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Study of the Interpeduncular Nucleus and the Trigeminal Sensory Complex as Paradigms of Neuronal Migration and Brain Segmentation in the Hindbrain

Estudio del Núcleo Interpeduncular y del Complejo Sensorial Trigeminal como Ejemplos de Procesos de Migración y Segmentación en el Rombencéfalo

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Estudio del núcleo interpeduncular y del complejo sensorial trigeminal como ejemplos de procesos de migración y segmentación en el rombencéfalo

Study of the interpeduncular nucleus and the trigeminal sensory complex as paradigms of neuronal migration and brain segmentation in the hindbrain

Memoria presentada para optar al grado de Doctor con Mención Internacional por:

Isabel María García Guillén
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A mis abuelos,

A mi madrina,

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PREFACE

This doctoral thesis is presented as a compendium of publications and it applies for the International Doctorate mention in accordance with the rules for the regulation of official doctoral studies of the University of Murcia (RD-99/2011 and R-310/2015) and with the approval of the thesis supervisors, the Academic Commission of The Doctoral Program in Signals Integration and Modulation in Biomedicine and the General Committee for Doctoral Studies. This thesis is composed of three research studies published in international journals indexed in Journal Citation Reports (JCR) ((García-Guillén et al., 2020, 2021a, 2021b)). Additionally, this document provides a general introduction, which presents the studies and justifies the scientific unity of the thesis, and an overall summary of the aims of the research and the final conclusions.

PREFACIO

Esta tesis doctoral es presentada como compendio de publicaciones y opta a la mención de Doctorado Internacional de acuerdo con el reglamento por el que se regulan las enseñanzas oficiales de doctorado de la Universidad de Murcia (RD-99/2011 y R-310/2015) y con la aprobación de los directores de tesis, la Comisión Académica del Programa de Doctorado en Integración y Modulación de Señales en Biomedicina y la Comisión General de Doctorado. Esta tesis está compuesta de tres trabajos de investigación publicados en revistas internacionales indexadas en *Journal Citation Reports* (JCR) ((García-Guillén et al., 2020, 2021a, 2021b)). Además, esta memoria consta de una introducción general que presenta los estudios y justifica la unidad científica de la tesis, así como un resumen de los objetivos y conclusiones de los trabajos de investigación mencionados.

RESUMEN EXTENDIDO EN CASTELLANO

De acuerdo con el reglamento por el que se regulan las enseñanzas oficiales de doctorado de la Universidad de Murcia se presenta en este anexo un resumen extendido traducido al castellano de la tesis doctoral, en el que se presentan los tres trabajos de investigación, y se justifica la unidad científica de la tesis doctoral:

Esta tesis doctoral se presenta como un compendio de publicaciones y opta a la mención de Doctorado Internacional. Esta tesis está compuesta de tres trabajos de investigación (García-Guillén et al., 2020; García-Guillén et al., 2021a; García-Guillén et al., 2021b) publicados en revistas internacionales indexadas en *Journal Citation Reports* (JCR). Todos los artículos configuran una unidad científica en el campo de la Neurobiología del Desarrollo.

El objetivo general ha sido profundizar en nuestro conocimiento sobre dos procesos fundamentales para la formación del encéfalo: la migración neuronal y la segmentación cerebral. Ambos son mecanismos del desarrollo que han contribuido al aumento de la complejidad del cerebro a lo largo de la evolución de los vertebrados. Nos hemos focalizado en dos estructuras del rombencéfalo: el núcleo interpeduncular (IPN) como un modelo para estudiar la migración neuronal y regionalización, y la columna sensitiva del trigémino como un modelo para estudiar el proceso de segmentación dentro de esta estructura. Tanto el IPN como la columna del trigémino son estructuras plurisegmentales, ya que tienen componentes en más de un segmento o neurómero. De acuerdo con bibliografía clásica (Vaage, 1969) y con el paradigma de segmentación actual, el modelo prosomérico (Puelles et al., 2013, 2018), estos neurómeros que forman el romboencéfalo se llaman rombómeros (r).

El IPN es una estructura cito- y quimio-arquitectónicamente compleja, y es la diana principal del tracto retroflejo que viene de la habénula medial (mHb) (Herkenham and Nauta, 1979; Contestabile and Flumerfelt, 1981). Aunque estudios clásicos habían localizado erróneamente el IPN en el mesencéfalo, si tenemos en cuenta criterios topológicos y embriológicos está realmente localizado en el rombencéfalo rostral (prepontino) (Lorente-

Cánovas et al., 2012). El IPN está constituido por tres componentes rostrocaudales: el prodromal (Pro) localizado en el istmo (Ist; también llamado r0), y el interpeduncular rostral (IPR) e interpeduncular caudal (IPC), localizados en r1 rostral (r1-r) y r1 caudal (r1-c), respectivamente (Lorente-Cánovas et al., 2012).

El IPN se forma íntegramente por migración neuronal de varias poblaciones neuronales con diferentes orígenes dorsoventrales y rostrocaudales. Estas poblaciones siguen rutas migratorias independientes tangenciales y/o radiales desde sus orígenes hasta su localización final en la placa del suelo del istmo y rombómero 1 (Lorente-Cánovas et al., 2012). El origen, rutas migratorias, y destino final de las poblaciones del IPN pudieron ser determinados gracias a que estas poblaciones expresan varios factores de transcripción (TF) (*Nkx6.1, Pax7, Otp, Otx2*) a lo largo de todo el proceso del desarrollo del IPN (Lorente-Cánovas et al., 2012). Estudios en mutantes de ratón demostraron el requerimiento de *Otx2* (Ruiz-Reig et al., 2019) y del morfógeno *Shh* (Moreno-Bravo et al., 2014), el gen responsable de la formación de la placa del suelo, para el desarrollo del IPN. Sin embargo, a parte de estos estudios, los mecanismos moleculares subyacentes a estas complejas migraciones neuronales seguían siendo en gran parte desconocidos.

Se sabe que el sistema de señalización Netrina-1/DCC dirige la migración de varias estructuras del rombencéfalo (Bloch-Gallego et al., 2005; Shi et al., 2008; Kratochwil et al., 2017). Considerando que el gen *Netrina-1* se expresa en la placa del suelo del rombencéfalo rostral, y que *Dcc* se expresa en las células $Otx2^{+}$ más dorsalmente ubicadas del IPN (Ruiz-Reig et al., 2019), decidimos explorar en el **estudio 1 (García-Guillén et al., 2020)** si el sistema de señalización Netrina-1/DCC está involucrado en la migración de las poblaciones del IPN. Para ello, analizamos de forma independiente por hibridación in situ (ISH) cada una de las poblaciones, caracterizadas por la expresión de *Nkx6.1, Pax7, Otx2, Otp* (identificados en estudios previos) e *Irx2* (identificado en el presente estudio), en ratones de tipo silvestre y mutantes de DCC. Nuestros resultados mostraron que en ausencia de DCC hay una deficiencia generalizada pero diferencial de la migración neuronal. Las poblaciones del Pro y del IPR fueron las más afectadas mientras que las poblaciones más caudales (del IPC) mostraron

una afectación más leve de su proceso migratorio. Sin embargo, a pesar del requerimiento general de Netrina-1/DCC para la migración de todas las poblaciones analizadas del IPN, algunas células fueron capaces de llegar al IPN, lo que sugiere mecanismos moleculares adicionales guiando estos procesos migratorios.

Los resultados del estudio 1 nos condujeron a explorar qué genes adicionales se expresan en el IPN. Así, en el **estudio 2 (García-Guillén et al., 2021a)**, nos propusimos identificar genes expresados diferencialmente en los dominios del IPN, así como también caracterizar sus posibles funcionalidades e interacciones. Para ello, realizamos un cribado in-silico de los 2038 genes cuyos experimentos de hibridación in situ (ISH) estaban disponibles en la base de datos Allen Developing Mouse Brain Atlas (ADMBA) (https://developingmouse.brain-map.org/). Elegimos analizar los experimentos en el estadio E18.5 ya que es una etapa gestacional tardía en la que los procesos migratorios involucrados en la constitución del IPN casi se han completado, y la gruesa morfología del IPN ya es evidente. En lo que respecta a la regionalización molecular del IPN, se habían identificado previamente varias poblaciones neuronales que expresan los factores de transcripción *Nkx6.1, Pax7, Otp, Otx2, Irx2,* tal y como se ha mencionado anteriormente (Lorente-Cánovas et al., 2012; Moreno-Bravo et al., 2014; García-Guillén et al., 2020). Sin embargo, considerando la complejidad estructural e histogenética del IPN, esperábamos que en este núcleo se expresaran muchos más factores de transcripción, así como otros genes involucrados en desarrollo neural.

A través del cribado en la base de datos ADMBA, identificamos 135 genes expresados en el IPN, y analizamos su patrón de expresión en relación con los dominios segmentarios rostrocaudales del IPN (Pro en el istmo, IPR en el r1-rostral, IPC en el r1-caudal) antes mencionados. Además, consideramos tres subdivisiones del IPR, que van de superficial (pial) a niveles más profundos, llamadas apical (IPRa), intermedia (IPRi) y basal (IPRb), ya que muchos genes mostraron expresión restringida en al menos una de estas regiones. Como resultado, encontramos 46 genes expresados en el prodromal, 17 en el IPRa, 36 en el IPRi, 47 en el IPRb y 45 en el IPC, así como otros 27 genes expresados en todo el IPR. Realizamos un test estadístico de sobrerrepresentación con la herramienta PANTHER

(http://pantherdb.org/) sobre el conjunto de los 135 genes, que resaltó familias de genes relacionadas con el desarrollo neuronal, morfogénesis celular y guía axonal. Adicionalmente, analizamos interacciones potenciales con la base de datos STRING (https://string-db.org/), obteniendo redes específicas que principalmente involucraban miembros de la familia de Efrinas y receptores de Efrinas (Ephs), Cadherinas, factores de transcripción y moléculas relacionadas con la neurotransmisión sináptica.

En conjunto, en este estudio proporcionamos un modelo genoarquitectónico del IPN que puede usarse para la integración de datos relacionados con la expresión génica o interacciones, así como una base para estudios futuros de conectividad y función de este núcleo. En lo que respecta a nuestra contribución sobre datos de expresión génica de esta estructura, nuestros resultados son relevantes considerando la compleja formación y morfología del IPN, así como el interés sobre la región rostral del rombencéfalo (Ist y r1) como parte de la unión mesencéfalo-rombencéfalo.

De forma análoga, en el **estudio 3 (García-Guillén et al., 2021b)**, usamos la base de datos Allen Mouse Brain Atlas (AMBA) (https://mouse.brain-map.org/) para estudiar la regionalización rostrocaudal de la columna sensitiva del trigémino, basada en la expresión diferencial de genes. La columna sensitiva del trigémino es una estructura columnar en la placa alar del rombencéfalo que recibe y analiza los aferentes somatosensoriales primarios que vienen del ganglio trigeminal. Está formada por el núcleo sensorial principal trigeminal (Pr5) localizado en r2 y r3 (Oury et al., 2006), y el núcleo espinal trigeminal (Sp5), que en consecuencia se extiende desde r4 hasta el límite rombencéfalo/médula espinal en el ratón. Convencionalmente, Sp5 ha sido subdividido en los subnúcleos oral, interpolar y caudal, en base a criterios citoarquitectónicos (Olszewski, 1950), o moleculares y de patrón de conectividad (Waite, 2004). Por otro lado, de acuerdo con el modelo prosomérico, esta columna estaría subdividida en segmentos transversales relacionados con los rombómeros subyacentes. Estudios de mapa de destino con quimeras de aves mostraron que efectivamente Sp5 está formado por unidades sucesivas derivadas de rombómeros (Marín and Puelles, 1995; Cambronero and Puelles, 2000). Sin embargo, hasta ahora no está totalmente clara la correspondencia entre los subnúcleos clásicos y el mapa rombomérico, ni la posible identidad molecular

de estos módulos segmentales que componen la columna sensitiva del trigémino. Así, nos propusimos reexaminar estas cuestiones en este estudio.

Con este objetivo, realizamos un cribado buscando genes expresados diferencialmente en la columna sensitiva del trigémino. Seleccionamos 12 genes (*Baiap3, Camk2a, Calb1, Calb2, Fn1, Mafb, Kcng4, Irx2, Tac1, Tac2, Pde1c, Zbtb16*), cuyos patrones de expresión estaban regionalizados a lo largo del eje rostrocaudal de la columna sensitiva del trigémino, y después los analizamos en relación con conocidas referencias morfológicas características de rombómeros específicos. Estos genes mostraron dominios de expresión regionalizados, cubriendo una o varias unidades romboméricas. Por ejemplo, encontramos que los genes *Mafb* y *Fn1* marcaban fuertemente r5 y r6, con poca o ninguna expresión en el resto de rombómeros, y que los genes *Pde1c* y *Zbtb16* eran específicos de r9. En conjunto, en este estudio proponemos un nuevo mapa segmentario de la columna sensitiva del trigémino en ratón de acuerdo con datos de expresión génica, caracterizándola como un complejo modular plurineuromérico en relación con una serie de dominios moleculares derivados de rombómeros.

Colectivamente, los **estudios 1 y 2** se enfocan en el desarrollo del IPN, analizando respectivamente los procesos de migración y regionalización necesarios para su constitución, mientras que el **estudio 3** estudia la estructura segmentaria de la columna sensitiva del trigémino de acuerdo con datos moleculares. En el estudio 1 demostramos que la señalización por Netrina-1/DCC está involucrada en parte de los procesos migratorios responsables de la formación del IPN. Los estudios 2 y 3 proporcionan, respectivamente, modelos genoarquitectónicos para el IPN y la columna sensitiva del trigémino, dentro del modelo neuromérico de desarrollo y estructura del encéfalo. Ambos modelos pueden ser usados para la integración de datos adicionales de expresión génica, y como base para estudios futuros funcionales y/o farmacológicos relacionados con estas estructuras.

Esta tesis doctoral está constituida por los siguientes trabajos de investigación publicados:

1. **Netrin-1/DCC Signaling Differentially Regulates the Migration of Pax7, Nkx6.1, Irx2, Otp, and Otx2 Cell Populations in the Developing Interpeduncular Nucleus**

    - Autores: IM. García-Guillén, A. Alonso, N. Morales-Delgado, B. Andrés, L. Puelles, G. López-Bendito, F. Marín and P. Aroca.
    - Revista científica: Frontiers in Cell and Developmental Biology
    - Fecha de publicación: Octubre del 2020
    - Volumen: 8
    - Página: 588851
    - Factor de impacto (JCR): 6.684 (2020)
    - Categoría de la revista: Developmental Biology (Biología del Desarrollo) and Cell Biology (Biología Celular)
    - Cuartil: Q1 en Developmental Biology y Q2 en Cell Biology
    - DOI: 10.3389/fcell.2020.588851
    - Disponible online en: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7606981/

2. **Multiple Regionalized Genes and Their Putative Networks in the Interpeduncular Nucleus Suggest Complex Mechanisms of Neuron Development and Axon Guidance**

    - Autores: IM. García-Guillén, A. Alonso, L. Puelles, F. Marín y P. Aroca
    - Revista científica: Frontiers in Neuroanatomy
    - Fecha de publicación: Febrero del 2021
    - Volumen: 15
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    - Factor de impacto (JCR): 3.856 (2020)
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- Cuartil: Q1 en Anatomy & Morphology y Q2 en Neurosciences
- DOI: 10.3389/fnana.2021.643320
- Disponible online en: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7921722/

3. **Molecular Segmentation of the Spinal Trigeminal Nucleus in the Adult Mouse Brain**

- Autores: IM. García-Guillén, M. Martínez-de-la-Torre, L. Puelles, P. Aroca y F. Marín
- Revista científica: Frontiers in Neuroanatomy
- Fecha de publicación: Diciembre del 2021
- Volumen: 15
- Página: 785840
- Factor de impacto (JCR): 3.856 (2020)
- Categoría de la revista: Anatomy & Morphology (Anatomía y Morfología) y Neurosciences (Neurociencias)
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INDEX

INTRODUCTION

1.1 PRESENTATION AND SCIENTIFIC UNIT

This doctoral thesis is presented as a compendium of publications and it applies for the International Doctorate mention. This thesis is composed of three research studies (García-Guillén et al., 2020; García-Guillén et al., 2021a; García-Guillén et al., 2021b) published in international journals indexed in *Journal Citation Reports* (JCR). All the articles configure a scientific unity in the field of Developmental Neurobiology.

The general objective has been to deepen our knowledge into two fundamental processes for brain formation: neuronal migration and brain segmentation. Both are developmental mechanisms that have contributed to the increased brain complexity over vertebrate evolution. We have focused on two hindbrain structures: the interpeduncular nucleus (IPN) as a model to study neuronal migration and regionalization, and the trigeminal sensory column as a model to study the segmentation within this structure. Both the IPN and the trigeminal column are plurisegmental structures, as they have components in more than one segment or neuromere. According to classical literature (Vaage, 1969) as well as the current segmental paradigm, the prosomeric model (Puelles et al., 2013, 2018), these neuromeres forming the hindbrain are called rhombomeres (r).

The IPN is a cyto- and chemoarchitectonically complex structure, and it is the principal target of the retroflex tract that comes from the medial habenula (mHb) (Herkenham and Nauta, 1979; Contestabile and Flumerfelt, 1981). Although classical studies had mislocated the IPN in the midbrain, it is actually placed in the rostral (prepontine) hindbrain according to topological and embryological criteria (Lorente-Cánovas et al., 2012). The IPN is constituted by three rostrocaudal components: the prodromal (Pro) located in the isthmus (Ist; also called r0), and the interpeduncular rostral (IPR) and interpeduncular caudal (IPC), placed in rostral r1 (r1-r) and caudal r1 (r1-c), respectively (Lorente-Cánovas et al., 2012).

The IPN is formed entirely by neuronal migration of various neuronal populations with different dorsoventral and rostrocaudal origins. These populations follow independent tangential and/or radial migratory routes from their origins until they reach their final location across the median floor

plate of Ist and r1 (Lorente-Cánovas et al., 2012). The origin, migratory trajectory, and fate of IPN populations could be determined by their expression of several transcription factors (TF) (*Nkx6.1, Pax7, Otp, Otx2*) throughout the entire developmental process of the IPN (Lorente-Cánovas et al., 2012). Studies in mouse mutants demonstrated the requirement of *Otx2* (Ruiz-Reig et al., 2019) and the morphogen *Shh* (Moreno-Bravo et al., 2014), the gene responsible for the formation of the floor plate, for IPN development. However, apart from these studies, the molecular mechanisms underlying these complex neuronal migrations remained largely unknown.

Netrin-1/DCC signaling is known to steer the migration of several structures in the hindbrain (Bloch-Gallego et al., 2005; Shi et al., 2008; Kratochwil et al., 2017). Considering that *Ntn1* (*Netrin-1)* is expressed in the floor plate of the rostral hindbrain, and that *Dcc* is expressed by the most dorsally located *Otx2*$^{+}$ IPN cells (Ruiz-Reig et al., 2019), we decided to explore in **study 1** (**García-Guillén et al., 2020**) if the Netrin-1/DCC signaling system is involved in the migration of IPN populations. For that, we independently analyzed by in situ hybridization (ISH) each population, characterized by the expression of *Nkx6.1, Pax7, Otx2, Otp* (identified in previous studies) and *Irx2* (identified in the present study), in wildtype and DCC$^{-/-}$ knockout mice. Our results showed that in absence of DCC there is a general but differential impairment of neuronal migration. The Pro and IPR populations were the most affected ones while more caudal populations (IPC) displayed mild impairment of their migratory process. However, despite the general requirement of Netrin-1/DCC for the migration of IPN populations, there were some cells that were able to reach the IPN, which suggests additional molecular mechanisms guiding this process.

Results from study 1 lead us to explore which additional genes are expressed in the IPN. Thus, in **study 2** (**García-Guillén et al., 2021a**), we aimed to identify genes differentially expressed across the domains of the IPN, and to characterize their putative functional roles and interactions. For that, we performed an in-silico screening of the 2038 genes whose experiments of in situ hybridization (ISH) were available in the Allen Developing Mouse Brain Atlas (ADMBA) database (https://developingmouse.brain-map.org/). From these, we chose to analyze E18.5 experiments because it is a late gestational stage when the migratory

processes involved in IPN constitution have virtually been completed and the gross morphology of the IPN is evident. Concerning the molecular regionalization of the IPN, several populations expressing *Nkx6.1, Pax7, Otp, Otx2, Irx2* had been previously identified, as commented above (Lorente-Cánovas et al., 2012; Moreno-Bravo et al., 2014; García-Guillén et al., 2020). However, considering the structural and histogenetic complexity of the IPN, we expected that many other transcription factors, as well as other genes involved in neural development, would be expressed in this nucleus.

Through the aforementioned screening in the ADMBA database, we identified 135 genes expressed in the IPN, and analyzed their expression pattern in relation to the rostrocaudally segmented domains of the IPN (Pro in Ist, IPR in r1-r, IPC in r1-c). We additionally considered three from deep to superficial (pial) subdivisions of the IPR called apical (IPRa), intermediate (IPRi) and basal (IPRb), since many of the genes showed restricted expression in at least one of these regions. As a result, we found 46 genes expressed in the Pro, 17 in the IPRa, 36 in the IPRi, 47 in the IPRb and 45 in the IPC, as well as other 27 genes detected in the entire IPR. We performed a statistical overrepresentation test with PANTHER tool (http://pantherdb.org/) over the set of 135 genes, which highlighted gene families related to neuron development, cell morphogenesis and axon guidance. Additionally, we performed an interactome analysis with STRING database (https://string-db.org/), which yielded specific networks that mainly involved members of the ephrin/Eph and Cadherin families, transcription factors and molecules related to synaptic neurotransmission.

On the whole, in this study we provide a genoarchitectonic model of the IPN that can be used for the integration of data related to gene expression or interactions, and as a basis for future studies on the connectivity and function of this nucleus. Regarding our contribution on gene expression data of this structure, these results are relevant considering the complex formation and morphology of the IPN as well as the developmental interest of the rostral hindbrain (Ist and r1) as part of the mid-hindbrain junction region.

Analogously, in **study 3** (**García-Guillén et al., 2021b**), we used the Allen Mouse Brain Atlas Database (AMBA) (https://mouse.brain-map.org/) to study the rostrocaudal regionalization of the trigeminal sensory column, based on the differential expression of genes. The trigeminal sensory complex

is a columnar (longitudinal) structure in the alar hindbrain that receives and analyzes the primary somatosensory afferents from the trigeminal ganglion. It is formed by the principal trigeminal sensory nucleus (Pr5) located in r2 and r3 (Oury et al., 2006), and the spinal trigeminal nucleus (Sp5) which accordingly extends from r4 down to the hindbrain/spinal cord junction in the mouse. Conventionally, Sp5 has been subdivided into the oral, interpolar and caudal subnuclei, based on cytoarchitectural (Olszewski, 1950) or molecular and connectivity pattern (Waite, 2004) criteria. On the other hand, according to the prosomeric model, this column would be subdivided into rhombomeres-related transverse segments. Fate mappings in avian chimeras showed that Sp5 is formed indeed by successive rhombomeric units (Marín and Puelles, 1995; Cambronero and Puelles, 2000). However, so far it is not fully clear the correspondence between the classical subnuclei and the rhombomeric map, nor the possible molecular identity of these segmental modules that compose the trigeminal sensory column. Thus, we aimed to reexamine this issue in this study.

With this objective, we performed a screening looking for genes differentially expressed within the trigeminal sensory column. We selected 12 genes (*Baiap3, Camk2a, Calb1, Calb2, Fn1, Mafb, Kcng4, Irx2, Tac1, Tac2, Pde1c, Zbtb16*), whose expression patterns were regionalized along the rostrocaudal axis of the trigeminal sensory column, and subsequently analyzed them in relation to known morphological landmarks characteristic of respective rhombomeres. These genes showed regionalized, segment-related expression domains covering one or several rhombomeric domains. For instance, we found that the genes *Mafb* and *Fn1* marked strongly r5 and r6, with little or no expression in the rest of the rhombomeres, and that the genes *Pde1c* and *Zbtb16* were specific of r9. On the whole, in this study we propose a novel segmental map of the mouse trigeminal sensory column according to gene expression, characterizing it as a plurineuromeric modular complex in relation to a series of rhombomere-derived molecular domains.

Collectively, **studies 1 and 2** focus on IPN development, analyzing respectively the migratory and regionalization processes needed for its constitution, whereas **study 3** addresses the segmental structure of the sensory trigeminal sensory column according to molecular data. In **study 1** we demonstrate that the Netrin-1/DCC pathway is involved in part of the

migratory processes responsible of the formation of the IPN. **Studies 2 and 3** provide, respectively, genoarchitectonic models for the IPN and the trigeminal sensory column, within the neuromeric model of brain development and structure. These can be both used for the integration of additional data on gene expression, and as a basis for future functional and/or pharmacological studies related to these structures.

The present doctoral thesis is constituted by the following published articles:

1. Netrin-1/DCC Signaling Differentially Regulates the Migration of Pax7, Nkx6.1, Irx2, Otp, and Otx2 Cell Populations in the Developing Interpeduncular Nucleus

- Authors: IM. García-Guillén, A. Alonso, N. Morales Delgado, B. Andrés, L. Puelles, G. López-Bendito, F. Marín and P. Aroca
- Journal: Frontiers in Cell and Developmental Biology
- Date of publication: October 2020
- Volume: 8
- Page: 588851
- Impact Factor (JCR): 6.684 (2020)
- Journal Category: Developmental Biology and Cell Biology
- Quartile: Q1 in Developmental Biology and Q2 in Cell Biology
- DOI: 10.3389/fcell.2020.588851
- Available online at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7606981/

2. Multiple Regionalized Genes and Their Putative Networks in the Interpeduncular Nucleus Suggest Complex Mechanisms of Neuron Development and Axon Guidance

- Authors: IM. García-Guillén, A. Alonso, L. Puelles, F. Marín and P. Aroca
- Journal: Frontiers in Neuroanatomy
- Date of publication: February 2021
- Volume: 15
- Page: 643320
- Impact Factor (JCR): 3.856 (2020)
- Journal Category: Anatomy & Morphology and Neurosciences
- Quartile: Q1 in Anatomy & Morphology and Q2 in Neurosciences
- DOI: 10.3389/fnana.2021.643320
- Available online at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7921722/

3. Molecular Segmentation of the Spinal Trigeminal Nucleus in the Adult Mouse Brain

- Authors: IM. García-Guillén, M. Martínez-de-la-Torre, L. Puelles, P. Aroca and F. Marín
- Journal: Frontiers in Neuroanatomy
- Date of publication: December 2021
- Volume: 15
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- Impact Factor (JCR): 3.856 (2020)
- Journal Category: Anatomy & Morphology and Neurosciences
- Quartile: Q1 in Anatomy & Morphology and Q2 in Neurosciences
- DOI: 10.3389/fnana.2021.785840
- Available online at: https://www.frontiersin.org/articles/10.3389/fnana.2021.785840/full

1.2 THEORETICAL CONCEPTUALIZATION

1.2.1 The prosomeric model

During development, the complexity of the brain is first laid out in the neural plate, a single-cell thick sheet of cells that forms within the ectoderm. Through the neurulation process, the neural plate closes to form the neural tube, which is subsequently subdivided as development proceeds. The anteroposterior (AP) subdivision of the neural tube follows three steps, going from tagmata to proneuromeres and neuromeres (**Figure 1**) (Puelles, 2018). The mechanism by which the brain is progressively regionalized is called segmentation, and it is one of the strategies involved in the increasing complexity of the brain anlagen.

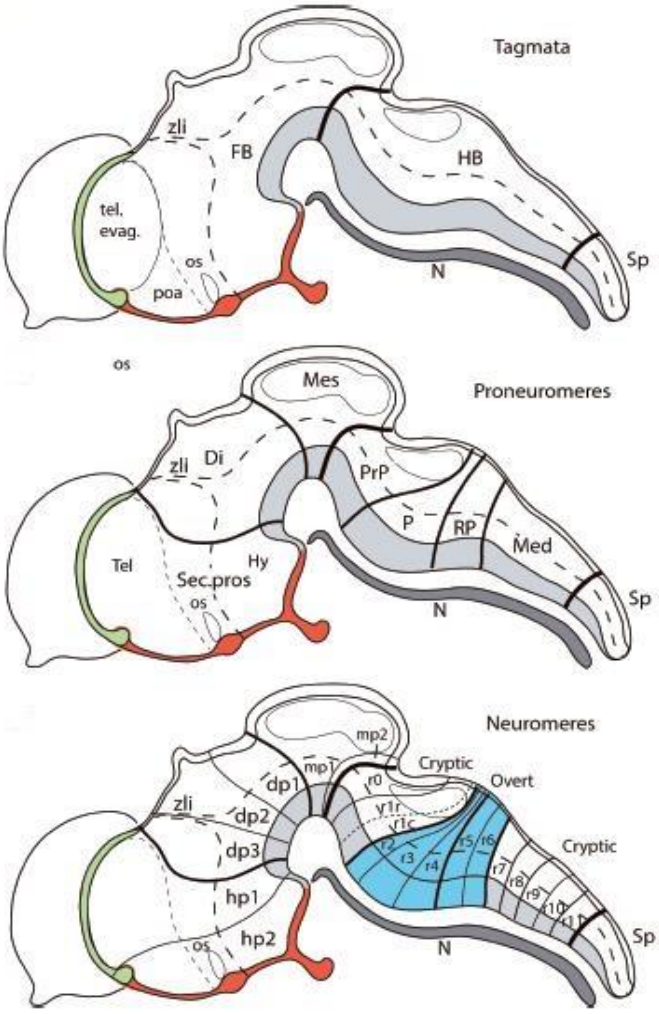


Figure 1. Steps in the anteroposterior subdivision of the neural tube according to the prosomeric model. Image taken from (Puelles, 2018).

Therefore, the developing vertebrate brain presents a series of segments along its rostrocaudal axis, following a metameric organization. This organization of the brain into several transverse metameric segments (which in the neural tube are called neuromeres) is analyzed by the prosomeric model, which integrates genetic, molecular, and morphological data. Currently, this model of brain segmentation considers 7 prosomeres in the forebrain (including two midbrain mesomeres), and the isthmus (Ist) plus 11 rhombomeres (r) in the hindbrain **(Figure 2)** (Watson et al., 2010; Puelles et al., 2013, 2018; Nieuwenhuys and Puelles, 2016; Ten Donkelaar, 2020).

In addition to the anteroposterior (rostrocaudal) segmentation, the brain is dorsoventrally subdivided into four major longitudinal zones (floor, basal, alar and roof plates) which are shared by all segments (see **Figure 2**). These longitudinal zones contain distinct progenitor domains along the dorsoventral axis that give rise to the different classes of postmitotic neurons.

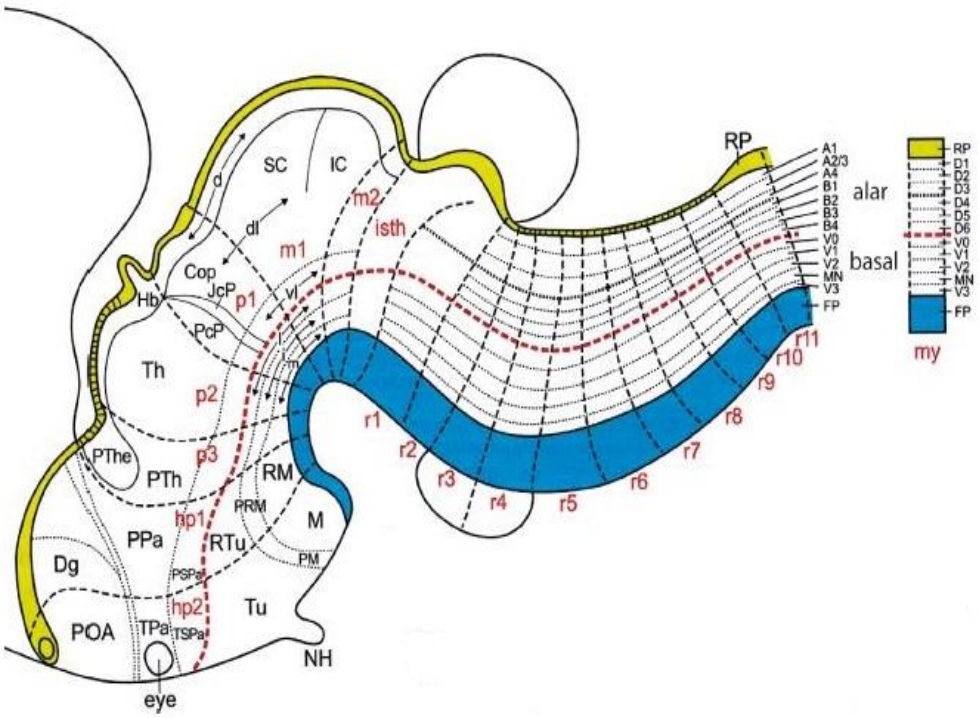


Figure 2. Brain organization according to the prosomeric model. The forebrain is subdivided in 7 prosomeres (hp1, hp2, p1, p2, p3, m1, m2) and the hindbrain is subdivided in 11 rhombomeres (r1-r11) plus the isthmus (isth). All the neuromeric subdivisions are named in red, with their rhombomeric limits in black dashed lines. The floor plate is colored in blue and the roof plate in yellow. A red dashed line marks the boundary between the alar and basal plates. Image taken from (Puelles, 2018).

1.2.2. Hindbrain segmentation

The rostrocaudal (transverse) segments of the hindbrain are named rhombomeres. The hindbrain is constituted by the isthmus (also called rhombomere 0) and 11 rhombomeres. In addition, some authors propose that rhombomere 1 can be further subdivided into rostral and caudal parts (Vaage, 1973; Aroca and Puelles, 2005; Puelles, 2013), so that the hindbrain would be composed by 12 or 13 molecularly defined transverse segments considering the r1 subdivisions.

Rhombomeres from r2 to r6 present overt segmentation while r0-r1 and r7-r11 do not (Lumsden, 1990; Cambronero and Puelles, 2000). The respective histogenetic identities of the r2-r6 classical rhombomeres were analyzed with fate mappings consisting either of avian chimeras (Marín and Puelles, 1995; Díaz et al., 1998) or transgenic mouse lines (Pasqualetti et al., 2007; Di Bonito et al., 2013). In turn, the nonbulging rhombomeres r7-r11 were initially known as "pseudorhombomeres" (Cambronero and Puelles, 2000) or cryptorhombomeres, that is, "hidden rhombomeres" (Puelles et al., 2018). However, they display respective morphological and molecular identities as shown by fate mapping and gene expression studies in chick and mouse embryos (Cambronero and Puelles, 2000; Marín et al., 2008; Tomás-Roca et al., 2016).

On the other hand, according to the current nomenclature that considers classical (morphological) together with developmental criteria, the hindbrain can be subdivided into prepontine, pontine, retropontine and medullary regions, which would correspond to r0-r1, r2-r4, r5-r6 and r7-r11, respectively However, r2 is sometimes included in the prepontine region, taking into account that the pontine nuclei are located in the ventral zone of r3 and r4 (Watson et al., 2017b, 2019).

Prepontine region (isthmus and r1)

The rostralmost hindbrain, also called prepontine or isthmocerebellar region is constituted by the isthmus and r1. It is under the influence of the isthmic organizer (Aroca and Puelles, 2005; Echevarria et al., 2005) and contributes to the formation of the cerebellum (Sgaier et al., 2005).

Several structures are located in the isthmus, such as the trochlear nucleus (4N), the dorsal raphe nucleus (Jensen et al., 2008; Alonso et al., 2013), the prodromal subnucleus of the interpeduncular nucleus (IPN) (Lorente-Cánovas et al., 2012), and the decussation of the superior cerebellar peduncle (Watson et al., 2017b).

On the other hand, r1 contains the interpeduncular rostral (IPR) and interpeduncular caudal (IPC) subnuclei of the IPN (Lorente-Cánovas et al., 2012), as well as the parabrachial complex (PB), the ventral nucleus of the lateral lemniscus (VLL) and the locus coeruleus (LoC), among other formations (Watson et al., 2010; Moreno-Bravo et al., 2014).

Pontine region (r2, r3, r4)

This region is mainly characterized by the presence of the pontine nuclei, which are located in the ventral surface of r3 and r4 after they have migrated from their origin in r6-r8 (Di Meglio et al., 2013; Tomás-Roca et al., 2016). In the sagittal plane, these rhombomeres appear wedge-shaped as a consequence of the pontine flexure.

Another significant structure is the motor trigeminal nucleus (5N), which is formed principally across r2 and r3 (Schneider-Maunoury et al., 1997; Song et al., 2006), with its rostral (major) part in r2 and a smaller caudal part in r3. Similarly, the principal trigeminal sensory nucleus (Pr5) is located in r2 and r3 in the mouse (Oury et al., 2006).

These rhombomeres, along with r5, contribute dorsally to the formation of the cochlear nuclei (Farago et al., 2006).

The fourth rhombomere contains also the exit point of the facial nerve (7n) (Di Bonito et al., 2017; Martínez-de-la-Torre et al., 2018) and the entry root of the vestibulocochlear nerve (8n) (Cordes, 2001).

Retropontine region (r5, r6)

In this region, r5 is characterized by the abducens motor nucleus (6N), the superior olive (SO), the trapezoid body (tb), the start of the spinal vestibular nucleus (which has been proposed to end in r9) and the facial nerve genus (7g). On the other hand, r6 contains the facial ascending fibers (asc7) plus the facial motor nucleus (7N), which has migrated tangentially from r4 (Cordes, 2001; Ju et al., 2004; Tomás-Roca et al., 2016; Martínez-de-la-

Torre et al., 2018). In addition, the nucleus raphe magnus lies across r5 and r6 (Alonso et al., 2013).

Medullary region (r7-r11)

This region is characterized by longitudinal plurisegmental structures such as the inferior olive (IO), found ventrally from r8 to r11, the complex formed by the hypoglossal nucleus (12N) and the migrated vagal preganglionic motor nucleus (10N) from r9 to r11, and the ambiguus branchiomotor nucleus (Amb), which extends from r7 to r10. On the other hand, the r9 segment contains the external cuneate nucleus (ECu), while r10 includes the area postrema (AP) and r11 the nucleus retroambiguus, as it has been proposed according to Hox gene mapping (Tomás-Roca et al., 2016; Watson et al., 2017b).

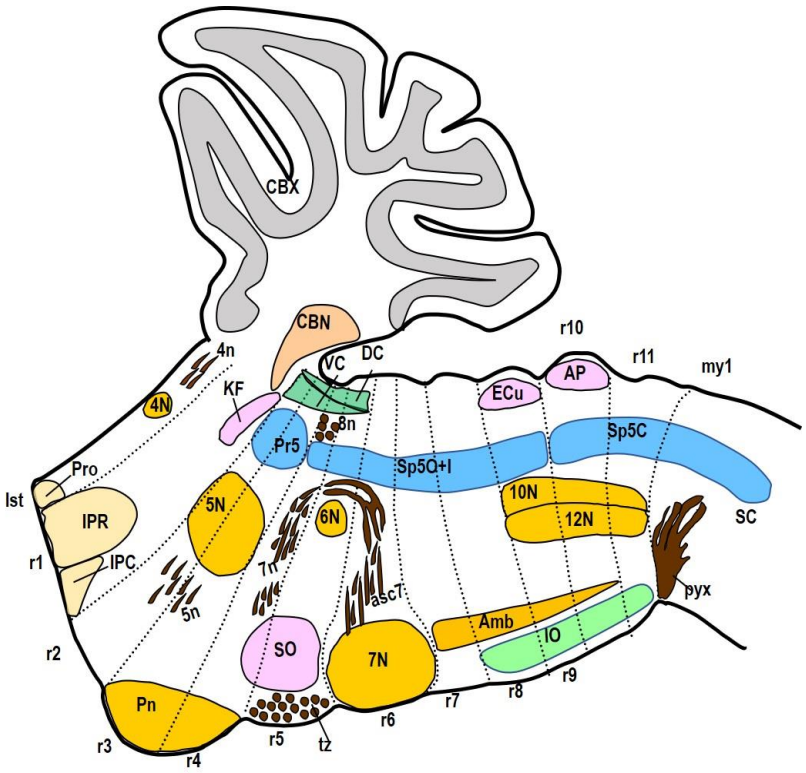


Figure 3. Schematic drawing showing a sagittal view of the hindbrain plus the first myelomere with remarkable morphological landmarks and nuclei and their rhombomeric location. Scheme slightly modified from (García-Guillén et al., 2021b). 4N, trochlear nucleus; 4n, trochlear nerve; 5N, trigeminal motor nucleus; 5n, trigeminal nerve root; 6N, abducens motor nucleus; 7N, facial motor nucleus; 7n, facial nerve; 8n, vestibulocochlear nerve; 10N, vagal motor nucleus; 12N, hypoglossal motor nucleus; Amb, ambiguous branchiomotor nucleus; AP, area postrema; asc7, facial ascending fibers; CBN, cerebellar nuclei; CBX, cerebellar cortex; DC, dorsal cochlear nuclei; ECu, external cuneatus nucleus; IO, inferior olive; KF, Kölliker-Fuse nucleus; my1, myelomere 1; Pn, pontine nuclei; Pr5, principal

trigeminal sensory nucleus; pyx, pyramidal decussation; r, rhombomere/s; SC, spinal cord; SO, superior olive; Sp5, spinal trigeminal sensory nucleus; Sp5C; subnucleus caudalis of the spinal trigeminal sensory nucleus; Sp5I, subnucleus interpolaris of the spinal trigeminal sensory nucleus; Sp5O, subnucleus oralis of the spinal trigeminal sensory nucleus; tz, trapezoid body; VC, ventral cochlear nuclei.

1.2.3. Neuronal migration

Neuronal migration is the mechanism by which neurons from different origins in the proliferative neuroepithelium are positioned into specific coordinates in the mantle of the mature brain. It is an essential process during brain development, although the existence of neuronal migrations in the adult brain has also been demonstrated (Luskin, 1993). Evolutionarily, neuronal migration is considered as a strategy to increase the complexity of the brain, since it allows that neurons reach specific locations (sometimes far away from their origins) as a previous step to the establishment of the proper connections.

Neurons originate from the proliferative epithelium named the ventricular zone (VZ), from where they migrate following a radial and/or tangential trajectory (**Figure 4**). The main difference between both migration strategies is that during radial migration neurons move perpendicularly to the ventricular surface, while in tangential migration the trajectories are parallel to the ventricular surface, either in the rostrocaudal- or the dorsoventral axis. In addition, contrary to tangentially migrating neurons, radially migrating neurons often use radial glial fibers as substrate (Metin et al., 2008; Marín et al., 2010). The tangential mode of migration is considered as a mechanism for neurons to reach locations far away from their origin, as opposed to the local glia-associated strategy.

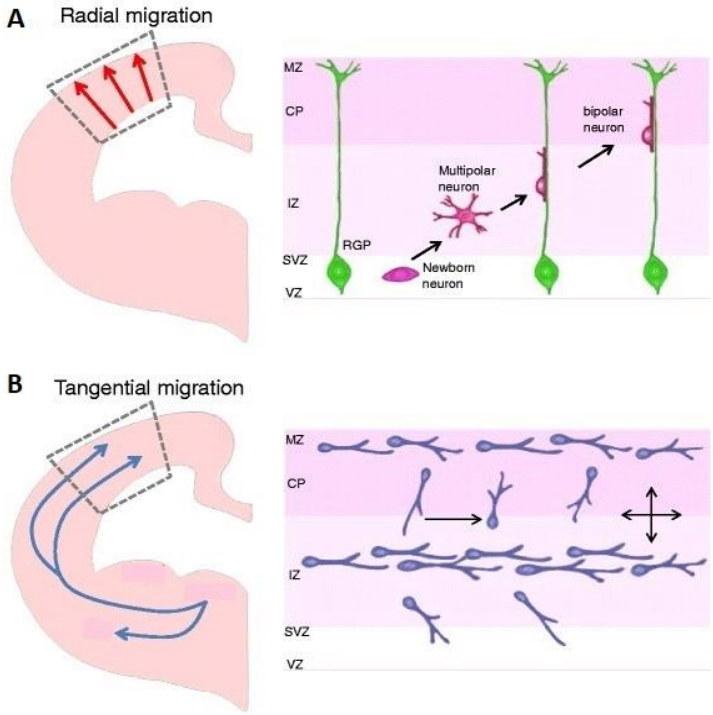


Figure 4. Radial (A) versus tangential (B) migration. In the radial mode, the newborn neuron (colored in purple) uses radial glial progenitors (RGP) (colored in green) as support to migrate perpendicularly to the ventricular zone (VZ). In the tangential mode, newborn neurons (colored in blue) migrate parallelly to the VZ, following one or multiple migratory streams. Scheme taken from (Moffat et al., 2015).

1.2.3.1. Neuronal migrations in the hindbrain

Several tangential migrations cause significant alterations to the anatomy of the hindbrain region. For instance, there are different populations of precerebellar neurons (PCN) derived from the caudal rhombic lip that perform tangential migrations; via extramural tangential stream into the external cuneate nucleus (ECu) and the lateral reticular nucleus (LRt), and via an intramural stream into the inferior olivary (IO) nuclei (Bloch-Gallego et al., 2005). Pontine neurons represent another classical example of tangential migration, in which caudal rhombic lip derivatives from r6-r7 migrate to their final fate in ventral r3-r4, where they form the pontine nuclei (Geisen et al., 2008; Zhu et al., 2009). In addition, the mammalian facial motor nucleus has its origin in the medial part of r4, from where it migrates to its final superficial position in r6 (Studer, 2001; Di Bonito et al., 2017).

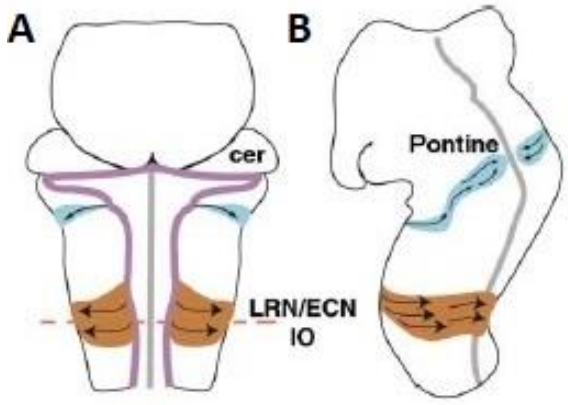


Figure 5. Dorsal (A) versus lateral (B) representations of the migratory pathways of precerebellar neurons (pontine, lateral reticular (LRN), external cuneatus (ECN) and inferior olivary (IO) neurons) in the hindbrain. Schema taken from (Marillat et al., 2004). All of them are originated in the rhombic lip (purple). Cer: cerebellum.

Regarding the rostral hindbrain, the interpeduncular nucleus (IPN) is a plurisegmental structure with components in the isthmus and r1 which is formed entirely by neurons migrated from different dorsoventral origins (Lorente-Cánovas et al., 2012). On the other hand, locus coeruleus (LoC) neurons tangentially migrate from its alar origin in r1 towards the basal plate, to become part of this nucleus (Aroca et al., 2006).

Netrin-1/DCC signaling directs the migration of hindbrain populations

Netrin-1 is expressed in the floorplate of the central nervous system. It is a secreted molecule that can function as chemoattractant or chemorepellent through its binding to several receptors that include DCC (Deleted in Colorectal Cancer), DSCAM (Down Syndrome Cell Adhesion Molecule) and UNC-5 (Rajasekharan and Kennedy, 2009). Netrin-1 acts as an attractive cue for the DCC expressing cells (Fearon et al., 1990; Hedrick et al., 1994; Keino-Masu et al., 1996).

Netrin-1/DCC signaling steers the migration of several neuronal populations in the hindbrain, such as noradrenergic neurons of the locus coeruleus (Shi et al., 2008) and precerebellar rhombic lip neurons (Alcántara et al., 2000; de Diego et al., 2002; Bloch-Gallego et al., 2005), including inferior olivary neurons (Bloch-Gallego et al., 2005; Marcos et al., 2009) and basilar pontine neurons (Kratochwil et al., 2017).

1.2.4. The interpeduncular nucleus

The interpeduncular nucleus (IPN) is a complex hindbrain structure highly conserved in all vertebrates. Although it is the principal target of the retroflex tract that comes from the medial habenula (mHb) (Herkenham and Nauta, 1979; Contestabile and Flumerfelt, 1981), the IPN has multiple connections with the rest of the brain (**Figure 6**), both ascending to limbic structures (e.g. to hypothalamus) and descending to other structures (e.g. to the median raphe nucleus) (Antolin-Fontes et al., 2015; Quina et al., 2017). Functionally, the mHb-IPN system is involved in several behavior-related activities, such as learning and memory, sleep, motor activity, stress, affective states (Klemm, 2004; Hikosaka, 2010) and mood-related psychiatric conditions (McLaughlin et al., 2017).

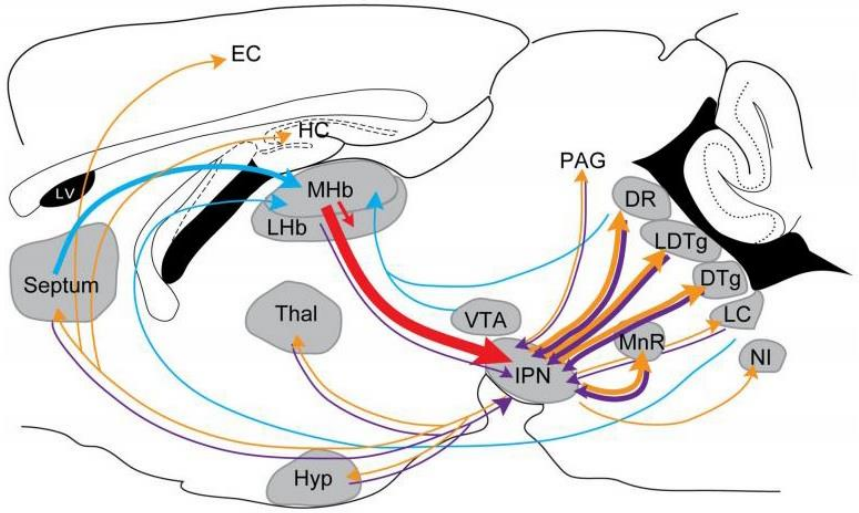


Figure 6. Scheme taken from (Antolin-Fontes et al., 2015) showing the afferences (in purple) and efferences (in orange) of the IPN. The retroflex tract is colored in red and comes from the medial habenula (MHb), the main IPN input. DR, dorsal raphe; DTg, dorsal tegmental nucleus; EC, entorhinal cortex; HC, hippocampus; Hyp, hypothalamus; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; LHb, lateral habenula; MnR, median raphe; NI, nucleus incertus; PAG, periaqueductal gray; Thal, thalamus; VTA, ventral tegmental area.

The development of the IPN has only recently been studied, in particular in the chick (Lorente-Cánovas et al., 2012) and mouse (Moreno-Bravo et al., 2014; Ruiz-Reig et al., 2019).

The study in chick (Lorente-Cánovas et al., 2012) demonstrated that the IPN is located subpially across the median floor plate of the rostral (prepontine) hindbrain, at the posterior end of the interpeduncular fossa. The

proposed model for the IPN consist of three rostrocaudal components: the rostralmost domain called prodromal (Pro) in the isthmus (Ist), and the interpeduncular rostral (IPR) and interpeduncular caudal (IPC) domains respectively located in rostral rhombomere 1 (r1-r) and caudal rhombomere 1 (r1-c). The latter two are further subdivided along the mediolateral and dorsoventral axes into several subnuclei. Thus, the IPR is formed by medial and lateral central parts (RCM, RCL), dorsally covered by a region with dorsomedial and apical subnuclei (RDM, RA), and laterally surrounded by the intermediate and lateral subnuclei (RI, RL). On the other hand, the IPC is composed of medial and lateral central subnuclei (CCM, CCL), surrounded by apical and lateral shell-like subnuclei (CA, CL) (**Figure 7**).

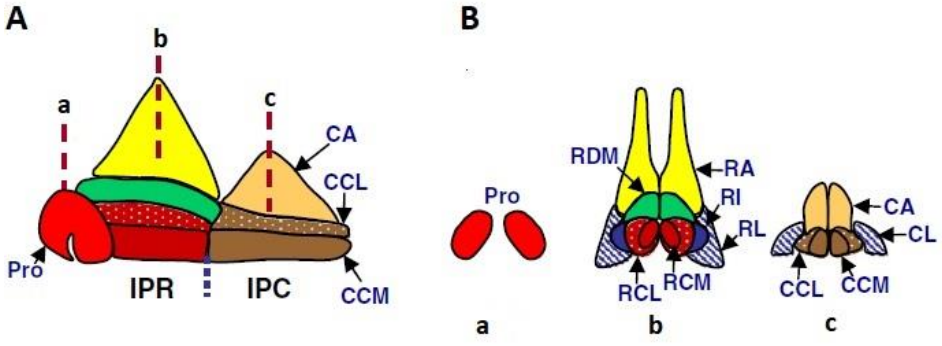


Figure 7. Sagittal (A) and frontal (B) views of the IPN domains and subnuclei in chick. In A, the section levels a, b and c correspond to the frontal views a, b and c represented in B. Scheme taken from Lorente-Cánovas et al., 2012.

In terms of the molecular regionalization of this nucleus, in the study in chick distinct neuronal populations were identified, characterized by the expression of the transcription factors *Nkx6.1*, *Pax7, Otp* or *Otx2* from their origins to their final fate within the IPN. These populations are originated in different dorsoventral progenitor domains, from where they follow specific dorsoventral tangential and/or radial migrations to reach their respective final position within the aforementioned segmental subdivisions of the mature IPN (Lorente-Cánovas et al., 2012). The same neuronal populations forming the chick IPN are also present in the mouse IPN, attending to their morphology, migratory routes, location, and gene expression (Moreno-Bravo et al., 2014; Ruiz-Reig et al., 2019).

Concerning the *Pax7*$^{+}$ cells, they have an alar origin in r1, from where they migrate tangentially towards the floor plate (Aroca et al., 2006; Lorente-Cánovas et al., 2012), contributing to both the IPR and the IPC. The *Otp*$^{+}$

population is generated in a subventricular region across the alar/basal plate boundary of r1-r, and it migrates to the IPR, intermixing with the *Pax7*$^+$ population. Both the *Nkx6.1*$^+$ and the *Otx2*$^+$ populations have an intermediate basal origin, respectively in the isthmus and r1-c ventricular zone, from where they subsequently migrate to the Pro (*Nkx6.1*$^+$) or IPC (*Otx2*$^+$) (Lorente-Cánovas et al., 2012; Moreno-Bravo et al., 2014; Ruiz-Reig et al., 2019) (**Figure 8**).

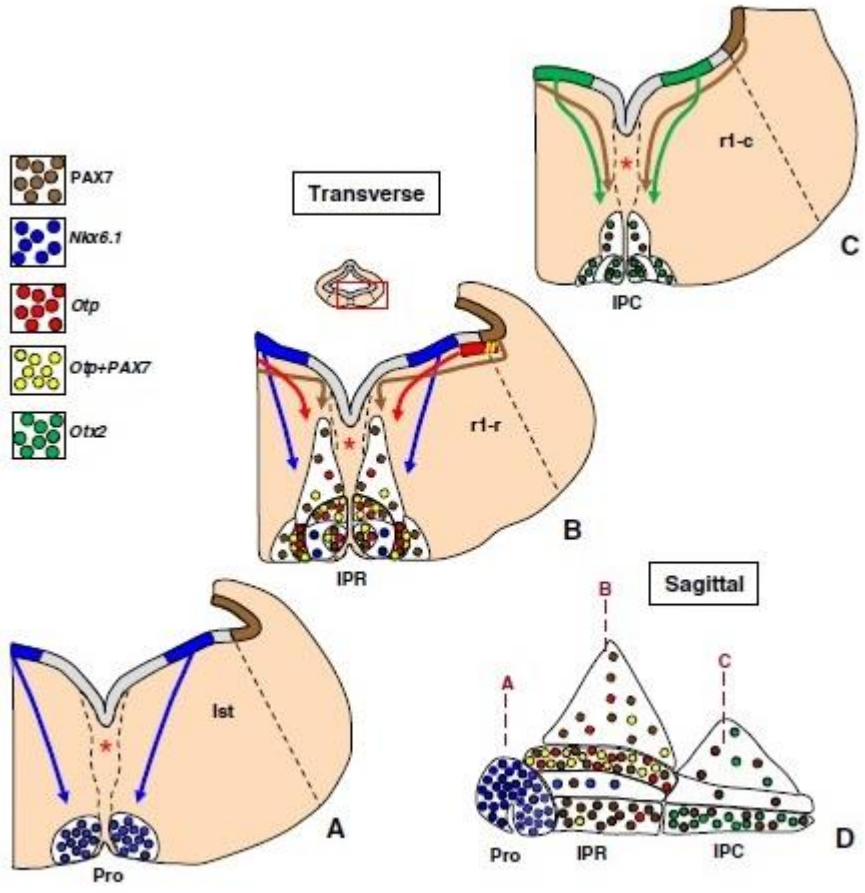


Figure 8. Schematic representation of the origins, migratory routes and final fates of the IPN populations identified in chick (Lorente-Cánovas et al., 2012). A, B and C represent schematic transverse sections at three rostrocaudal levels (Ist, r1-r, r1-c), where the Pro (A), IPR (B) and IPC (c) are respectively located. D represents a schematic sagittal view of the IPN, where the levels of A, B and C are indicated with red dash-lines. The 5 populations identified have different dorsoventral (alar, *Pax7* (brown); basal, *Nkx6.1* (blue) and *Otx2* (green); alar-basal, *Otp* (red), *Otp*+*Pax7* (yellow)) and rostrocaudal (Ist, *Nkx6.1*; r1-r, *Pax7*, *Otp*, *Otp*+ *Pax7*; r1-c, *Pax7*, *Otx2*) origins, from where they migrate following independent tangential and/or radial migratory routes to reach their proper fate within the IPN locus (Pro, IPR, IPC). The Pro is formed exclusively by *Nkx6.1*$^+$ cells, while in the IPR coexist mainly *Pax7*$^+$ and *Otp*$^+$ cells, with few *Otp*$^+$**+***Pax7*$^+$ and *Nkx6.1*$^+$ cells. In the IPC there are *Pax7*$^+$ and *Otx2*$^+$ cells.

Regarding the development of the IPN in rodents, a study with a r1-conditional mouse mutant for *Shh*, the gene responsible for the formation of the floorplate, demonstrated that in absence of *Shh* function the development of the interpeduncular nucleus is impaired. In this mutant, the migration of the *Pax7*$^+$, *Nkx6.1*$^+$, *Otp*$^+$ and *Otx2*$^+$ IPN populations is altered, so that the neuronal populations with a basal origin (*Nkx6.1+, Otx2+, Otp+*) are more affected than the populations with an alar origin (*Pax7*$^+$).

Another study in mouse demonstrated the requirement of *Otx2* for the IPN and the mHb development. In wildtype mouse, *Otx2*$^+$ cells follow 2 main steps (first they migrate tangentially and later radially) in their migration to the IPC. When *Otx2* is deleted, the IPN cells that would normally form the IPC skip the first step of tangential migration, and do not reach the IPC properly. Therefore, *Otx2* is required for the tangential migration of IPC cells that express this gene (Ruiz-Reig et al., 2019). Interestingly, IPN cells that had lost *Otx2* showed downregulation of *Dcc* gene (Netrin-1 receptor), which could underly the defective tangential migration, as proposed by the authors (Ruiz-Reig et al., 2019).

Thus, the IPN emerges as a plurisegmental heterogeneous formation whose development depends on the proper migration of multiple neuronal populations, characterized by the expression of different transcription factors. However, apart from the need of *Shh* (Moreno-Bravo et al., 2014) and *Otx2* (Ruiz-Reig et al., 2019), the molecular mechanisms underlying these independent migratory events remain largely unknown.

Considering that *Netrin-1* is expressed in the floor plate of the rostral hindbrain where this nucleus develops, and that *Dcc* is expressed (at least) by the migrating *Otx2*$^+$ cells that form the IPC (Ruiz-Reig et al., 2019) we decided to explore in **study 1** (García-Guillén et al., 2021) the possible involvement of the Netrin-1/DCC signaling system in the migration of the IPN populations. For that, we analyzed independently each IPN population (*Pax7*$^+$, *Nkx6*.1$^+$, *Otp*$^+$, *Otx2*$^+$ and *Irx2*$^+$) in DCC mutant mice, as compared to wildtype mice. Our results show that, in absence of DCC, there is a general but differential impairment of the IPN migrations, so that in study 1 we demonstrate that the Netrin-1/DCC system is one of the mechanisms involved in steering the migration of the neuronal populations of this nucleus.

In the DCC$^{-/-}$ mutant mouse, the rostral populations (*Nkx6.1*$^{+}$, *Pax7*$^{+}$, *Otp*$^{+}$, *Irx2*$^{+}$) are more affected than the caudal ones (*Pax7*$^{+}$, *Otx2*$^{+}$), as can be deduced by the number of cells reaching the IPN locus, which is higher for the populations forming the IPC. The Pro and IPR loci are almost completely devoid of cells in the mutant. Therefore, since some few cells are able to reach the IPN in absence of the Netrin-1 receptor, there must be additional mechanisms guiding the neuronal migrations of the IPN.

This hypothesis lead us to the **second study** (García-Guillén et al., 2021a), where we looked for additional genes expressed in the IPN at late developmental stages (E18.5, P4). For this purpose, we first searched for genes expressed in the IPN using the Allen Developing Mouse Brain Atlas (ADMBA) (https://developingmouse.brain-map.org/), and we found 135 genes regionalized within the IPN. Then, through bioinformatic tools, we performed and interaction (with STRING; https://string-db.org/) and a functional (with PANTHER; http://pantherdb.org/) analysis. The interactome analysis within each IPN subunit (Pro, apical IPR, intermediate IPR, basal IPR and IPC) gave rise to different networks (interactomes) that mainly involved several families of transcription factors, molecules related to neurotransmission, as well as ephrins/Ephs and Cadherins. On the other hand, the functional analysis highlighted an overrepresentation of gene families related to cell morphogenesis, axon guidance and neuron development.

On the whole, studies 1 and 2 focus on IPN development, respectively on its migration and molecular regionalization. Study 1 demonstrates that Netrin-1/DCC is one of the mechanisms involved in IPN neuronal migrations, and study 2 provides a genoarchitectonic IPN model with 135 genes differentially expressed in this nucleus, and their putative interactions and functionalities.

1.2.5. The trigeminal sensory column

The trigeminal sensory column is a structure located in the hindbrain that receives the afferences from the trigeminal ganglionic nerve fibers, as well as other somatosensory afferent fibers from the facial, glossopharyngeal, and vagal nerves. The trigeminal nerve inputs come from 3 different branches,

called ophthalmic, maxillary, and mandibular branches, that innervate different regions of the head and face (**Figure 9**) (Waite, 2004).

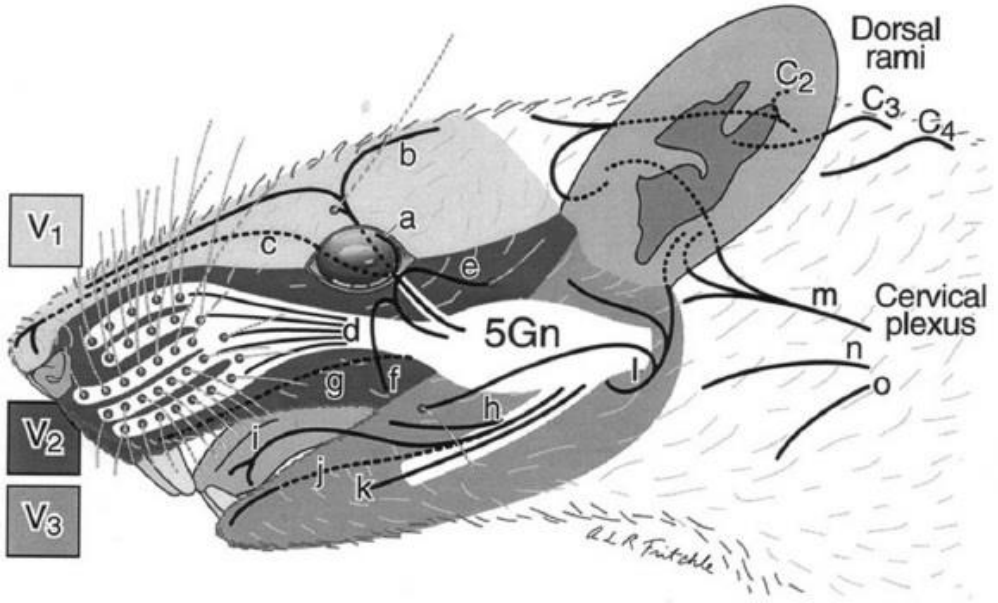


Figure 9. Trigeminal nerve branches in the rat. The three trigeminal subdivisions (V_1, ophthalmic; V_2, maxillary; V_3, mandibular) originate in the trigeminal ganglion (5Gn) and innervate different regions of the head. Schema taken from (Waite, 2004).

The somatosensory inputs coming from the three branches are received and integrated by the trigeminal column, which is composed by the principal sensory nucleus (Pr5) located in the rostral hindbrain, and the spinal trigeminal nucleus (Sp5) (**Figure 10**), which extends caudalwards to the hindbrain/spinal cord boundary. Sp5 is further subdivided into subnucleus oralis (Sp5O), subnucleus interpolaris (Sp5I) and subnucleus caudalis (Sp5C) (Olszewski, 1950). In addition to these nuclei, the trigeminal system comprises other structures such as the mesencephalic nucleus (Me5) and the trigeminal motor nucleus (5N).

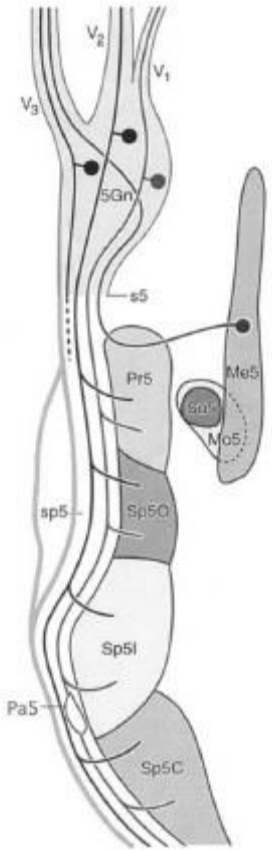


Figure 10. Trigeminal sensory column subnuclei and their innervation by the three trigeminal branches, in a schematic representation in the horizontal plane. Rostral is up and medial is to the right side of the image. In this representation, the trigeminal motor nucleus 5N is labeled as Mo5. Scheme taken from (Waite, 2004).

Although the subnuclei Sp5O, Sp5I and Sp5C were first defined according to cytoarchitectural differences (Olszewski, 1950), they also display different pattern of connections, projecting to different brain structures (Waite, 2004). In addition, the afferents received by the spinal subnuclei are organized following a precise topography, in the rostrocaudal, dorsoventral and mediolateral axes (Waite, 2004).

Thus, the aforementioned subdivisions of the trigeminal sensory column were stablished principally according to their pattern of connections, and to morphological and cytoarchitectural criteria. On the other hand, according to the prosomeric model, this plurisegmental hindbrain structure would be composed by successive transverse segments that would each derive from the respective rhombomeres. At this respect, Pr5 has been experimentally located in r1 in the chick (Marín and Puelles, 1995; Aroca et al., 2006; Rhinn et al., 2013), and in r2 and r3 in the mouse (Oury et al., 2006). Consequently, Sp5, which limits rostrally with Pr5, extends from r2 in

chick or r4 in mouse, to the hindbrain/spinal cord boundary, thus covering most of the rhombomeric units of the hindbrain.

This composition of the trigeminal column (and other hindbrain structures) by rhombomere-derived units was experimentally demonstrated with fate mappings in avian chimeras, where the neuronal derivatives and anatomical fate of each rhombomere were analyzed (**Figure 11**) (Marín and Puelles, 1995; Cambronero and Puelles, 2000).

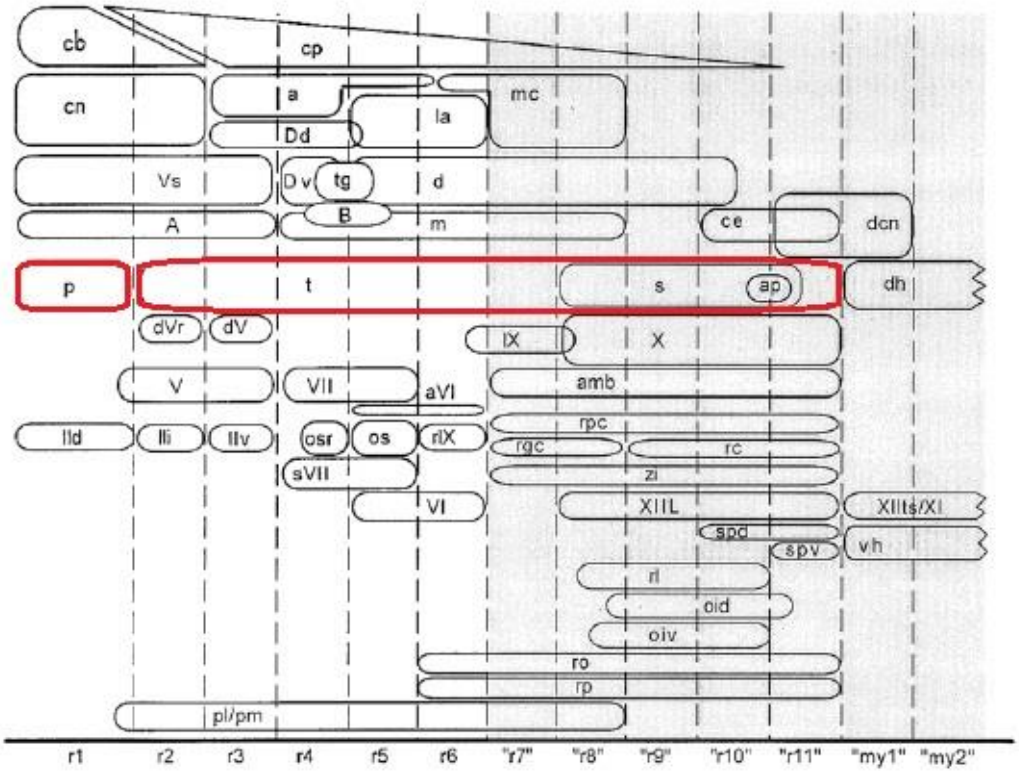


Figure 11. Morphological fate of each rhombomere in chick, summarizing the fate-mapping results from (Marín and Puelles, 1995) for r2-r6, and from (Cambronero and Puelles, 2000) for r7-r11. Scheme slightly modified from (Cambronero and Puelles, 2000) with the trigeminal column highlighted in red. p, principal sensory nucleus (Pr5); t: descending trigeminal nucleus (Sp5).

As previously commented, the prepontine (isthmus and r1) and the medullary (r7-r11) regions of the hindbrain lack visible intersegmental boundaries, contrary to the overtly segmented pontine (r2-r4) and retropontine (r5, r6) territories. However, the study of (Cambronero and Puelles, 2000) showed that the empirically defined segments from the caudal hindbrain which were fate-mapped (at that moment called pseudorhombomeres) gave rise to discrete portions of the hindbrain, with neuronal nuclei whose morphological transverse borders largely fitted the

limits between pseudorhombomeres (see **Figure 11**). Therefore, this study highly supported a hidden segmentation for this brain region.

The transverse segments in the hindbrain (rhombomeres and cryptorhombomeres) express specific combinations of developmental genes, which control the neuronal differentiation and specification processes within each territory (Addison and Wilkinson, 2016; Parker and Krumlauf, 2020). Some cell trespassing of interrhombomeric boundaries may be caused by neural migration (Watson et al., 2017a), including the tangential migration processes as previously commented as well as discrete cell migration or intermingling across the boundaries (Birgbauer and Fraser, 1994). In addition, both the overtly segmented part of the hindbrain and the caudal hindbrain (as well as the spinal cord) display graded differential expression of Hox genes. Thus, the rostrocaudal molecular regionalization of the hindbrain is molecularly defined at least by the nested and striped expression of Hox genes: 3' Hox genes from groups 1 to 3 for the overtly segmented region (Nolte and Krumlauf, 2013) and Hox genes from groups 4 to 7 to the medulla oblongata (caudal hindbrain) (**Figure 12**) (Marín et al., 2008; Tomás-Roca et al., 2016).

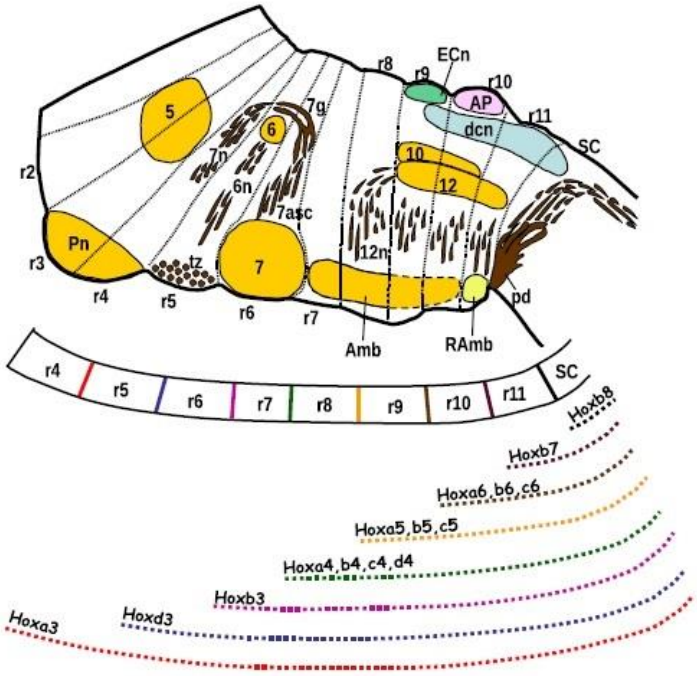


Figure 12. Schema from (Tomás-Roca et al., 2016) showing Hox genes expression pattern in the mid-gestational/perinatal mouse hindbrain. The anterior borders of expression of Hox genes coincide with the interrhombomeric limits.

The aforementioned studies regarding segmentation and Hox expression within the trigeminal sensory column were performed in embryonic stages. In **the third work (García-Guillén et al., 2021b)** of this doctoral thesis, we wanted to reexamine the issue of the molecular segmentation of the trigeminal sensory column in the adult mouse brain.

Although we mainly focused on the spinal trigeminal nucleus (Sp5), we also included in the study the principal trigeminal nucleus (Pr5) as it is the immediate rostral continuation of Sp5. For our purpose, we searched the Allen Mouse Brain Atlas (AMBA) database (https://mouse.brain-map.org/) looking for genes differentially expressed within the trigeminal sensory column. We performed the screening at P56 (adult stage), selecting genes whose expression pattern was sustained in the coronal plane and in postnatal stages (P4, P14, P28). As a result, we selected 12 genes, and analyzed their expression pattern along the rostrocaudal axis of the trigeminal column in relation to known morphological landmarks (**Figure 3**). In addition, we tried to correlate their expression pattern with the classical vs. rhombomeric subdivisions of the trigeminal column. Some of the selected genes (*Calb1, Calb2, Camk2a, Tac1, Tac2*) had been previously related with the trigeminal column, so that in study 3 we provide the description of their segmentary pattern in this structure. On the other hand, for the rest of analyzed genes (*Irx2, Mafb, Fn1, Pde1c, Zbtb16, Kcng4,* and *Baiap3*) we describe for the first time their relationship with the trigeminal column, as well as their segmentary pattern. On the whole, study 3 provides a genoarchitectonic model for the spinal trigeminal nucleus in the adult mouse brain, which can be used for the integration of additional data on gene expression, as well as a basis for future functional studies about this structure and its relationship with brains disorders such as the migraine.

AIMS AND OBJECTIVES

2.1. GENERAL AIM:

The main objective of this thesis was to deepen the knowledge about two fundamental processes for brain formation: neuronal migration and brain segmentation. For this, we focused on two hindbrain structures: the interpeduncular nucleus (IPN) and the trigeminal sensory column. Studies 1 and 2 respectively focused on the migration and regionalization processes in the IPN, while study 3 addressed the molecular regionalization and segmentation of the sensory trigeminal column.

2.2. AIMS IN STUDY 1:

The general aim in this work was to determine if the Netrin-1/DCC signaling pathway is involved in the migration of the IPN populations. We aimed to analyze each of the IPN populations identified by the respective expression of *Nkx6.1, Pax7, Otp, Irx2* and *Otx2*, in brains of a knock-out mouse line for the *Dcc* gene ($DCC^{-/-}$) as compared to its wildtype (WT) counterpart. Objectives:

1.1. To analyze the expression of *Dcc and Netrin-1* in the rostral hindbrain of wildtype mice at several stages (from E11.5 to E18.5) of development, and to determine their expression either in IPN cells or in relation to their migratory pathways or final location.

1.2. To analyze and compare the expression of *Nkx6.1, Pax7, Otp, Irx2* and *Otx2* in the IPN of wildtype versus $DCC^{-/-}$ mice, and determine the possible affectation of the analyzed populations in the mutant.

2.3. AIMS IN STUDY 2:

The general aim of this work was to identify genes that are differentially expressed in the domains of the IPN, in order to provide new insight into the morphological and molecular characterization of this hindbrain structure. In addition, we wanted to characterize their putative functional roles and

interactions. On the whole, we aimed to provide a genoarchitectonic and molecular model of the IPN that could be used as a basis for further functional experiments. Objectives:

2.1. To perform a screening of 2038 genes with available E18.5 in situ hybridization (ISH) data in the Allen Developing Mouse Brain Atlas (ADMBA) in order to select those genes with a significant regionalized expression within the IPN.

2.2. To analyze the expression pattern of the selected genes, determining their positive or negative expression within the different domains of the IPN.

2.3. To perform an in silico functional analysis in order to discern the possible overrepresented functional categories among the selected genes.

2.4. To analyze potential (predicted) interactions among the set of genes expressed within each IPN domain, through the mining of available databases.

2.4. AIMS IN STUDY 3:

Analogously to study 2, the general aim of this third study was to identify genes that are differentially expressed along the trigeminal sensory column, and correlate their respective expression domains with the different rhombomeric subdivisions of the hindbrain, in order to discern the possible segmentary organization along the hindbrain. Objectives:

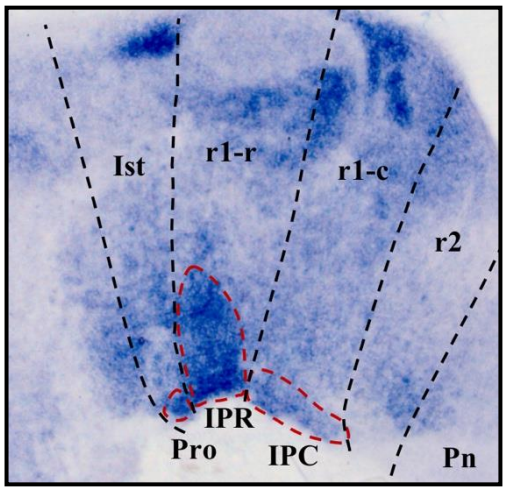
3.4. To perform a screening in the ADMBA and AMBA databases to find genes with differential expression along the sensory trigeminal column of the adult and juvenile mouse brain.

3.5. To analyze the expression pattern of the selected genes, determining their positive or negative expression within the different rostrocaudal domains of the sensory trigeminal column.

3.6. To study the possible correspondence of these molecular rostrocaudal domains to classical versus rhombomeric subdivisions of the trigeminal column.

PUBLICATIONS

3.1. STUDY 1



Netrin-1/DCC Signaling Differentially Regulates the Migration of Pax7, Nkx6.1, Irx2, Otp, and Otx2 Cell Populations in the Developing Interpeduncular Nucleus

García-Guillén IM, Alonso A, Morales-Delgado N, Andrés B, Puelles L, López-Bendito G, Marín F and Aroca P

Journal: Frontiers in Cell and Developmental Biology

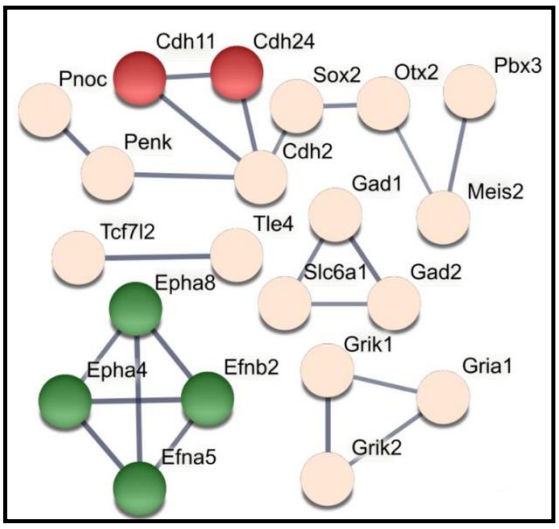
Doi: 10.3389/fcell.2020.588851

https://www.frontiersin.org/articles/10.3389/fcell.2020.588851/full

Abstract

The interpeduncular nucleus (IPN) is a hindbrain structure formed by three main subdivisions, the prodromal (Pro) domain located at the isthmus (Ist), and the rostral and caudal interpeduncular domains (IPR, IPC) within rhombomere 1 (r1). Various cell populations can be detected in the IPN through the expression of the *Nkx6.1, Otp, Otx2, Pax7*, and/or *Irx2* transcription factors. These cell populations follow independent dorsoventral tangential and radial migratory routes targeting the ventral paramedian region of Ist and r1. Here we set out to examine the influence of the Netrin-1/DCC pathway on these migrations, since it is known to regulate other processes of neuronal migration in the brain. To this end, we analyzed IPN development in late gestational wild-type and $DCC^{-/-}$ mice, using mainly in situ hybridization (ISH) to identify the cells expressing each of the aforementioned genes. We found that the migration of *Nkx6.1*$^+$ and *Irx2*$^+$ cells into the Pro domain was strongly disrupted by the loss of DCC, as occurred with the migration of *Pax7*$^+$, *Irx2*$^+$, and *Otp*$^+$ cells that would normally form the IPR. In addition, there was mild impairment of the migration of the *Pax7*$^+$ and *Otx2*$^+$ cells that form the IPC. These results demonstrate that the Netrin-1/DCC signaling pathway is involved in the migration of most of the IPN populations, mainly affecting those of the Pro and IPR domains of this nucleus. There are psychiatric disorders that involve the medial habenula (mHb)-IPN system, so that this experimental model could provide a basis to study their neurodevelopmental etiology.

3.2. STUDY 2



Multiple Regionalized Genes and Their Putative Networks in the Interpeduncular Nucleus Suggest Complex Mechanisms of Neuron Development and Axon Guidance

García-Guillén IM, Alonso A, Puelles L, Marín F and Aroca P

Journal: Frontiers in Neuroanatomy

Doi: 10.3389/fnana.2021.643320

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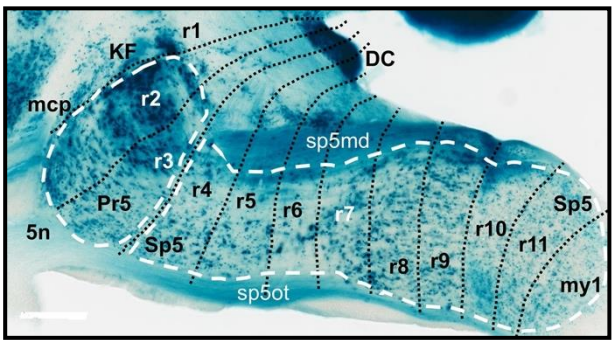
Abstract

The interpeduncular nucleus (IPN) is a highly conserved limbic structure in the vertebrate brain, located in the isthmus and rhombomere 1. It is formed by various populations that migrate from different sites to the distinct domains within the IPN: the prodromal, rostral interpeduncular, and caudal interpeduncular nuclei. The aim here was to identify genes that are differentially expressed across these domains, characterizing their putative functional roles and interactions. To this end, we screened the 2,038 genes in the Allen Developing Mouse Brain Atlas database expressed at E18.5 and we identified 135 genes expressed within the IPN. The functional analysis of these genes highlighted an overrepresentation of gene families related to neuron development, cell morphogenesis and axon guidance. The interactome analysis within each IPN domain yielded specific networks that mainly involve members of the ephrin/Eph and Cadherin families, transcription factors and molecules related to synaptic neurotransmission. These results bring to light specific mechanisms that might participate in the formation, molecular regionalization, axon guidance and connectivity of the different IPN domains. This genoarchitectonic model of the IPN enables data on gene expression and interactions to be integrated and interpreted, providing a basis for the further study of the connectivity and function of this poorly understood nuclear complex under both normal and pathological conditions.

3.3. STUDY 3



Molecular segmentation of the spinal trigeminal nucleus in the adult mouse brain

García-Guillén IM, Martínez-de-la-Torre M, Puelles L, Aroca P and Marín F

Journal: Frontiers in Neuroanatomy

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https://www.frontiersin.org/articles/10.3389/fnana.2021.785840/full

Abstract

The trigeminal column is a hindbrain structure formed by second order sensory neurons that receive afferences from trigeminal primary (ganglionic) nerve fibers. Classical studies subdivide it into the principal sensory trigeminal nucleus located next to the pontine nerve root, and the spinal trigeminal nucleus which in turn consists of oral, interpolar and caudal subnuclei. On the other hand, according to the prosomeric model, this column would be subdivided into segmental units derived from respective rhombomeres. Experimental studies have mapped the principal sensory trigeminal nucleus to pontine rhombomeres (r) r2-r3 in the mouse. The spinal trigeminal nucleus emerges as a plurisegmental formation covering several rhombomeres (r4 to r11 in mice) across pontine, retropontine and medullary hindbrain regions. In the present work we reexamined the issue of rhombomeric versus classical subdivisions of this column. To this end, we analysed its subdivisions in an AZIN2-lacZ transgenic mouse, known as a reference model for hindbrain topography, together with transgenic reporter lines for trigeminal fibers. We screened as well for genes differentially expressed along the axial dimension of this structure in the adult and juvenile mouse brain. This analysis yielded genes from multiple functional families that display transverse domains fitting the mentioned rhombomeric map. The spinal trigeminal nucleus thus represents a plurisegmental structure with a series of distinct neuromeric units having unique combinatorial molecular profiles.

CONCLUSIONS

4.1. CONCLUSIONS FROM STUDY 1

1.1. *Netrin-1* is expressed strongly by the floor plate in the isthmus and rhombomere 1 where the IPN develops, in accordance with its proposed role as attractor or target for the migratory cells of the IPN.

1.2. The Netrin-1 receptor *Dcc* is expressed by cells of the interpeduncular nucleus during development. At least from E15.5 onwards, *Dcc* is expressed by IPN neurons migrating to the IPN to become part of the three rostrocaudal portions of the nucleus (Pro, IPR, and IPC) at E18.5.

1.3. The migration of the five independent neuronal populations identified in the IPN is differentially disrupted in the $DCC^{-}/^{-}$ mouse mutant line. In particular, the migration of the Pro and IPR populations (*Nkx6.1, Irx2, Otp, Pax7*) is more compromised than that of the IPC cells (*Pax7, Otx2*).

1.4. Netrin-1/DCC signaling pathway is one of the mechanisms involved in the migration of the IPN. However, considering that some IPN cells are able to reach the IPN locus in absence of DCC, there must be additional mechanisms guiding the migration of these populations.

4.2. CONCLUSIONS FROM STUDY 2

2.1. The 3 known domains of the IPN (prodromal, rostral and caudal IPN) can be characterized by their respective molecular identity, according to the differential expression of 135 genes at least from E18.5 to P4.

2.2. Many of these genes are additionally differentially expressed within deep to superficial subdomains (apical, intermediate, and basal) of the interpeduncular rostral domain (IPR).

2.3. The functional analysis of these 135 genes highlights an overrepresentation of gene families related to neuron development (*neuron development* (GO: 0048666)), cell morphogenesis (*cell morphogenesis* (GO: 0000902), *cellular component morphogenesis* (GO: 0032989), axon guidance (*axon guidance* (GO: 0007411), and *neuron projection guidance* (GO: 0097485).

2.4. The interactome analysis within each IPN domain yields specific networks that mainly involve members of the ephrin/Eph and Cadherin

families, transcription factors and molecules related to synaptic neurotransmission.

2.5. Collectively, the results from both the predicted interactomes and the functional overrepresentation analysis indicate that genes active in processes like axon guidance or neuronal development and morphogenesis, would be relevant to the formation and connectivity of the IPN.

4.3. CONCLUSIONS FROM STUDY 3

3.1. The result of the screening of the genes expressed in the trigeminal sensory column was the identification of 12 genes from different functional families with a regionalized expression within this structure. These genes are expressed in one or more transverse domains fitting the rhombomeric organization of the hindbrain.

3.2. The principal trigeminal sensory nucleus (Pr5) can be differentiated in molecular domains located respectively in r2 and r3, thanks to the stronger expression of *Irx2* and *Baiap3* in r2.

3.3. Within the spinal sensory trigeminal nucleus (Sp5) there appear as differentiated domains those formed by r5 plus r6 (with strong expression of *Mafb* and *Fn1*), r9 (with clearcut expression of *Pde1c* and *Zbtb16* as compared to adjacent domains) and r10 together with r11 (with strong expression of *Baiap3*, *Tac1* and *Calb2*).

3.4. The r9/r10 boundary (junction between the interpolar and caudal subnuclei of Sp5) is marked by the expression of 8 of the 12 analyzed genes whose respective expression domains abut this boundary either rostrally or caudally.

3.5. Within the caudal subnucleus of Sp5, that corresponds to r10 and r11, there is an apparent homogeneity of its cytoarchitecture (laminar structure) and gene expression, that extends caudalwards at least into the dorsal horn of the first myelomere (my1). Nevertheless, from the analyzed markers, *Tac2* differentiated r11 from r10, showing higher number of positive cells in the former.

3.6. The trigeminal sensory column appears therefore as a multineuromeric structure with rostrocaudal molecular domains that correspond to respective rhombomeres or pairs of them.

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García-Guillén, I. M., Martínez-de-la-Torre, M., Puelles, L., Aroca, P., and Marín, F. (2021b). Molecular Segmentation of the Spinal Trigeminal Nucleus in the Adult Mouse Brain. *Front. Neuroanat.* 15, 107. doi:10.3389/fnana.2021.785840.

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APPENDIXES

Supervisors' Approval

UNIVERSIDAD DE
MURCIA

Dª. Mª Pilar Aroca Tejedor, Catedrática de Universidad del Área de Anatomía y Embriología Humana en el Departamento de Anatomía Humana y Psicobiología, AUTORIZA:

La presentación de la Tesis Doctoral titulada "Estudio del núcleo interpeduncular y del complejo sensorial trigeminal como ejemplos de procesos de migración y segmentación en el rombencéfalo/Study of the interpeduncular nucleus and the trigeminal sensory complex as paradigms of neuronal migration and brain segmentation in the hindbrain", realizada por Dª. Isabel María García Guillén, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 13 de diciembre de 2021

UNIVERSIDAD DE
MURCIA

D. Faustino Marín San Leandro, Profesor Titular de Universidad del Área de Anatomía y Embriología Humana en el Departamento de Anatomía Humana y Psicobiología, AUTORIZA:

La presentación de la Tesis Doctoral titulada "Estudio del núcleo interpeduncular y del complejo sensorial trigeminal como ejemplos de procesos de migración y segmentación en el rombencéfalo/Study of the interpeduncular nucleus and the trigeminal sensory complex as paradigms of neuronal migration and brain segmentation in the hindbrain", realizada por Dª. Isabel María García Guillén, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 13 de diciembre de 2021

MARIN SAN LEANDRO FAUSTINO - 22965628J

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UNIVERSIDAD DE
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CERTIFICADO DE ESTANCIA DE INVESTIGACIÓN FUERA DE ESPAÑA PARA OPTAR A MENCIÓN DE «DOCTORADO INTERNACIONAL»
INTERNATIONAL Ph.D. VISITING RESEARCH CERTIFICATE

1. DATOS DEL DOCTORANDO QUE HA REALIZADO LA ESTANCIA / Ph.D. STUDENT´S PERSONAL DATA

Nombre y apellidos del doctorando/a / Ph.D. student name and surname: Isabel María García Guillén
Centro de origen / Institution of origin: Escuela Internacional de Doctorado de la Universidad de Murcia
Programa de Doctorado / Doctoral Programme: Integración y Modulación de Señales en Biomedicina
Título de la tesis / Thesis title: Búsqueda de las moléculas de guía axonal responsables de la migración de las poblaciones neuronales que forman el núcleo interpeduncular: Estudio en ratones DCC.

2. DATOS DEL CENTRO EN EL QUE SE HA REALIZADO LA ESTANCIA / HOST INSTITUTION

Nombre del Centro de Educación Superior o Instituto de Investigación / Name of the host institution: Kent State University
Departamento/Centro / Department/Centre: Biological Sciences
Dirección del Centro / Address: 256 Cunningham Hall, Kent State University
Localidad y país / City and country: Kent, OHIO 44242-0001

3. DATOS DEL TUTOR/INVESTIGADOR RESPONSABLE DE LA ESTANCIA / RESEARCH SUPERVISOR AT HOST INSTITUTION

Nombre y apellidos del tutor/investigador / Research supervisor name and surname: Kristy Welshhans
DNI/Pasaporte nº. / I.D. / Passport nº: 467812535
E-mail: kwelshha@kent.edu
Departamento/Centro al que pertenece / Department/Centre: Biological Sciences

4. CERTIFICADO DE LA ESTANCIA / VISITING RESEARCH CERTIFICATE (1):

(1)A cumplimentar por el tutor investigador responsable de la estancia / To complete by the research supervisor.

El abajo firmante certifica que el doctorando/a arriba mencionado/a ha realizado una estancia en este Centro bajo mi supervisión en las siguientes fechas: desde el 01 de 05 de 2019 hasta el 31 de 07 de 2019, realizando un estudio sobre las migraciones neuronales de las poblaciones que forman el núcleo interpeduncular de ratón en el modelo de Síndrome de Down (Ts65Dn).

Kristy Welshhans 2/24/2020

COMISIÓN GENERAL DE DOCTORADO DE LA UNIVERSIDAD DE MURCIA (España)

UNIVERSIDAD DE
MURCIA

(indicar la investigación realizada en relación con su tesis doctoral)

The person who sign this document certify that the Ph.D. student above-mentioned has visited this institution under my supervision in the following dates: from 01 of 05 of 2019 to 31 of 07 of 2019, carrying out a study of the neuronal migrations of the populations that form the mouse interpeduncular nucleus in a Down Syndrome model (Ts65Dn).

(research carried out related to the thesis)

En/In Kent a 24/02 de 2020

Firmado y sellado / Signed and stamped*. Dr/a Dº./Dª.:

(*) Debe firmar y sellar todas las hojas del informe / You must sign and seal all the pages of the report

COMISIÓN GENERAL DE DOCTORADO DE LA UNIVERSIDAD DE MURCIA (España)

Página **2** de **2**

UNIVERSITY OF
South Carolina
Department of Biological Sciences

September 27, 2021

Re: Isabel Maria Garcia Guillen

To Whom It May Concern,

This letter certifies that Isabel Maria Garcia Guillen (ID number 23306757Y) received research training for 3 months (May 1, 2019 through July 31, 2019) in my laboratory at Kent State University as a visiting scholar. The scientific training Isa received in my laboratory is part of her PhD/doctoral studies. During her visit, Isa gained proficiency in experimental design and implementation of multiple techniques, including in situ hybridization, PCR, and cell culture. Furthermore, Isa became proficient in animal and culture models that increase our understanding of the intellectual disability phenotype of Down syndrome. While Isa was in my laboratory, I was an assistant professor at Kent State University. However, I accepted a new faculty position and moved my laboratory to the University of South Carolina in August 2021.

Should you need further information, do not hesitate to contact me through any of the means below.

Sincerely,

Kristy Welshhans

Kristy Welshhans, PhD

Assistant Professor
Department of Biological Sciences
University of South Carolina
715 Sumter Street
Columbia, SC 29208
Phone: 803.777.5957
Email: kristyw@mailbox.sc.edu

UNIVERSIDAD DE MURCIA

INFORME JUSTIFICATIVO SOBRE LA CONTRIBUCIÓN DEL DOCTORANDO A LAS PUBLICACIONES INTEGRADAS EN LA TESIS DOCTORAL

D.ª Isabel María García Guillén, a efectos de obtención de autorización de la Comisión General de Doctorado para la presentación de la tesis doctoral "Estudio del núcleo interpeduncular y del complejo sensorial trigeminal como ejemplos de procesos de migración y segmentación en el romboencéfalo", justifica su contribución a las publicaciones que la integran, en los aspectos que se detallan a continuación, con el aval de sus directores, D. ª Mª Pilar Aroca Tejedor y D. Faustino Marín San Leandro.

Artículo 1:

García-Guillén, I. M., Alonso, A., Morales-Delgado, N., Andrés, B., Puelles, L., López-Bendito, G., Marín, F., and Aroca, P. (2020). Netrin-1/DCC Signaling Differentially Regulates the Migration of Pax7, Nkx6.1, Irx2, Otp, and Otx2 Cell Populations in the Developing Interpeduncular Nucleus. Front. Cell Dev. Biol. 8. doi:10.3389/fcell.2020.588851.

El doctorando, y primer autor de esta publicación, ha contribuido de forma relevante en:

-Contextualización teórica y análisis del estado de la cuestión.
-Realización de los experimentos (obtención de embriones y procesamiento, obtención de ribosondas, realización de las hibridaciones in situ, obtención de imágenes al microscopio).
-Montaje de las planchas del artículo.
-Análisis e interpretación de los resultados, y discusión.
-Redacción del manuscrito.

Artículo 2:

García-Guillén, I. M., Alonso, A., Puelles, L., Marín, F., and Aroca, P. (2021). Multiple Regionalized Genes and Their Putative Networks in the Interpeduncular Nucleus Suggest Complex Mechanisms of Neuron Development and Axon Guidance. Front. Neuroanat. 15. doi:10.3389/fnana.2021.643320.

El doctorando, y primer autor de esta publicación, ha contribuido de forma relevante en:

-Contextualización teórica y análisis del estado de la cuestión.
-Realización de la parte experimental. Por un lado, el cribado exhaustivo en bases de datos de expresión génica, análisis de imágenes, test de sobrerrepresentación y análisis de interacciones con herramientas bioinformáticas. Por otro lado, obtención de embriones y procesamiento, obtención de ribosondas, realización de las hibridaciones in situ, y obtención de imágenes al microscopio (para imágenes incluidas en material suplementario de la publicación).
-Montaje de las planchas del artículo.
-Análisis e interpretación de los resultados, y discusión.
-Redacción del manuscrito.

Artículo 3:

García-Guillén, I. M., Martínez-de-la-Torre, M., Puelles, L., Aroca, P., and Marín, F. (2021 accepted). Molecular segmentation of the spinal trigeminal nucleus in the adult mouse brain. Front. Neuroanat.

El doctorando, y primer autor de esta publicación, ha contribuido de forma relevante en:

-Contextualización teórica y análisis del estado de la cuestión.

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-Realización de la parte experimental (cribado exhaustivo en bases de datos de expresión génica, análisis de imágenes).
-Montaje de las planchas del artículo.
-Análisis e interpretación de los resultados, y discusión.
-Redacción del manuscrito.

Firmado por GARCIA GUILLEN ISABEL MARIA - 23306757Y el día 26/11/2021 con un certificado emitido por AC FNMT Usuarios