



ORIGINAL ARTICLE

# Gene expression analysis in the mouse brainstem identifies Cart and Nesfatin as neuropeptides coexpressed in the Calbindin-positive neurons of the *Nucleus papilio*

Franck Girard<sup>1,\*</sup>, Michelle von Siebenthal<sup>1</sup>, Fred P. Davis<sup>2,3</sup> and Marco R. Celio<sup>1</sup>

<sup>1</sup>Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland, <sup>2</sup>Janelia-Farm Research Campus, Howard Hughes Medical Institute, Ashburn, VA and <sup>3</sup>Present address: Molecular Immunology and Inflammation Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institute of Health, Bethesda, MA.

\*Corresponding author. Franck Girard, Anatomy and Program in Neuroscience, Department of Medicine, Faculty of Science, University of Fribourg, Route A. Gockel 1, CH1700 Fribourg, Switzerland. Email: [franck.girard@unifr.ch](mailto:franck.girard@unifr.ch).

## Abstract

**Study Objectives:** The brainstem contains several neuronal populations, heterogeneous in terms of neurotransmitter/neuropeptide content, which are important for controlling various aspects of the rapid eye movement (REM) phase of sleep. Among these populations are the Calbindin (Calb)-immunoreactive NP<sup>Calb</sup> neurons, located in the *Nucleus papilio*, within the dorsal paragigantocellular nucleus (DPGi), and recently shown to control eye movement during the REM phase of sleep.

**Methods:** We performed in-depth data mining of the in situ hybridization data collected at the Allen Brain Atlas, in order to identify potentially interesting genes expressed in this brainstem nucleus. Our attention focused on genes encoding neuropeptides, including Cart (Cocaine and Amphetamine Regulated Transcripts) and Nesfatin 1.

**Results:** While nesfatin 1 appeared ubiquitously expressed in this Calb-positive neuronal population, Cart was coexpressed in only a subset of these glutamatergic NP<sup>Calb</sup> neurons. Furthermore, an REM sleep deprivation and rebound assay performed with mice revealed that the Cart-positive neuronal population within the DPGi was activated during REM sleep (as measured by c-fos immunoreactivity), suggesting a role of this neuropeptide in regulating some aspects of REM sleep.

**Conclusions:** The assembled information could afford functional clues to investigators, conducive to further experimental pursuits.

## Statement of Significance

Several physiological and behavioral features are characteristics of the rapid eye movement (REM) phase of sleep, also called paradoxical sleep. These include muscle atonia, desynchronized EEG activity, vivid dreaming, and rapid eye movements. A small cluster of Calbindin-immunoreactive neurons (namely, the *Nucleus papilio*) has been recently identified in the brainstem and shown to be both necessary and sufficient for triggering eye movement during REM sleep. In the present study, we performed data mining of the in situ hybridization data collected at the Allen Brain Atlas, in order to identify genes expressed in these neurons. Our data show that the neuropeptide Cart (Cocaine and Amphetamine Regulated Transcript) is expressed in some of these Calbindin-immunoreactive neurons and that these Cart-neurons are activated during REM sleep.

**Key words:** *Nucleus papilio*; NP<sup>Calb</sup>; REM sleep; DPGi; calbindin; Cart; nesfatin

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## Introduction

Several physiological and behavioral features are characteristics of the REM phase of sleep, also called paradoxical sleep. These include rapid eye movements, vivid dreaming, desynchronized electroencephalogram activity, and atonia of the postural muscles [1]. Among several brainstem structures that have been shown to be involved during REM sleep [2–6], the dorsal paragigantocellular nucleus (DPGi) appears as a very crucial area in sleep regulation, as it contains neurons of different nature, which apparently play particular roles in regulating some aspects of REM sleep. Indeed, GABAergic neurons of the DPGi were proposed to inhibit the noradrenergic wake-promoting neurons of the *Locus ceruleus*, the dorsal raphé nucleus, and the ventrolateral periaqueductal gray, thereby favoring the initiation of REM sleep [4, 7, 8]. Within the DPGi we recently identified the *Nucleus papilio* (NP<sup>Calb</sup>) as a bilateral, symmetric cluster of glutamatergic neurons expressing the calcium-binding protein Calbindin-D28k (Calb) [9]. Calb immunoreactivity in this nucleus is conserved in rodents (mouse and rat), monkey, and human. In mouse, it densely projects to the three contralateral eye-muscle nuclei (abducens, trochlear, and oculomotor), but also to several brain areas contributing to REM sleep control including the MCH-neurons of the lateral hypothalamus, the subcoeruleus nucleus (SubC), the pontine reticular formation (PnC), and the gigantocellular reticular nucleus (Gi). Noteworthy, activating or inactivating these neurons by means of optogenetics demonstrated both the necessity and sufficiency of the NP<sup>Calb</sup> for triggering eye movement during REM sleep [9].

The automated ALLENMINER search [10] of in situ hybridization (ISH) images in the Allen Brain Atlas (ABA) can be implemented to identify genes that are expressed in rather small agglomerations of cells, such as the PV1/Parvafox nucleus (parvalbumin-FoxB1 immunoreactive nucleus) in the lateral hypothalamus [11]. Using this protocol, potentially interesting information respecting very small neuronal populations can be elicited. By this means, ISH on adjacent sections has hitherto revealed most of the genes that were tested to be coexpressed with the mRNA for *Pvalb* [11]. We were therefore sanguine that a similar search would facilitate molecular and potentially functional characterization of the Calb-expressing neurons of the NP<sup>Calb</sup>. In addition, we screened the AGEA (Anatomic Gene expression Atlas) [12] at the ABA, focusing on genes expressed in the NP<sup>Calb</sup>/DPGi area.

## Methods

### Animals

For analyzing the expression of several proteins potentially coexpressed in the NP<sup>Calb</sup>, 15 C57BL/6J mice (from our animal

facility) of both sexes, aged 10–14 weeks, as well as 3 Wistar rats (Janvier, Lyon, France), were used. For the analysis of the neurotransmitter status of the Cart-expressing neurons, two mice of each genotype were used (*Slc17a6::Cre* and *Slc32a1::Cre* encoding, respectively, VGlut2 glutamate and VGat GABA transporters, both obtained from the Jackson Laboratory; *Slc6a5-GFP::Cre* mice encoding the Glyt2 glycine transporter, obtained from Dr Zeilhofer, Pharmacology, Zurich). Fifteen C57BL/6J female mice were included in the REM sleep deprivation and rebound assay.

All animals were housed in our animal facilities and in accordance with the relevant Swiss laws. The Veterinary Commission for Animal Research of the Canton of Fribourg (Switzerland) approved this study.

Animals were anesthetized with pentobarbital (100 mg/kg of body weight) and then perfused via the left ventricle, first with chilled (4°C) physiological (0.9%) saline and then with chilled (4°C) 4% paraformaldehyde. The brains were excised and post-fixed overnight at 4°C in 4% paraformaldehyde and subsequently immersed in 0.1 M Tris buffer (pH 7.3) containing 20% sucrose in preparation for cryo-sectioning.

### Immunohistochemistry

The various brain specimens were cryo-sectioned into 30, or 40, µm coronal sections and collected directly in 0.1 M Tris buffer containing 0.02% sodium azide, within which they were maintained until the time of analysis. The sections were immunostained according to standard protocols. Free-floating sections were incubated for 1–3 days at 4°C with primary antibody mixture diluted in TBS containing 0.1% Triton X-100 and 10% calf serum. The primary antibodies used are described in Table 1. Depending on the experiment, secondary antibodies included Cy3- or Cy2-conjugated anti-rabbit/mouse, Alexa488-conjugated anti-rabbit/mouse, Cy3- or Cy2-conjugated Streptavidin (Jackson ImmunoResearch, Suffolk, UK), biotinylated anti-rabbit/mouse (Vector Laboratories, Servion, Switzerland), all used at the dilution recommended by the suppliers.

### Stereotactic injections in mouse brains

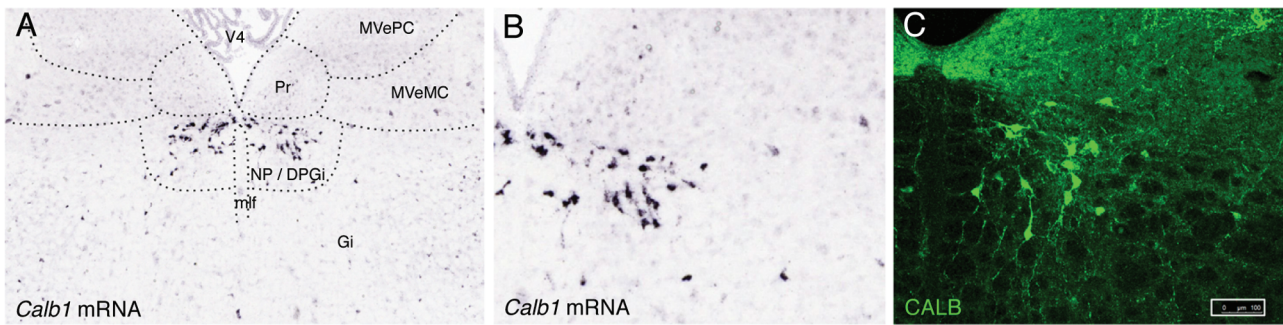
The experiment was conducted essentially as already described [9, 13]. Briefly, AAV2/1.CAG.Flex.Tomato.WPRE.bGH viral construct (Vector Core, University of Pennsylvania, USA) was stereotactically injected in the brain of either *Slc17a6::Cre* or *Slc32a1::Cre* mice. Injections were performed in the NP<sup>Calb</sup>, at the following Bregma coordinates: rostro-caudal: –6.36 mm, medio-lateral: –0.2 mm, and dorso-ventral: –4.35 mm. Two weeks after the stereotactic injections, the animals were anesthetized

**Table 1.** Description of Primary Antibodies Used for Immunohistochemistry

| Antibody to     | Host species | Antigen                          | Manufacturer                                | Catalog number | Dilution used |
|-----------------|--------------|----------------------------------|---|----------------|---------------|
| Calbindin D-28K | Mouse        | Whole chicken protein from gut   | Swant, Marly, Switzerland                   | CB300          | 1–2,000       |
| Calbindin D-28K | Rabbit       | Recombinant rat calbindin D-28K  | Swant, Marly, Switzerland                   | CB38           | 1–2,000       |
| Cart            | Rabbit       | Rat Cart aa 55–102               | Phoenix Pharmaceuticals, Karlsruhe, Germany | H-003-62       | 1–2,000       |
| Nesfatin        | Rabbit       | Rat Nesfatin aa 1–82             | Phoenix Pharmaceuticals, Karlsruhe, Germany | H-003-22       | 1–1,000       |
| c-fos           | Mouse        | Recombinant human c-fos aa 1–380 | Abcam, Cambridge, UK                        | ab208942       | 1–2,000       |
| ChAT            | Rabbit       | Pig ChAT aa 150–250              | Abcam, Cambridge, UK                        | ab178850       | 1–1,000       |

The antigen, host species, manufacturer, catalog number, and working dilution are given for all primary antibodies used in this study.



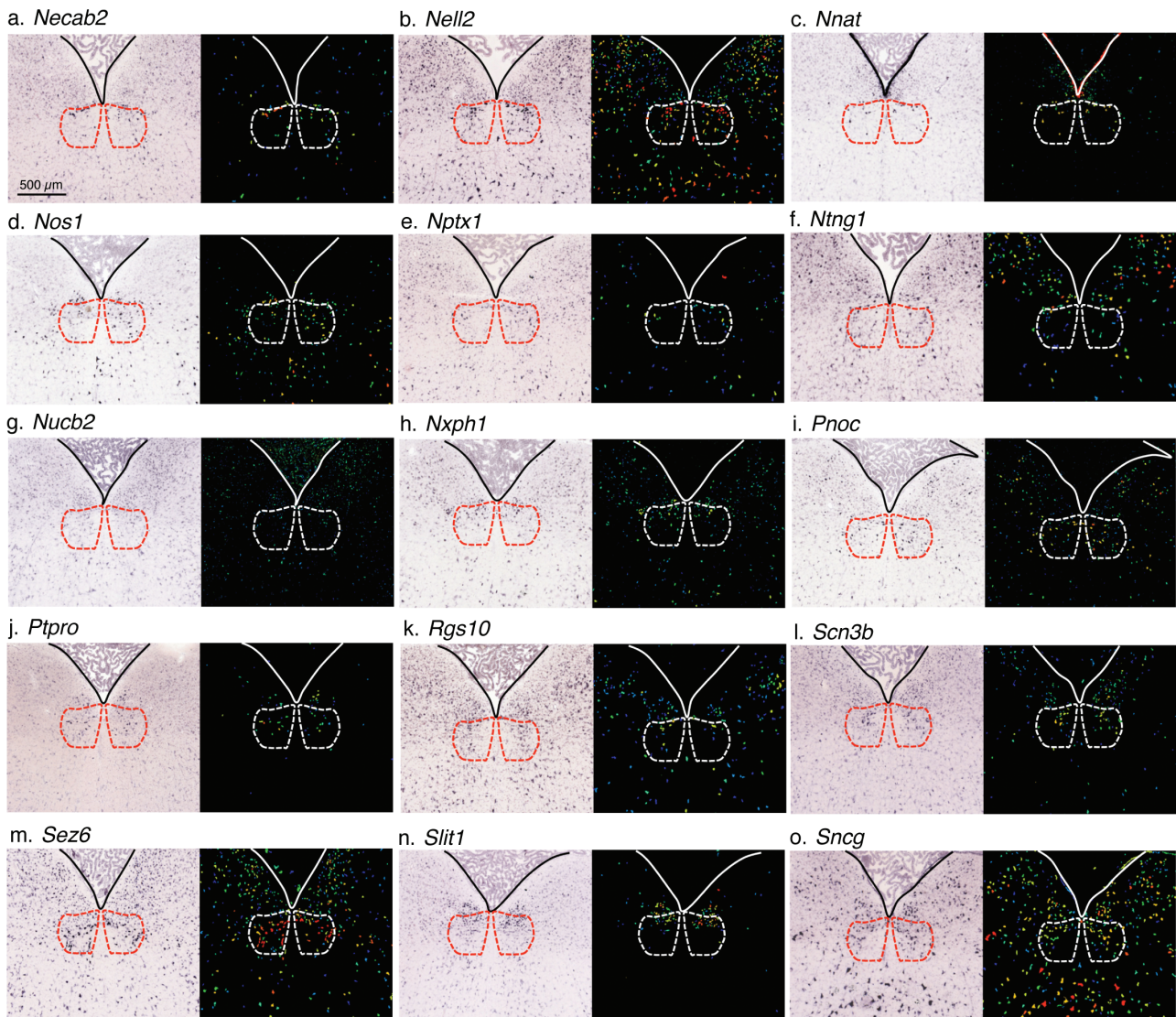


**Figure 1.** Expression of *Calb1* mRNA (A and B) and protein (C) in the NP<sup>Calb</sup>. ISH images (A and B) were taken from the Allen Brain Atlas (Image credit: Allen Institute; <http://mouse.brain-map.org/experiment/show/79556672>). (B) It is a higher magnification of the image shown in (A), focusing only on one hemisphere. (C) It is a confocal image of a mouse brain coronal section immunostained for Calb protein, showing immunoreactivity in neuronal cell bodies within the DPGi and in neurites in surrounding areas (Pr and Gi). DPGi, dorsal paragigantocellular nucleus; Gi, gigantocellular reticular nucleus; MVeMC, medial vestibular nucleus, magnocellular; MVePC, medial vestibular nucleus, parvocellular; NP, Nucleus papilio; Pr, prepositus nucleus; V4, fourth ventricle.



**Figure 2.** Genes showing expression in the area of the NP<sup>Calb</sup>—Part 1. The expression profile of the following genes is shown: (A) *Calb1*, (B) *Cacna1g*, (C) *Cartpt*, (D) *Cck*, (E) *Cnr1*, (F) *Crh*, (G) *Crhr1*, (H) *Ecel1*, (I) *Fxyd7*, (J) *Grik1*, (K) *Grm8*, (L) *Htr2c*, (M) *Kcng3*, (N) *Kcnip3*, and (O) *Ly6h*. For the complete name of the genes shown, as well as the function of the encoded proteins, see Tables 1–3. Red, respectively white, dashed lines delimit the NP<sup>Calb</sup> area. Black, respectively white, lines delimit the fourth ventricle. Data for the *Calb1* gene are given in panel (A) as reference. For each gene are shown both the ISH image (left; obtained with a digoxigenin-based method) and the corresponding colored image (right; ranging from blue to red, respectively from low to high expression) (Image credit: Allen Institute; *Calb1*: <http://mouse.brain-map.org/experiment/show/79556672>; *Cacna1g*: <http://mouse.brain-map.org/experiment/show/71587822>; *Cartpt*: <http://mouse.brain-map.org/experiment/show/72077479>; *Cck*: <http://mouse.brain-map.org/experiment/show/200>; *Cnr1*: <http://mouse.brain-map.org/experiment/show/79591675>; *Crh*: <http://mouse.brain-map.org/experiment/show/292>; *Crhr1*: <http://mouse.brain-map.org/experiment/show/297>; *Ecel1*: <http://mouse.brain-map.org/experiment/show/70231305>; *Fxyd7*: <http://mouse.brain-map.org/experiment/show/73592536>; *Grik1*: <http://mouse.brain-map.org/experiment/show/75749751>; *Grm8*: <http://mouse.brain-map.org/experiment/show/73771227>; *Htr2c*: <http://mouse.brain-map.org/experiment/show/73636098>; *Kcng3*: <http://mouse.brain-map.org/experiment/show/71717451>; *Kcnip3*: <http://mouse.brain-map.org/experiment/show/71587887>; *Ly6h*: <http://mouse.brain-map.org/experiment/show/71924388>).





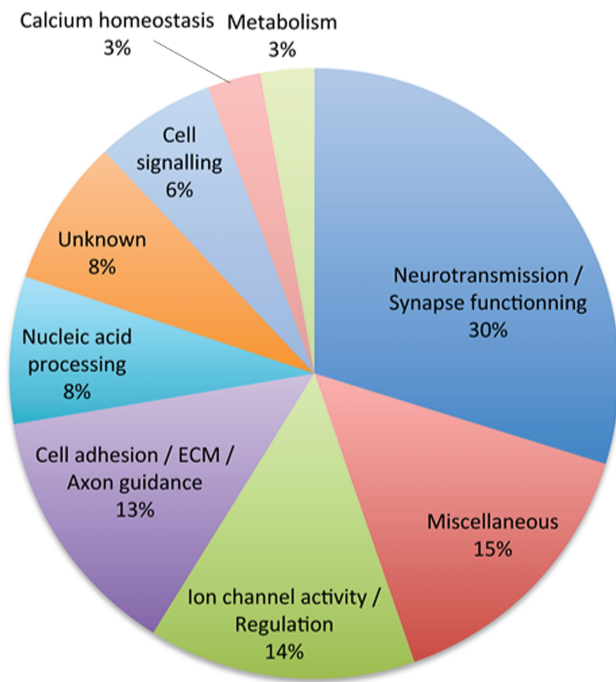
**Figure 3.** Genes showing expression in the area of the NP<sup>Calb</sup>—Part 2. The expression profile of the following genes is shown: (A) *Necab2*, (B) *Nell2*, (C) *Nnat*, (D) *Nos1*, (E) *Nptx1*, (F) *Ntnng1*, (G) *Nucb2*, (H) *Nxph1*, (I) *Pnoc*, (J) *Ptpro*, (K) *Rgs10*, (L) *Scn3b*, (M) *Sez6*, (N) *Slit1*, and (O) *Sncg*. See the legend of **Figure 2** for details (Image credit: Allen Institute; *Necab2*: <http://mouse.brain-map.org/experiment/show/73788010>; *Nell2*: <http://mouse.brain-map.org/experiment/show/72103854>; *Nnat*: <http://mouse.brain-map.org/experiment/show/77887874>; *Nos1*: <http://mouse.brain-map.org/experiment/show/75147762>; *Nptx1*: <http://mouse.brain-map.org/experiment/show/73520998>; *Ntnng1*: <http://mouse.brain-map.org/experiment/show/71924185>; *Nucb2*: <http://mouse.brain-map.org/experiment/show/75774683>; *Nxph1*: <http://mouse.brain-map.org/experiment/show/75084479>; *Pnoc*: <http://mouse.brain-map.org/experiment/show/75038402>; *Ptpro*: <http://mouse.brain-map.org/experiment/show/72340109>; *Rgs10*: <http://mouse.brain-map.org/experiment/show/74511849>; *Scn3b*: <http://mouse.brain-map.org/experiment/show/71064082>; *Sez6*: <http://mouse.brain-map.org/experiment/show/71063725>; *Slit1*: <http://mouse.brain-map.org/experiment/show/73788105>; *Sncg*: <http://mouse.brain-map.org/experiment/show/72081426>).

and perfused with 4% paraformaldehyde. The brains were excised and cryo-sectioned, and the specimens were analyzed immunohistochemically for *Cart* and *Tomato* expression. In these conditions, a specific and accurate *Tomato* expression is obtained in *Cre*-expressing neurons, as previously shown in *Calb1::Cre* mice [9].

### REM sleep deprivation and rebound assay

Mice were deprived of REM sleep by implementing a modified version of the flower-pot technique, which spared the animals of major stress [14, 15]. Three groups were established: in the first group (“REM sleep deprivation and rebound” = REMS-D + R), the animals ( $n = 5$ ) were maintained together for 72 h on six

small stone platforms ( $7 \times 4$  cm for rats,  $3 \times 3$  for mice), placed in a water tank. The surface of the platform was 1 cm above the water level. During this 72 h period, the animals had free access to food and water. Owing to the loss of the muscular tone that characterizes the onset of REM sleep, the animals fell into the water and were thereby deprived of REM sleep. After 72 h, the animals were transferred to a conventional cage in a quiet room and were permitted for 3 h to undergo REM sleep (=rebound). In the second group (“REM sleep deprivation” = REMS-D), the animals ( $n = 5$ ) were sacrificed immediately after the termination of the 72 h REM sleep deprivation period, without recovery. In the third group (“control” = C), the animals ( $n = 5$ ) were maintained in their cages under standard conditions for 72 h prior to sacrifice. The animals were anesthetized and perfused with



**Figure 4.** Proportions of the several categories of genes expressed in the NP<sup>Calb</sup> area. See also Tables 1–3 for details on gene name and function.

fixative as described above under the “Animals” section. The brains were then excised and cryo-sectioned. The sections were immunostained for Cart as well as for *c-fos*, a surrogate marker of neuronal activity [16]. The number of double-stained *c-fos*/Cart was determined on alternating 30  $\mu$ m coronal sections, on the following brain areas: dorsal motor nucleus of vagus (10N)/nucleus of the solitary tract (Sol), Nucleus prepositus (Pr), DPGi, gigantocellular reticular nucleus (Gi), medial longitudinal fasciculus (mlf), and lateral paragigantocellular nucleus (LPGi). Only cells with strong Cart immunoreactivity were taken into account. The percentage of Cart+Fos+ relative to the total Cart+ cells was statistically compared between the three different conditions using a one-tailed Student’s *t*-test, for each of the anatomical areas investigated. Data from  $n = 4$  animals in each of the conditions were used.

### Image analysis

The specimens were evaluated either in a Leica epifluorescence microscope, a Nikon Eclipse Ni fluorescence microscope, a Leica TCS SP5 confocal laser microscope, or a Hamamatsu Nanozoomer scanner. Postprocessing of the images and contrast adjustments therein were performed using the Adobe Photoshop and Nanozoomer slide-processing software.

### Informatics

A search of the adult mouse ABA (<https://portal.brain-map.org/>) was undertaken to identify genes that might be coexpressed with *Calb1* in the targeted NP<sup>Calb</sup>. First, we downloaded three-dimensional expression data measured by ISH from coronal sections of the adult mouse brain using ALLENMINER (v2.0) [10]. Since the *Calb1* gene expression in the target nucleus was restricted to a small region, we only used data from the coronal

ISH series (4,216 datasets available for 3,968 genes), which are more densely sampled than the sagittal ones. Next, we defined a region of interest (ROI), which bilaterally encompassed the *Calb1*-expressing cells and the local neighborhood as a cube with boundaries in ABA coordinate space of 11–12 mm rostral-caudal, 5.4–6.4 mm dorso-ventral, and 5–6.4 mm medio-lateral. Within this ROI, the patterns of gene expression were then ranked relative to that for *Calb1* (ABA image series 71717640, <https://mouse.brain-map.org/experiment/show/71717640> and 79556672, <https://mouse.brain-map.org/experiment/show/79556672>), as measured by the Pearson’s correlation of the expression energy reported in corresponding voxels in the region (ALLENMINER run mode—sim search). An additional search was performed using the AGEA facility available at the ABA, allowing users to screen for genes expressed in a selected ROI [12]. The area investigated, focused on the DPGi, corresponded to AGEA coordinates: 11.154/5.422/5.887.

## Results

### Data mining for genes expressed in the Nucleus papilio in the mouse brain

The NP<sup>Calb</sup> was previously defined as a symmetric cluster of *Calb*-expressing neurons, lodged in the DPGi, which in the mouse brain spans a distance of  $\sim 0.6$  mm, from Bregma levels  $-5.8$  to  $-6.4$  mm [9]. The ABA ISH data available for the *Calb1* gene show that *Calb1* mRNA expression fully recapitulates the protein expression (Figure 1) [9, 17].

For the ALLENMINER search, we drew only on data that were derived from coronally sectioned ISH series (4,216 datasets available for 3,968 genes—see the “Methods” section). Consequently, they relate to only a fraction of the murine genome. Additional data mining using AGEA yielded 107 pages each comprising 20 genes, with ISH data also corresponding to coronal sections. The genes that were selected by both automated searches were further screened by visual inspection of the imaged ISH sections that traversed the NP<sup>Calb</sup> area. After this second round of screening, we eliminated genes for which the signals were so low as to raise doubts respecting their specificity and those with ubiquitous expression in the *medulla oblongata*.

The screens identified 141 genes, which are restrictedly expressed in discrete regions of the *medulla oblongata*, including the area comprising the NP<sup>Calb</sup>. Figures 2 and 3 illustrate examples of genes that manifest such restricted expression patterns, with a focus on the area corresponding to the NP<sup>Calb</sup>. The genes fall into several main categories (Figure 4; Tables 2–4):

- “Neurotransmission”: Neuropeptides: *Adcyap1*; *Cartpt*, *Cck*, *Crh*, *Nucb2*, *Nxph1*, *Nxph4*, *Penk*, *Pnoc*. Neurotransmitter/neuropeptide receptor: *Chrm2*, *Chrm3*, *Cnr1*, *Crhr1*, *Gabra1*, *Gla1*, *Gla4*, *Grid1*, *Grik1*, *Grin3a*, *Grm8*, *Htr2c*. Neurotransmitter synthesis/transport/release/exocytosis: *Apba1*, *Baiap3*, *Gad1*, *Gad2*, *Nos1*, *Nos1ap*; *Slc17a6*, *Slc17a7*, *Slc32a1*, *Sv2b*, *Sv2c*, *Syt4*.
- “Synapse functioning”: *Cadps2*, *Nrn1*, *Ptpro*, *Sez6*, *Sez6l*, *Sv2b*, *Sv2c*.
- “Ion channel”: For calcium: *Cacna1g*, *Cacna1h*, *Cacna2d1*, *Cacna2d3*, *Cacng5*. For potassium: *Hcn1*, *Kcna1*, *Kcnb1*, *Kcnc2*, *Kcnc3*, *Kcng3*, *Kcng4*, *Kcnj3*. For sodium: *Asic2*, *Scn3b*, *Scn4b*. Ion channel regulation: *Fxyd6*, *Fxyd7*, *Kcnp1*, *Kcnp4*.
- “Cell adhesion/Extracellular matrix/Axon guidance”: *Ajap*, *Cdh8*, *Cdh13*, *Cd24a*, *Cntnap2*, *Col6a1*, *Col27a1*, *Crta1*, *Igsf21*, *Megf11*, *Nell2*, *Npnt*, *Ntng1*, *Sdk2*, *Sema3a*, *Sema6a*, *Slit1*, *Slit2*, *Spp1*.

Table 2. Genes Expressed in the DPGi/NP<sup>Calb</sup> Region—Part 1

| Gene            | Complete name  | Molecular activity                        | Biological process  |
|-----------------|--|---|---|
| <i>Adarb1</i>   | Adenosine deaminase, RNA-specific, B1  | Enzyme                                    | Nucleic acid processing   |
| <i>Adcyap1</i>  | Adenylate cyclase activating polypeptide 1 (=PACAP)                          | Neuropeptide                              | Neurotransmission/synapse functioning (neuropeptide) [18, 19]         |
| <i>Adk</i>      | Adenosine kinase   | Enzyme                                    | Metabolism (adenine) [20]   |
| <i>Ajap1</i>    | Adherens junction associated protein 1 (=Shrew1)                             |   | Cell adhesion/ECM/axon guidance                                       |
| <i>Apba1</i>    | Amyloid beta (A4) precursor protein binding, family A, member 1 (=X11/Mint1) | Adaptator protein                         | Neurotransmission/synapse functioning (neurotransmitter release)      |
| <i>Arl10</i>    | ADP-ribosylation factor-like 10  | GTPase activity                           | Multiple  |
| <i>Asic2</i>    | Acid-sensing (proton-gated) ion channel 2                                    | Sodium channel                            | Ion channel   |
| <i>Baiap3</i>   | BAI1-associated protein 3  |   | Neurotransmission/synapse functioning (SNARE-dependent exocytosis)    |
| <i>Btbd11</i>   | BTB (POZ) domain containing 11   |   |   |
| <i>Cacna1g</i>  | Calcium channel, voltage-dependent, T type, alpha 1G subunit (=Cav3.1)       | Calcium channel                           | Ion channel [21–23]   |
| <i>Cacna1h</i>  | Calcium channel, voltage-dependent, T type, alpha 1H subunit (=Cav3.2)       | Calcium channel                           | Ion channel [21–23]   |
| <i>Cacna2d1</i> | Calcium channel, voltage-dependent, alpha2/delta subunit 1 (=a2d1)           | Calcium channel                           | Ion channel   |
| <i>Cacna2d3</i> | Calcium channel, voltage-dependent, alpha2/delta subunit 3 (=a2d3)           | Calcium channel                           | Ion channel   |
| <i>Cacng5</i>   | Calcium channel, voltage-dependent, gamma subunit 5                          | Calcium channel                           | Ion channel   |
| <i>Cadps2</i>   | Ca <sup>2+</sup> -dependent activator protein for secretion 2                |   | Neurotransmission/synapse functioning (dendritic spine maintenance)   |
| <i>Calb1</i>    | Calbindin 1 (=Calbindin D28k)  | EF-hand Ca binding; calcium sensor/buffer | Calcium homeostasis [9]   |
| <i>Calb2</i>    | Calbindin 2 (=Calretinin)  | EF-hand Ca binding; calcium sensor/buffer | Calcium homeostasis   |
| <i>CamkV</i>    | CaM kinase-like vesicle-associated   | Enzyme                                    | Neurotransmission/synapse functioning                                 |
| <i>Cartpt</i>   | CART prepropeptide   | Neuropeptide                              | Neurotransmission/synapse functioning (neuropeptide) [24–26]          |
| <i>Cck</i>      | Cholecystokinin  | Neuropeptide                              | Neurotransmission/synapse functioning (neuropeptide) [27, 28]         |
| <i>Cdh8</i>     | Cadherin 8   | Protein binding                           | Cell adhesion/ECM/axon guidance                                       |
| <i>Cdh13</i>    | Cadherin 13 (=Tcad)  | Protein binding                           | Cell adhesion/ECM/axon guidance                                       |
| <i>Cd24a</i>    | CD24a antigen  | Protein binding                           | Cell adhesion/ECM/axon guidance                                       |
| <i>Chrm2</i>    | Cholinergic receptor, muscarinic 2, cardiac (=AChR-M2)                       | GPCR                                      | Neurotransmission/synapse functioning (cholinergic receptor) [29–31]  |
| <i>Chrm3</i>    | Cholinergic receptor, muscarinic 3, cardiac (=AChR-M3)                       | GPCR                                      | Neurotransmission/synapse functioning (cholinergic receptor) [29–31]  |
| <i>Cnr1</i>     | Cannabinoid receptor 1 (brain) (=CB1)  | GPCR                                      | Neurotransmission/synapse functioning (cannabinoid receptor) [32, 33] |
| <i>Cntnap2</i>  | Contactin associated protein-like 2 (=Gaspr2)                                | Protein binding                           | Cell adhesion/ECM/axon guidance [34]                                  |
| <i>Coch</i>     | Cochlin  | Protein binding                           | Immunity  |
| <i>Col6a1</i>   | Collagen, type VI, alpha 1   | ECM structural component                  | Cell adhesion/ECM/axon guidance                                       |
| <i>Col27a1</i>  | Procollagen, type XXVII, alpha 1   | ECM structural component                  | Cell adhesion/ECM/axon guidance                                       |
| <i>Cpne6</i>    | Copine VI  | Ca binding; Ca sensor                     | Neurotransmission/synapse functioning                                 |
| <i>Crh</i>      | Corticotropin releasing hormone  | Neuropeptide                              | Neurotransmission/synapse functioning (neuropeptide) [35]             |
| <i>Crhr1</i>    | Corticotropin releasing hormone receptor 1                                   | GPCR                                      | Neurotransmission/synapse functioning (neuropeptide receptor)         |
| <i>Crtac1</i>   | Cartilage acidic protein 1 (=Lotus)  | Ca binding; protein binding               | Cell adhesion/ECM/axon guidance                                       |
| <i>Ctxn1</i>    | Cortixin 1   |   |   |
| <i>Cux2</i>     | Cut-like homeobox 2  | Transcription factor                      | Nucleic acid processing   |
| <i>Cyp26b1</i>  | Cytochrome P450, family 26, subfamily b, polypeptide 1                       | Enzyme                                    | Metabolism  |
| <i>Deptor</i>   | DEP domain containing MTOR-interacting protein (=Depdc6)                     |   | Cell signaling  |
| <i>Dkk3</i>     | Dickkopf WNT signaling pathway inhibitor 3                                   | Secreted ligand                           | Cell signaling  |
| <i>Dpp10</i>    | Dipeptidylpeptidase 10   | Enzyme                                    | Proteolysis   |
| <i>Ecel1</i>    | Endothelin converting enzyme-like 1  | Enzyme                                    | Multiple  |
| <i>Esyt1</i>    | Extended synaptotagmin-like protein 1  | Ca/lipid/protein binding                  | Intracellular lipid dynamics  |
| <i>Fbxw7</i>    | F-box and WD-40 domain protein 7   | Protein binding                           | Cell signaling  |
| <i>Foxa1</i>    | Forkhead box A1  | Transcription factor                      | Nucleic acid processing   |
| <i>Foxp1</i>    | Forkhead box P1  | Transcription factor                      | Nucleic acid processing   |
| <i>Fxyd6</i>    | FXYD domain-containing ion transport regulator 6                             | Na-K ATPase regulator                     | Ion channel regulation  |
| <i>Fxyd7</i>    | FXYD domain-containing ion transport regulator 7                             | Na-K ATPase regulator                     | Ion channel regulation  |

A list of the genes that are restrictedly expressed in discrete regions of the murine *medulla oblongata*, including the region embracing the *Nucleus papilio*, as revealed by ALLENMINER and AGEA searches of the ISH images in the ABA. The abbreviated as well as the full name of each gene are given, together with their known or putative molecular activity and functions (in ontologic terms). In gray: genes that have been experimentally implicated in the regulation of the sleep/wake cycle, with references indicated.



Table 3. Genes Expressed in the DPGI/NP<sup>Calb</sup> Region—Part 2 (see the footnote of Table 2)

| Gene          | Complete name   | Molecular activity                         | Biological process  |
|---------------|---|--|---|
| <i>Gabra1</i> | Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 1                        | Transmitter gated ion channel activity     | Neurotransmission/synapse functioning (GABA receptor)               |
| <i>Gad1</i>   | Glutamic acid decarboxylase 1   | Enzyme                                     | Neurotransmission/synapse functioning (GABA synthesis)              |
| <i>Gad2</i>   | Glutamic acid decarboxylase 2   | Enzyme                                     | Neurotransmission/synapse functioning (GABA synthesis)              |
| <i>Glr1</i>   | Glycine receptor, alpha 1 subunit   | Transmitter gated ion channel activity     | Neurotransmission/synapse functioning (glycine receptor)            |
| <i>Glr4</i>   | Glycine receptor, alpha 4 subunit   | Transmitter gated ion channel activity     | Neurotransmission/synapse functioning (glycine receptor)            |
| <i>Gpr125</i> | G protein-coupled receptor 125 (=Adgra3)  | GPCR                                       | Cell signaling  |
| <i>Gpr137</i> | G protein-coupled receptor 137  | GPCR                                       | Cell signaling  |
| <i>Grid1</i>  | Glutamate receptor, ionotropic, delta 1   | Transmitter gated ion channel activity     | Neurotransmission/synapse functioning (glutamate receptor)          |
| <i>Grik1</i>  | Glutamate receptor, ionotropic, kainate 1   | Transmitter gated ion channel activity     | Neurotransmission/synapse functioning (kainate/glutamate receptor)  |
| <i>Grin3a</i> | Glutamate receptor ionotropic, NMDA3A   | Transmitter gated ion channel activity     | Neurotransmission/synapse functioning (NMDA/glutamate receptor)     |
| <i>Grm8</i>   | Glutamate receptor, metabotropic 8  | GPCR                                       | Neurotransmission/synapse functioning (glutamate receptor)          |
| <i>Grsf1</i>  | G-rich RNA sequence binding factor 1  | RNA binding                                | Nucleic acid processing   |
| <i>Gsta4</i>  | Glutathione S-transferase, alpha 4  | Enzyme                                     | Metabolism (glutathione metabolism)                                 |
| <i>Hap1</i>   | Huntingtin-associated protein 1   | Protein binding                            | Axonal transport  |
| <i>Hcn1</i>   | Hyperpolarization-activated, cyclic nucleotide-gated K <sup>+</sup> 1             | Potassium channel                          | Ion channel   |
| <i>Htr2c</i>  | 5-Hydroxytryptamine (serotonin) receptor 2C                                       | GPCR                                       | Neurotransmission/synapse functioning (serotonin receptor) [36]     |
| <i>Igsf21</i> | Immunoglobulin superfamily, member 21   | Protein binding                            | Cell adhesion/ECM/axon guidance                                     |
| <i>Itm2c</i>  | Integral membrane protein 2C  |  |   |
| <i>Kcna1</i>  | Potassium voltage-gated channel, shaker-related subfamily, member 1 (=Kv1.1)      | Potassium channel                          | Ion channel   |
| <i>Kcnab1</i> | Potassium voltage-gated channel, shaker-related subfamily, beta member 1 (=Kv1.3) | Potassium channel                          | Ion channel   |
| <i>Kcnc2</i>  | Potassium voltage gated channel, Shaw-related subfamily, member 2 (=Kv3.2)        | Potassium channel                          | Ion channel [37]  |
| <i>Kcnc3</i>  | Potassium voltage gated channel, Shaw-related subfamily, member 3 (=Kv3.3)        | Potassium channel                          | Ion channel [38]  |
| <i>Kcng3</i>  | Potassium voltage-gated channel, subfamily G, member 3 (=Kv6.3)                   | Potassium channel                          | Ion channel   |
| <i>Kcng4</i>  | Potassium voltage-gated channel, subfamily G, member 4 (=Kv6.4)                   | Potassium channel                          | Ion channel   |
| <i>Kcnj3</i>  | Potassium inwardly rectifying channel, subfamily J, member 3                      | Potassium channel                          | Ion channel   |
| <i>Kcnip1</i> | Kv channel-interacting protein 1 (=KCHIP1)  | K channel regulator (Ca binding)           | Ion channel regulation  |
| <i>Kcnip4</i> | Kv channel interacting protein 4 (=KCHIP4)  | K channel regulator (Ca binding)           | Ion channel regulation  |
| <i>Lrrn1</i>  | Leucine rich repeat protein 1, neuronal   |  |   |
| <i>Ly6h</i>   | Lymphocyte antigen 6 complex, locus H   | Prototoxin                                 | Neurotransmission/synapse functioning (modulation of AchR activity) |
| <i>Megf11</i> | Multiple EGF-like-domains 11  |  | Cell adhesion/ECM/axon guidance                                     |
| <i>Mesdc2</i> | Mesoderm development candidate 2  | Chaperone LDL                              | Cell signaling  |
| <i>Myo5b</i>  | Myosin VB   | Multiple                                   | Cytoskeleton dynamics   |
| <i>Ndst4</i>  | N-deacetylase/N-sulfotransferase (heparin glucosaminyl) 4                         | Enzyme                                     | Metabolism (glycosaminoglycan)                                      |
| <i>Necab2</i> | N-terminal EF-hand calcium binding protein 2                                      | EF-hand Ca binding                         |   |
| <i>Necab3</i> | N-terminal EF-hand calcium binding protein 3                                      | EF-hand Ca binding                         |   |
| <i>Nell2</i>  | NEL-like 2 (neural EGF like 2)  | Ca/protein/ECM binding                     | Cell adhesion/ECM/axon guidance                                     |
| <i>Nnat</i>   | Neuronatin  |  | Multiple  |
| <i>Nos1</i>   | Nitric oxide synthase 1, neuronal   | Enzyme                                     | Neurotransmission/synapse functioning (NO synthesis) [39–41]        |
| <i>Nos1ap</i> | Nitric oxide synthase 1 (neuronal) adaptor protein (=Capon)                       | Protein binding                            | Neurotransmission/synapse functioning (NO synthesis)                |
| <i>Npnt</i>   | Nephronectin  | Ca/protein/ECM binding                     | Cell adhesion/ECM/axon guidance                                     |
| <i>Nptx1</i>  | Neuronal pentraxin 1 (=NP1)   |  | Neurotransmission/synapse functioning                               |
| <i>Nrg1</i>   | Neuregulin 1  | Protein binding                            | Cell signaling  |
| <i>Nrn1</i>   | Neuritin 1  |  | Neurotransmission/synapse functioning                               |
| <i>Ntn1</i>   | Netrin G1   | Protein binding                            | Cell adhesion/ECM/axon guidance                                     |
| <i>Nucb2</i>  | Nucleobindin 2  | Ca <sup>2+</sup> /DNA binding/neuropeptide | Neurotransmission/synapse functioning (neuropeptide) [42, 43]       |
| <i>Nxph1</i>  | Neurexophilin 1   | Neurexin ligand                            | Neurotransmission/synapse functioning (neuropeptide-like)           |
| <i>Nxph4</i>  | Neurexophilin 4   | Neurexin ligand                            | Neurotransmission/synapse functioning (neuropeptide-like)           |

Table 4. Genes Expressed in the DPGi/NP<sup>Ca1b</sup> Region—Part 3 (see the footnote of Table 2)

| Gene            | Complete name  | Molecular activity                        | Biological process   |
|-----------------|--|---|--|
| <i>Pcp4</i>     | Purkinje cell protein 4  | Ca/protein binding                        | Multiple   |
| <i>Pcp4l1</i>   | Purkinje cell protein 4-like 1   | Ca/protein binding                        | Multiple   |
| <i>Penk</i>     | Preproenkephalin   | Neuropeptide                              | Neurotransmission/synapse functioning (neuropeptide)                   |
| <i>Pnoc</i>     | Prepronociceptin   | Neuropeptide                              | Neurotransmission/synapse functioning (neuropeptide)                   |
| <i>Psd</i>      | Pleckstrin and Sec7 domain containing (=EFA6)  | GEF activity                              | Axonal transport   |
| <i>Ptpro</i>    | Protein tyrosine phosphatase, receptor type, O   | Receptor/enzyme                           | Neurotransmission/synapse functioning (promotes synapse formation)     |
| <i>Pvalb</i>    | Parvalbumin  | EF-hand Ca binding; calcium sensor/buffer | Calcium homeostasis [44]   |
| <i>Rec8</i>     | REC8 meiotic recombination protein   | Chromatin binding                         | Nucleic acid processing  |
| <i>Rgs4</i>     | Regulator of G-protein signaling 4   | GTPase activator                          | Cell signaling   |
| <i>Rgs10</i>    | Regulator of G-protein signaling 10  | GTPase activator                          | Cell signaling   |
| <i>Scn3b</i>    | Sodium channel, voltage-gated, type III, beta  | Sodium channel                            | Ion channel  |
| <i>Scn4b</i>    | Sodium channel, type IV, beta  | Sodium channel                            | Ion channel  |
| <i>Scr1</i>     | Scratch family zinc finger 1   | Transcription repressor                   | Nucleic acid processing  |
| <i>Sdk2</i>     | Sidekick homolog 2 (chicken)   |   | Cell adhesion/ECM/axon guidance  |
| <i>Slc6a7</i>   | Solute carrier family 6 (neurotransmitter transporter, L-proline), member 7            | Proline transporter                       | Multiple   |
| <i>Slc8a1</i>   | Solute carrier family 8 (sodium/calcium exchanger), member 1 (=Ncx1)                   | Ca/Na antiporter                          | Calcium homeostasis  |
| <i>Slc17a6</i>  | Solute carrier family 6 (sodium-dependent inorganic phosphate cotransporter), member 6 | Vesicular neurotransmitter transporter    | Neurotransmission/synapse functioning (=VGlut2, glutamate transporter) |
| <i>Slc17a7</i>  | Solute carrier family 6 (sodium-dependent inorganic phosphate cotransporter), member 7 | Vesicular neurotransmitter transporter    | Neurotransmission/synapse functioning (=VGlut1, glutamate transporter) |
| <i>Slc32a1</i>  | Solute carrier family 32 (GABA vesicular transporter), member 1                        | Vesicular neurotransmitter transporter    | Neurotransmission/synapse functioning (=VGAT, GABA transporter)        |
| <i>Slc36a1</i>  | Solute carrier family 36 (proton/amino acid symporter), member 1                       | Transmembrane transporter                 | Multiple   |
| <i>Sema3a</i>   | Semaphorin 3A  | Protein/ECM binding                       | Cell adhesion/ECM/axon guidance  |
| <i>Sema6a</i>   | Semaphorin 6a  | Protein/ECM binding                       | Cell adhesion/ECM/axon guidance  |
| <i>Sez6</i>     | Seizure related gene 6 (=BSRP-C)   |   | Neurotransmission/synapse functioning (shaping dendritic arborization) |
| <i>Sez6l</i>    | Seizure related 6 homolog like   |   | Neurotransmission/synapse functioning (shaping dendritic arborization) |
| <i>Sh3bgrl2</i> | SH3 domain binding glutamic acid-rich protein like 2                                   |   |  |
| <i>Slit1</i>    | Slit guidance ligand 1   | Ca/protein/ECM binding                    | Cell adhesion/ECM/axon guidance  |
| <i>Slit2</i>    | Slit guidance ligand 2   | Ca/protein/ECM binding                    | Cell adhesion/ECM/axon guidance  |
| <i>Snca</i>     | Synuclein, alpha   | Protein binding                           | Multiple (involved in synucleinopathies including PD; RBD) [45, 46]    |
| <i>Sncg</i>     | Synuclein, gamma   | Protein binding                           | Multiple   |
| <i>Sphkap</i>   | SPHK1 interactor, AKAP domain containing (=SKIP)                                       | A kinase anchoring protein                |  |
| <i>Spp1</i>     | Secreted phosphoprotein 1 (=OPN)   | Cytokine/ECM binding                      | Cell adhesion/ECM/axon guidance  |
| <i>Steap2</i>   | Six transmembrane epithelial antigen of prostate 2                                     | Enzyme                                    | Multiple   |
| <i>Sv2b</i>     | Synaptic vesicle glycoprotein 2 b  | Transmembrane transporter                 | Neurotransmission/synapse functioning (vesicular transport/exocytosis) |
| <i>Sv2c</i>     | Synaptic vesicle glycoprotein 2c   | Transmembrane transporter                 | Neurotransmission/synapse functioning (vesicular transport/exocytosis) |
| <i>Syt4</i>     | Synaptotagmin IV   | Ca/protein/lipid binding                  | Neurotransmission/synapse functioning (vesicular transport/exocytosis) |
| <i>S100a10</i>  | S100 calcium-binding protein A10 (=calgizzarin)  | EF-hand Ca binding/protein binding        | Multiple   |
| <i>S100b</i>    | S100 protein, beta polypeptide, neural   | EF-hand Ca binding/protein binding        | Multiple (marker in sleep disturbance syndromes and PD)                |
| <i>Tesc</i>     | Tescalcin  | EF-hand Ca binding/protein binding        | Multiple   |
| <i>Tmem65</i>   | Transmembrane protein 65   |   |  |
| <i>Tpbp</i>     | Trophoblast glycoprotein   |   |  |
| <i>Usp11</i>    | Ubiquitin-specific peptidase 11  | Enzyme                                    | Proteolysis  |
| <i>Vat1l</i>    | Vesicle amine transport protein 1 homolog-like   |   |  |
| <i>Whrn</i>     | Whirlin  |   | Cytoskeleton dynamics  |
| <i>Zfp385b</i>  | Zinc finger protein 385B   | Transcription factor                      | Nucleic acid processing  |
| <i>Zfx4</i>     | Zinc finger homeodomain 4  | Transcription factor                      | Nucleic acid processing  |
| <i>Zfp365</i>   | Zinc finger protein 365  | Transcription factor                      | Nucleic acid processing  |
| <i>Zkscan16</i> | Zinc finger with KRAB and SCAN domains 16  | Transcription factor                      | Nucleic acid processing  |

- “Nucleic acid processing”: *Adarb1*, *Cux2*, *Foxa1*, *Foxp1*, *Grsf1*, *Rec8*, *Scrt1*, *Zfp365*, *Zfp385b*, *Zfhx4*, *Zkscan16*.

Some other less represented categories included metabolism, cell signaling, and calcium homeostasis.

### Identifying neurotransmitters and neuropeptides expressed in the Nucleus papilio

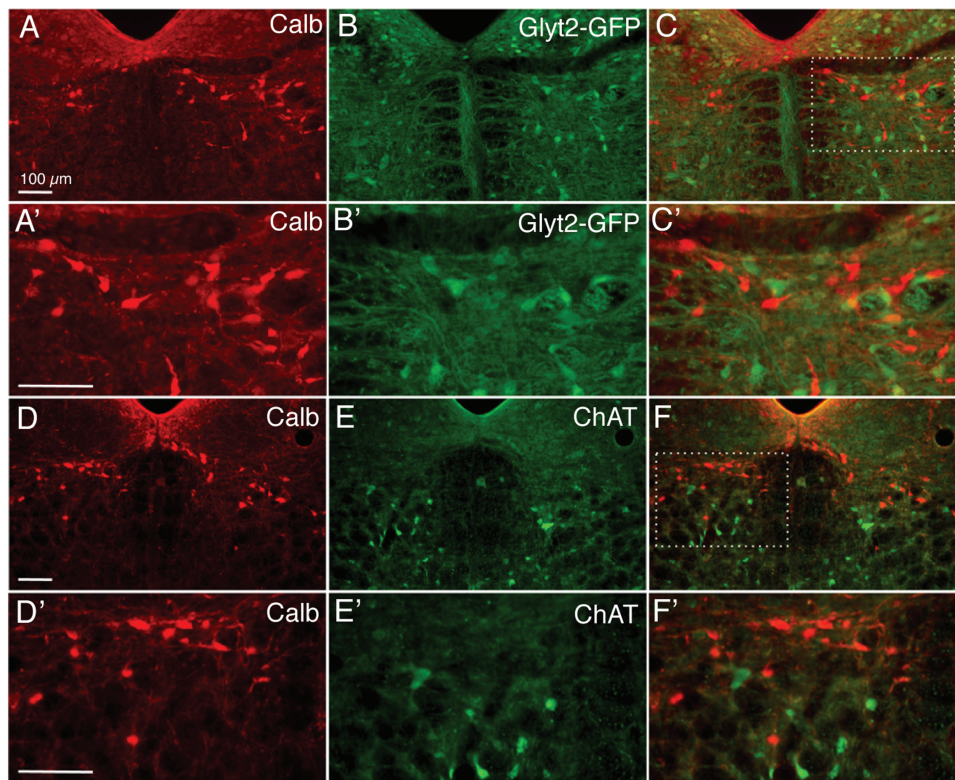
We have started previously to investigate the neurotransmitter status of NP<sup>Calb</sup> neurons, by showing that a significant proportion (33.8%) of Calb-positive neurons were glutamatergic and that none were GABAergic [9]. Analyzing the ABA ISH data on sagittal sections revealed that the *Slc6a5* gene, encoding the Glyt2 glycine vesicular transporter, and the *Chat* gene, encoding choline acetyltransferase, were also expressed in the region of the *medulla oblongata*. Immunostaining for Calb on coronal sections from GlyT2-GFP mouse brain revealed that NP<sup>Calb</sup> neurons were not glycinergic (Figure 5A–C). Similarly, double immunostaining for Calb and Chat revealed the absence of Chat expression in NP<sup>Calb</sup> neurons (Figure 5D–F).

Our search identified several genes encoding neuropeptides that are likely to be coexpressed with *Calb1*, namely, *Cartpt* (encoding Cart, cocaine-and-amphetamine-regulated transcript), *Cck* (encoding cholecystokinin), *Crh* (encoding corticotrophin-releasing hormone), *Nucb2* (encoding nesfatin 1), *Penk* (encoding preproenkephalin), and *Pnoc* (encoding prepronociceptin) (see Figures 2 and 3 for the ABA ISH data). For the immunohistochemical revelation of coexpression, coronal sections through murine brains were double-stained for Calb and two of these neuropeptides. Nesfatin immunoreactivity

was present in all Calb-immunoreactive neurons of the NP<sup>Calb</sup> (Figure 6A–C and A'–C'), whereas that for Cart (Figure 6D–F and D'–F') was apparent in some, but not all. In addition, some cells lying close to the Calb-immunoreactive neurons were positive for each of the tested peptides, but not for Calb. Noteworthy, similar observations were made using sections from rat brains (not shown). We estimated that  $27.2\% \pm 5.6\%$  of the DPGi Calb+ cells were Cart+ (counting performed on each second sections in  $n = 8$  mice) and that  $65.9\% \pm 13.7\%$  of the DPGi Cart+ cells were Calb+ (counting performed in  $n = 3$  mice).

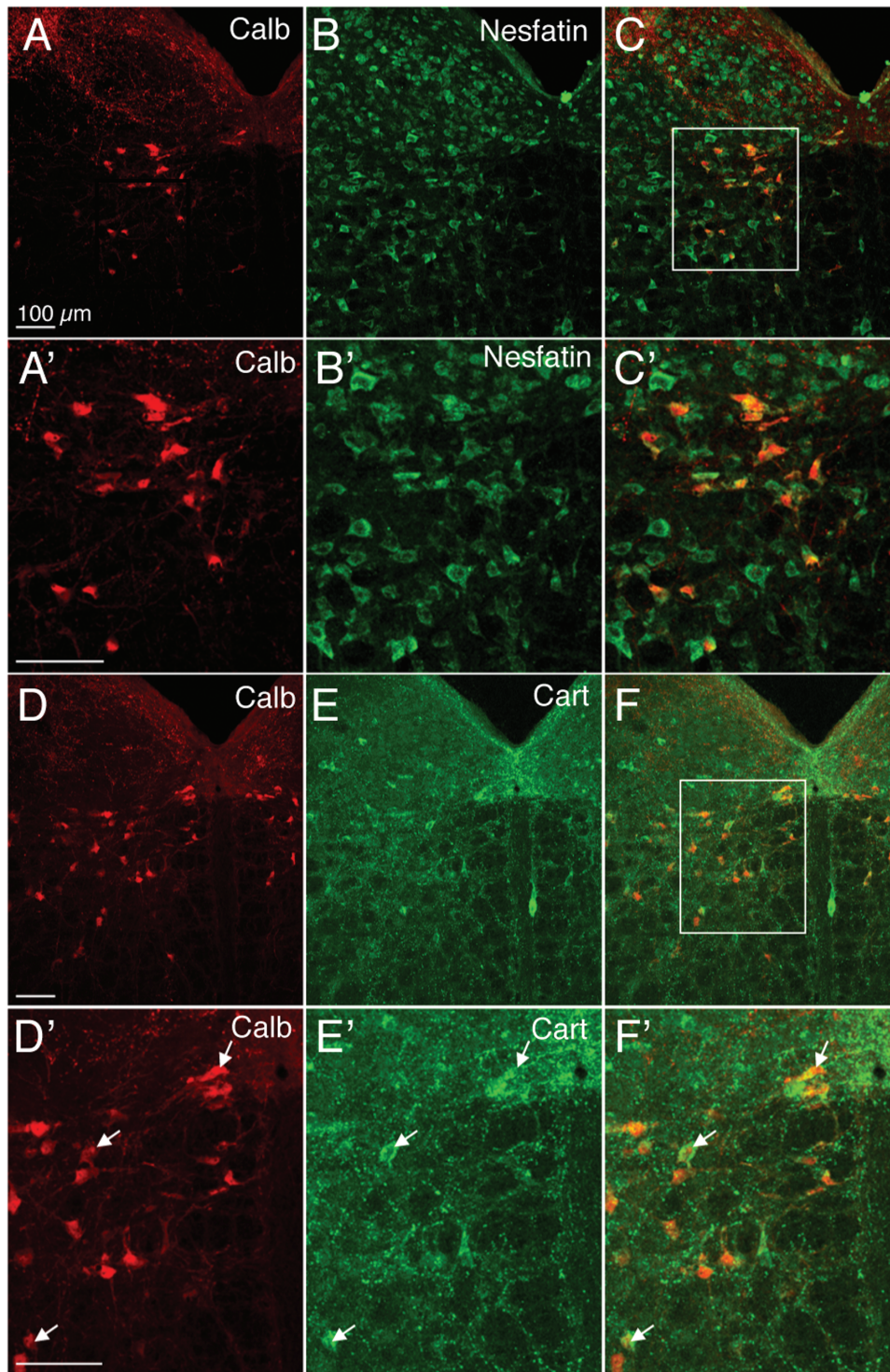
### Cart-expressing neurons within the DPGi are glutamatergic

With the aim of analyzing the neurotransmitter status of the Cart-expressing neurons in the NP<sup>Calb</sup>/DPGi area, glutamatergic, respectively GABAergic, neurons within the *medulla oblongata* were labeled by means of fluorescent adenovirus tracer injection in *Slc17a6::Cre* mouse brains (encoding the VGlut2 glutamate transporter) and *Slc32a1::Cre* (encoding the VGAT GABA transporter). In these conditions, glutamatergic, respectively GABAergic, cell bodies could be easily identified by their strong fluorescence. Co-staining with anti-Cart antibody revealed that all Cart-positive cells within the DPGi were VGlut2-positive and that none was VGat-positive, highlighting their glutamatergic nature (Figure 7). In addition, Cart-positive cells located in either the adjacent *Nucleus prepositus* or the gigantocellular reticular nucleus were also mostly glutamatergic, while only very few Cart-positive cells within the *Nucleus prepositus* appeared as GABAergic.



**Figure 5.** NP<sup>Calb</sup> neurons are neither glycinergic nor cholinergic. (A–C) Coronal sections from a GlyT2-GFP mouse brain, stained for Calb (red) and GFP (green). The dashed square in (C) marks the area shown at higher magnification in panels (A'–C'). (D–F) Coronal sections from a C57Bl6 mouse brain, stained for Calb (red) and ChAT (green). The dashed square in (F) marks the area shown at higher magnification in panels (D'–F'). Bars represent 100  $\mu\text{m}$ .





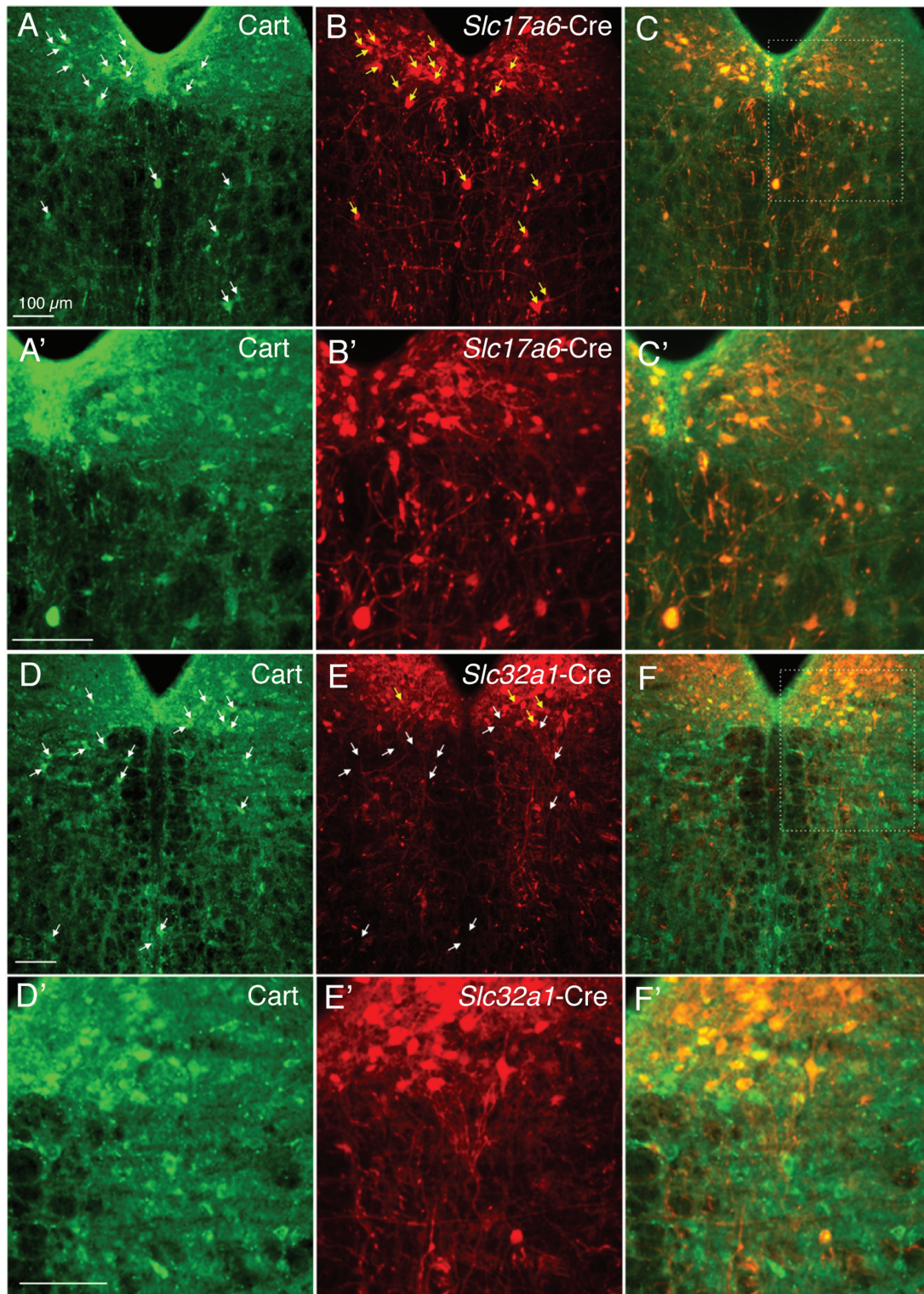
**Figure 6.** Cart and Nesfatin neuropeptide expression in NP<sup>Calb</sup> neurons. Coronal sections from a C57Bl6 mouse brain, stained for Calb and either Nesfatin (A–C) or Cart (D–F) in the NP<sup>Calb</sup>. All Calb-positive NP<sup>Calb</sup>-neurons coexpress Nesfatin, while coexpression with Cart is limited to few neurons (marked by arrows in D'–F'). Panels (A'–C') and (D'–F') are higher magnifications. All images were obtained with confocal microscope. Bars represent 100  $\mu$ m.

### Cart-expressing neurons within the DPGi are activated during REM sleep

In an experimental paradigm consisting of REM sleep rebound following a 72 h REM sleep deprivation, we could demonstrate that neurons of the DPGi [47–49], and particularly the NP<sup>Calb</sup> neurons [9], were activated during REM

sleep, as demonstrated by neuronal c-fos immunoreactivity. A similar test performed on mice revealed that Cart-positive neurons localized within the DPGi of the *medulla oblongata* were activated during REM sleep too. Only in the DPGi significant differences between the three different groups analyzed could be observed (REM sleep deprivation group; REM sleep deprivation + rebound group; Control group) (Figure 8).

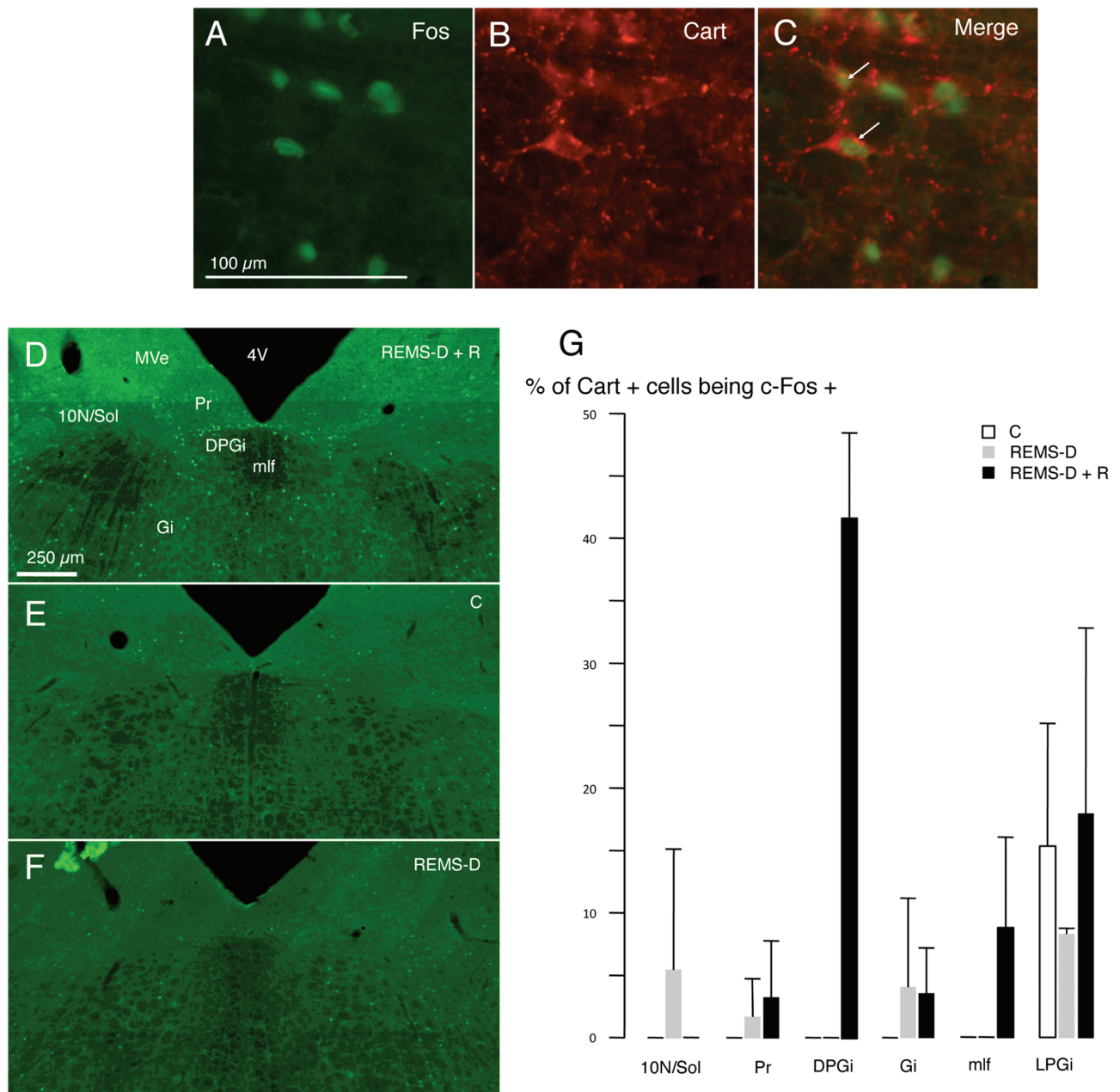




**Figure 7.** Cart-expressing neurons within the DPGi are glutamatergic. Immunostaining with Cart antibody reveals the glutamatergic nature of all Cart-expressing neurons in the DPGi. Shown are representative coronal sections through the *medulla oblongata* of a brain from (A–C) *Slc7a6::Cre* (VGlut2-Cre) and (D–F) *Slc32a1::Cre* (VGAT-Cre) mice, injected with Cre-dependent AAV-Tomato. In (C), respectively (F), a dashed square indicates the area presented at higher magnification in (A'–C'), respectively (D'–F'). White arrows in (A) and (D) point to Cart<sup>+</sup> cells. Within the DPGi and the Gi, all Cart<sup>+</sup> neurons are glutamatergic (yellow arrows in B), while none are GABAergic (white arrows in E). On the contrary, in the *prepositus nucleus* both glutamatergic and GABAergic Cart<sup>+</sup> neurons are visible (yellow arrows pointing to Pr neurons in B and E). Bars represent 100  $\mu$ m.

Indeed, in the REM sleep rebound group, we quantified  $41.6\% \pm 6.9\%$  of DPGi Cart-positive neurons displaying c-fos immunoreactivity and  $31\% \pm 6.5\%$  of DPGi c-fos<sup>+</sup> cells being Cart<sup>+</sup>. Analyzing the other areas (including the Gi, the LPGi,

the mlf, the 10N/Sol), no significant difference between the three groups was observed (Figure 8). This experiment suggests that Cart-expressing neurons in the DPGi are activated during the REM phase of sleep.



**Figure 8.** Analysis of c-fos immunoreactivity in Cart-expressing neurons in the *medulla oblongata* during REM sleep. (A–C) Representative coronal section from the brain of an animal of the group “REM sleep deprivation and rebound,” stained for Cart (red) and c-fos (green). The two arrows in panel C point to Cart+c-fos+ neurons. (D–F) Representative brain coronal sections from animals of each three groups used for the REM sleep deprivation and rebound assay (REMSD + R, REM sleep deprivation and rebound; REMSD, REM sleep deprivation; C, Control), immunostained for c-fos. (G) Percentage of c-fos immunoreactive cells within the Cart+ population, in different brain areas. Counting was performed on alternating coronal sections from three animals of each three groups (REMSD + R; REMSD; C). Statistical significance at  $p < 0.05$  between the different groups was achieved only in the DPGi; group REMSD-D+R versus group C:  $t = 10.78389$  and  $p = 0.000019$ ; group REMSD-D+R versus group REMSD-D:  $t = 10.78389$  and  $p = 0.000019$ . DPGi, dorsal paragigantocellular nucleus; Gi, gigantocellular reticular nucleus; LPGi, lateral paragigantocellular nucleus; mlf, medial longitudinal fasciculus; MVe, medial vestibular nucleus; Pr, prepositus nucleus; 4V, fourth ventricle; 10N, dorsal motor nucleus of vagus/Sol: nucleus of the solitary tract.

## Discussion

The *Nucleus papilio* is a recently described brainstem structure, characterized by Calb immunoreactivity, and by its involvement in triggering eye movement during the REM sleep period [9]. Through an extensive data mining of the ABA, we propose here a list of genes that are likely to be expressed in the NP<sup>Calb</sup>. Several of these genes encode proteins involved in the synthesis/transport of neurotransmitters, namely, *Slc17a7* and *Slc17a6* (encoding VGlut1- and VGlut2-glutamate transporter, respectively), *Gad1/2* (encoding glutamic

acid decarboxylase, which is responsible for the production of GABA), and *Slc32a1* (encoding a GABA transporter). By injecting Cre-dependent AAV-Tomato virus in *Slc17a6::Cre* and *Slc17a7::Cre* (both specific for glutamatergic neurons) and *Slc32a1::Cre* mice (specific for GABAergic neurons), we were able to show the absence of Calb immunoreactivity in the GABAergic neurons of the DPGi, whereas a significant proportion of the Calb-positive neurons composing the NP<sup>Calb</sup> were of glutamatergic nature [9]. Analyzing the list of potential genes expressed in the NP<sup>Calb</sup>, several glutamate receptors and a single GABA receptor were found (Tables 2–4).



Of special interest for us were the genes encoding neuropeptides. Nesfatin 1 is derived from a precursor, nucleobindin 2, which via posttranslational cleavage, yields either the neuropeptide nesfatin 1 or the DNA/Ca<sup>2+</sup>-binding proteins nesfatin 2 and 3. Nesfatin 1 has been identified as a satiety molecule in the hypothalamus [50]. Albeit so, its widespread extra-hypothalamic expression indicates that it might exert endocrine and autonomic effects on energy expenditure [51]. Cart peptides have been implicated in the regulation not only of food intake and body weight, but also of a variety of physiological processes, including drug reward/reinforcement and stress [52, 53], findings that are consistent with the complex pattern of Cart immunoreactivity in the rat brain [54]. Cart has been shown to coexist with Calb-D28k in granule cells of the dentate gyrus [55] and to be coexpressed with nesfatin in several hypothalamic and non-hypothalamic areas of the rat brain [51, 56, 57], including MCH-neurons. Data presented in the present study indicate that both peptides are coexpressed also in some Calb-immunoreactive neurons of the NP<sup>Calb</sup>. Several studies that have been recently conducted afford evidence for a role of nesfatin 1 in the regulation of REM sleep. Disruption of nesfatin signaling in the tuberal hypothalamic neurons, by the intra-cerebroventricular administration of either an antiserum against the neuropeptide or *Nucb2* antisense, has been shown to suppress REM sleep [24]. Similarly, deprivation of REM sleep led to a downregulation of nesfatin 1 expression, which was reverted during REM rebound [25]. And finally, the activity of these hypothalamic nesfatin-positive neurons (which are also MCH positive), as monitored by *c-fos* immunostaining, was correlated with REM sleep [24, 25]. Clear evidence in favor of an involvement of Cart in the regulation of the sleep/wake regulation has not been forthcoming [26]. Indeed, although an intra-cerebroventricular injection of the Cart55-102-peptide promotes the wake phase in rats [18], both Cart-positive and Cart-negative MCH-immunoreactive neurons in the hypothalamus are activated during REM sleep [19, 20]. Our finding that a significant proportion of Cart-expressing neurons in the DPGi were *c-fos*-positive following REM sleep rebound is thus particularly interesting and suggests a possible involvement of Cart in regulating some aspects of REM sleep.

The Calb-expressing neurons forming the NP<sup>Calb</sup> appear to form a heterogeneous population, involved partly in controlling eye movement during REM sleep [9], with a substantial number being excitatory glutamatergic neurons, and with the neuropeptides Cart and nociceptin being expressed only in a subset of these neurons while nesfatin being present in all of them (this study). In addition, the surrounding DPGi also contains another pool of inhibitory GABAergic neurons involved in the initiation of REM sleep, as well as glycinergic and cholinergic neurons [4, 7, 8]. Deciphering the specific neuronal connections made by these particular neuronal populations will be challenging toward a better understanding of the functions of this nucleus in regulating various aspects of REM sleep. Indeed, apart from their connections to the three eye motor nuclei, we observed strong efferent connections of the NP<sup>Calb</sup> neurons to several of the brain areas involved in the initiation and the maintenance of REM sleep (including the subcoeruleus nucleus and the pontine reticular nuclei) [9], suggesting additional roles for the NP<sup>Calb</sup> in regulating some aspects of REM sleep.

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## Disclosure Statement

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