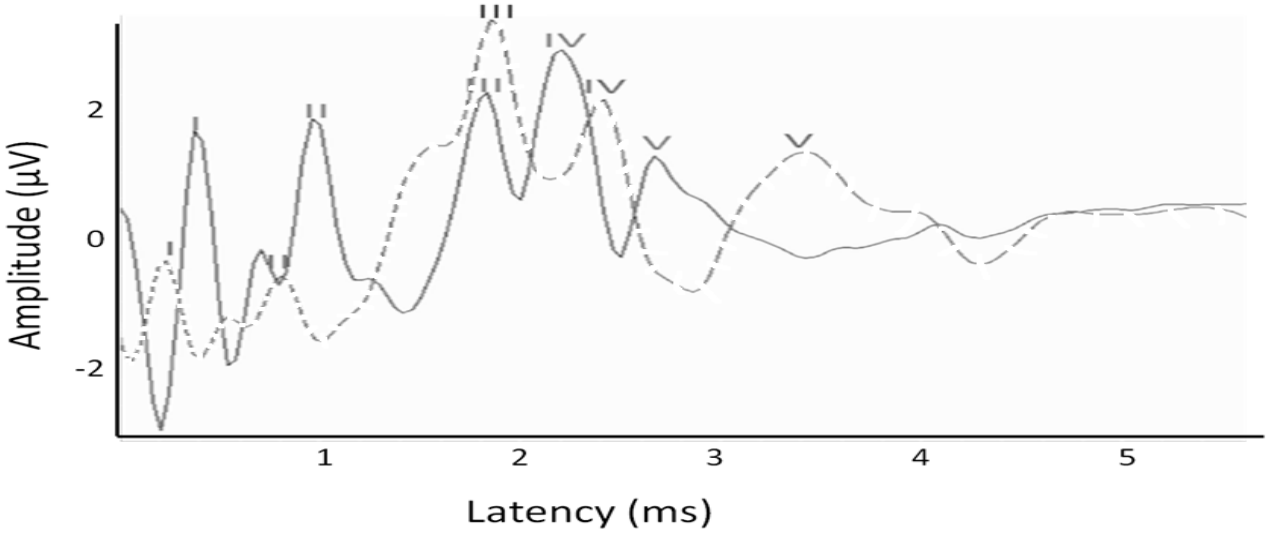

Fig. 5 Representative image of the auditory threshold of a control 7-day-Sprague-Dawley rat (continue line) and a rat with hypoxic ischemia injury (dash line). We can identify a similar pattern in both situations, but in the injured rat the waves are displaced in latency. The most delayed one in latency is the wave V that corresponds to the response of the inferior colliculi.

vulnerable to a physiological change probably attributed to generators lying primarily within the midbrain. So, the more central part (III–V interval) of the auditory pathway is more affected by the HI insult than the more peripheral part (I–III interval) of the pathway (Jiang et al., 2004). The progressive increase in ABR latencies and interpeak intervals, showed in Fig 5, with increasing click rate may be attributed to a cumulative decrease in the efficacy of synaptic transmission at high stimulus rates, resulting in prolonged synaptic delays along the brainstem auditory pathway (Jiang et al., 2010).

Amplitude reduction in the ABR reflects neuronal impairment and/or death in the brainstem following HI insult. This impairment results in fewer neurons contributing to ABR wave amplitudes and/or smaller contribution from each neuron (neural asynchrony) and a decrease in the membrane potential of neurons. The persistent amplitude reduction during the first month of life after perinatal asphyxia suggests that HI insult causes sustained neuronal impairment, which is unlikely to recover within relatively short period, or even death.

These electrophysiological findings are consistent with previous histopathological observations that perinatal asphyxia often causes discrete lesions in brainstem auditory nuclei.

3.1 Histological changes in brainstem after hypoxic-ischemia

Hipoxia- ischemia is an important injury that gives rise to damage in the inner ear and can develop many hearing disorders, such as sudden sensorineural hearing loss, presbyacusis and noise-induced hearing loss that are suspected to be related to alterations in blow flow (Mazurek et al., 2006).

There have been documented diverse studies related to the regional neuronal damage in the hippocampus, striatum and cortex after a perinatal asphyxia (Alonso-Alconada et al., 2012), but little attention has been given to the brainstem. The studies about the effect of HI injury in the cochlea, focused mostly in the susceptibility of the hair cells of the organ of Corti. According to these studies, hair cell loss will increase significantly if the cochlea is deficient in both oxygen and glucose (Gross et al., 2014).

Histopathological studies have provided evidence that brainstem of the human neonate is highly vulnerable to anoxia with a predominant damage effect on various brainstem nuclei and inferior colliculi (Misra et al., 1997), showed in Fig 6.

The decrease in ATP levels after perinatal asphyxia lead to a downstream cascade of pathological mechanisms that include; membrane depolarization, increases in intracellular Ca^{2+} accumulation of excitotoxic

amino acids, generation of free radicals, delivery of cytokines, inflammation, activation of phospholipases, proteases and nucleases and activation of caspases. These events lead to necrotic or apoptotic cell death in many brain regions affected. Injury induced between postnatal days 1-7 in the rat lead to white matter damage, and also to damage in the corpus callosum, cerebral cortex, hippocampus, striatum, globus pallidus, thalamus, and brainstem (McClure et al., 2006).

Tomimatsu et al., (2002), showed the presence of apoptotic cells in the ipsilateral inferior colliculus after HI injury, showed in Fig. 7. He also claimed that the increase of the latency in the wave V could be due to the ipsilateral lesions in the inferior colliculus ([Jewett & Williston, 1971).

CONCLUSION

The HI event produces several cellular mechanisms that contribute to cell damage or even cell death through apoptosis or necrosis depending on the severity of the damage, maturative state and region affected. One of the regions that can be affected by this injury is the brainstem, and in particular the inferior colliculus.

There is an electrophysiological change in the auditory threshold of HI rat with an increase of ABR latencies and an interpeak interval and also histological damages that confirm previous studies. These damages can contribute to hearing disorders so it can be an interest start point for the future assays in order to reduce neonatal hypoxic-ischemic induced hypoacusia.

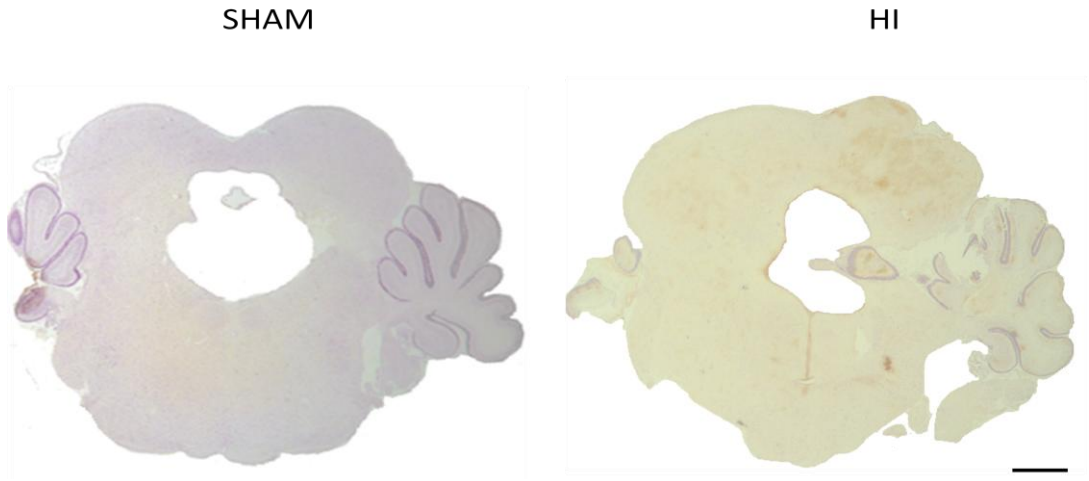

Fig.6 Representative image of hipoxiprobe staining in the brainstem of a control and HI Sprague-Dawley rat. Sample were obtained from P14 after ischemia (permanent left carotid occlusion) and hypoxia (reduction of 02 to 8%) by Rice & Vannucci (1981) method and the SHAM group were animals with no ischemic or hypoxic injury. The hypoxiprobe kit stained cell exposure to a reduction of the oxygen, so we observe in the HI group a different staining comparing to the SHAM group due to the hypoxic event. Besides, we observe a difference in the morphology of the brainstem in HI group comparing to the SHAM group owing to the ischemic event. Stereomicroscopical image (Bar=50µm).

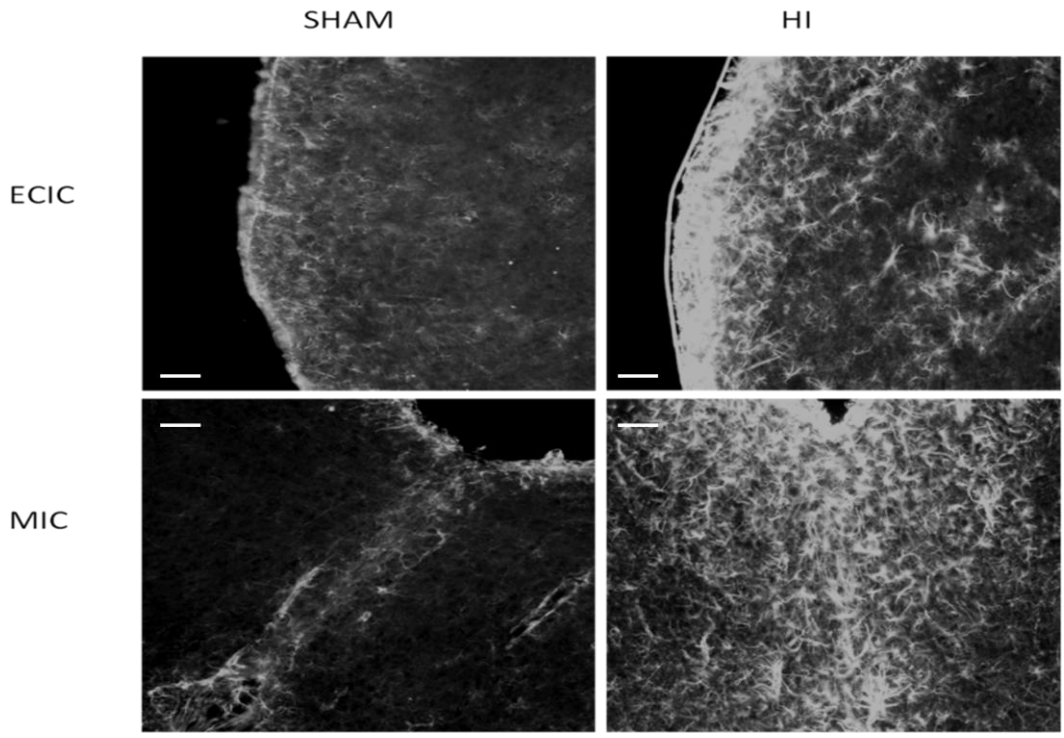

Fig. 7 Representative image of the Glial Fibrilary Acidic Protein (GFAP) inmunoreactivity in the external cortex of the inferior colliculus (ECIC) and in the middle of the inferior colliculus (MIC) in control an HI Sprague-Dawley rat. Samples were obtained from P14 after the HI event. Increase in fluorescence in the HI groups is due to more GFAP positive cells. As a consequence of high levels of extracellular glutamate and intracellular calcium astrocytes stimulate phosphorylation of GFAP and create new cytoplasmatic processes into the injured site creating a glial scar (Panickar & Norenberg, 2005; Sullivan et al., 2012). Florescence microscope image (Bar: 50µm).

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6.2 I. ARTIKULUA

BRAIN PATHOLOGY

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Antioxidant treatments recover the alteration of auditory evoked potentials and reduce morphological damage in the inferior colliculus after perinatal asphyxia in rat

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Keywords

auditory brainstem response (ABR), auditory pathway, hypoxic ischemic injury, inferior colliculus, morphological brainstem damage, neuronal injury, rat, white matter injury.

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Abstract

Maturation of the auditory pathway is dependent upon central nervous system myelination and it can be affected by pathologies such as neonatal hypoxic-ischemic encephalopathy. Our aim was to evaluate the functional integrity of the auditory pathway and to visualize, by histological and cellular methods, the damage to the brainstem using a neonatal rat model of hypoxic-ischemic brain injury. To carry out this evaluation, we studied the effects of the administration of the antioxidants nicotine, melatonin, resveratrol and docosahexaenoic acid after hypoxia-ischemia on the inferior colliculus and on the auditory pathway. We found that the integrity of the auditory pathway in the brainstem was altered as a consequence of the hypoxic ischemic insult. Thus, the auditory brainstem response (ABR) showed increased I-V and III-V wave latencies. At a histological level, hypoxia-ischemia altered the morphology of the inferior colliculus: neurons had altered axonal prolongations and condensed cytoplasm; astrocytes were reactive with increased glial fibrillary acidic protein expression, and oligodendrocytes exhibited reduced myelin basic protein expression. At molecular level, the mitochondria membrane potential and integrity was altered during the first hours after the HI and ROS activity is increased 12h after the injury in the brainstem. Following antioxidant treatment, ABR interpeak latency intervals were restored and the body and brain weight was recovered as well as the morphology of the inferior colliculus that was similar to the control group. At molecular level the antioxidant have different responses, being melatonin the most effective one against ROS activation after the damage. Our results support the hypothesis that antioxidant treatments have a protective effect on the functional changes of the auditory pathway and on the morphological damage which occurs after hypoxic ischemic insult.

INTRODUCTION

Despite improvements in neonatology, perinatal hypoxic-ischemic (HI) encephalopathy remains one of the main causes of disabilities in term-born infants (Pin, et al., 2009). This specific pathology underlies many neurological disorders such as learning difficulties, language and attention deficit, hyperactivity disorders and cerebral palsy (Volpe, 2008). Moreover, it is also a notable risk factor for hearing impairments which affect neonates (Chayasirisobhon, et al., 1996; Misra, et al., 1997).

The auditory brainstem response (ABR) has been shown to be very sensitive to low blood oxygen concentrations, which result in damage to the neurosensory cells of the Organ of Corti or loss of brainstem neurons, such as those of cochlear nuclei or the inferior colliculus (IC) (Freeman, 1991; Jiang, et al., 2004). This neuronal damage compromises nerve conduction in the brain, leading to acute auditory impairment (Jiang et al., 2000). ABRs are the most sensitive and reliable methods used to evaluate such functional impairments (Tomimatsu, et al., 2003; Smit, et al., 2013). In particular, the two most widely used parameters are wave V latency and its I-V interval, because they intimately reflect neuronal conduction, brainstem conduction time, myelination and synaptic function, both in the brainstem and in the central auditory pathway (Jiang, et al., 2008).

Following a perinatal asphyxia event, ABR abnormalities have been reported, including an elevated response threshold, an increase in wave latencies, brainstem conduction time and interpeak intervals; reduced wave amplitudes and V/I amplitude ratio (Hecox and Cone, 1981; Wilkinson and Jiang, 2006; Jiang, et al., 2009). These electrophysiological findings have been correlated with histopathological observations which indicated that perinatal asphyxia often causes discrete lesions

in brainstem auditory nuclei (Tomimatsu, et al., 2002).

The effects of HI injury are due to diverse mechanisms which ultimately produce cell damage (Goñi de Cerio, et al., 2007). Thus, neural cells are particularly susceptible to energy failure (decreased ATP levels), cellular excitotoxicity and oxidative stress which can in turn promote cell death (Vexler and Ferriero, 2001; Alvarez Diaz, et al., 2007) and in this sense, death receptor-activated pathways, altered mitochondrial function (Northington, et al., 2001). HI events are also known to perturb neuron metabolism and depress synaptic function between cells (Jiang, et al., 2008). This function is vital for the synthesis, release and uptake of neurotransmitters during neural development (Benveniste et al., 1984; Johnston, 2001; Jiang et al., 2004). Although astrocytes can also be affected by HI which can compromise their capacity for neurotransmitter uptake, they are more resistant to HI events than neurons (Chen and Swanson, 2003; Alvarez Diaz, et al., 2007). In contrast, oligodendrocytes are particularly vulnerable to perinatal asphyxia which affects myelination leading to white matter lesions and damaging gray matter

oligodendrocyte progenitors (Rosthstein and Levison, 2005). These cells are responsible for myelin production. Thus, reduced expression of myelin basic protein (MBP), the major myelin protein, is considered a hallmark of inflammation-associated diffuse white matter damage in fetal rodents (Wang, et al., 2007) and in preterm infants (Inder, et al., 2003).

One of the key events in HI pathogenesis is the early generation of reactive oxygen species (ROS) (Li, et al., 2009). Increased ROS production leads to lipid and protein oxidation, loss of endogenous antioxidants, and damage to neurons (Kelly, 1993; Margaill, et al., 2005). Consequently, supplementation or treatment with antioxidants has been proposed to be neuroprotective, and may be an appropriate target area for novel therapies (Moosmann and Behl, 2002; McMahon, et al., 2006).

There are many possible candidates for antioxidant therapies. Here, we have chosen four agents that have shown good protective effects in brain damage after HI, but their effects on auditory impairments after HI injury are unknown. Nicotine (N) exerts its effects via specific nicotinic acetylcholine receptors (nAChR), the stimulation of which has various effects including antioxidant functions. In addition, nicotine can inhibit glutamate neurotoxicity and increase the expression of BCL-2 and other antiapoptotic proteins. Melatonin (M) (N-acetyl-5-methoxytryptamine) is an endogenous indolamine generated primarily by the pineal gland and released into the blood stream and cerebrospinal fluid. It exerts a wide range of physiological functions including the removal of free radicals (Tan, et al., 2007) and the inhibition of the oxidation of biomolecules (Reiter, 1998). Resveratrol (RV) (3,5,4′-trihydroxystilbene) is a non-flavonoid polyphenolic compound consisting of two aromatic rings attached by a methylene bridge. It is produced by 72 different plant species, including grapevines, pines, legumes, peanuts, soybeans and pomegranates but the most common dietary source of resveratrol is red wine (Liu, et al., 2007). The neuroprotective effects of RV are due to its antioxidant activity associated with its stilbene structure with two phenol rings (Lopez-Miranda, et al., 2012). DHA (Docosahexaenoic acid) (22:6n-3) is a long-chain omega-3 fatty acid, commonly found in fish such as salmon and tuna. In humans, DHA is present in low concentrations in blood, but in very high concentrations in the brain, retina and spermatozoa. DHA provides plasma membrane fluidity at synaptic regions, so it is crucial for maintaining membrane integrity and consequently, neuronal excitability and synaptic function (Wurtmar, 2008; Davis-Bruno and Tassinari, 2011). A DHA-enriched diet during pregnancy has been shown to provide neuroprotection against neonatal brain injury by inhibiting oxidative stress (Suganuma, et al., 2013).

The aim of the present work was to evaluate morphofunctionally the effect of a panel of antioxidants on HI-induced auditory deficits. To this end, we studied the effects of Nicotine, Melatonin, Resveratrol and DHA on the neonatal

auditory system via measurement of auditory evoked potentials and characterization of the morphological integrity of the IC.

MATERIALS AND METHODS

Subjects

7-day-old Sprague-Dawley rats were used for histological and functional studies which were carried out in compliance with the animal research regulations specified in the European Communities Directive [UE 86/609], and were approved by The Basque Country University Animal Care and Use Committee. Meanwhile, flow cytometer experiments were studied at different points of time after the HI insult (3, 12 and 24h).

Hypoxia Ischemia

The hypoxic ischemic event was induced in perinatal rat pups by the Rice-Vannucci method (Rice and Vannucci, 1981). Briefly, Sprague Dawley rat pups were anesthetized with isoflurane (3.5% for induction and 1.5% for maintenance) in oxygen. The left common carotid artery was permanently ligated with 6-9 surgical silk and cauterized to block blood flow through the carotid. Animals were returned to their mother for 2 hours to recover from anesthesia and then placed in a bath at 36º during the hypoxic event. They were locked in a glass bottle and were subjected to 8% oxygen in a nitrogen gas mixture for 135 minutes. In order to verify the occurrence of brain hypoxia, some of the animals were injected with hypoxyprobe-1 just before the hypoxic event and were sacrificed immediately after the hypoxic stress. The rest of the animals were returned to their biological mothers until they were 14 days-old. Following measurement of the auditory brainstem response they were euthanized.

Experimental groups

Pups were randomly assigned to six experimental groups (n=8): control, HI and the four HI groups administered with the different drugs (Table 1). Drugs were intraperitoneally administered and Nicotine, Resveratrol and DHA were administered before the hypoxic event while melatonin was administered 10 min after the hypoxic event (table 1).

The time interval of treatment injections pre- and post-HI and treatment doses were selected because of existing good results rat data on other brain areas after a perinatal asphyxia. DHA was previously described as neuroprotective antioxidant when administered 1h or 3h before the HI event (Pan, et al., 2009), Resveratrol when is given 10-15 min before, (Huang, et al., 2001; West, et al., 2007) and Nicotine 2h before the damage (Chen, et al., 2013). Meanwhile, Melatonin is neuroprotective when is administered just after the damage (Carloni, et al., 2008; Cetinkaya, et al., 2011; Alonso-Alconada, et al., 2013).

Auditory brainstem response (ABR) measurements

Animals were anesthetized with ketamin-xylacin (80 and 10 mg/kg, respectively) to measure the

auditory evoked potentials. GSI Audera equipment with software version 1.0.3.4 was used to record the ABR. Measurements were performed in a sound-proofed room to ensure minimization of background noise. ABR latencies and amplitudes were recorded using three gold-plated disk electrodes placed at the middle forehead (positive), ipsilateral earlobe (negative) and middle body (ground) respectively.

Drug	**Dose**	**Solvent**	**Administration time**
Nicotine Hydrogen tartrate (N)	1.2 mg/kg (Chen, et al., 2013)	Saline	2 h before Hypoxia (Chen, et al., 2013)
Melatonin (M)	15 mg/kg (Carloni, et al., 2008)	Saline and DMSO 5%	10 min after Hypoxia (Carloni, et al., 2008; Cetincaya, et al., 2011; Alonso-Alconada, et al., 2013)
Resveratrol (RV)	20 mg/kg (West, et al., 2007)	DMSO	10 min before hypoxia (Huang, 2000; West, et al., 2007)
Docosahexaenoic acid (DHA)	1 mg/kg (Berman, et al., 2009)	HSA 25% diluted in normal saline	10 min before hypoxia (Pan , 2009)

Table 1. Administered drugs with dose, dilution, administration time, mode and commercial house from each one. Each drug was administered intraperitoneally and were purchased from Sigma Aldrich Co, St Louise, USA.

Measurement acquisition was calibrated with the following parameters: sweep time 10 ms with 150 and 3000 Hz filters for low and high frequencies, respectively. Stimulation was performed by means of 11.1/s clicks ABR and averages were taken of 2006 responses. We used continuous clicks at intensities of 100 dB. Repeated ABRs were recorded for both ears. The parameters analyzed were peak amplitudes (µV), peak latencies (ms) and intervals between peaks (ms).

Tissue processing for histological study

After ABR recordings, animals were weighed, sacrificed with sodium pentobarbital overdose and perfused with saline-heparin followed by 4% formaldehyde prepared with fresh paraformaldehyde. The brain was removed and weighed before being fixed in 4% formaldehyde overnight. The brainstem was isolated, dehydrated and embedded in paraffin and then sectioned with a microtome (5µm) at stereotaxic standard level of bregma 9.30 mm, at the level of the mesencephalon interaural 0.30 mm (Paxinos, 1986). Sections were collected on polylysine-coated slides and processed for

hematoxylin-eosin staining. The same procedure was carried out for hypoxyprobe-1 staining, but P7 pups were used instead of using P14 animals in this case. For MBP expression analysis, at least three sections per brain were assayed for the central nuclei of the inferior colliculus (CIC) and three more sections per brain for the external cortex of the inferior colliculus (ECIC) (Sun et al., 2011).

Assessment of brain damage

To quantify cell damage in the inferior colliculus with the hypoxia marker staining, astrogliosis or white matter injury assessments, we only used samples with a reduced infarct area or without it. To represent the infarct area that occurs in some animals we used the hematoxylin-eosin staining.

2.6.1 Hypoxia marker

To evaluate the existence of hypoxia in the brain, we used the hypoxia marker hypoxyprobe-1 (Hypoxyprobe Inc. Burlington MA, USA). Hypoxyprobe™-1 (60 mg/kg) was intraperitoneally injected immediately after the HI event. Hypoxyprobe™ - 1 distributes to all tissues including the brain but it only binds thiol-containing proteins in those cells which have an oxygen concentration less than 14 □m. After blocking endogenous peroxidase with H_2O_2 (3%), dewaxed and rehydrated sections were incubated with Hypoxyprobe-1 MAb1 mouse primary antibody (1/50) for one hour. Following abundant washing in saline, sections were indicated for 10 min with peroxidase-conjugated anti-mouse antiserum (1/500). The presence of bound primary antibody was manifest with diaminobenzidine and sections were finally weakly counterstained with hematoxylin.

Neuronal injury

The neuronal injury was evaluated in sections stained with hematoxylin-eosin (Sigma-Aldrich Co. St. Louis, MO, USA).

Astrogliosis

Astrogliosis was evaluated using glial fibrillary acidic protein (GFAP) immunohistochemistry. Sections were rehydrated and blocked with endogenous peroxidase (1%), incubated with mouse anti- GFAP primary antibody (1:500, Dako, Denmark) overnight and then with peroxidase-labeled second antibody (HRP anti-mouse 1:100, Santa Cruz Biotechnology, CA, USA) for one hour. Sections were stained with diaminobenzidine and counterstained with hematoxylin.

White matter injury assessment

For myelin basic protein (MBP) immunohistochemistry, sections were incubated with mouse primary anti-MBP antibody (1:200, Santa Cruz Biotechnology, CA, USA) overnight and then with peroxidase-labeled second antibody (HRP anti-mouse 1:100, Santa Cruz Biotechnology, CA, USA) for one hour. The sections were stained with diaminobenzidine and counterstained with hematoxylin. White matter injury was analyzed by densitometry of MBP immunostaining, using a computerized video-camera-based image-analysis system

(National Institutes of Health Image software, public domain, (http://rsb.info.nih.gov/nih-image/) as described by Liu, et al. (2002). Unaltered TIFF images were digitized, segmented (using a consistent arbitrary threshold -50%), and binarized (black versus white). Total black pixels per hemisphere were counted, and average values were calculated per brain, and expressed as pixels per hemisphere. Hemisphere areas were also outlined and measured for each section that was analyzed by densitometry. At least eight sections per brain were analyzed and only sections with obvious technical artifacts related to the staining procedure were excluded. Densitometry values were expressed as the ratio of left to right hemispheric measurements; for each brain sample (Left hemisphere /Right hemisphere) pixels of MBP was calculated.

Flow cytometry analysis

For the flow cytometry analysis, animals were sacrificed with pentobarbital sodium overdose and perfused with Ringer lactate solution. Ipsilateral brainstem sections were isolated and disaggregated with a blade in a lactate solution always kept in ice cold and then samples were put in a cell strainer with 4ml collagenase (1.5 mg/ml diluted in Hank´s (HBSS;
Sigma-Aldrich, St Louis, Mo, EEUU) in each dish and incubated at 37°C during 20 min. Cell suspension was washed with Hank´s in a centrifugation at 15000 rpm during 5 min, and after removing the supernatant, the pellet was suspended in 5ml of Hank´s. Then, cell suspensions (600-1000 µl) were incubated with different fluorochromes. The samples were incubated during 30 min at 37°C and after washing twice with centrifugation during 5 min at 1500 rpm in Hank´s solution, pellet suspended in 350µl of Hank´s were taken to the cytometry. Analyses were determined by an EPICS ELITE Flow Cytometry (Colter, Inc., Florida, USA). To exclude debris and cellular aggregates, samples were gated based on light scattering properties, in side scattering (SSC), which correlates with cell complexity, and forward scattering (FSC), which correlates with cell size, and 10,000 events per sample within a gate (R1) were collected. Events within R1, which corresponded to individual cells, were plotted for their fluorescence. An unstained sample was used as a control to remove autofluorescence. Data analysis was performed using the Summit v4.3 software and statistical analyses with GraphPad prism 5 software.

To study the membrane integrity and potential the level of cardiolipin was observed by using the fluorochrome Nonyl Acridine Orange (NAO, Invitrogen, The Netherlands) and the level of Rhodamine 123 (Rh-123, Invitrogen, The Netherlands) a lipophilic cationic fluorochrome. In the first marker, cell suspensions were incubated with NAO (10^{-2} M) in PBS at 4 °C and in dark conditions for 30 min and later cells were washed twice in buffer before loading to the flow cytometry. For Rhodamine 123 study, cell suspension was incubated with Rh123 (4µl) in

100 µl Hanks' solution for 30 min at 37 °C followed by washing and incubated during, 30 min more at 37°C before taking to the flow cytometry for the analysis. Intracellular ROS were detected using fluorochrome 2′,7′-dichlorofluorescein diacetate (DCFH-DA Invitrogen, The Netherlands). Cell suspension was incubated with DCFH-DA fluorochrome (10 µM) in HBSS for 30 min at 37 °C and taken directly to the flow cytometry for the analysis.

Statistical analysis

Values are represented as means ± SD. Group differences were studied by one-factor analysis of variance with Bonferroni-Dunn correction. The statistical analysis of data was performed using GraphPad prism 5 software version 5.01 (GraphPad Software, Inc. CA, USA).

RESULTS

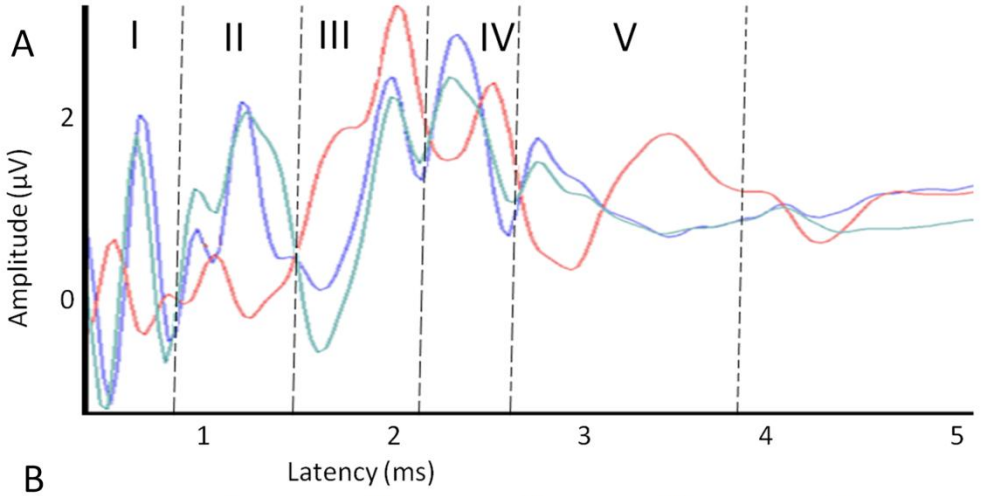
Alterations in ABR

Wave amplitudes

The amplitudes of the peaks were different in most of the animals and the standard deviation was very high in the groups to compare them, so we did not consider them for analyzing.

Wave latencies

In the pups with neonatal HI, the latency of



B

	I	II	III	IV	V
Control	0,370±0,03	0,94±0,07	1,74±0,149	2,275±0,06	2,752±0,08
HI	0,36±0,019	0,99±0,038	1,84±0,137	2,315±0,09*	3,035±0,11].*
Nicotine	0,37±0,043	0,90±0,135	1,72±0,186	2,23±0,114#	2,713±0,09#
Melatonine	0,392±0,03	0,99±0,07	1,84±0,09	2,25±0,100#	2,723±0,01#
Resveratrol	0,38±0,055	0,99±0,09	1,74±1,18	2,237±0,14#	2,73±0,149#
DHA	0,39±0,038	0,93±0,06	1,83±0,115	2,148±0,14#	2,669±0,25#

Figure 1. Typical auditory brainstem response (ABR) recording of 14-day-old rats (n=8). (A) Representative ABR responses of control (blue), hypoxia-ischemia (red) and treated rats (green) (n=8 in each group). A representative rat treated with melatonin was use for the illustration. (B) Results of the ABR expressed as mean ± standard deviation in all experimental groups. *p<0.05 vs. Control and #p<0.05 vs HI, ANOVA.

waves I, II and III (0.36 ms ± 0.02; 0.99 ms ± 0.04; 1.84 ms ± 0.14, respectively) were similar to those of the control group. In contrast, wave IV and V latencies in the hypoxic-ischemic animals were significantly longer than in control animals, from 2.28 ms ± 0.06 to 2.32 ± 0.09 and from 2.75 ± 0.08 to 3.04 ± 0.11 respectively (Fig. 1B). All antioxidant treated groups showed wave latencies which were similar to those of the control group with slight differences which were not statistically significant (Fig. 1A, B). Since there were no differences between the left and right ears, we evaluated the data together.

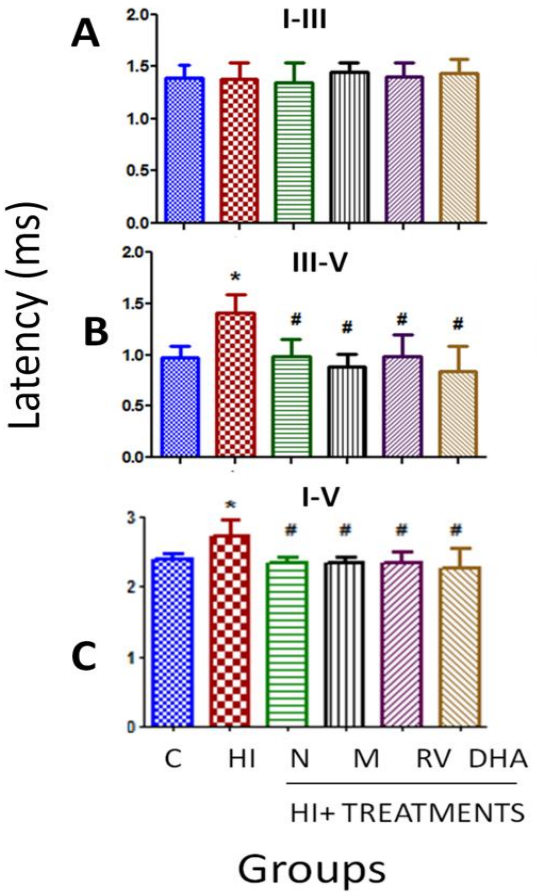


Figure 2. Interpeak latency of ABRs in 14 day-old control, HI and treated rats. Data are expressed as means ± SD (*p < 0.05 vs. controls and # p< 0.05 vs HI, ANOVA). Abbreviations: C, control; HI, hypoxia-ischemia; N, nicotine; M, melatonin; RV, resveratrol; DHA, docosahexaenoic acid.

Interpeak intervals

The HI event induced a statistically significant decline in ABR latency intervals (Fig. 2) in the latency III-V and in the I-V intervals between the peaks, while there were no statistical differences between the treatments and control groups. In the I-III interval, there were no statistical differences between the peaks (mean latency; 1.4 ms in all groups). In contrast, wave V latency after HI tended to be slightly longer than in the control or treated groups and a statistically significant enhancement of the interval was observed in III-V intervals with a 35% decrease in the interpeak interval of the latency in almost all treated groups compared to the HI group. In the I-V interpeak interval, a 14% decrease was measured in the treated groups compared to the HI group.

Body weight

We compared the body weight of each animal in each group, and no differences were found among the different groups at postnatal day 7 (P7). However, after experiments (day 14) differences in body weight were observed (p< 0.05) (Fig. 3). The HI group showed a 14% loss of body weight during these days, (29.13 g ± 2.2 vs 25.19g ± 1.34, control and HI groups respectively), while all antioxidant treated groups showed similar body weights to the control group.

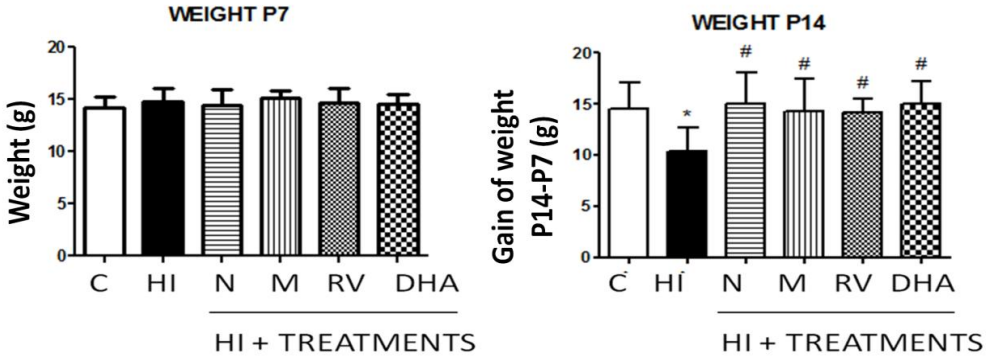


Figure 3. Body weight changes in 7 and 14 day old neonatal rats (n=8 for each group). (A) Body weight (in g) of P7 animals before experimental intervention. (B) Body weight of 14 day old rats after hypoxia ischemia and recovery following antioxidant treatment. Body weight loss due to hypoxia-ischemia was blocked by all antioxidant treatments. Abbreviations as in Fig. 2. Bar represents mean ± standard deviation. * p<0.05 versus control; # P<0.05 versus HI, ANOVA.

Assessment of brain damage

We also weighed brains after the sacrifice of the animals. There were statistical differences between the experimental groups (p<0.05) (Fig. 4), in that we found a 28% brain weight reduction in the HI group compared to the control group (14.49 g ± 2.54 vs 10.40 g ± 2.25) while antioxidant treatment with nicotine, melatonin and resveratrol abolished this decrease. Treatment with DHA had a similar effect, but the difference was not significant.

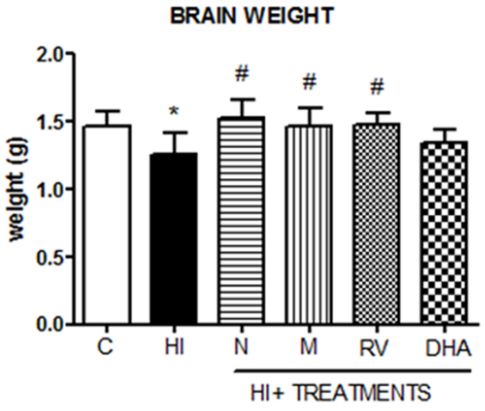


Figure 4. Brain weight of 14 day-old animals after hypoxia ischemia and recovery following administration of antioxidants (n=8 for each group). Loss in brain weight due to hypoxia ischemia was prevented by the different antioxidant treatments, with the exception of DHA. Abbreviations as in Fig. 2. Bars represent mean ± standard deviation. * p<0.05 vs control; # P<0.05 vs HI, ANOVA.

We examined the presence of tissue hypoxia in samples stained with the immunohistochemical hypoxia marker hypoxyprobe-1 (Fig. 5). Examination of histological sections at the midbrain levels 7 days after left carotid ligation and 2 h and 15 min of hypoxia revealed the absence of hypoxyprobe-1 labeling in control midbrain sections (Fig. 5A).

However, intense hypoxia labeling was apparent in the HI group, especially in the ipsilateral side where morphological damage to the ischemic event is obvious (Fig. 5B). In the treated groups,

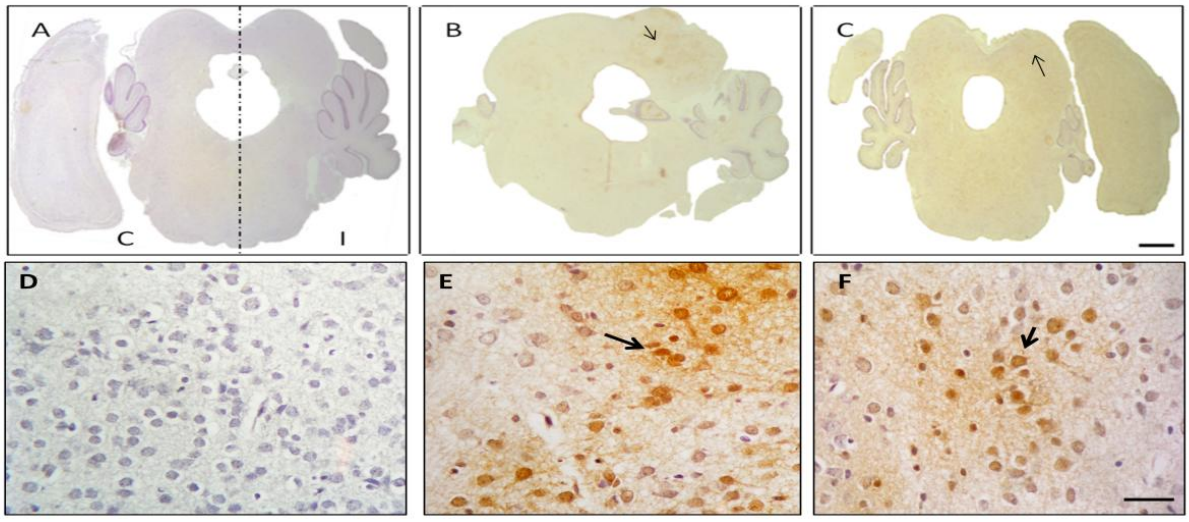


Figure 5. Representative stereo microscope image (A-C) and microphotographs (D-F) of hypoxyprobe-1 labeling in midbrain sections (n=8). (A, D) No hypoxyprobe-1 labeling was apparent in the control group; I, ipsilateral side; C, control side. (B, E) Hypoxyprobe-1 labeling together with morphological damage was apparent on the ipsilateral side of the inferior colliculus in the HI group, with reactive cells (arrow). (C, F) Hypoxyprobe-1 labeled cells appear in the treatment group (nicotine), but there were no morphological differences between the ipsi- and contralateral sides. The contralateral side is to the left in all cases. Stereo microscope images bar: 2.5 mm; microphotographs bar: 500µm.

hypoxyprobe-1 labeled cells could also be observed but no morphological damage was apparent (Fig. 5C). The image of the treated group (image of HI+ nicotine rat) is a representative image for all of treatments. In more augmented images, reactive cells are observed in HI and treated groups while there are no evidence of this reactivity in the Control group (Fig 5. D-F).

Hematoxylin-eosin staining

Staining of brain sections with hematoxylin-eosin macroscopically revealed signs of early neuronal damage induced by the hypoxic- animals or treated animals did not (Fig. 6).

In the inferior colliculus (Fig. 7), hypoxic-ischemic insult induced a significant alteration in neuron

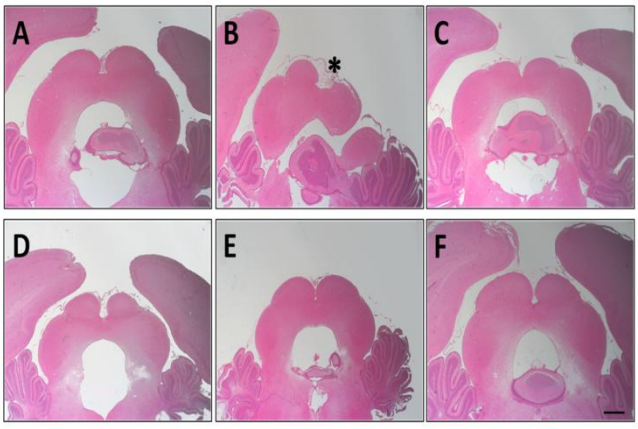


Figure 6. Representative stereo microscope photographs of the inferior colliculus of P14 perinatal rat brains (interaural distance 0.30 mm and bregma 9.30 mm) stained with hematoxylin-eosin (n=8 for each group). (A) Control group. (B) HI group with the infarction area indicated with an asterisk). (C-F) HI + treatment: nicotine, melatonin, resveratrol and DHA, respectively. Bar: 1 mm

morphology, as indicated by an asterisk. Indeed, asphyctic animals showed swollen and deformed neurons in the CIC at the ipsi-lateral side, also with axonal prolongations and condensed cytoplasm. There were no signs of neuron damage in the slices from treated animals, in the CIC.

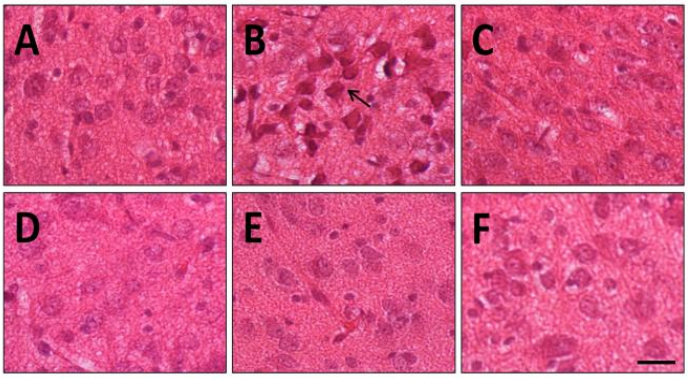


Figure 7. Representative microphotographs illustrating the alteration in neuron morphology in the central nuclei of the inferior colliculus in hematoxylin-stained brain sections from P14 rat pups. (A) Control, (B) HI, (C-F) HI+ treated groups, nicotine, melatonin, resveratrol and DHA, respectively. Condensate and bigger cells after hypoxia-ischemia (arrow) are apparent while after treatment administration the cells are similar to the control group. Bar: 100 µm.

immunohistochemistry to test if there was astrocyte reactivity in the inferior colliculus after the HI event (Fig. 8). Low levels of GFAP expression were detected in control animals (Fig. 8A). In contrast, we found remarkable reactivity in the ipsilateral inferior colliculus of HI animals (Fig. 8B). Astrogliosis was diminished in all cases after treatment (Fig. 8 C-F).

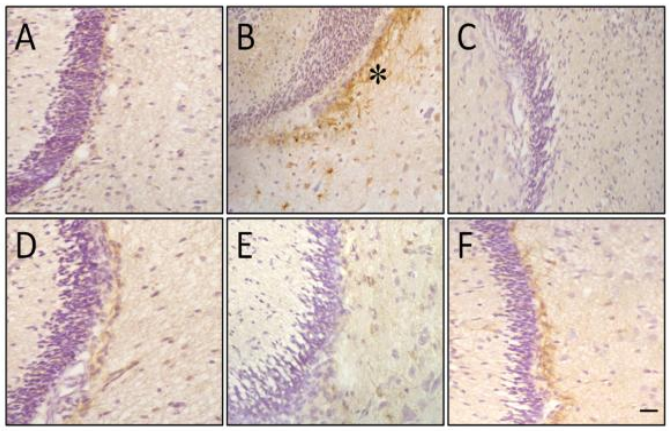


Figure 8. Increase in reactive gliosis (measured as GFAP expression) in injured animals and reduction of reactivity after treatment administration.Representative microphotographs illustrating GFAP immunostaining in brain sections from the central nucleus of the inferior colliculus (CIC) in control (A) HI with reactive astrogliosis (asterisk) (B), HI+ antioxidant treatment: nicotine, melatonin, resveratrol and DHA, respectively (C-F) . Samples were obtained from P14 animals. Bar: 100 µm.

Myelin basic protein immunoreactivity

MBP immunostaining was found to be reduced in subcortical white matter at the level of the ECIC and also in the CIC. We quantified the expression of MBP in the ipsilateral (L) and contralateral (R) hemispheres of the IC and established the MBP ratio as expression level in the left/right hemispheres. This L/R MBP ratio of HI pups was significantly decreased ($p<0.05$) when compared with control animals in the ECIC (1.02 ± 0.22 vs 0.53 ± 0.19 control and HI groups, respectively) and in the CIC (0.98 ± 0.06 vs 0.68 ± 0.14, control and HI groups respectively). Treated animals showed a lesser degree of MBP loss in the ipsilateral hemisphere, with the L/ R

MBP ratio being similar to that observed in the control group (Fig. 9). Thus, antioxidant treatment resulted in the maintaining of MBP levels in the subcortical white matter of the ipsilateral hemisphere, in both anatomical regions.

Flow cytometer results

Just after the HI event, the membrane integrity is affected in the HI group and also in melatonin and resveratrol treated groups, but treated groups recover the membrane integrity in 3 hours while the HI group has an statistical significant decrease in the percentage of positive cells to fluorocrome Nonyl Acridine Orange comparing to the control group. 12 hours after the HI injury, all groups showed the same percentage of positive cells to this cardiolipin binding marker (Fig. 10)

As in the case of the membrane integrity study, the membrane potential is affected in the HI groups with a statistically significant decline in the percentage of positive cells in the Rhodamine 123 in vivo marker.

This reduction is remarkable just after the damage and also 3 hours after the damage but it seems that the membrane potential returns to

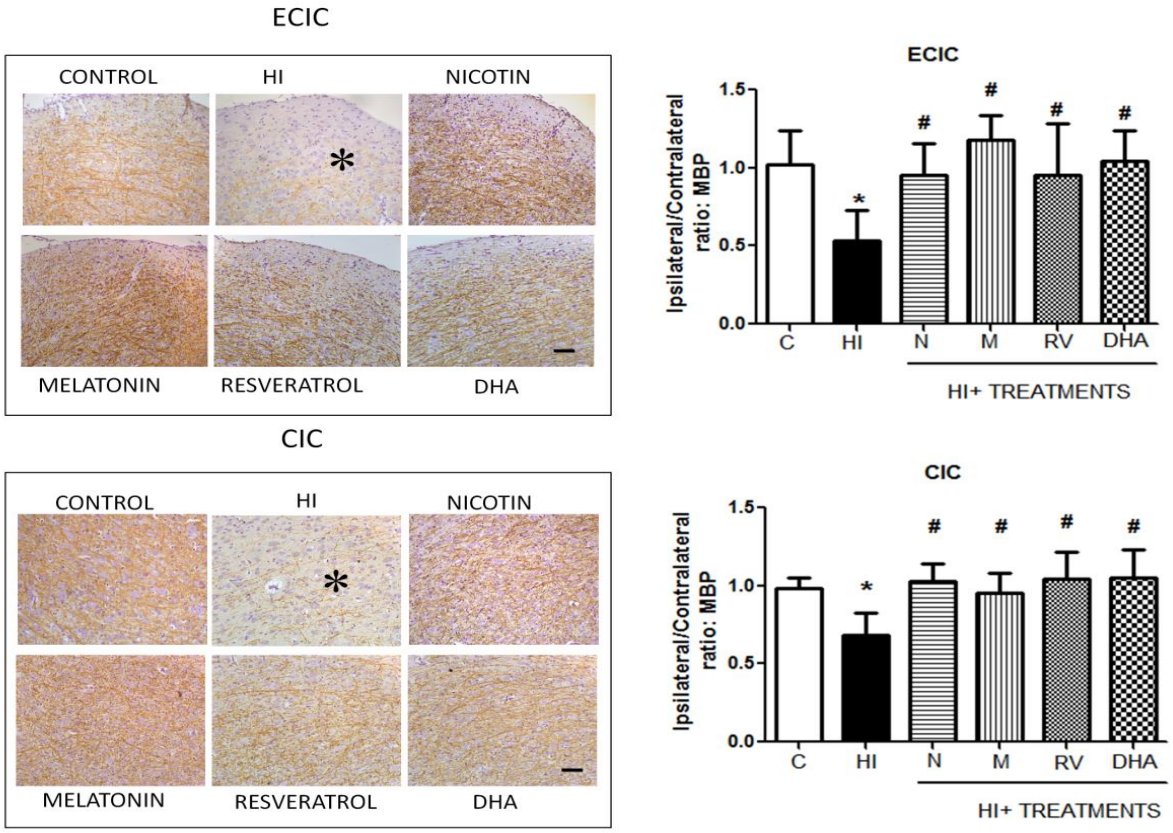


Figure 9. Loss of MBP immunostaining at the level of the inferior colliculus in injured animals and recovery after antioxidant treatment. Microphotographs illustrating the disruption of MBP immunostaining in the external cortex of the inferior colliculus (ECIC) (B) (asterisk) compared to the control group and in the CIC (C). In (A, D) the histograms represent the ipsilateral/contralateral ratio of MBP expression in the ECIC and CIC, respectively. Bars represent the mean and SD (± standard deviation) with n=8 for each group. * $p<0.05$ vs control; # $P<0.05$ vs HI, ANOVA. Bar: 100 µm

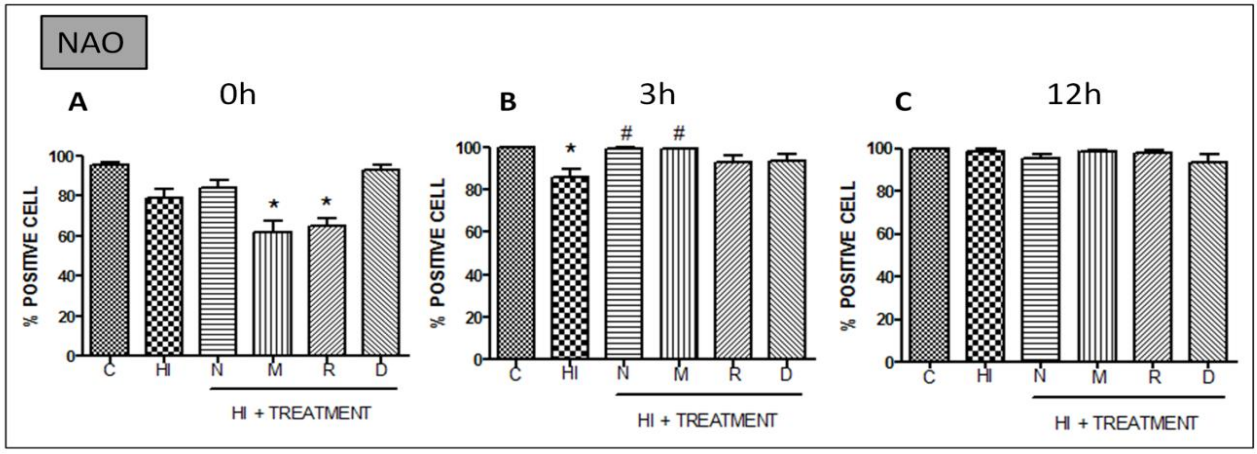


Figure 10: Percentage of positive cells with in vivo markers Nao at different points after HI (n=5). Data are expressed as means ± SEM (*p < 0.05 vs. controls and # p< 0.05 vs HI, ANOVA). Abbreviations: C, control; HI, hypoxia-ischemia; N, nicotine; M, melatonin; RV, resveratrol; DHA, docosahexaenoic acid.

the normality after 12 hours, as in the previous cas. Antioxidants treatments act different in the first hours after the injury but they also recover the normal percentage of positive cells and fluorescence 12h after the HI event (Fig. 11). According to the oxygen reactive species (ROS), there is also a statistical significant decrease in the HI and DHA treated groups comparing to the control group just after the damage. 3h after the damage all the groups have more or less the same positive cells of DCFH marker, so the HI group and DHA group has increased the positive cells during this hours. But the main difference comparing to the previous results is that 12 hours after the injury in all groups except from the melatonin treated group the ROS production has increased in almost 20% comparing to the control group (Fig. 12).

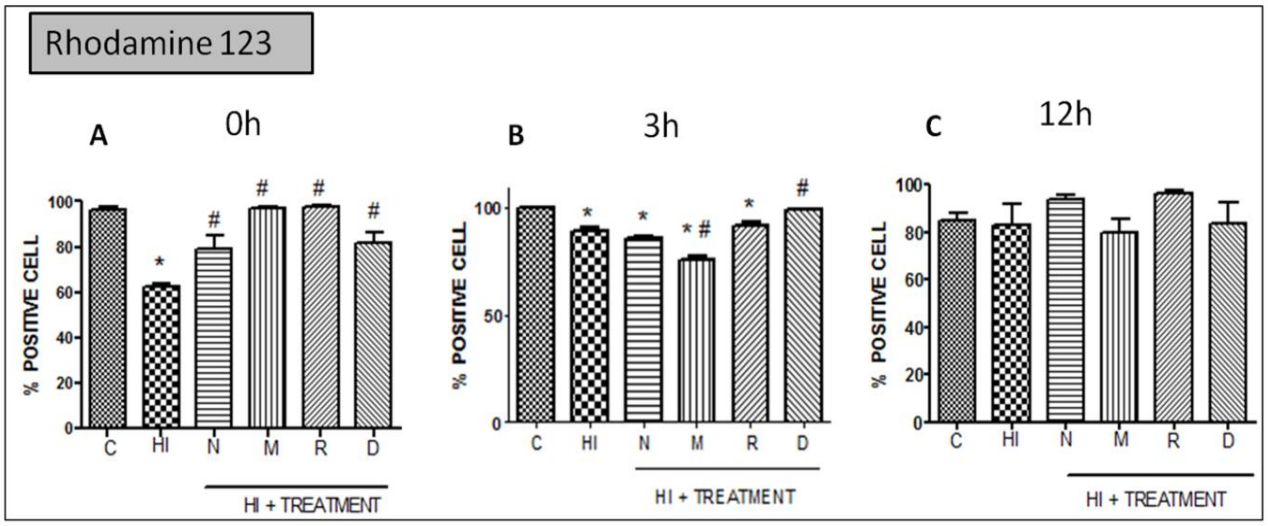


Figure 11. Percentage of positive cells with in vivo markers Rhodamine 123 at different points after HI (n= 5) in P7 rats. Data are expressed as means ± SEM (*p < 0.05 vs. controls and # p< 0.05 vs HI, ANOVA). Abbreviations: C, control; HI, hypoxia-ischemia; DHA, docosahexaenoic acid; N, nicotine; M, melatonin; RV, resveratrol.

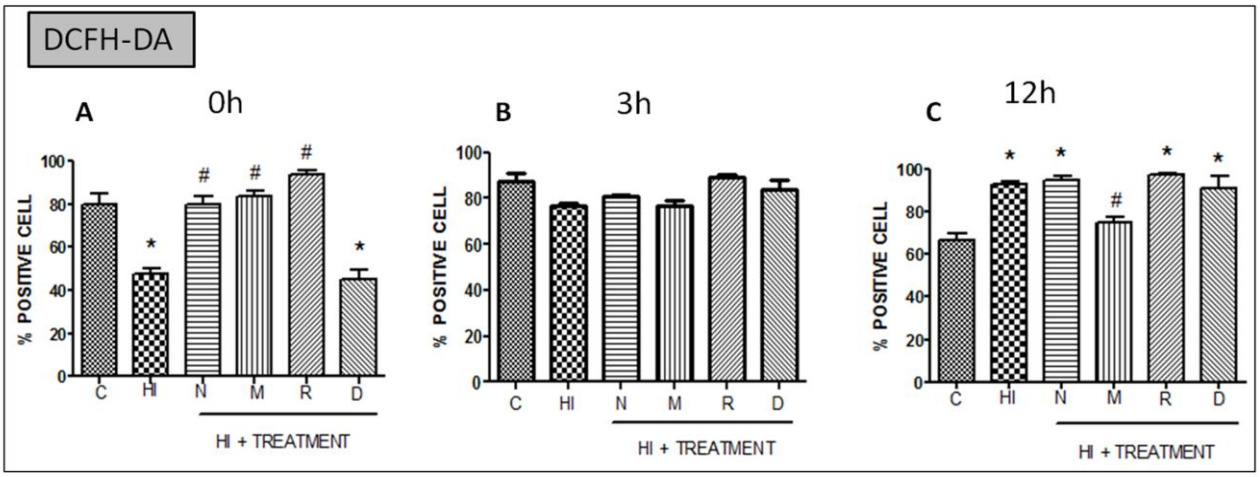


Figure 12. Percentage of positive cells with in vivo markers DCFH-DA at different points after HI (n= 5) in P7 rats . Data are expressed as means ± SEM (* p < 0.05 vs. controls and # p< 0.05 vs HI, ANOVA). Abbreviations: C, control; HI, hypoxia-ischemia; DHA, docosahexaenoic acid; N, nicotine; M, melatonin; RV, resveratrol.

DISCUSSION

The present work supports the hypothesis that hypoxia ischemia induces significant functional, histological and cellular damage to the brainstem and auditory system. We provide additional evidence that antioxidant treatment can be a powerful tool in order to minimize HI-associated damage.

Hypoxia- ischemia is one of the most important causes of damage to the inner ear and can develop many hearing disorders such as sudden sensorineural hearing loss, presbycusis and noise-induced hearing loss that are suspected to be related to alterations in blow flow (Ross and Pawlina, 2011). Diverse studies have characterized the damage to neurons in the hippocampus, striatum and cortex after a perinatal asphyxia event (Alvarez-Diaz, et al., 2007; Alonso-Alconada, et al., 2012), but relatively little attention has been given to the auditory system. Besides, studies of the effect of HI in the cochlea have focused mostly on the susceptibility of the hair cells of the organ of Corti. According to these studies, hair cell loss is correlated with the deficiency in both oxygen and glucose in the cochlea (Gross, et al., 2014).

The auditory brainstem response (ABR) is an important diagnostic method to evaluate brainstem functionality and is widely used to detect HI auditory impairments (Kinely, et al., 1980; Yasuhara, et al., 1986; Tomimatsu, et al., 2002). In this study, we have used the ABR to observe the existence of changes in auditory function in response to HI. There were differences in the amplitude of the ABR between the animals (the intensity of the response was different among animals in the same group). However, the latency of the peaks showed the same pattern in all animals, indicating that ABR wave latency is a stable and objective parameter. Alterations in this pattern could

therefore be indicative of brain damage. The latency of the wave V and the I–V interval latency are the two most widely used variables that reflect neural conduction. These are associated with myelination and synaptic function in the brainstem or central auditory pathway (Jiang, 1998; Jiang, et al., 2009). We observed a significant increase both in the I–V and in the III–V intervals; although the increase in the III–V interval was larger than that of the I-III interval. It seems that wave V following perinatal HI may be particularly vulnerable to physiological changes (Tomimatsu, et al., 2003). Thus, our finding corroborates the findings of others in that the more central part (III–V interval) of the auditory pathway is more affected by HI insult than the more peripheral part (I–III interval) of the pathway (Jiang, et al., 2009). The progressive increase in ABR latencies and interpeak intervals may be attributed to a cumulative decrease in the efficacy of synaptic transmission at high stimulus rates, resulting in prolonged synaptic delays along the brainstem auditory pathway (Jiang, et al., 2010).

The brainstem receives blood from different sources but is mainly supplied by the vertebral artery, and the common carotid arteries play a minor role in this region. For this reason, it has been suggested that the brainstem is largely unaffected in the Rice Vannucci model, which is a perinatal asphyxia model broadly used to investigate cerebral damage because the maturity of the CNS of P7 rats is similar to term human babies (Volpe, 1995; Hagberg, et al., 1997). Nevertheless, Tomimatsu has demonstrated reduced blood flow to the ipsilateral inferior colliculus during the HI event with this method, thereby validating its extensive use as a model of perinatal asphyxia by many research teams including our own (Hilario, et al., 2005; Alvarez-Diaz, et al., 2007; Goñi de Cerio, et al., 2007).

To evaluate the effects of HI, here we have also measured the parameters of animal body and brain weight. Neonatal hypoxia-ischemia not only causes brain damage and neurological deficits, but also decreases somatic growth. Accordingly, we found somatic growth retardation in HI rats at P14 weeks compared to the control group, and that antioxidant treatments significantly improved body weight. Moreover, we also show that antioxidant treated pups maintained brain weight values, suggesting a beneficial effect of these compounds. The decrease in brain weight was likely due to the loss of cerebral tissue, an observation which corroborates that of other authors who described the formation of brain cavities associated with long HI episodes. Indeed, in some authors observed an important variability in the extension of the damaged area derived from HI insult which leads to ipsilateral cerebral infarction (Renolleau, et al., 1998) and manifests as a dramatic loss of cerebral tissue (Towfighi, et al., 1991; Biran, et al., 2006). In response to the administration of antioxidant treatments, neurons in the inferior colliculus appear to be less damaged and this may underlie

the decreased infarct volume of antioxidant treated animals after a HI event.

In addition to considering functional perturbations, we consider that an optimal method to evaluate neuroprotection is the combination of functional approaches with histological evaluation. With this view in mind, we prospectively studied the effects of neonatal hypoxia ischemia on the three neural cell types; neurons, astrocytes and oligodendrocytes. Depending on its severity and duration, brain injury may cause either infarction or selective neuronal death. Neurons are known to be the brain cells which are most sensitive to the lack of oxygen; they also exhibit selective vulnerability (Hilario, et al., 2005; Johnston, et al., 1998). Moreover, in this study, we found that neuronal injury was more susceptible in the inferior colliculus than in the rest of the brainstem, such as, cochlear nucleus or vestibular nucleus. Indeed, it is widely known that astrocytes are generally more resistant to ischemia and other stressors than neurons because they are able to survive and function for extended periods under hypoxic conditions (Chen and Swanson, 2003). However, astrocyte reactivity was observed in the form of GFAP upregulation during the hypoxic ischemic event in the inferior colliculus, while this reactivity was seen to be ameliorated in the rest of antioxidant-treated groups.

White matter injury is a clinical hallmark of Hypoxic-Ischemic Encephalopathy (HIE) (Volpe, et al., 2011; Jellema, et al., 2013). We also observed a reduction in MBP expression in the HI group and recuperation in the treated groups, results that corroborate the findings of other authors (Alonso-Alconada, et al., 2012). MBP is the major myelin protein in the brain, constituting 30% of all myelin proteins. It is known to play a major role in myelin compaction during central nervous system development by intercalating between phospholipidic sheets and interacting with lipids and proteolipids. Oligodendrocytes are the major cellular component of white matter and are the cells responsible for myelin formation in the central nervous system (Barres, 2008). Thus damage to these cells can lead to a dysfunction which alters the formation of the myelin sheath after hypoxia-ischemia (Alonso-Alconada, et al., 2012). Maturation of oligodendroglia is known to be altered in HIE due to three mechanism, microglial activation, excitotoxicity and free radical attack. Excitotoxicity likely leads to oligodendrocyte injury by promoting Ca^{2+} influx, and as a result, the generation of reactive oxygen and nitrogen species (ROS/RNS) (Volpe, et al., 2011).

The role of mitochondria in apoptosis seems to be very important. During prolonged hypoxia the mitochondria in the majority of neonatal neurons were slowly depolarized, and during a brief period of hypoxia followed by reoxygenation, the majority of the neonatal mitochondria demonstrated a partial depolarization followed by recovery, that correlate with previous studies (Larsen, et al.,

2008). In the study of mitochondrial integrity, we observed a decrease in the percentage of positive cells to NAO in the HI group in the first hours in the brainstem which supports the idea that the oxidation of cardiolipin is implicated in mitochondrial dysfunction and can be the consequence of release of cytochrome C from the mitochondria to the cytoplasm, a process which is involved in the apoptotic cell death cascade (Goñi-de-Cerio, et al., 2007; Fernandez-Gomez, et al., 2005; Ramirez, et al., 2003; Ferriero, et al., 2001). Moreover, the same results was found in the percentage of positive cells to Rhodamine 123 suggesting that both membrane potential and integrity are altered during the HI event in the first moments.

Reactive oxygen species (ROS) play a critical role in hypoxic ischemic injury. ROS generated during the HI event, cause cell damage not only via direct action on the cell but also via activation of inflammatory pathway (Zhao, et al., 2013). We observed an increase in ROS production 12h after the HI event, this could be because mitochondria are both a source and target of ROS (Zhao, et al., 2009), so after the mitochondrial first injury, there is an increase of ROS production and that can lead to cell death (Lara-Celador, et al., 2012).

Taking into account that antioxidant treatments have been previously described as a type of neuroprotective therapy, we consider if it could be also effective at this level. So antioxidant treatments for this injury are well documented. Antioxidant effect was observed in all treatments but the protective mechanism is unknown. In the functional and histological studies, all treatments show neuroprotective effect while these results are not so clear in the molecular studies. The mitochondrial membrane seem to act in the same way in all treated groups after 12h but ROS production after 12h to the injury is only decreased in the melatonin treated group suggesting that the antioxidant effect of melatonin is faster comparing to the rest of the studied groups.

Melatonin is one of the most well-known neurohormones that are used as a neuroprotective agent for several brain injuries (Alonso-Alconada, et al., 2012; Carloni, et al., 2014). It has been shown to exhibit neuroprotective effects against transient or permanent ischemic brain injury (Pei, et al., 2002; Kilic, et al., 2005), making it a potentially good tool for the treatment of neonatal hypoxia-ischemia (Welin, et al., 2007; Carloni, et al., 2008). RVT and DHA treatments have also been demonstrated to be effective against hypoxic-ischemic brain injury in the neonatal rat model, reducing infarct volume and neuronal loss, minimizing lipid and DNA peroxidation, blocking some apoptotic pathways, decreasing inflammation, inhibiting free radical production and increasing the production of some antioxidant enzymes such as GPx and SOD (Arteaga, et al., 2014). Apart from the well-known antioxidant treatments for the hypoxic ischemic injury, activation of neuronal nicotinic

acetylcholine receptors (nAChR) by nicotine has also been suggested to protect neurons against hypoxic insult (Hejmadi, et al., 2003). Our findings confirm this potentially protective effect of nicotine, by showing reduced brain damage following HI, when nicotine is administered.

Conclusions

Taken as a whole, the present prospective study presents for the first time a correlation between the functional, morphological and molecular aspects underlying the antioxidant induced amelioration of HI induced brainstem damage. Thus, antioxidant treatments were found to provide effective neuroprotection to the immature auditory system before a perinatal hypoxic-ischemic event. To our knowledge, this is the first study which demonstrates the neuroprotective effects of nicotine, melatonin, resveratrol and DHA on the functional changes of the auditory pathway and on the morphological and cellular damage which occurs after neonatal hypoxic ischemic insult.

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6.3 II. ARTIKULUA (submitted)

Characterization of gene expression in the rat brainstem after neonatal hypoxic-ischemic injury and antioxidant treatment

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Keywords
Gene expression, brainstem, rat, flow cytometer, perinatal asphyxia and brainstem.

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Abstract

The perinatal brainstem is known to be very vulnerable to hypoxic ischemic events which can lead to deafness, swallowing dysfunction, and defective respiratory control. The aim of the present work was to evaluate the potential neuroprotective effects of nicotine, melatonin, resveratrol and docosahexaenoic acid on the expression of a panel of genes in the brainstem following hypoxic ischemic damage.

Quantitative PCR was used to examine gene expression 3 and 12 hours after the damage and immunohistochemistry was employed to evaluate neurons, astrocytes and synaptic vesicles 24h post insult.

We found that the expression of some immediate early genes, as well as that of inflammatory genes TNFα, COX2 and caspase 3 was upregulated in response to the insult. 24 h after the damage, the % of NeuN and synaptophysin immunolabeled cells was found to be reduced while GFAP expression was upregulated. No differences were observed in ROS gene expression following treatments.

INTRODUCTION

Most term neonatal encephalopathies, 1-6/1000 cases, are due to hypoxic-ischemic (HI) events which happen under some intrapartum conditions or in utero (Bellot et al., 2014; de Vries et al., 2010). The corresponding injuries are associated with a high incidence of death or motor and sensory disabilities in children such as cerebral palsy, severe learning impairment, cortical visual loss or hearing impairment (Shankaran et al., 2012; Martinez-Biarge et al., 2011; Marlow et al., 2005; Anderson & Doyle, 2003).

Regional damage in the hippocampus, striatum and cortex after perinatal asphyxia have been studied using a variety of approaches (Alonso-Alconada et al., 2012; Johnston et al., 2001), but little attention has been paid to the brainstem. While the mature brainstem is remarkably resistant to the hypoxia-ischemia, in early life it seems to be very vulnerable and such insults can lead to clinical abnormalities in survivors, including deafness, swallowing dysfunction, and in most severe cases, defective respiratory control necessitating mechanical assistance (Reinebrant et al., 2012; Panigrahy et al., 1995).

In recent years, immediate early genes (IEG) have received attention because many of their protein products are transcription factors. Cerebral ischemia is known to induce increased synthesis of a number of specific proteins such as heat shock proteins or “stress” proteins and although the function of these stress proteins is not well understood, their induction has been correlated with increased resistance to injury in many cell types (Tang et al., 1997). Some of the upregulated genes include the c-

fos and c-jun proto-oncogenes; the protein products of these immediate early genes (Fos and Jun) are thought to regulate target genes which participate in fundamental biological responses (Chen et al., 1996).

Heat shock factor 1 (HSF1) is another gene expressed immediately after the damage; this factor positively regulates the expression of inducible heat shock protein (HSP70). HSF1 is believed to protect cells and organisms from the ill effects of physiological and environmental stress (Li et al., 1992), whereas hsp70 gene activation has been linked to protection against brain damage (Kim et al., 2001). Egr-1 is another member of the family of immediate-early genes which encode transcription factors associated with vascular occlusion. The IEGs translate pathological stimuli into the activation of appropriate effector molecules and their expression is known to be acutely up-regulated within minutes of stimulation by multiple forms of tissue stress and injury (Tyree et al., 2006; Zhang et al., 2006). EGR-1 in turn has been shown to directly or indirectly upregulate the expression of numerous vascular growth factors, including vascular endothelial growth factor (VEGF), which is capable of inducing or supporting angiogenesis and which may in

concert be capable of inducing collateral vessel formation (Schalch et al., 2004; Marti et al., 2000).

One of the most known transcription factors which is a key regulator of redox and oxygen homeostasis is hypoxia inducible factor 1 (HIF1) which facilitates adaptation to oxygen deficiency and other redox stresses (Kanngiesser et al., 2014). Activation of HIF-1 by hypoxia/hypoxemia facilitates metabolic adaptation to environmental challenge; glucose uptake by the non-insulin-dependent glucose transporter (GLUT1) is enhanced, and expression of glycolytic enzymes, erythropoietin and vascular endothelial growth factor (VEGF) is also amplified (Matsuda et al., 2005; fang et al., 2000).

Neurons are known to be the brain cells which are most sensitive to the lack of oxygen (Hilario et al., 2005) but HI injury can also affect the normal activity of astrocytes and oligodendrocytes. Astrocytes can be activated under hypoxic conditions resulting in their proliferation and morphological and functional changes. Recent evidence suggests that astrocytes are activated and contribute to the generation of neuroinflammation during the neurodegenerative process (Yang et al., 2014). Evidence suggests that reactive astrocytes are present within the lesion, and they can also be an important barrier between the lesion and the surrounding tissue (Pekny et al., 2014).

In the ischemic brain, activated astrocytes can synthesize and secrete cytokines and neurotrophic factors which are important for the development and progression of ischemic brain damage. These secreted factors have different roles in neuronal apoptosis; for example, tumor necrosis factor (TNF) may contribute to injury-induced neuronal apoptosis and play important roles in the pathogenesis of the injury (Li et al.,2014; Kalay et al.,2013; Cojocaru et al.,2009).

The pre-term period in humans, and early postnatal development in rodents, is a time of active oligodendrogenesis, myelination and axonal organization in the developing sub-cortical white matter and this stage of development is really vulnerable to insults such as hypoxia ischemia with associated inflammation and oxidative stress (Ritter et al., 2013; Schmitz et al., 2012; Bradl and Lassmann, 2009; Volpe et al., 2009).

Apart from cell damage, brain ischemia causes neurological disturbances. Moreover, brain-derived neurotrophic factor expression is markedly reduced following ischemia in rats and also a rapid loss of synapse formation. The release of brain-derived neurotrophic factor enhances presynaptic activity and induces activity-dependent regulation of synaptic structure, particularly in glutamatergic synapses (Tsai et al., 2013). This activity can be measured in terms of the levels of expression of synaptophysin a glycoprotein in presynaptic

vesicles whose function is the liberation of Ca2+ dependent neurotransmitters.

The importance of antioxidant treatments has increased during the last decade as it has been shown that supplementation treatment can reduce the damage produced by the early generation of reactive oxygen species (ROS) during hypoxic ischemic injury (Li et al., 2009; McMahon et al., 2006; Guaiquil et al., 2004). The therapeutic effects of many treatments have been examined in the hippocampus and the cortex, but their effects in the brainstem are less known.

2001). It seems that resveratrol is able to interfere with advanced glycosylated end products, mediating oxidative DNA damage. Moreover fatty acids are important for the histological, anatomical and biochemical integrity of the brain. Docosahexaenoic acid (DHA) affects cell viability and protects against stress insults. DHA activates positive regulators of cell survival by up-regulating Akt, extracellular-signal-regulated kinase (ERK) and/or Bcl-2 (Shimazawa et al., 2009; Pan et al., 2008). We have shown that this antioxidant has beneficial effects at functional and histological levels (Revuelta et al., 2015), but to date there is no evidence of its molecular effects in the brainstem after hypoxia-ischemia or after treatment with these antioxidants.

The aim of the present work was to evaluate the effects of nicotine, melatonin, resveratrol

Nicotine (N) exerts its effects via specific nicotinic acetylcholine receptors (nAChR) and it is known to have antioxidant functions. In addition, nicotine can inhibit glutamate neurotoxicity and increase the expression of BCL-2 and other anti-apoptotic proteins (Hejmadi, et al., 2003). Melatonin in the hippocampus and cortex are known to have diverse pharmacological activities, including anti-inflammation, antioxidation, anti-apoptotic and analgesic properties (Cilio et al., 2010). Resveratrol is another powerful antioxidant, but its neuroprotective effects following hypoxic-ischemic injury have not been documented (Huang et al.,
and DHA in the brainstem following hypoxia-ischemia. To this end, we studied the effect of the damage in some immediate early genes expression and inflammation, neuronal death and cellular activation related gene expression just after the damage, 3 and 12 hours after the damage by qPCR and by immunohistochemistry the effect of the damage in neurons, astrocytes and synaptic vesicles 24hours after the damage. Indeed, we also evaluate the neuroprotective effect of the treatments previously described in the hypoxic ischemic injury in rat brainstem.

MATERIALS AND METHODS

Animals

Sprague-Dawley rat pups were used in this study. All the experimental procedures received previous approval of the Animal Welfare Committee of the University of Basque Country

(UPV/EHU) and complied with the National Institutes of Health guidelines for the care and use of laboratory animals, and the European Communities Directive 86/609/EEC regulating animal research. For histological studies, we used 14 day-old rats (7 day-old animals subjected to HI + 7 day recovery); the infarct area and immunohistochemistry by flow cytometry was studied 24 hours after the damage and molecular studies were performed at different time points by qPCR analysis (0 h, 3 h and 12 h after the damage).

Experimental procedures

The Rice–Vannucci experimental procedure (Rice et al., 1981) was used to cause hypoxic–ischemic brain injury in 7-day-old (P7) Sprague Dawley rats. Pups were anesthetized with isofluorane (3.5% for induction and 1.5% for maintenance) in oxygen, and the left common carotid artery was isolated and ligated at two locations with 6–0 surgical silk. The common carotid was then cauterized to block blood flow through the carotid and then pups were returned to their cages and allowed to recover for an hour. After recovery from anesthesia, pups were placed in a humidified container maintained at 36°C and hypoxia was induced by perfusing the container with humidified 8% oxygen in nitrogen gas mixture for 135 minutes. Control animals underwent anesthesia and the common carotid artery was exposed without ligation and hypoxia.

Experimental groups and drug administration

The various antioxidants were intraperitoneally administered as follows: nicotine hydrogen tartrate (1.2 mg/kg; Sigma-Aldrich, St Louis, Mo, EEUU) diluted in saline (Chen, et al., 2013) 2 h before the HI insult; melatonin (15 mg/kg; Sigma-Aldrich, St Louis, Mo, EEUU) diluted in saline and DMSO 5% (Carloni et al., 2008) just after the damage; resveratrol, 20 mg/kg; Sigma-Aldrich, St Louis, Mo, EEUU) diluted in DMSO, 10 min before the HI event (West et al., 2007) and docosahexaenoic acid (DHA; 1mg/kg; Sigma-Aldrich, St Louis, Mo, EEUU) diluted in DMSO and also 10 min before the HI event (Berman et al., 2009). Subsequent analyses were performed at different time points after the HI insult (0, 3, 12, 24 h and 7 days).

Animals were assigned to the following experimental groups (n=5): control, hypoxic–ischemic group (HI), hypoxic–ischemic group with nicotine (N), melatonin (M), resveratrol (R) or with DHA (D). A group “control +vehicle” (saline or DMSO) was initially examined to test the response to the vehicle but no difference was shown with respect to the control group (n=3).

Nissl staining

7 days after HI insult (when animals were now P14), changes in neuronal morphology were evaluated in sections stained with Nissl (Sigma-Aldrich Co. St. Louis, MO, USA) using an optical microscope.

Brainstem damage assessment

2,3,5-triphenyltetrazolium chloride (TTC) staining

24 h after the injury, rats brain were removed after sacrifice and the brainstem was sectioned into 2 mm slices which were then incubated in 2,3,5-triphenyltetrazolium chloride 2% (TTC; Sigma-Aldrich Co. St. Louis, MO, USA) at 37°C during 30 min and fixed in 4% formaldehyde. Photographs of sections were taken with a digital camera in a stereoscopic microscope.

Flow cytometry

For flow cytometry analysis, animals were sacrificed with pentobarbital sodium overdose and perfused with glucosaline solution 24 h after HI insult. Brainstem sections were isolated and disaggregated with a blade in an ice cold glucosaline solution. They were then placed in a cell strainer with 4 ml collagenase (1.5 mg/ml diluted in HBSS; Sigma-Aldrich, St Louis, Mo, EEUU) in each dish and incubated at 37°C during 20 min. The cell suspension was washed with HBSS and centrifuged at 2000 rpm for 5 min. After removing supernatant, the pellet was resuspended in 5 ml of fresh HBSS. The cell suspension was fixed with 70% ethanol and stored at 4ºC.

Flow cytometry was used for the quantification of GFAP, NeuN and synaptophysin (SYP) immunofluorescence. We washed the fixed cell suspension twice with cold PBS-BSA (0.5%). 450 µl samples were then incubated with PBS-Tween20 (1%) for 10 min and then washed with PBS-BSA (0.5%) twice by means of centrifugation at 2000 rpm for 5 min. The cell suspension was incubated with primary antibodies: GFAP (1:500, DAKO, Glostru, Denmark); NeuN (1:500, Millipore, Massachusetts, EEUU) or SYP (1:500, Sigma-Aldrich, Co. St. Louis, MO, USA) in PBS- 0.25% BSA + 0.1% Tween20 overnight at 4ºC. The next day we washed twice the cell suspension with PBS-BSA 0.5% 5 min at 2000 rpm, and incubated with the 488 anti-rabbit secondary antibody for GFAP detection and 488 anti-mouse antibodies for NeuN and SYP detection in PBS-BSA 0.5% for 1 h at room temperature with no light. After washing the samples three times, we suspend the cells in PBS for flow cytometry analyses.

As a control, parallel cell suspensions were processed as above except for the omission of the primary antibody. The percentage of immunopositive cells and the means of fluorescent intensity were measured in an EPICS ELITE flow cytometer. To exclude debris and cellular aggregates, samples were gated based on light scattering properties in the side scattering (SSC) mode which correlates with cell complexity, and forward scattering (FSC) which correlates with cell size, and 10,000 events per sample within a gate (R1) were collected. An unstained sample was used as a control to establish autofluorescence background values.

Semi-quantitative RT-PCR

The RNA isolation procedure was performed with a PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) strictly adhering to the manufacturer's indications. RNA concentration and purity were estimated using NanoDrop ND-1000 UV-VIS kit. cDNA was synthesized with a cDNA Preparation with Fluidigm® Reverse Transcription Master Mix (Fluidigm). qPCR was performed by Fast Gene Expression Analysis using EvaGreen® with the BioMark HD Nanofluidic qPCR System combined with GE 96.96 Dynamic Arrays IFC System.

Table 1: Primers used in q RT-PCR.

	Gene	GenBank ID
Housekeeping genes	**B2M**	NM_012512(1)
	PPIA	NM_017101(1)
	TBP	NM_001004198(1)
	HPRT1	XM_003752155(2)
	PGK1	NM_053291(1)
	GAPDH	NM_017008(1)
	RPLP0	NM_022402(1)

Analyzed genes	Gene	GenBank ID
Cellular activation	**Neurabin/Ppp1r9a**	NM 053473
	GFAP	NM 017009
	Vimentin	NM 031140
	NG2	NM 031022
	Synaptophysin	NM 012664
Cell death	**Caspase 3**	NM 012922
	Bax	NM 017059
	Bcl-2	NM 016993
	Cytochrome C oxidase	NM 012839
Early gene expression	**C-FOS**	NM 022197
	Egr-1	NM 012551
	VEGF	NM 001110334
	HSF1	NM 024393
	Hif-1a	NM 024359
Inflammation	**TNFα**	NM 012675
	COX2	NM 017232
Reactive oxygen species (ROS)	**Sod2**	NM 017051

For the specification of primers, see Table 1). For mRNA detection, Prime Time qPCR predesigned primers were used (IDT Integrated DNA Technologies) and B2M and PGK1 were chosen as appropriate reference genes for the normalization of gene expression. For raw data and input data analysis, the manufacturer's instructions (Fluidigm Real-Time PCR Analysis Software version 3.1.3) were used and Triple Ct values were analyzed by using the comparative Ct (ΔΔCt) method with software GenEx (MultiD). The different time points studied were 0, 3 and 12h post HI.

Statistics

Values are represented as means ± SEM. Differences between two groups were studied by T test and group differences were studied by one-factor analysis of variance with Bonferroni-Dunn correction. Statistical analysis of data was performed using GraphPad prism 5 software version 5.01 (GraphPad Software, Inc. CA, USA.

RESULTS

Histological assessment of brain damage

Nissl staining in the brainstem of P14 rats

7 days after the damage (P14), Nissl staining was performed to observe macroscopic signs of neuronal damage. Staining of brain sections with Nissl revealed signs of early neuronal damage induced by the hypoxic-ischemic event, with the brainstem displaying significant evidence of infarction in the ipsilateral side, whereas sections of non-ischemic control animals or treated animals did not (Fig. 1). In the images with higher magnification, HI animals showed condensed cytoplasm and swollen and deformed neurons comparing to those of the control group. Treated animals showed similar neuronal morphology to the control group although resveratrol treated animals showed neuronal deformation and condensed cytoplasm, similar to HI group neurons.

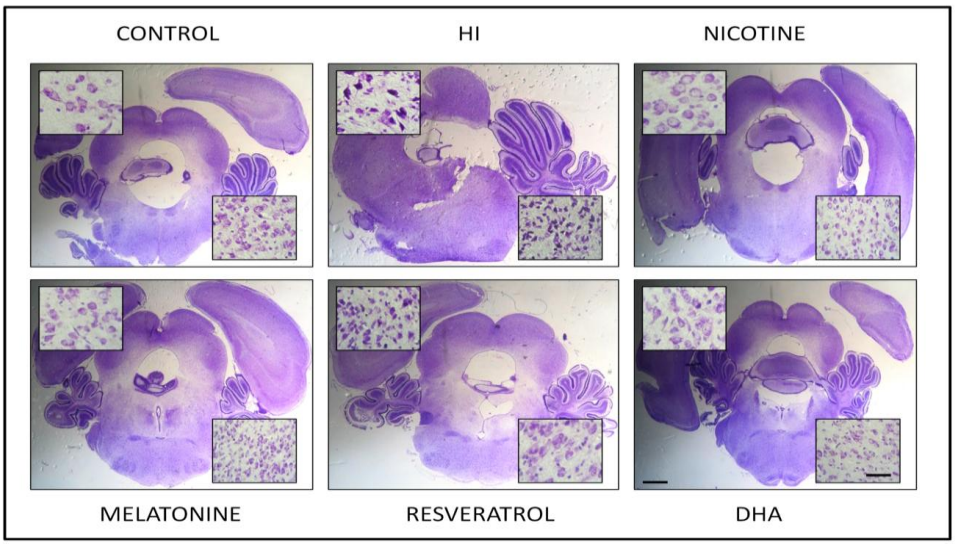

Figure 1: Representative stereo microscope photographs and microphotographs of the brainstem of P14 perinatal rat brains (interaural distance 0.30 mm and bregma 9.30 mm) stained with Nissl (n=8 for each group) and illustrating the alteration in neuron morphology. Stereo microscope images, bar: 2.5 mm; microphotographs bar: 500 µm.

Infarct volume 24 h post HI after antioxidant administration

As we observed evidence of neuronal damage in P14 rats, we analyzed the infarct volume 24 h after the HI event. Infarct volume was significantly increased after HI as can be seen in Fig. 10B. This infarct volume was also observed in animals treated with resveratrol (Fig. 2 E) but with less intensity while in the rest of the antioxidant treated groups (Fig. 2 C,D,F) the infarct volume was almost nonexistent, being similar to the control group (Fig. 2 A).

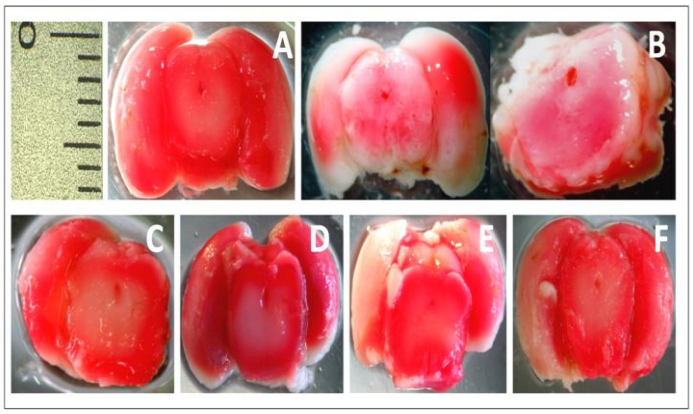

Figure 2: TTC staining of the infarct area 24 h after of HI event (n=8) in P14 pups seven days following HI. A) control group, B) HI group, C, D, E and F) HI+ antioxidant treatment with nicotine, melatonin, resveratrol and DHA respectively.

Flow cytometry analysis

% of positive cell and fluorescence intensity of astrocytes, neurons and synapses 24 h after the HI event

Astrocytes are one of the cell groups that is thought to be altered after hypoxia-ischemia, so

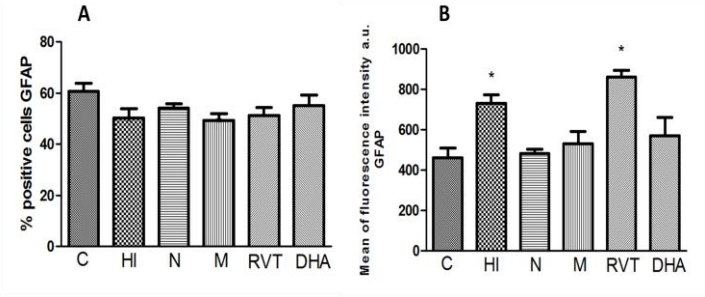

Figure 3: Percentage of GFAP immunopositive cells (A) and the mean of the fluorescence intensity (B) in arbitrary units of these GFAP expressing cells (n=5 for each group). Data are expressed as means ± SD (*p < 0.05 vs. controls and # p< 0.05 vs. HI, ANOVA). Abbreviations: C, control; HI, hypoxia-ischemia; N, nicotine; M, melatonin; RV, resveratrol; DHA, docosahexaenoic acid.

we analyzed by flow cytometry the percentage of GFAP+ cells and the florescence intensity of GFAP+ cells in the brainstem. There was no statistically significant difference in the percentage of GFAP positive cells after the hypoxic ischemic event, although there was a slight decrease in the number of positive cells after the event. This tendency was also observed in the antioxidant treated groups compared to the control group (Fig. 3).

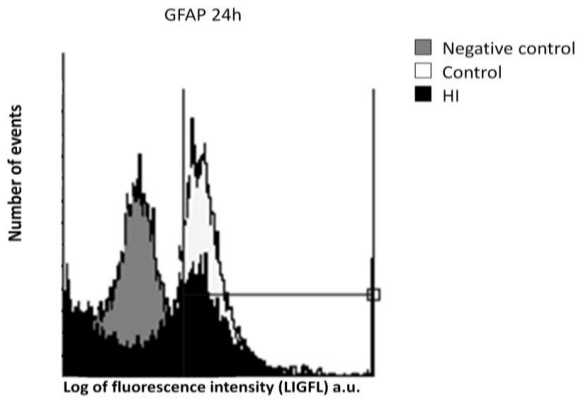

Figure 4: Representative fluorograms obtained after flow cytometry analysis showing the logarithm of fluorescence intensity of GFAP positive cells in arbitrary units 24h after the hypoxic-ischemic event. Only control and HI groups are represented and the negative control consisted of cell suspensions not exposed to the primary antibody.

When we analyzed the florescence intensity of these GFAP positive cells in brainstem, we found a statistically significant increase following HI, but this GFAP reactivity was antagonized by most of the antioxidants with the exception of RVT (Fig. 4).

Flow cytometry revealed that there was a significant decrease in the % of NeuN+ neurons 24 h after the insult (Fig. 5A). Thus, with all treatments the % of NeuN+ cells decreased after this period although this decrease was statistically significant only in the groups treated with melatonin and resveratrol. Comparing the fluorescence intensity of these cells for the different groups, we found that treatment with resveratrol led to increased NeuN fluorescence compared to the control group, but this increase was not statistically significant, while there was no difference in the fluorescence intensity between the other analyzed groups (Fig. 5B).

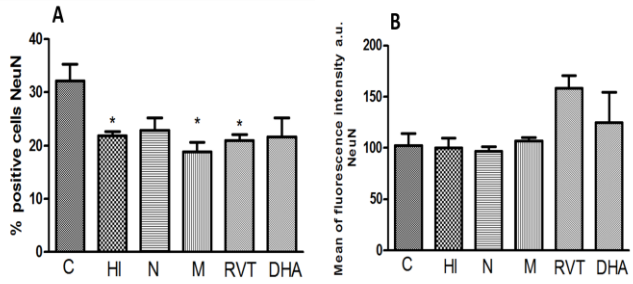

Figure 5: Percentage of NeuN positive cells (A) and the mean of the fluorescence intensity (B) in arbitrary units of these NeuN positive cells (n=5 for each group). Data are expressed as means ± SD (*p < 0.05 vs. controls and # p< 0.05 vs. HI, ANOVA). Abbreviations: C, control; HI, hypoxia-ischemia; N, nicotine; M, melatonin; RV, resveratrol; DHA, docosahexaenoic acid.

In the representative figure comparing the logarithm of fluorescence intensity between the control and HI groups, this decrease in intensity and also in the percentage of NeuN+ cells can be observed (Fig. 6).

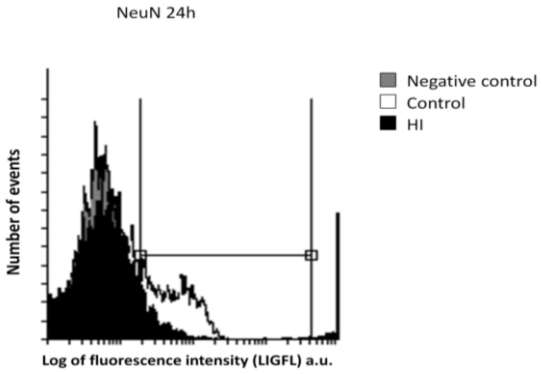

Figure 6: Representative fluorograms obtained after flow cytometry analysis showing the logarithm of fluorescence intensity of NeuN positive cells in arbitrary units 24 h after the hypoxic-ischemic event. Only control and HI groups are represented and the negative control consisted of cell suspensions not exposed to the primary antibody.

Regarding synaptophysin (Fig. 7), we analyzed the % of Syp+ cells 24 h after HI. At this time point, the % of SYP positive cells was found to be reduced by around 10% in the HI group and in groups treated with melatonin and resveratrol. Animals treated with nicotine and DHA did not exhibit a reduction in the % of SYP positive cells compared to the control group. Regarding the mean fluorescence intensity, there were no statistical differences between groups, although this intensity increased in all groups except for the group treated with melatonin, whose fluorescence intensity was similar to that exhibited by the control one.

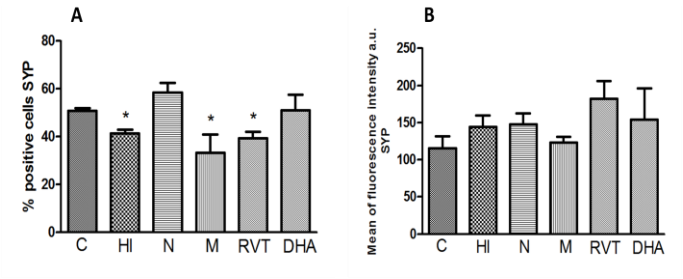

Figure 7: Percentage of SYP positive cells (A) and the mean of the fluorescence intensity (B) in arbitrary units of these positive cells (n=5 for each group). Data are expressed as means ± SD (*p < 0.05 vs. controls and # p< 0.05 vs. HI, ANOVA). Abbreviations: C, control; HI, hypoxia-ischemia; N, nicotine; M, melatonin; RV, resveratrol; DHA, docosahexaenoic acid.

Altered gene expression in response to HI 0 h, 3 h and 12 h after the HI event

Immediate early genes (IEG)

To investigate whether the expression of genes is changed between groups, we analyzed changes in the expression of five genes at three different time points; from previous studies it was found that these genes are expressed immediately after stress or injury.

Early growth response 1 (EGR1), FBJ osteosarcoma oncogene (Fos), hypoxia inducible factor a (HIFa), heat shock factor 1 (HSF1) and vascular endothelial growth factor a (VEGFa) gene expression was analyzed by qPCR 0, 3 and 12 hours after HI. Results are compared with control group (Figure 8).

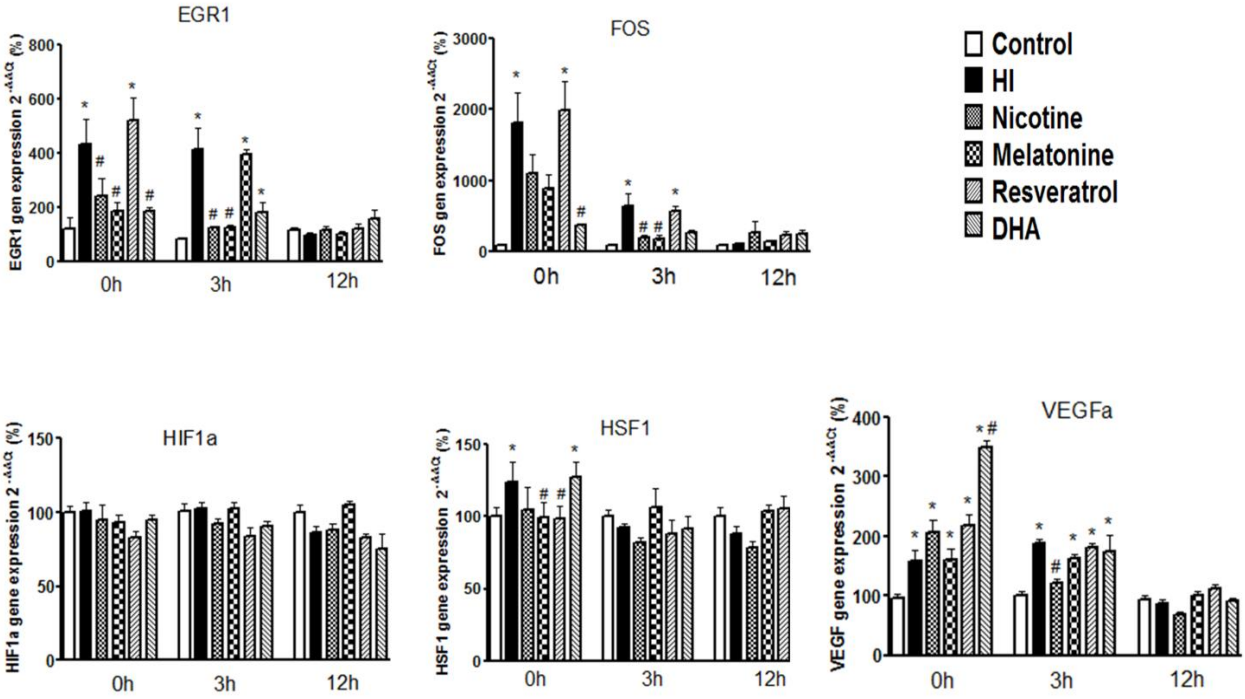

Figure 8: Alteration in the expression of immediate early genes in response to HI insult and various antioxidant treatments in 14 day-old pups who had been subjected to HI insult at 7 days of age. These findings show that there is an increase in EGR1, FOS, HSF1 and VEGFa gene expression just after the damage. Data are expressed as means ± SEM (*p < 0.05 vs. controls and # p< 0.05 vs. HI, ANOVA). Abbreviations: EGR1, early growth response 1; HIF1a, hypoxia inducible factor a; HSF1, heat shock factor 1; VEGFa, vascular endothelial growth factor a.

Directly after the HI insult, altered gene expression of several immediate early genes was observed. In the HI group EGR1, Fos, HSF1 and VEGFa gene expression was increased just after HI insult and this increase was maintained for at least the next 3 hours except for HSF1 gene expression in which there was no statistical difference between groups 3 h after the insult.

The pattern of altered gene expression was different for each gene. Thus, resveratrol upregulated the expression of the EGR1 and Fos genes and this increase was maintained also 3 h later.

The expression of HSF1 was also increased just after the insult in the DHA group, but not in the rest of treated groups. However, this expression returned to normal levels in the rest of the studied time points.

Meanwhile, VEGFa gene expression was found to be upregulated in most groups just after the HI insult and 3 h later compared to the control group with an increase of 100% for almost all groups in the first moment, except from nicotine group that is similar to the control one 3h after the damage. This increase was reduced later.

12h after the insult, there were no significant differences in gene expression between the studied groups, indicating that these changes occurred just after the damage, as is appropriate for immediate early genes. We also observed that expression of HIF1a was unaffected at any of the studied time points (Fig. 8).

Cell death

To investigate the impact of HI insult on cell death, we quantified the expression of common

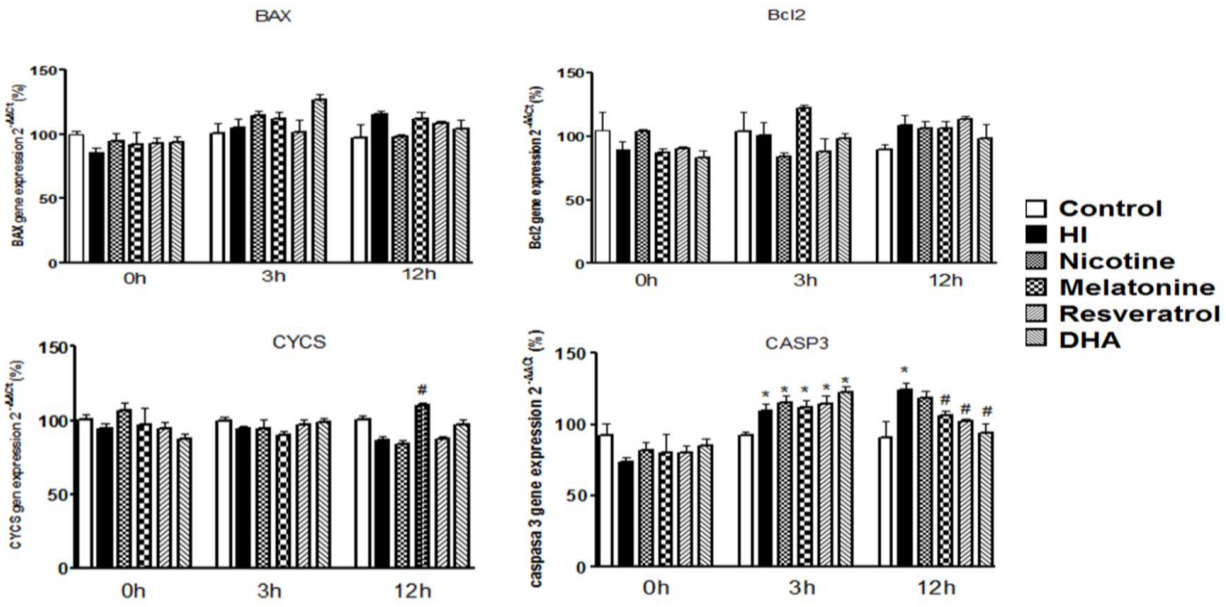

Figure 9: Expression of cell death related genes BAX, BCL2, CYCS and caspase 3 genes which are implicated in cell death, 0, 3 and 12 h after the hypoxic-ischemic event. Data are expressed as means ± SEM (*p < 0.05 vs. controls and # p< 0.05 vs. HI, ANOVA).

cell death genes such as BCL2-asociated X protein (BAX), B-cell CLL/lymphoma 2 (Bcl 2) and caspase 3 (CASP3) by qPCR (Figure 9). Only Casp3 gene expression was significantly altered in response to the HI insult. Expression of this gene was increased 3 and 12 hours after the event in the HI group and in treated groups, its expression was reducing 12 hours post-insult in all groups except for the group treated with nicotine in which 12 hours after insult, the increase was still visible (Figure 9).

Inflammation and ROS

Although we found no statistically significant difference between groups for SOD2 gene expression at any time point (Fig. 10), there was an intense increase in the expression of the inflammation related TNFα gene 3 h after the HI event in the Hi, nicotine and melatonin groups. The increase was not so clear in the rest of the antioxidant treated groups, but 12 h after the insult, this increase was observed in the resveratrol and melatonin treated groups (Fig. 10).

Concerning the PTGS2/COX2 gene, its expression was increased in the HI group just after the damage, while in treated groups its expression was similar to that of the control group at all studied time points (Fig. 10).

Cellular activation evidence

According to previous studies, after a hypoxic ischemic event, several elements can be altered, such as neurons, astrocytes, oligodendrocytes, synapses and structural elements. To this end, we analyzed the effect of hypoxic ischemic injury on the expression of

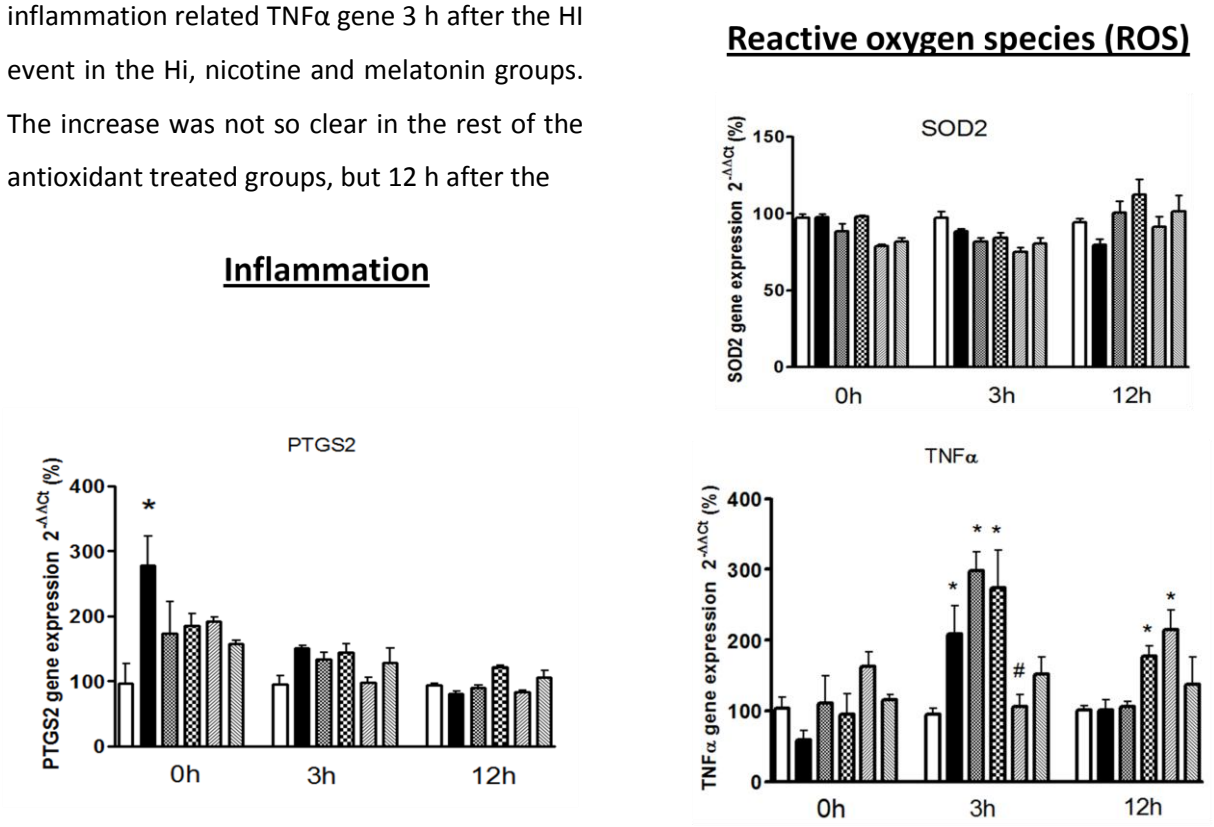

Figure 10: Expression of inflammation and ROS related genes; SOD2, PTGS2 / COX2 and TNFα at different time points after the HI event. Data are expressed as means ± SEM (*p < 0.05 vs. controls and # p< 0.05 vs. HI, ANOVA).

glial fibrilary acidic protein (GFAP), chondroitin sulfate proteoglycan 4 (cspg4/NG2), protein phosphatase 1, regulatory subunit 9B (PPP1R9B/Neurabin II), vimentin (VIM) and synaptophysin (SYP) genes (Fig. 11).

The expression levels of most of the analyzed genes were substantially altered after the insult. GFAP, NG2, neurabin II and Syp gene expression was upregulated just after the insult in the HI group compared to the control group and this increase was maintained until at least 3 h after the insult in all genes except for Syp.

Antioxidant treatments had different effects on the expression of each gene. Regarding GFAP gene expression, all treated groups manifested increased expression during the first hours except from the group treated with resveratrol and nicotine that are similar to the control group, it seem that resveratrol and nicotine inhibit GFAP increased expression in response to HI. 12 h after the insult, GFAP expression is decreased in all groups to control levels, except from melatonine group, that still has increased gene expression. Taken together, these results indicate that a massive astrogliosis occurs in almost all groups after the damage.

However, expression of the NG2 gene in the antioxidant treated groups was similar to that of the control group during all studied time

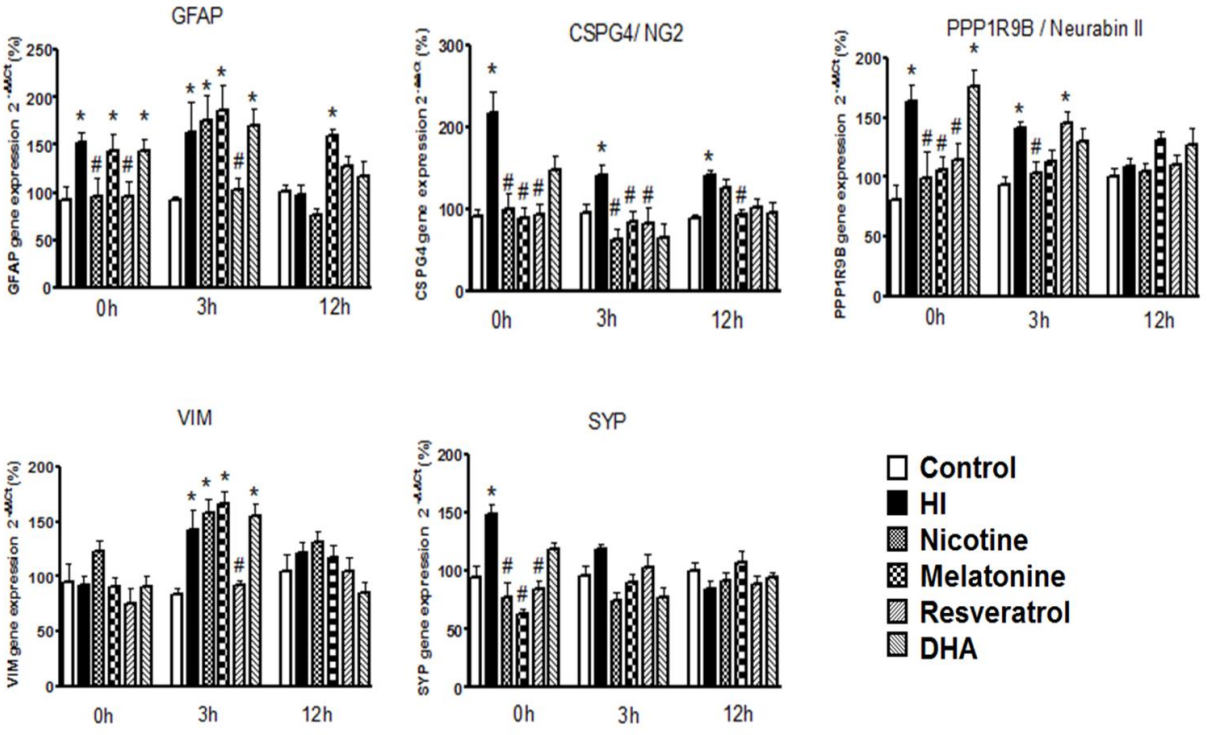

Figure 11: Expression of genes indicative of cellular activation during the hypoxic ischemic event including GFAP, CSPG4/NG2, pp1r9b/Neurabin II, VIM and SYP at different time points after the HI event. Data are expressed as means ± SEM (*p < 0.05 vs. controls and # p< 0.05 vs. HI, ANOVA).

points, indicating that these antioxidants inhibited NG2 gene upregulation in response to the HI insult.

Meanwhile, neurabin II gene expression is increased in DHA and Resveratrol treated groups, the fist one just after the insult and the second one 3 h after the insult compared to the control group.

Vimentin is the only gene whose expression was not altered just after the insult, but 3 h after the ischemic event all groups except for the resveratrol treated group exhibited increased expression compared to the control group. Nevertheless, this difference disappeared 12 h after the insult.

Syp gene expression upregulation in response to the HI event was completely antagonized by antioxidant treatments, since Syp expression levels were not significantly different to those of the control group (Fig. 11).

All examined genes (except for Vimentin) show immediate activation following HI insult, behaving also like immediate early genes in the brainstem.

DISCUSSION

Here, we report alteration in the expression of a panel of relevant genes in the brainstem immediately after hypoxic-ischemic insult and 3 h later, as well as 24 h later in the infarct area; 7 days after the insult, damage was observed in neuronal morphology, so the damage was maintained during the time. Although the effect of candidate neuroprotective substances was different for each gene, protection was observed in the infarct area 24 h after the insult and also in neuronal morphology 7 days after the HI.

Our work also documents evidence of cellular activation, in terms of structural gene expression upregulation from as early as the first moments post HI insult to at least 24 hours later. The activation markers we examined include markers for brainstem neurons, astrocytes, oligodendrocytes, intermediate filaments and synapses.

Glial cells expressing nerve/glial antigen 2 (NG2 cells) are widespread cell populations identified by their specific expression of NG2 chondroitin sulphate proteoglycan (CSPG), which in the central nervous system (CNS) accounts for approximately 8% to 9% of the total cell population in adult white matter and 2% to 3% of total cells in adult grey matter. These cells mainly differentiate into oligodendrocytes which participate in myelination; a dramatic increase in early OL progenitors in HI animals has recently been reported to be a reactive response to injury (Yang et al., 2013). Other studies have demonstrated that neurabin, a protein involved in neurite outgrowth (Orioli et al., 2006) is upregulated after middle carotid artery occlusion (MCAO); the same was found for vimentin, a protein involved in intermediate

filament structure maintenance (Benitez et al., 2014). This result is consistent with ours in which the expression of these two genes is increased, 0 and 3 h after the insult and 3 h after the insult respectively.

Synaptophysin (Syp), a surface protein present in synaptic vesicles, was analyzed to study the effect of the hypoxic ischemic injury to the synaptic transmision in tha brainstem. Increased levels of syp gene expression have been found to be associated with an increased number of synaptic vesicles (Tagliaferro et al., 2006; Tsai et al., 2013).

Astrocytes become reactive after CNS injury and they show increased gene expression, including increased expression of glial fibrillary acidic protein (GFAP), nestin, and vascular endothelial growth factor (VEGF) (Na et al., 2015). We found similar results in which just after the injury and 3 hours after the injury the expression of GFAP and VEGF was increased compared to the control group. In all treated groups, we found caspase activation 3 hours after the insult and also VEGFa upregulation in the first moments, but this alteration was reduced 12 h after the insult.

After observing this cellular activation in some gene expression just after the damage, we analyzed by immunofluorescence if the damage persists over time. 24 h after the HI event, we found fewer neuronal positive cells and synaptic vesicles but the fluorescence intensity of GFAP marker was increased in the HI group compared to the control group. This results correlates with the findings of previous studies (Xiong et al., 2009; Donega et al., 2014) in which there was a progressive reduction in neuron numbers 1 to 7 days after HI as well as significant astrocyte activation at 0 and 1 day post HI. Regarding synaptophysin, previous studies found that syp immunoreactivity was reduced around areas of infarction in keeping with our results (Tuor et al., 2001).It seem that synaptic transmission is altered 24h after the injury

Hypoxia ischemia altered the expression of most genes from the first moments after the insult and EGR1, NG2 and Casp3 gene expression similar to the control group in the rest of estudied groups during all studied time point. Surprisingly, there was no alteration in the expression levels of Sod2 or HIF1a or in the expression of the BAX, BCL2 and CYCS genes which are related to cell death. In the case of the TNFα and Vim genes, changes in expression were not seen immediately after the insult, but rather they were found to occur 3 hours after the damage (See table 2).

Group	Gene	HI	TREATMENTS			
			Nicotine	Melatonine	Resveratrol	DHA
Early gene expression	*EGR1*	0,3,12	-	-	0,3	-
	FOS	0,3	-	-	0,3	-
	HIF1a	-	-	-	-	-
	HISF1	0	-	-	-	0
	VEGFa	0,3	0	0,3	0,3	0,3
ROS	*Sod2*	-	-	-	-	-
INFLAMMATION	*TNFα*	3	3	3	-	-
	Cox2	0	-	-	-	-
CELLULAR ACTIVATION	*GFAP*	0,3	3	0,3,12	-	0,3
	NG2	0,3,12	-	-	-	-
	Neurabin II	0,3	-	-	3	0
	VIM	3	3	3	-	3
	Syp	0	-	-	-	-
CELL DEATH	*BAX*	-	-	-	-	-
	Bcl2	-	-	-	-	-
	Cycs	-	-	12	-	-
	Casp3	3,12	3	3	3	3

Table 2: Summary of the genes whose expression was significantly altered compared to the control group. Abbreviations: 0, 0 h after the HI insult; 3, 3 h after the insult; 12, 12 h after the insult and "-", no difference compared to the control group. In all cases the difference was an increase in the gene expression.

Changes in immediate early gene expression

IEGs have received attention because many of their protein products are transcription factors and play a crucial role in the effect of several stresses in long term changes in the nervous system. However, the precise role of IEGs in neonatal HI is unclear. In HI models, the maximal induction of IEG mRNA was found at 1 to 2 hours after the insults, which correlates well with the present findings. EGR1 is an important mediator of fibroblast activation and its upregulation is followed by the activation of a multitude of signaling cascades vital for growth, differentiation and apoptosis (Zins et al., 2014). EGR1 also activates expression of multiple target downstream genes, especially those associated with the inflammatory response, such as TNFα (Yan et al., 2000; Okada et al., 2002) or COX2 in keeping with our results, in which TNFα expression levels were increased 3 h after the insult and Cox2 just after the insult. Previous studies have demonstrated that EGR1 is upregulated in many organs after HI (Wu et al., 2013) and overexpression of EGR1 induces apoptosis with caspase activation (Zins et al., 2014) which also correlates with our results in which we found an increase in expression of EGR1 immediately after and 3 h after the insult and with the upregulation of caspase 3, 3 h and 12 h after the HI event. Moreover, it has been reported that both mRNA and protein levels of caspase-3 are increased in vulnerable brain regions following transient global ischemia (Wang et al., 1999). Studies of neuronal activity after hypoxia in the brainstem have revealed that

stimulation of these receptors can induce Fos expression in both the catecholaminergic and serotoninergic neurons in the brainstem (Erickson &Millhorn, 1994). In keeping with this finding, we also found a huge

increase in Fos expression just after the insult and 3 h later.

Apart from the mentioned immediate early genes, hypoxia-inducible factor 1alpha (HIF-1a) is considered to be a major oxygen sensor (Goetzenich et al., 2014). Our results indicate that there was no difference between groups at any analyzed time point in the brainstem after the HI event. This finding may indicate that the expression of this gene is not relevant in the brainstem compared to the rest of the brain. Pascual et al. (2012) reported that HIF-1a protein levels increased under hypoxia in regions involved in cardiorespiratory control but in contrast, HIF-1a accumulation was not detected in other brainstem regions (Olivetto et al., 2015).

Other genes that are expressed after damage are heat shock factor1 (HSF1) and vascular endothelial growth factor (VEGF). The first one is the master regulator of the heat shock response (Fujimoto and Nakai et al., 2010). Activation of HSF1 leads to its nuclear translocation and induction of more than 3,000 genes, including the classical molecular chaperone genes such as HSP70 (Islam et al., 2013). Our results suggest that HSF1 is upregulated just after the HI event, but this increased expression is short lasting and by 3 h has returned to control levels. Accordingly, Sun et al. (2015) have reported that in mature neurons (P26), HI leads to nuclear translocation of HSF1 and induction of molecular chaperones, while chaperones are induced minimally in P7 immature neurons, but moderately in P7 microglia after HI. As GFAP and NG2 gene expression was found to be strongly upregulated just after the insult, we can assume that HSF1 upregulation in our case is due to microglial reactivity. Meanwhile, an increase in the expression of VEGFa during the first 3 hours was also observed after the HI injury. In this regard, VEGFa has been reported to be a hypoxia-inducible signal glycoprotein that causes vascular endothelial cell growth and proliferation and leads to the regeneration of the vascular network in brain regions damaged by ischemia. Several studies have found that VEGF can act as a direct stimulator of neurogenesis (Sunet al., 2003; Kovacs et al., 1995) in keeping with the present findings.

Effects of antioxidants on HI-induced gene upregulation

The antagonistic effects of the various antioxidants on gene expression were different for each gene. Thus, nicotine pre-treatment hardly altered the expression of the immediate early genes compare to the control group. The mayor difference was 3 hours after the insult when astrogliosis, inflammation and structural changes begin to occur and the corresponding

genes were found to be activated; this upregulation disappeared 12 hours after the insult. Previous studies confirm that the nicotinic acetylcholine receptor has anti-inflammatory (Tarras et al., 2013) effects and can induce upregulation of HIF-1α and VEGF (Ma et al., 2014).

Melatonin seems to have retarded effects on gene activation. There was also astrogliosis during all studied time points, inflammation, structural change and cytochrome C oxidase upregulation 12 hours after the insult while there was almost no early gene expression compare to the control group. This could be because melatonin was administered just after the damage and it needs more time to respond to the alterations. Melatonin achieves powerful neuroprotective effects via anti-oxidant, anti-apoptotic and anti-inflammatory processes and by promoting neuronal and glial development (Acuna-Castroviejo et al., 2001; Hassell et al., 2015).

Regarding resveratrol, IEG expression was activated after the HI event. EGR1 and fos were upregulated just after the insult and 3 hours later. Meanwhile there were no differences in inflammation or structural changes compared to the control group. Several studies confirm that resveratrol treatment promotes an upregulation of Egr-1 expression (Rossler et al.,2015) and that resveratrol-mediated changes in neurotrophin factor expression in the hippocampus and in levels of proinflammatory cytokines in circulation promote neuroprotective and anti-inflammatory effects (Yazir et al., 2015) apart from promoting angiogenesis in cerebral endothelial cells (Simao et al., 2012) in keeping with our results.

Meanwhile, the effects of DHA seem to be related to cellular activation. There is astrogliosis, structural damage and neurite activation and HSF1 is upregulated just after the insult. However, there is no inflammation change (Velten et al., 2014).

However we did not find any difference concerning to ROS gene expression in the HI group. This suggests that the neuroprotective effect of administered treatments in the brainstem after the HI injury is not related to their antioxidant effects, but rather may be related to other neuroprotective mechanisms which should be investigated.

In conclusion, the present work support the hypothesis that the brainstem is also affected by hypoxic ischemic injury with alterations in the expression of several key genes and in the activation of some cellular responses. Loss of neuronal and synaptic vesicles ensues and astrocyte activation occurs, leading to the infarct area days after the damage. The studied antioxidant treatments do seem to confer neuroprotection to the brainstem after the injury but the underlying mechanism is not well understood. However, the present findings indicate that their neuroprotective

effect is not due to their antioxidant properties, at least in the brainstem. It can be that depending on the area the protective mechanism is different.

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6.4 III. ARTIKULUA

PLOS ONE

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Effect of neonatal asphyxia on the impairment of the auditory pathway by recording auditory brainstem responses in newborn piglets. A new experimentation model to study the perinatal hypoxic-ischemic damage on the auditory system.

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Keywords

Hypoxia-Ischemia; Perinatal Asphyxia; Auditory Pathway; Auditory Brainstem Responses.

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Abstract

Hypoxia–ischemia (HI) is a major perinatal problem that results in severe damage to the brain impairing the normal development of the auditory system. The purpose of the present study is to study the effect of perinatal asphyxia on the auditory pathway by recording auditory brain responses in a novel animal experimentation model in newborn piglets. Hypoxia-ischemia was induced to 1.3 day-old piglets by clamping 30 minutes both carotid arteries by vascular occluders and lowering the fraction of inspired oxygen. We compared the Auditory Brain Responses (ABRs) of newborn piglets exposed to acute hypoxia/ischemia (n=6) and a control group with no such exposure (n=10). ABRs were recorded for both ears before the start of the experiment (baseline), after 30 minutes of HI injury, and every 30 minutes during 6 h after the HI injury. Auditory brain responses were altered during the hypoxic-ischemic insult but recovered 30-60 minutes later. Hypoxia/ischemia seemed to induce auditory functional damage by increasing I-V latencies and decreasing wave I, III and V amplitudes, although differences were not significant. The described experimental model of hypoxia-ischemia in newborn piglets may be useful for studying the effect of perinatal asphyxia on the impairment of the auditory pathway.

INTRODUCTION

Hearing, mostly developed over the first few years of life, is a fundamental sense for communication skills. In this sense, a healthy auditory system from birth is needed to develop correct hearing and avoid the language delays associated with perinatal hearing loss. Otherwise, perinatal hearing loss will be associated with language delay. At the same time, the maduration of the auditory system finishes at 18 months and is related to the process of myelination of the central nervous system. Normal development, maduration and myelination of the auditory system can be impaired by certain pathological conditions such as viral infections, hyperbilirubinemia, meningitis or perinatal asphyxia and is related to the process of myelination of the central nervous system. Specifically, in kernicterus, the auditory system is damaged at the level of the cochlear nuclei and when hypoxia is severe, it is associated with mental retardation and neuromuscular disorders (Lefebvre, et al., 2002; Cao et al., 2010).

In this context, the synaptic nuclei of the brainstem are an important part of the auditory system and represent the first synapse of the acoustic nerve. The nerve bundles that innervate the cochlear nuclei are the anatomical substrate that has a decisive role in modulating the acoustic information from afferent and efferent nerves transmitted to the brainstem and higher cortical levels (Rubel and Fritzsch, 2002; Kishan et al., 2011; Appler and Goodrich, 2011).

Hypoxia–ischemia (HI) is a major perinatal problem that results in severe damage to the brain. In humans, 80% of the growth of the central nervous system (CNS) takes place in the last two months of gestation and the first few months of life, periods during which this system is particularly damaged by the effects of a low blood flow (du-Plessis and Volpe, 2002; Soehle et al., 2003). In line with this, hypoxic/ischemic brain injury that takes place during this perinatal period is one of the most common causes of long-term severe neurological impairments. (Volpe, 2001; Perlman, 2004). Auditory brainstem evocated potentials are very useful in the diagnosis of these injuries because they can quantify the degree of damage. (Martinez Ibargüen et al., 1993). Neurons are the elements that are the most vulnerable to oxygen deprivation but if exposure is sufficiently severe other cell systems, in particular the glial cells, are also affected (Hilario et al, 2005; Goñi de Cerio et al, 2007). Further, the immature brain is made more vulnerable by a series of specific factors (Inder and Volpe, 2000, Alvarez A et al. 2007), including a greater susceptibility to excitotoxicity and to free radicals and a greater tendency to cell death by apoptosis (Echteler et al., 2005). So, plasticity of the synaptic connections depends on various factors such as perisynaptic glial cells and pre- and post-synaptic neuronal mechanisms (Todt K.J, et al, 2006).

The newborn piglet is considered an ideal experimental model for studying metabolism

and brain circulation. Specifically, the development of the brain of a newborn piglet, < 5 days old, is similar to the development of the brain of a newborn human infant, making it well suited for studying lesions observed in premature infants who have experienced a period of HI. Further, this model allows the use of instruments required to study responses over not just the short (hours) but also the long (days or weeks) term, unlike other animal models given their small size and weight (e.g., the rat fetus). In the newborn piglet model, various methods have been used to induce brain injury, occlusion of the carotid while reducing the inhaled oxygen fraction being the most widely used (Foster KA, 2009). In the present work we studied HI piglets up to 360 min post-HI insult. This period is commonly selected in this model because the biochemical, neuropathological, and neurobehavioral consequences of HI are well established over that interval of time (Foster KA, 2009; Schubert S, 2005; Peeters-Scholte C, 2003).

The objective of the present study was to develop a new animal experimental model in piglets for studying the effect of perinatal asphyxia on the auditory pathway. For this, we studied the integrity of the brainstem after HI investigating whether such damage produces electrophysiological changes in Auditory Brainstem Response (ABR) recordings.

METHODS

Animal preparation

The study was performed in the laboratories of the Research Unit on Experimental Perinatal Physiopathology of Cruces Hospital. The experimental protocol met European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005) and was approved by the Ethical Committee for Animal Welfare of Cruces Hospital and has been extensively described elsewhere (Alvarez FJ, 2008).

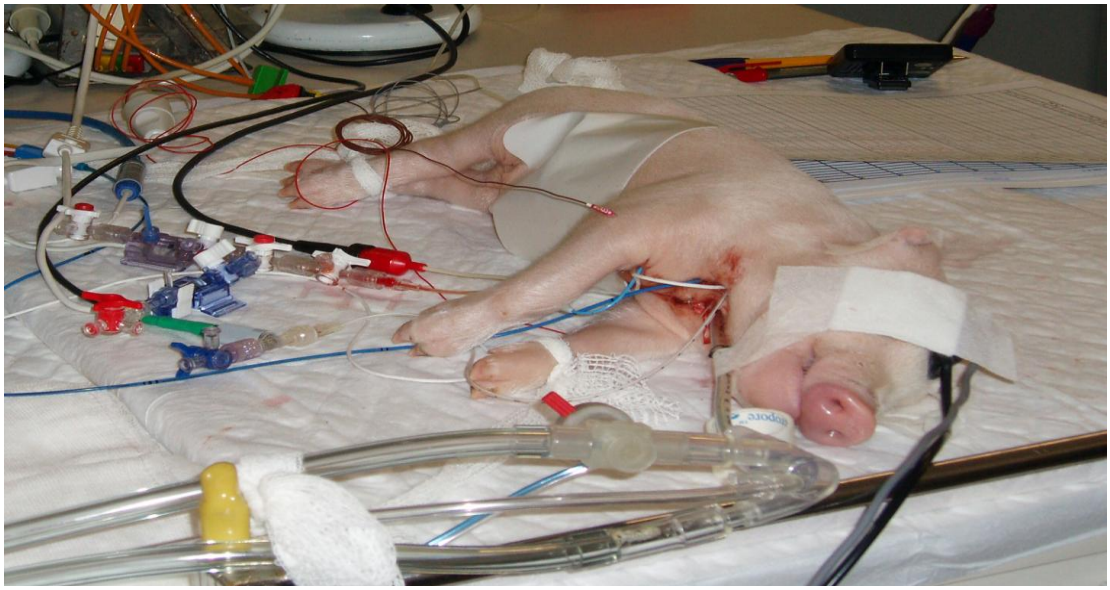


Fig. 1. Piglet. Panoramic view of the experimental model in newborn piglet.

NEUROPHYSIOLOGICAL ASSESSMENT (mean±SD)	Basal	20 minutes	1 hour	3 hours	6 hours
aEEG Control (µV)	21,00 ± 1,10	22,00 ± 1,10	21,00 ± 0,57	22,00 ± 1,23	23,00 ± 1,19
aEEG HI (µV)	23,50 ± 1,32	1,92 ± 0,55	2,33 ± 0,57	2,72 ± 0,81	3,12 ± 0,77
TOI Control (%)	50,00 ± 0,66	51,67 ± 0,66	52,33 ± 0,95	52,33 ± 1,06	52,67 ± 0,49
TOI HI (%)	52,00 ± 1,12	26,63 ± 1,12	50,25 ± 1,42	52,00 ± 0,48	53,29 ± 0.69
nTHI Control (%)	95,67 ± 2,94	96,33 ± 3,00	95,00 ± 4,29	93,33 ± 3,53	93,00 ± 5,35
nTHI HI (%)	99,13 ± 2,75	122,80 ± 3,93	107,63 ± 2,70	110,00 ± 3,76	104,57 ± 2,24

Table 1. Neurophysiological assessment of piglets: amplitude-integrated electroencephalography aEEG, tissue oxygenation index (TOI) and normalized tissue hemoglobin index (nTHI) in control and Hypoxic-Ischemic (HI) groups. HI Hypoxia–ischemia. TOI: tissue oxygenation index. nTHI: normalized tissue hemoglobin index. SD: standard deviation.

Briefly, 1- to 3-d-old piglets, previously anesthetized and paralyzed with a perfusion of fentanyl, propofol and midazolam in dextrose 5% (0.004, 3 and 0.5 mg/kg/h, respectively) and vecuronium (3 mg/kg/h), were intubated and mechanically ventilated (Bourns BP200; CA), Fig. 1. The femoral artery was cannulated to monitor blood pressure (Ominare CMS24; HP, Göblingen, Germany) and to obtain blood samples. Blood oxygen saturation was monitored by transcutaneous pulse oximetry. Blood gases and glycemia were regularly checked to adjust the ventilator settings and/or to add dextrose or vasoactive drugs to correct deviations from appropriate levels. Rectal temperature was maintained between 37.5 and 38.5°C with heat lamps.

Experimental procedure

HI group (n=6): HI was induced by clamping carotid arteries bilaterally with vascular occluders and lowering the fraction of inspired oxygen to 8–10% over 30 minutes (age 1.3 ± 0.3 d, and weight 1.7 ± 0.1 kg). (Fig. 1). Control group (n=10): Piglets were similarly anesthetized and intubated but were kept with normal oxygen levels and their carotids were not clamped (age 1.7 ± 0.5 d, and weight 1.6 ± 0.1 kg). Animals in each group were kept ventilated with sedation and analgesia up to 6 h after HI. At the end of the experiment, anesthetized piglets were sacrificed with intravenous potassium chloride.

Neurophysiological and neurobehavioral assessment of animals: The tissue oxygenation index expressed as percentage, and normalized tissue Hb index were continuously monitored using a near-infrared spectroscopy (NIRS) system (NIRO-200; Hamamatsu Photonics KK, Joko Cho, Japan). The NIRS sensor was placed on the skull frontoparietally at the midline and fixed with ndages. Brain activity was monitored using a two-channel bedside amplitude-integrated electroencephalography (aEEG) monitor

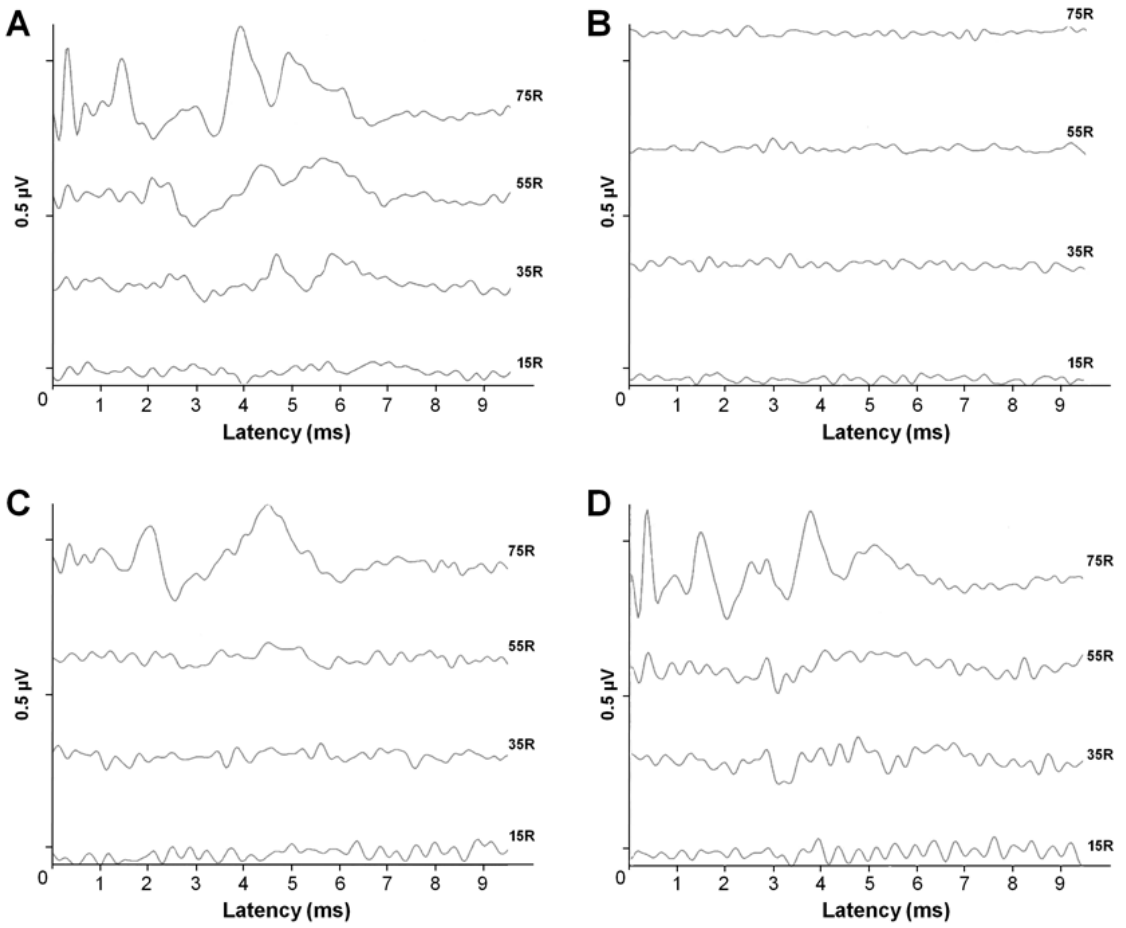


Fig. 2. ABRs responses. Acoustic brainstem responses before, during and after hypoxia/ischemia exposure. A) Basaline. B) During HI event. C) 30 min. after HI event. D) 360 min. after HI event. We can observe four different traces associated to different intensities of ABR presentations showing that the responses disappear during exposure, partially recover after 30 minutes and completely recover after 360 minutes

(BRM2BrainZ Instruments, Auckland, New Zealand) using five needle electrodes placed at C3–P3, C4–P4 and near the shoulder with lead directing towards the head as recommended in the user guide by the manufacturer (International 10–20 system). NIRS and aEEG were continuously recorded throughout the period of mechanical ventilation and sedation and analgesia. The aEEG from each pair of electrodes was processed with a pass-band of 1 Hz to 50 Hz (single pole high-pass filter at 1 Hz, 4-pole low-pass Butterworth filter at 50 Hz) to exclude muscle artifacts. A neurological examination was performed using an adapted standardized approach for scoring piglets (Schubert S, 2005). The aEEG from each pair of electrodes was processed with a pass-band of 1 Hz to 50 Hz (single pole high-pass filter at 1 Hz, 4-pole low-pass Butterworth filter at 50 Hz) to exclude artifacts. Table 1 shows aEEG, TOI and nTHI in control and Hypoxic-Ischemic groups.

Auditory Brainstem Responses (ABR)

A GSI Audera device running software version 1.0.3.4 was used to record auditory steady-state evoked potentials. The measurements were

performed in a sound-proofed room to ensure that background noise did not affect our results. The specifications of the acquisition were: sweep time 10 ms with 150 and 3000 Hz filters for low and high frequencies, respectively.

Stimulation was performed by means of clicks at 1000 Hz carrier frequency and 10 stimuli/second repetition rate. The averages were taken of 2006 responses. The study started with continuous clicks at intensities of 75 dB nHL, this being progressively reduced by 20 dB (to 55, 35 and 15 dB nHL) until identification of the auditory threshold.

Repeated auditory brainstem responses (ABRs) were recorded for both ears of all of the animals in the following conditions: 1) baseline, before the start of the experiment; 2) during the stabilization phase of the animal, after the carotid and femoral surgery; 3) After 30 minutes of HI injury, and 4) every 30 minutes during 6 h after the HI injury. Wave components of the responses were analysed to assess brainstem auditory electrophysiology. The parameters analysed were hearing thresholds, peak amplitudes (µV), peak latencies (ms) and interpeak latencies.

Statistical analysis

SPSS 15.0.0 software was used for all statistical analyses. Data are presented as means ± standard deviation (SD). Median values have been compared using Mann-Whitney U test. A p value < 0.05 was considered to be significant.

Ethical considerations and limitations

The experimental protocol was overseen by the Ethics Committee for Animal Welfare and the Research Commission of Cruces Hospital in accordance with EU directive 86/609/EEC and the NIH Guide. Once experiments had been completed, animals were sacrificed according to the spanish and european guidelines.

RESULTS

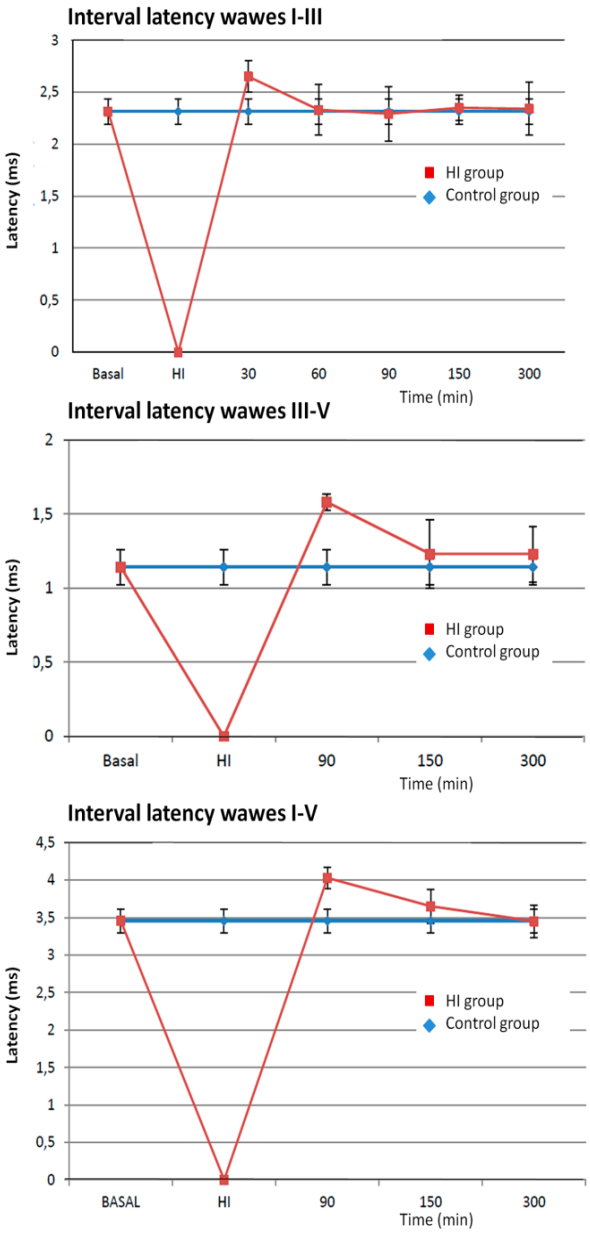
Fig. 2 shows ABR recordings before, during and after inducing HI. It can be seen that the ABRs in newborn piglets in the control group followed a normal pattern, all with the same morphology and thresholds of 15-35 dB. Further, the waves of the ABRs completely disappeared during the HI exposure and start recovering over a period of 30-60 minutes following the end of the HI conditions. 360 minutes after the hypoxic ischemic event, the waves practically return to baseline latency and amplitude.

ABR VARIABLES	CONTROL mean ± SD	30 min HI mean ± SD	60 min HI mean ± SD	90 min HI mean ± SD	150 min HI mean ± SD	360 min HI mean ± SD
I Amplitude (µV)	0,139 ± 0,025	0,106 ± 0,004	0,130 ± 0,026	0,131 ± 0,033	0,142 ± 0,026	0,148 ± 0,014
III Amplitude (µV)	0,182 ± 0,042	0,161 ± 0,011	0,161 ± 0,021	0,162 ± 0,045	0,176 ± 0,027	0,158 ± 0,03
I Latency (ms)	1,598 ± 0,16	1,716 ± 0,244	1,66 ± 0,112	1,66 ± 0,272	1,74 ± 0,221	1,58 ± 0,035
III Latency (ms)	3,913 ± 0,12	4,366 ± 0,303	3,99 ± 0,214	3,95 ± 0,165	4,09 ± 0,167	3,92 ± 0,208
V Latency (ms)	5,056 ± 0,174	-	-	5,46 ± 0,127	5,185 ± 0,2	5,1 ± 0,028
Interval latency I-III (ms)	2,315 ± 0,12	2,65 ± 0,152	2,33 ± 0,242	2,293 ± 0,26	2,35 ± 0,121	2,34 ± 0,254
Interval latency III-V (ms)	1,142 ± 0,12	-	-	1,58 ± 0,056	1,23± 0,23	1,23 ± 0,188
Interval latency I-V (ms)	3,457 ± 0,156	-	-	4,03 ± 0,141	3,65 ± 0,23	3,452 ± 0,218

Table 2. Means and standard deviations (SD) of amplitudes, latencies and intervals of waves I, III and V in the control group and damaged group during and after the hypoxic ischemic event. HI: Hypoxia–ischemia. min: minutes. SD standard deviation.

Table 2 shows the mean and SD of the amplitudes, latencies and intervals for waves I, III and V in the control group and HI groups during and after the event. Diminished amplitudes and increased latencies were observed in the HI group relative to the control group in the first minutes after the HI event, although these differences were not significant in any case, $p>0.05$. ABR recordings remained steady with no further variations through the 6-hour post-HI period. While I and III waves disappeared during the damage and recovered in the first 30 minutes following the end of the HI conditions, the wave V disappeared in the same point but the recovery occurred after 90 minutes following the end of the damage.

Fig. 3 shows graphics of the interval latency that represent the time conduction between the wave's I-III, III-V and I-V in control and HI group. In all the cases, interval latency increased relative to the control group after the HI event, but it returned to baseline in the following hours.



DISCUSSION

Meyer et al. and Newton authors (Newton, 2001; Meyer 2001) have studied auditory impairment in infants and children, attempting to identify the causes of the impairment. They found that there were often several potential causative factors for the impairment and that it could not be attributed to any one cause. In relation to this, sensory-neural hearing loss (SNHL) is a common feature in the post-asphyxial syndrome in newborns. In this study, we used ABR in newborn piglets to examine brainstem neural conduction. The present work supports the utility of this novel hypoxic-ischemic experimental model to study the effect of perinatal asphyxia on the auditory system.

Previous experiments in animals indicated that a normal ABR requires the integrity of an anatomically diffuse system comprising a set of neurons, their axons, the synapses between them, and the neurons on which each of the ABR components terminate (Zaaroor, 1991). Disruption of any portion of the system will alter the amplitude and/or the latency of that component.

Jiang et al. (2009) compared 68 preterm infants exposed to perinatal HI with healthy preterm infants. The preterm infants after perinatal HI showed a significant increase in the I–V interval, while the III–V interval and III–V/I–III interval ratio also increased significantly. Compared with normal term controls, these preterm infants showed similar, but slightly more significant, abnormalities. These results demonstrated that functional integrity of the brainstem was impaired mainly in the most central part. These findings agree with our results in the newborn piglet model, in which that wave V was more severely affected than waves III and I. These authors concluded that HI damage to the preterm brainstem is unlikely to completely

recover in a relatively short period after the insult, which is also in accordance with our findings. In a previous study (Jiang, 2006) the same group found I–V and III–V intervals and the III–V/I–III interval ratio increased significantly after perinatal HI compared with values in controls. These findings reflect abnormalities in neural conduction related to neural synchronization, myelination and synaptic function in the HI brainstem (Jiang, 2008). These data provide clear evidence that the functional integrity of the brainstem and auditory system are damaged after perinatal HI and that ABRs detect the impairment. This is in agreement with their previous findings in term infants after HI and animals with experimental HI (Jiang, 2008, 2003, 2000).

Smit et al. (2013) used an animal model with lambs to study the functional impairment of the auditory pathway after perinatal asphyxia. They observed significantly elevated mean thresholds, diminished amplitudes, and elevated latencies in the asphyxia group relative to the control group through the observation period. They also found that propofol anesthesia reduces auditory impairment after perinatal asphyxia. Their results support the hypothesis that some drugs might prevent the functional changes to the auditory pathway in the event of perinatal asphyxia.

Hence, the model we have developed will facilitate future research into the effect of different treatments for the prevention of injury to the central nervous system. In relation to this, we have recently reported that post-HI administration of cannabinoid cannabidiol to newborn piglets, for example, produces beneficial effects in the very early hours after HI, as reflected by the improvement in brain activity monitored by aEEG and metabolism as measured by NIRS (Alvarez, 2008). These beneficial effects are sustained 72 h after HI and they are associated not only with histological improvement but also with neurobehavioral normalization [Alonso-Alconada, 2011; Lafuente, 2011; Alonso-Alconada, 2010].

CONCLUSIONS

The hypoxic-ischemic experimental model in newborn piglets is a suited model for studying the functional effects of perinatal asphyxia on the brainstem. Moreover, the development of this model will allow us in the future to develop interventions to minimise the injury during acute episodes study and to study the useful of new strategies for the prevention of cerebral impairment in neonates.

Conflicts of Interest

Authors disclose any sponsorship or funding arrangements relating to their research. Authors indicate that they have not conflicts of interest.

Acknowledgments

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EZTABAIDA OROKORRA

Asfixia perinatalak ikasketetan, lengoaian eta arreta gaitasunean arazoak sor ditzake eta baita hiperaktibitate desordenak edo oraindik larriagoa den garun paralisia ume jaio berrietan (Anand et al., 1991). Gainera, entzefalo enborrari eragin ahal dion entzumen ezgaitasunak ere kaltetuak gerta daitezke (Chayasirisobhon et al., 1996, Misra et al., 1997).

Garai perinatalean gertatzen den asfixiak hipokanpoan, estriatuan edo kortexean sortzen dituen neuronen kalteri buruz hainbat ikerketa egin dira (Alonso-Alconada et al., 2012), baina entzefalo enborrak atentzio gutxi izan du. Entzumen-bidea odoleko oxigeno kontzentrazioaren gutxitzearekiko oso sentikorra da. Hipoxia iskemiak koklean duen eragina ikusteko orain arte egindako ikerketak, Kortiren Organoko estereozilioen sentikortasunean jarri dute arreta. Ikerketa hauen arabera, kokleak oxigeno eta glukosa defizita duenean, estereozilioen galera gertatzen da (Gross et al., 2014). Entzefalo enborraren mailan, Freemanek (1991) eta Jiangek (2004) nerbioaren garraio eta sinapsian aldaketak gertatzen direla adierazi dute eta ondorioz entzumen defizita gertatuko litzakeela (Jiang et al., 2000).

Entzumen-sistemaren funtzionaltasuna aztertzeko, Auditory Brainstem Response (ABR) delakoak, diagnostikorako metodo garrantzitsuak dira, entsegu klinikoetan erabiltzen direnak hipoxia iskemia pairatu duten ume jaio berriei ezgaitasunak detektatzeko. ABRetan gehien aztertzen diren aldagaiak V.uhinaren latentzia eta I-V uhin tartearen latentziak dira. Izan ere, aldagai hauek neuronen eroapena, eroapenaren denbora eta mielinizazio zein funtzio sinaptikoarekin erlazionaturik daude, bai entzefalo enborrean zein entzumen-sistema zentralean (Jiang et al., 2008).

Gure ikerketetan, kaltea eragin eta 7 egunetara, I-V uhin tartearen eta III-V uhin tartearen handitzea ikusi dugu, nahiz eta lehenengo uhin tartearen handitzea handiagoa izan den.Dirudienez, V.uhina sentikorragoa da asfixia perinatal baten ostean gertatzen diren aldaketa txikiekiko (Tomimatsu, et al., 2003). Gainera, gure emaitzak beste ikertzaile batzuek ikusi dutenarekin bat datoz, hauen arabera, entzunbideko erdiko zonaldea (III-V uhin tarteari dagokiona) kaltetuagoa gertatzen da hipoxia iskemia baten ostean entzunbide periferikoko zonaldea baino (I-III uhin tarteari

dagokiona) (Jiang, et al., 2009). ABR latentzia eta uhin tarte hauen latentziaren handitzea, entzunbidean zeharreko sinapsi transmisioaren efikazia gutxitzearekin erlazionatu da (Jiang, et al., 2010).

Gure lanean, entzunbidearen funtzionaltasuna aztertzeaz gain, garunean dauden 3 zelula moten azterketa morfologikoa egin da kaltea gertatu eta 7 egunetara: Neuronak, astrozitoak eta oligodendrozitoak. Ikerketa hauetan, behe kolikuluko neuronak kalte hipoxiko iskemikoari sentikorrak direla ikusi dugu. Aldaketak denboran zehar mantentzen direla ikusi dugu eta kaltea gertatu eta 24 ordutara zelulen kalte eta galera dagoela ikusi dugu, neuronen eta sinapsi besikulen gutxitzearekin, astrozitoen erreaktibitatea handituz eta MBP maila jaitsiz. Aldaketa hauek garuneko beste gune batzuetan aurkituak izan dira gure talde eta beste hainbat talde batzuengandik gertaera hipoxiko iskemiko baten ostean (Alonso-Alconada, et al., 2012). Tratamendua jaso zuten taldeetan berriz, ez zegoen aldaketa hauen arrastorik.

Funtzio zelularraren ikerketan, mitokondrio mintzaren integritatea eta potentziala aztertu ziren. Azterketa hau kaltearen ostean 0, 3 eta 12 ordutara egin zen entzefalo enborrean eta NAO zein Rhodamina 123 fluorokromoekiko positibo ziren zelula kopurua gutxitu zela ikusi zen, honek mitokondrioaren integritatea eta mintzaren potentzialean aldaketak daudela esan nahi du. Aldaketa hauek tratatutako taldeetan ez ziren ikusi. Emaitza hauek gure taldeak zein beste talde batzuk garuneko beste eremu batzuetan aurkitutakoarekin bat datoz (Larsen, et al., 2008, Lara-celador et al., 2012).

Mitokondrioan ikusitako aldaketez gain, hainbat geneen adierazpenean aldaketak ikusi ziren denbora berdinetan entzefalo enborrean ere. Hipoxia iskemiak inflazioarekin (TNFa eta COX2) eta heriotza zelularrarekin (Caspasa 3) erlazionaturik dauden hainbat gene aktibatzen dituzte. Gainera, estres baten ostean aktibatzen diren geneen adierazpenean (IGR1, FOS, VEGFa, HSF) eta hainbat zelula moten aktibazioan (GFAP, NG2, VIM, SYP, NEURABIN II) eragina dute hainbat geneen aldaketak ikusi dira.

Hala ere eta guretzat harrigarria izan dena, entzefalo enborrean gertaera hipoxiko iskemiko baten ostean ez genuen ikusi Reactive Oxygen Species (ROS) erlazionaturik zeuden geneen (SOD2) aktibazioa.

Tratamendua jaso zuten taldeetan, geneen adierazpen hau nahiko aldakorra izan zen entzefalo enborrean. Honela, Nikotina tratamendua jaso zuten animaliek, estres baten ondoren aktibatzen diren geneen adierazpena ez zen ikusi baina hantura, aldaketa estruktural edota astrozitoen aktibazioarekin erlazionaturik zeuden geneak. Lehenago egindako ikerketen arabera, nikotinaren hartzaile azetilkolinikoak hantura kontrako efektuak ditu eta VEGFa bezalako geneak aktibatzen ditu, gure emaitzekin bat datozenak (Tarras et al., 2013, Ma et al., 2014).

Melatonina berriz, erantzun atzeratuagoa duela dirudi, bere administrazioa kaltearen ostean egiten delako izan daiteke eta ez ikertutako beste tratamenduetan bezala administrazio hau kaltearen aurretik egiten dela. Astrozitoen aktibazioarekin erlazionaturik dauden geneak aktibatzen dira eta baita hanturarekin eta sinaptofisinarekin erlazionaturik daudena, hala ere, estres baten ostean aktibatzen diren geneen adierazpenean ez da aldaketarik ikusi. Badira hainbat ikerketa, (Acuna-Castroviejo et al., 2001; Hassell et al., 2015) zeintzuek, Melatoninak efektu antioxidatzaileaz gain, beste hainbat efektu izan ditzakeela uste dutenak, hala nola, eragin antiapoptotikoa, hanturaren kontrakoa eta neurona eta gliaren garapenarenarena, ikerketa hauek gure hipotesiaren alde egiten dute. Zentzu honetan, tratamendu hauek eragin bat baino gehiago izan dezaketela kontuan hartu beharko da, gure emaitzekin bat etorriko zena.

Resberatrolak ordea, estresaren ostean aktibatzen diren geneen adierazpena aldatzen du. Honela, EGR1 eta FOS geneen adierazpena oso altua da eta hanturarekin edo zelulen estrukturarekin erlazionaturik dauden geneen adierazpena ordea, kontrol taldearen antzekoak dira. Hainbat ikerketen arabera, Resberatrolak EGR1 aren adierazpena bultzatzen du eta hantura eragiten duten zitokinak aktibatzen dira honen eraginez hipokanpoan, honela garuneko zelula endotelialen angiogenesia gertatuz (Simão et al., 2012; Rössler et al., 2015, Yazir et al., 2015). Era berea, DHA tratamendua

jaso duten animaliak, dirudienez, zelulen aktibazioarekin erlazionaturik dauden hainbat gene aktibatzen dituzte. Astrogliosiarekin, neuriten aktibazioarekin eta Vimentinarekin erlazionaturik dauden geneak ere aktibatzen dituzte baina hanturarekin erlazionaturiko geneak ez (Velten et al., 2014). Melatoninaren kasuan gertatzen den bezala, hainbat ikerketek, tratamendu hauen eragina antioxidatzaileaz gain beste hainbat izan daitezkeela adierazten dute.

Gure ikerketetan ez dugu aldaketarik ikusi ROS arekin erlazionaturik dauden geneetan HI taldearen eta kontrol taldearen artean. Tratamendu hauek antioxidatzaile talde orokor barruan sartzen direnez, hasiera batean behintzat, gure emaitzak harritu gintuzten, baina guztiz erlazionaturik daude fluxuzko zitometrian izan genituen emaitzekin. Honegatik entzefalo enborrean behintzat, emandako sustantzia hauen efektua, bere efektu antioxidatzaileaz gain beste efekturen batengatik izan behar dela uste dugu eta honegatik beste ikerketa batzuk egitea beharrezkoa dela uste dugu.

Arratoietan egindako ikerketez gain, entzunbidearen ikerketa hauek gure taldean egiten ziren beste asfixia perinatal modelo batean (Alvarez et al., 2008). aztertu nahi izan genituen, txerri jaio berrietan hain zuzen ere. ABRak aztertu ziren hauena ere eta hipoxia iskemia momentuan, entzumenaren erantzunak nuluak ziren eta berreskuratzeko lehen uneetan, I-V uhin tarteen latentziak luzatuak agertzen ziren.

Emaitza hauek baieztatzen dute, entzefalo enborraren funtzionaltasunaren integritatean arazoak daudela, batez ere entzunbidearen erdiko zonaldean. Arratoian gertatzen den bezala, V. uhinaren latentziaren luzapena ikusi zen Jiangek (2006, 2008) egindako ikerketekin bat datorrena.

Emaitza hauek neuronen garraioan arazoak daudela esan nahi du sinkronizazio, mielinizazio eta sinapsi funtzioarekin erlazionatuak daudenak. Zentzu honetan, txerrian egindako asfixia perinatal modelo hau, modelo egokia izan daiteke entzumen-bideko ezgaitasunak neurtzeko eta ondorengo azterketa histologiko, molekular zein zelularrak egiteko.

ONDORIOAK /CONCLUSIONS

Lortutako emaitzek ondorengoa ondorioztatzen dute:

1- Entzumen-bidean kalte elektrofisiologikoa dago hipoxia iskemia eragin diegun arratoietan, ABR latentzien eta latentzien arteko tartearen luzapenarekin.

2- Entzefalo enborra eta konkretuki behe kolikulua kalteturik gertatzen dira asfixia perinatal baten ostean, mitokondrioen integritatean aldaketak egonik, mitokondrioaren mintzaren potentziala murriztuz, neuronetan kalteak eta aldaketa morfologikoak gertatuz bai astrozito, erreaktibitatea handituz, zein oligodendrozitoentan, mielina gutxituz.

3- Entzefalo enborreko hainbat geneen adierazpena aldatua dago hipoxia iskemia baten ostean eta hainbat zelula taldeen aktibazioa gertatzen da. Gainera, neuronen eta besikula sinaptikoen galera gertatzen da, infartua eragiten dutenak.

4- Antioxidatzaileen bidezko tratamenduek efektu neurobabestzaile dute entzumen-sisteman gertaera hipoxiko iskemiko baten ondoren, baina beraiek akzio mekanismoa ez da guztiz ezagutzen, izan ere, ez dirudi babesaren eragilea beraien efektu antioxidatzailea dela entzefalo enborraren kasuan behintzat.

5- Asfixia perinatal baten ostean entzumen-bidearen funtzioa kaltetua gertatzen da txerrietan ere, beraz txerria ere hipoxia iskemia baten ostean entzumen-sistemako aldaketak ikertzeko modelo egokia izan daiteke.

The results obtained in the present work suggest the next conclusions:

1- There is an electrophysiological change in the auditory threshold of HI rat with an increase of ABR latencies and an interpeak interval.

2- The HI event produces several cellular mechanisms that contribute to cell damage or even cell death through apoptosis or necrosis depending on the severity of the damage, maturative state and region affected. One of the regions that can be affected by this injury is the brainstem, and in particular the inferior colliculus.

3- Brainstem is also affected by the hypoxic ischemic injury with alterations in several gene expressions and some cellular activation. There is an evident neuronal and synaptic vesicles loose and astrocytes activation which provokes the infarct area days after the damage.

4- Antioxidant treatments were found to provide effective neuroprotection to the immature auditory system before a perinatal hypoxic-ischemic event, but the mechanism is not well understood although it seems that the antioxidant effect is not the mechanism they use for this protection in the brainstem. It can be that the mechanical action is different depending on the area.

5- The hypoxic-ischemic experimental model in newborn piglets is a suited model for studying the functional effects of perinatal asphyxia on the brainstem.

TESIA /THESIS

Lan honetan zehar lortutako emaitzetatik hipoxia iskemia baten ostean kalte elektrofisiologiko, histologiko, molekular eta zelularra dagoela ondorioztatu dezakegu bai mesentzefaloan eta zehazkiago behe kolikuloan eta Nikotina, DHA, Resberatrola eta Melaonina bidezko antioxidatzaileen tratamenduak kalte honen murriztea eragiten duela.

Guzti honegatik, antioxidatzaileen bidezko berreskuratze terapeutikoak heriotz zelularra murrizten duela esaten amaitu dezakegu, batez ere, mitokondrioaren funtzioa mantentzen delako eta hainbat geneen alterazioak, kaltearekiko babesle diren hainbat akzio garrantzitsu eragin ditzakeelako. Honela, antioxidatzaileek neuronen kaltea eta gliaren erreaktibitate maila murrizten dute. Hala ere, ez da guztiz argi gelditzen tratamendu hauen funtzioa, izan ere, beraien eragin antioxidatzailea ez dirudi babespen honen eragile denik mesentzefaloan.

After the results obtained in the present work, we can suggest that there is a elecrofisiological, histological, molecular and cellular alteration in the brainstem and particularly the inferior colluculus after a perintal asfixia and that antioxidant treatments, such as, Nicotine, DHA, Resveratrol and Melatonin have neuroprotective effects.

In conclusion, antioxidant treatment produce a remarkable amelioration of the brainstem injury, resucing apoptotic cell death, contributing to the mainteinance of the mitochondrial functionality and alterating some gene expresion that can be important for neuroprotection. Indeed, antioxidant treatment reduce neuronal damage an glial reactivity. However, the mechanism of studied treatments is not well understood although it seems that the antioxidant effect is not the main mechanism they use for this protection in the brainstem.

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