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## How does apelin affect LH levels? An investigation at the level of GnRH and KNDy neurons

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Short title : Apelin and central control of reproduction

**Conflict of interest:** none

Keywords : adipokines, kisspeptin, dynorphin, neurokinin B, hypothalamus, reproduction, rodents.

## Highlights

- Apelin terminals are present both on KNDy and GnRH neurons in male rats and mice
- Apelin chronic infusion does not decrease LH blood levels in male mice
- Apelin chronic infusion does not affect gene expression in KNDy neurons
- Apelin acute treatment modifies electrical activity in 18% of GnRH neurons

## Abstract

Hypothalamic control of reproduction relies on GnRH and kisspeptin (KP) secretions. KP neurons are sensitive to sex steroids and metabolic status and their distribution overlaps with neurons producing apelin, a metabolic hormone known to decrease LH secretion in rats. Here, we observed neuroanatomical contacts between apelin fibers and both KP and GnRH neurons in the hypothalamus of male rodents. Intracerebroventricular apelin infusion for 2 weeks in male mice did not decrease LH levels nor did it affect gene expression for KP, neurokinin B and dynorphin. Finally, increasing apelin concentrations did not modulate  $Ca^{2+}$  levels of cultured GnRH neurons, while 10 $\mu$ M apelin infusion on forskolin pretreated GnRH neurons reevoked a rhythmic activity in 18% of GnRH neurons. These results suggest that acute apelin effect on LH secretion does not involve modulation of gene expression in KP neurons but may affect the secretory activity of GnRH neurons.

## 1. Introduction

### *Apelin system and its central functions*

Deorphanization study of the APJ receptor led to the discovery of apelin (Tatemoto et al., 1998), a polypeptide originating from a 77-amino acids polypeptide and cleaved into several bioactive forms ranging from 12 to 36 amino acids (Kawamata et al., 2001). APJ receptors belong to the G-Protein Coupled Receptor (GPCR) family and were reported to activate both  $G_{\alpha i}$  and  $G_{\alpha q}$  pathways (Chapman et al., 2014). Apelin is produced both peripherally (adipose tissue, intestine...) and centrally within the central nervous system (for review see: (Knauf et al., 2013)). The highest density of APJ receptor transcripts are found in the hypothalamus and most specifically in the paraventricular, supraoptic and arcuate (ARC) nuclei (De Mota et al., 2000, Pope et al., 2012). In rats, the highest density of apelin producing neurons was found in the rostro-caudal extent of the ARC (Reaux et al., 2002). Apelin belongs to the family of adipokines and exerts, similarly to the others family members, a prominent

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role in metabolic regulation. In the hypothalamus, the effects of apelin on glucose homeostasis are dose-dependent and vary depending on the nutritional status in mice (Duparc et al., 2011). In addition, hypothalamic apelin levels are significantly increased in obese/diabetic mice (Reaux-Le Goazigo et al., 2011) and participate to the establishment of a hyperglycemic state characteristic of a type 2 diabetes phenotype (Drougard et al., 2014). In fact, a physiological dose of apelin (i.e. low dose of apelin) had beneficial effects on glucose homeostasis while a high dose of centrally-infused apelin (similar to the level measured in the hypothalamus of diabetic mice) was suggested to be involved in the transition from normal to diabetic state (Duparc et al., 2011). In addition, apelin was also suggested to be involved in the central regulation of reproduction as an acute intracerebroventricular infusion of apelin 13 to male rats induced a rapid decrease of LH levels (Sandal et al., 2015, Taheri et al., 2002) and was associated with a decrease of the number of Leydig cells in testes of treated rats (Sandal et al., 2015).

### *Central control of reproduction*

LH secretion is under the direct control of GnRH release from neurons submitted to the stimulatory or inhibitory influence of other neuronal populations relaying negative and/or positive sex steroid feedbacks. Among those, the supremacy of kisspeptin (KP) neurons in the central regulation of reproduction has been thoroughly demonstrated. Indeed, absence of KP or of its receptor KISS1R is associated with hypogonadotropic hypogonadism (de Roux et al., 2003, Seminara et al., 2003) and results in infertility. Kisspeptin neurons are observed in two diencephalic areas in rodents namely in the ARC and more rostrally in the antero ventral periventricular area (AVPV). This latter area is sexually dimorphic with females displaying significantly more KP expressing neurons than males (Clarkson et al., 2006). KP neurons are the target of sex steroids and depending on their distribution relay either positive (AVPV) or negative (ARC) feedback to GnRH neurons (for a review see (Goodman et al., 2012)). KP neurons of the ARC coexpress dynorphin (Dyn) and neurokinin B (NKB) in several species including sheep (Goodman et al., 2007), rats (True et al., 2011) and mouse (Navarro et al., 2009) and have therefore been dubbed KNDy neurons. Of note, KP neurons are the recipient of metabolic regulation that affects peptide release. For example, a short period of fasting in rodents resulted in a decrease expression of genes coding for KP and its receptor (Luque et al., 2007) and obese mice also presented a decreased of both KP expression and immunoreactivity related to controls (Quennell et al., 2011). These data showing reduced levels of KP synthesis under opposite metabolic imbalance suggest that KP neurons may represent metabolic sensors allowing the occurrence of successful reproductive function under well balanced metabolic diet relayed by several hormonal and peptidergic signals.



## *Potential involvement of apelin on central control of reproduction*

The effect of apelin on GnRH neuron activity has not been documented yet but the overlap between apelin and KP neuronal populations at the level of the medio basal hypothalamus suggests that apelin may affect KP neuron activity and hence GnRH secretion leading to reduced LH release. Indeed KP and proopiomelanocortin (POMC) neurons originate from the same cell lineage (Sanz et al., 2015) and the electrical activity of approximately 50% of proopiomelanocortin (POMC) neurons is increased by apelin 13 (Lee et al., 2015). Taken together, these data reinforce the hypothesis that apelin may also target KP neurons. However, a direct effect of apelin on GnRH neuron activity cannot be excluded.

To unravel how apelin affects the central control of reproduction, the objectives of this study were therefore to assess in male rodent brains whether 1) neuroanatomical relationships exist between GnRH or KNDy and apelin neurons, 2) a chronic intracerebroventricular infusion of apelin 13 for two weeks affects LH levels and *Kiss*, *Tac2* (the gene coding for NKB) and *Dyn* gene expressions at the hypothalamic level and 3) apelin directly affects GnRH neurons by measuring calcium activity on olfactory placode cultured explants.

## **2. Material and Methods**

### *2.1 Experiment1: Double immunofluorescence to detect KP or GnRH neurons and apelin fibers.*

To check for the presence of apelin terminals on GnRH or KP neurons, supernumerary coronal brain sections from two male rats stored at -20°C from a previous study (Dufourny et al., 2018) and from 8-weeks old C57 BL/6J male mice (n=5; experiments performed under Ethics committee approval number APAFIS 2019102914184478) were used in this work. Briefly, rats (40µg/animal) and mice (30µg/animal) were intracerebroventricularly injected with colchicine 24-27h before animal euthanasia. Brains were fixed by intraaortic perfusion of heparinised 0.9% saline (50 mL for rats/20 mL for mice) followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH=7.4 (300 mL for rats/50mL for mice). Following dissection, brains were soaked in sucrose for 24h and subsequently frozen before being cut using a cryostat to obtain coronal 14µm thick sections. Sections were submitted to a double immunocytochemistry to detect either GnRH and apelin at the level of the organum vasculosum of the lamina terminals (OVLT) or KP and apelin at the level of the ARC. Sections were washed (3 x 5 min; room temperature) in 0.01 M phosphate-buffered saline (PBS; pH = 7.4) and incubated for 48 h at 4°C in humid atmosphere in PBS containing 0.3% Triton X100 (Sigma), 5% normal donkey serum (Sigma) and a cocktail of primary antibodies: a rabbit polyclonal anti-apelin antibody (orb247041, Biorbyt,

1 Cambridge, UK; 1:1000) and a sheep anti-rat KP (AC067; a gift from Dr A. Caraty, INRA, Nouzilly, France;  
2 1:1000; (Franceschini et al., 2013)) for rat sections or a sheep anti-mouse KP (AC024; Dr. Caraty; 1:5000  
3 (Porteous et al., 2011)) for mice sections. Regarding GnRH/apelin double staining, rats sections were  
4 incubated with rabbit anti-apelin antibody and a mouse anti-GnRH antibody (#19301, QED Bioscience,  
5 San Diego, CA, USA; 1:5000 (Dufourny et al., 2005)) and mice sections were incubated with a sheep  
6 anti-GnRH antibody (AC517, gift from Dr. A. Caraty; 1:5000 (Geller et al., 2017)) in conjunction with  
7 the rabbit anti-apelin antibody. Before and after each incubation step, sections were washed for 3 x 5  
8 min in PBS at room temperature. Sections were incubated for 90 min at room temperature with Texas  
9 red donkey anti-sheep IgG (1:500, Jackson immunoresearch, West Grove, PA, USA) for KP detection in  
10 both species or with Texas red donkey anti-mouse IgG (1:500, Jackson) for GnRH detection in rats, then  
11 in biotinylated donkey anti-rabbit IgG (1:500, Jackson) followed by FITC-linked streptavidin (1:500;  
12 Vector laboratories, Burlingame, CA, USA) for 1 h at room temperature to detect apelin. Following this  
13 last incubation step, sections were thoroughly washed in PBS, covered with Vectashield (Vector) and  
14 coverslips were applied. Omission of the primary antibodies or their replacement with normal serum  
15 from the same species, or the use of secondary antibodies raised against inappropriate species  
16 resulted in a total absence of immunostaining.

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29 Sections were then examined with a confocal microscope (Zeiss LSM 700) equipped with 488 nm and  
30 555 nm diode laser lines. KP neurons were randomly chosen within the ARC under the inverted  
31 fluorescence microscope linked to the confocal system and presence of apelin fibres in close proximity  
32 with KP neurons was observed with the confocal microscope using sequential mode, using dichroic  
33 mirrors to avoid altering fluorescence intensity and any “bleed-through”. Similarly, GnRH neurons from  
34 the OVLT were randomly chosen and observed using the same mode. Twenty-four-bit confocal images  
35 were acquired with a frame size of 1,024 x 1,024 pixels. Quantitative analysis was performed on mice  
36 sections and 9-12 GnRH neurons and 15-18 KP neurons were randomly chosen for each animal. All  
37 images were then captured as 7-12  $\mu\text{m}$  thick z-stacks with a 1  $\mu\text{m}$  gap between images in sequential  
38 mode, using dichroic mirrors to avoid altering fluorescence intensity and any “bleed-through”. The  
39 number of apelin appositions for each neuron was then determined. Percentage of GnRH neurons with  
40 apelin terminals and mean number of terminals per GnRH neuron were then calculated. Similarly,  
41 percentage of KP neurons with apelin terminals and mean number of terminals per KP neuron were  
42 then determined.

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56 *2.2 Experiment 2: Influence of apelin chronic infusion on LH levels and KP, Tac2 and Dyn gene*  
57 *expressions in male mice*  
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### 2.2.1 Animals and treatment

All animal procedures were performed following Regional Ethics Committee's approval.

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the local Animal Care and Use Committee (under ethical protocol n°2016031610579875 approved in 2016).

Sixteen 9-week-old C57BL/6 male mice (Charles River laboratories, L'Arbresle, France) were housed conventionally at constant temperature and humidity with a 12h:12h light/dark cycle. Animals had free access to food and water and were divided into two experimental groups: control or treated groups on Day (D) 0. Animals were weighed and glycemia was measured with a gluco-meter (Accu-Chek Active, Roche, Meylan, France) at D0, D7 and D14.

An indwelling intracerebroventricular (i.c.v.) catheter (Alzet Brain Perfusion Kit 3, 1-3 mm, Charles River, Wilmington, PA, USA; -1mm lateral, -0.2mm anteroposterior from the bregma and 1.7mm below the skull surface) was implanted in mice anesthetized with isoflurane (Abbott, Rungis, France). Then, the i.c.v. catheter was directly connected to a subcutaneous osmotic mini-pump (osmotic Alzet pump 2004 0,25MICL/H-28J; Charles River). The osmotic pump delivered either artificial cerebrospinal fluid or apelin-13 (20nM; Sigma, St Louis, MO, USA) over 2 weeks, at a rate of 0.25 µl/h. The mice were housed individually during the 2 weeks. The dose of apelin was chosen from previous experiments as it was known not to affect glycaemia in fed mice (Duparc et al., 2011).

### 2.2.2 Sample collections

Blood was collected from the caudal vein using capillaries to assay LH levels on three occurrences: before the start of apelin infusion at D0, and after the start of apelin infusion at D7 and D14.

Following animal's euthanasia, the brain was quickly dissected out and a hypothalamic block encompassing the ARC was delineated and quickly frozen in liquid nitrogen and stored at -80°C for further RT-qPCR analyses.

Testes were also removed and weighed.

### 2.2.3 LH assay

LH was assayed using "LH detect® for rodents" kit (Repropharm Vet, Nouzilly, France) consisting in an ELISA assay. The sensitivity of the assay was 0.01 ng/mL, and the intra- and interassay CVs were 4% and 8%, respectively. (Chung et al., 2011).

### 2.2.4 RNA extraction, standard RT-PCR and RT-qPCR analysis

RNA was extracted from each hypothalamic block using TriReagent (Sigma). Concentration and purity of individual samples were determined with Nanodrop 2000 (ThermoScientific, Waltham, MA, USA) and integrity was checked by standard agarose gel electrophoresis. For both standard RT-PCR and RT-qPCR, cDNA was synthesised using Omniscript RT kit (Qiagen, Hilden, Germany) and Oligo-dT primers (Eurofins, Luxembourg). For RT-qPCR, the optimal cDNA dilution and calibration curves were established using cDNA synthesized from an equimolar mix of all individual RNA. As a negative control, the same mix with water instead of RT was prepared. Quantitative PCR was performed using CFX-96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and Sso Adv Universal SYBR Green Supermix (Bio-Rad). All samples were assessed in triplicate and Gapdh was used as a housekeeping gene to normalize expression of candidate genes; Gapdh expression did not display any statistical differences between groups. The four primer pairs had efficiencies between 95 and 100%. The quantification of mRNA level was obtained by the 2- $\Delta$ CT method and data are presented as fold-change compared to the control condition. Sequences of the primers used for cDNA amplification in the quantitative RT-PCR experiments are detailed in Table 1.

**Table 1: Primer sequences**

Gene-Primer	NCBI reference	Oligo Sequence (5'3')	Reference
mGAPDH Fwd	XM_011241214.1	ACCACAGTCCATGCCATCAC	(Xu et al., 2017)
mGADPH Rev		TCCACCACCCTGTTG CTGTA	
mKP Fwd	XM_006497292.2	AGCTGCTGCTTCTCCTCTGT	(Cravo et al., 2011)
mKP Rev		GATTCCTTTTCCCAGGCATT	
mNKB Fwd	NM_001199971.1	TTCCACAGAAACGTGACATGC	(Popa et al., 2013)
mNKB Rev		GGGGGTGTTCTCTTCAACCAC	
mPDyn Fwd	NM_018863	GAGGTTGCTTTGGAAGAAGGC	(Popa et al., 2013)
mPDyn Rev		TTTCCTCTGGGACGCTGGTAA	

### 2.2.5 Analysis of results

Out of the 16 mice, 3 lost their Alzet pump before the end of the experiment and were not considered in this study.

Therefore, results obtained in 6 apelin-treated mice were compared to data from 7 control mice related to their body weight, body weight gain, fed glycemia, and testis weight using unpaired t test (GraphPad Prism). P<0.05 was considered significant.

1 Regarding LH secretion, raw data obtained at D0, D7 and D14 (LH levels in ng/mL) were compared  
2 within each group using a repeated measures two-way ANOVA followed by Tukey post-hoc tests  
3 adjusted by Bonferroni method using time and treatment as variables after data passed normality  
4 tests, and were checked for homogeneity and equal variance (GraphPad Prism).  
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7 RT-qPCR data were analyzed using GraphPad Prism and are reported as mean  $\pm$  sem. RT-qPCR data  
8 were analysed by Mann-Whitney non-parametric tests using treatment as variable.  $P < 0.05$  was  
9 considered significant.  
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### 15 *2.3 Experiment 3: Calcium activity on cultured olfactory placodes following apelin exposure*

#### 16 *2.3.1 Culture of olfactory placode explants*

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18 Mice were maintained under a 12:12 light/dark cycle. To obtain dated pregnancies, one male  
19 was introduced in a cage with two females (at pro-estrus or estrus stage) at 17:30 and was removed  
20 at 7:30 the following day. Vaginal plugs were detected the following morning. Olfactory explant  
21 dissection and cultures were made according to the procedure described by (Fueshko et al., 1994).  
22 Briefly, embryonic day 11.5 (E11.5) mouse embryos were obtained from pregnant Swiss mice killed by  
23 cervical dislocation. Embryos were placed in ice cold Gey's balanced salt solution (Eurobio, les Ullis,  
24 France) enriched with glucose. Olfactory pits were dissected out and put onto glass coverslips coated  
25 with chicken plasma / thrombin (Sigma). Cultures were maintained in serum free medium (SFM)  
26 (Fueshko and Wray, 1994). On culture day 3, fresh SFM containing uridine (5mg / l) and 5'-fluoro-2-  
27 deoxyuridine (2mg/ml) (Sigma) was added to inhibit non-neuronal cells' proliferation. On culture day  
28 6 and twice a week afterwards, the medium was changed.  
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#### 41 *2.3.2 Calcium imaging*

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43 Intracellular calcium ( $[Ca^{2+}]_i$ ) imaging was performed on nasal explant cultures from E11.5  
44 embryos aged between 7 and 14 days in vitro (DIV) (N = 9 embryos, n = 264 neurons). Fluo-4 AM  
45 (Molecular probes, ThermoFisher, France) was used at 1  $\mu$ M final concentration in SFM 80%  
46 dimethylsulfoxide 20% pluronic F-127 solution (Molecular Probes) 1:1000. Nasal explant cultures were  
47 placed in a perfusion chamber (Warner Instruments, Hamden, USA) and incubated in 1 $\mu$ M Fluo-A AM  
48 – SFM solution for 30 min at room temperature in total darkness. The chamber was then washed with  
49 fresh SFM maintained at 37°C under 95% air, 5% CO<sub>2</sub> perfusion at 50  $\mu$ l / min for 10 min (peristaltic  
50 pump Ismatec, Wertheim, Germany) to allow the culture to equilibrate before imaging. Calcium probe  
51 fluorescence was visualized using an inverted microscope (DM-IRB Leica Microsystem, GmbH,  
52 Germany) through a x10 fluorescence objective. One second time-lapse images were acquired by a  
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1 cooled intensified charge-coupled device camera (CoolSNAP HQ2, Roper Instruments Photometrics,  
2 USA) piloted by the Metafluor software (Molecular Devices, USA) controlling the shutter and  
3 acquisition parameters. Excitation wavelength was provided by a xenon lamp through a GFP excitation  
4 filter (XF1042 485DF15 Omega, USA). Fluorescence emission went through a dichroic filter  
5 (XF2050[385-485-560] TBDR Omega, USA) and an emission filter (XF3003 520DF40 Omega, USA)  
6 before reaching the camera. We used a 100 ms acquisition duration time.  
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10 [Ca<sup>2+</sup>]<sub>i</sub> fluctuations were analyzed with Metafluor and FIJI softwares. Each neuron was  
11 individually identified on the basis of its morphology (round soma with two neurites) as a circular  
12 region of interest and/or on the presence of regular [Ca<sup>2+</sup>]<sub>i</sub> transients lasting 2-10 sec. Fluo-4 mean  
13 fluorescence intensity for each region of interest was calculated and plotted against time using  
14 Microsoft Excel (Microsoft Corporation) and R Studio (<http://www.R-project.org>). We defined a  
15 transient if the fluorescence value detected at time t was greater than or equal to the average value  
16 of the five previous points plus 3 x standard deviation of the five previous points (adapted from  
17 (Constantin et al., 2009)).  
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26 Set 1: Effect of apelin on the frequency of [Ca<sup>2+</sup>]<sub>i</sub> transients in GnRH neurons  
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28 In order to search for a stimulatory effect of apelin through coupling of APJ receptor to Gα<sub>q</sub>,  
29 we examined the effect of increasing concentrations of apelin-13 (Sigma): 1, 10, 100 and 1000 nM, on  
30 [Ca<sup>2+</sup>]<sub>i</sub> transients. All treatments were delivered through continuous perfusion at 50 μl / min.  
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34 Set 2: Effect of apelin on the frequency of [Ca<sup>2+</sup>]<sub>i</sub> transients in forskolin-stimulated GnRH  
35 neurons  
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37 In order to detect an inhibitory effect of apelin through the coupling of APJ receptor to Gα<sub>i</sub>, we  
38 examined the effect of 10 μM apelin on [Ca<sup>2+</sup>]<sub>i</sub> transients in 5 μM forskolin (FSK; Tocris UK) pre-treated  
39 olfactory explant cultures. FSK is used to increase cAMP levels through the stimulation of adenylate  
40 cyclase. Cultures were recorded in control condition (SFM perfusion) for 5 min, then incubated  
41 (perfusion) with 5μM FSK for 15 min, only the last 5 min were recorded and then recorded in the  
42 presence of 10 μM apelin + 5μM FSK for 15 min.  
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### 53 3. Results

#### 54 3.1 Apelin appositions on GnRH and KP neurons

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Based on previously published data (Sandal et al., 2015), we searched on sections from two male rat brains for the occurrence of anatomical relationships between apelin system and GnRH or KNDy neuronal populations. Sections processed for double fluorescent immunohistochemistry directed against apelin and GnRH (Fig. 1 A-B) and apelin and KP (Fig. 1 C-E) were observed using a confocal microscope to perform z-stacks with a 1  $\mu\text{m}$ -thick steps. All examined neurons from the male rat OVLT displaying red fluorescence associated with GnRH showed small apelin appositions labelled in green (Fig. 1 A-B). Similarly, in the ARC, most neurons displaying red fluorescence associated with KP had close associations with apelin terminals marked in yellow-green (Fig. 1 C-E). Similar observations were made in male colchicine-treated mice (Fig. 1-F-G), where quantitative analysis performed in 5 animals revealed 89.54 $\pm$ 3.27% of GnRH neurons showed apelin appositions and each GnRH neuron had

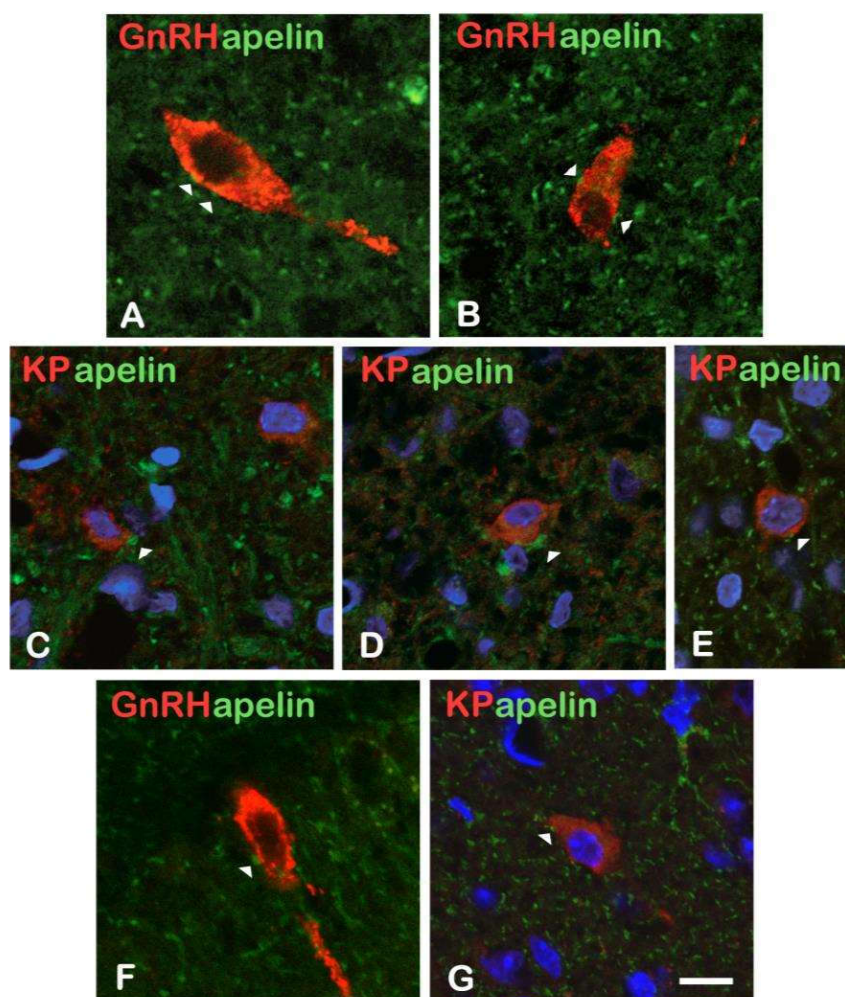


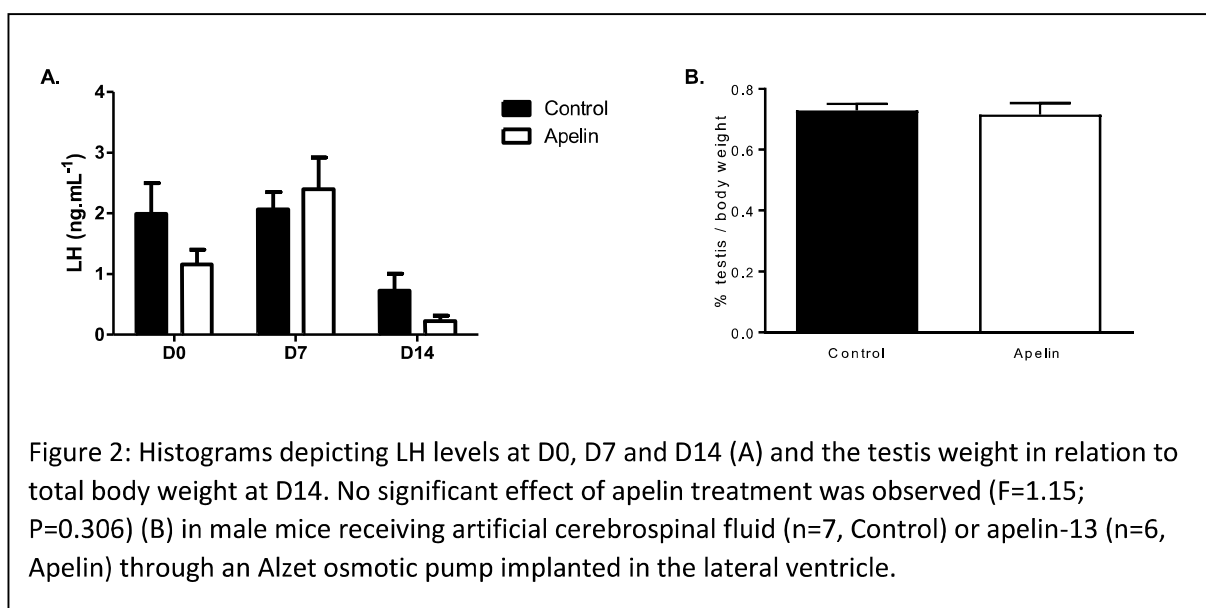
Figure 1: Microphotographs of rat (A-E) and mouse (F-G) frontal hypothalamic sections that underwent a double immunohistochemical procedure to detect either GnRH and apelin (A-B, F) or KP and apelin (C-E, G). Each image corresponds to the merge image of a 1 $\mu\text{m}$ -thick slice of a z-stack. Please note the presence of apelin buttons (arrowheads; stained in green from FITC) in close proximity with GnRH neurons or KP neurons (both stained in red from Texas red). Scale bar :14  $\mu\text{m}$  in A-B, 10  $\mu\text{m}$  in C-E and G and 11 $\mu\text{m}$  in F.

1 between 1 and 4 apelin terminals on its perimeter (mean number of appositions per neuron was  
2 2.41±0.09). A few appositions between GnRH fibers and apelin fibers were also discernible on these  
3 sections. Regarding KP and apelin interactions, 98.74±1.26% of KP neurons displayed apelin buttons  
4 and each KP neuron was surrounded by 1-8 apelin terminals (mean number was 4.04±0.65).  
5 Appositions between KP fibers and apelin fibers were also observed on these sections. Those results  
6 suggested that both GnRH and KP neurons may have direct anatomical connections with apelin fibers  
7 and may therefore have their activities impacted by apelin leading us to perform experiments 2 and 3  
8 in well-known murine models to solve this issue.  
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### 10 3.2 Influence of apelin chronic infusion on LH levels and KP, Tac2 and Dyn gene expressions in male 11 mice 12

#### 13 3.2.1 LH secretion, testes weight and metabolic status 14

15 As a chronic apelin central infusion was validated in our experimental mouse model (Duparc et al.,  
16 2011), we checked whether apelin also modified LH levels in that model. Repeated measures two way  
17 ANOVA revealed that time accounts for 39.96% of the total variance of LH levels over the course of the  
18 experiment (2way ANOVA “Time factor”: F=12.87, P=0.0002) irrespective to the group of mice (control  
19 vs. treated). In contrast with two previous studies measuring LH after an acute apelin icv injection  
20 (Sandal et al., 2015, Taheri et al., 2002), there was no statistical effect of long-term apelin-treatment  
21 on LH levels between groups as treatment accounts for 2.11% of the total variance (2way ANOVA  
22 “Treatment factor”: F=1.15, P=0.306). There was no statistical difference between groups for the  
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24 Figure 2: Histograms depicting LH levels at D0, D7 and D14 (A) and the testis weight in relation to  
25 total body weight at D14. No significant effect of apelin treatment was observed (F=1.15;  
26 P=0.306) (B) in male mice receiving artificial cerebrospinal fluid (n=7, Control) or apelin-13 (n=6,  
27 Apelin) through an Alzet osmotic pump implanted in the lateral ventricle.  
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29 interaction between time and treatment (2way ANOVA “Interaction”: F=1.47, P=0.25) At D0, before  
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the start of any infusion ( $p>0.05$ ), LH levels were ranging between  $1.99\pm 0.50$  ng/mL in controls and  $1.16\pm 0.24$  ng/mL in animals set for the apelin treatment. At D7, LH levels were slightly elevated but statistically similar between groups ( $2.06\pm 0.28$  ng/mL in controls vs.  $2.39\pm 0.53$  ng/mL in apelin-treated;  $p>0.05$ ) and at D14, LH levels dropped in both groups with no significant difference between them ( $0.72\pm 0.27$  ng/mL in controls vs.  $0.22\pm 0.08$  ng/mL in treated;  $p>0.05$ ). At D14, both groups showed LH levels lower than those measured at D0 (Fig. 2A).

Testes weight were also not significantly different between control ( $189\pm 4$  mg) and apelin ( $181\pm 7$  mg) treated animals (unpaired t test,  $P=0.32$ ) (Fig. 2B) thereby supporting the absence of changes in body

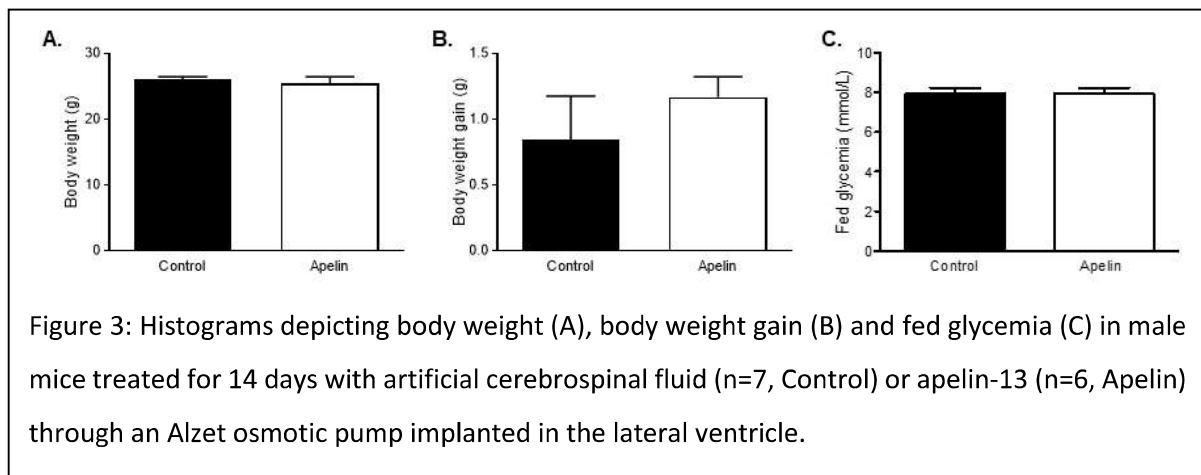
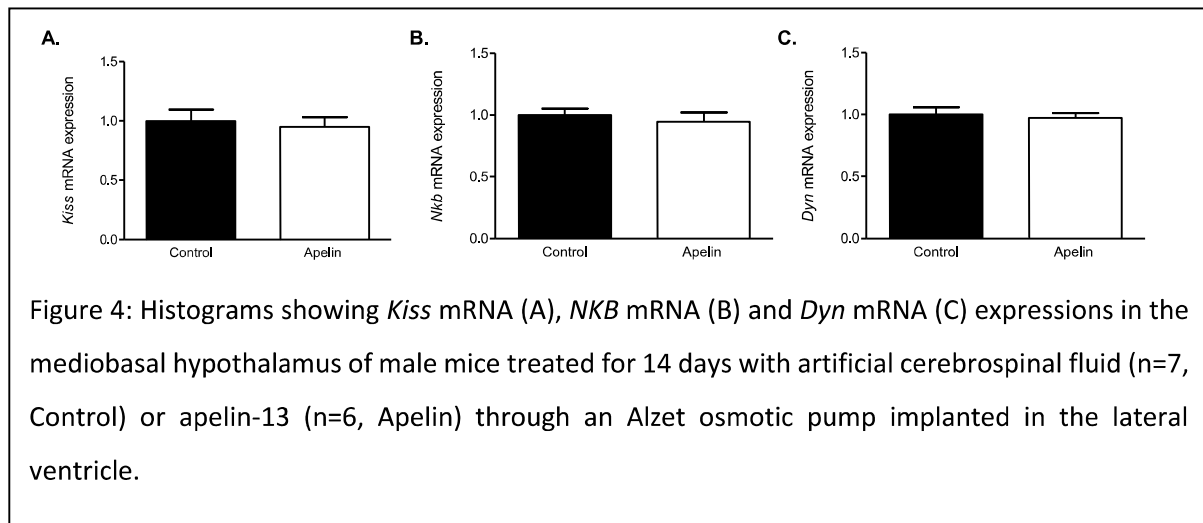


Figure 3: Histograms depicting body weight (A), body weight gain (B) and fed glycemia (C) in male mice treated for 14 days with artificial cerebrospinal fluid ( $n=7$ , Control) or apelin-13 ( $n=6$ , Apelin) through an Alzet osmotic pump implanted in the lateral ventricle.

weight (Fig. 3A), body weight gain (Fig 3B), and fed glycemia (Fig. 3C) following two-weeks of apelin infusion into the brain as previously described (Duparc et al., 2011).

### 3.2.2 Gene expression levels of Kiss1, Tac2 and Nkb

RT-qPCR were performed on samples extracted from dissected medio-basal hypothalamus. No significant difference was detected for the expression of *Kiss1*, *Tac2* and *Dyn* genes between control and apelin-treated groups (Mann-Whitney test: *Kiss1*, P=0.7; *Tac2*, P=0.8 and *Dyn*, P=0.52; Fig.4).



### 3.3 Effect of apelin on intracellular calcium signaling in GnRH neurons

Since APJ receptor can activate either  $G\alpha_{q/11}$  or  $G\alpha_{i/o}$  (Masri et al., 2006, Szokodi et al., 2002) dependent pathway, we tested the effect of apelin on GnRH neurons intra-cellular calcium in basal conditions (Set 1) or in Forskolin (FSK)-stimulated GnRH neurons (Set 2) in our well-validated explant culture model.

#### 3.3.1 Set 1: effect of increasing concentrations of apelin on $[Ca^{2+}]_i$ transients frequency in GnRH neurons

We tested apelin at 1, 10, 100 and 1000 nM on a total of 238 neurons from 4 cultures (Table 2). We observed no significant effect of apelin from 1 to 1000 nM on  $[Ca^{2+}]_i$  (figure 5 A and B). The frequency of  $[Ca^{2+}]_i$  transients was not significantly affected by apelin 1-1000 nM (Kruskal-Wallis chi-squared = 7.12, p = 0.1297).

**Table 2: Effect of increasing apelin concentrations on  $[Ca^{2+}]_i$  transients frequency in GnRH neurons**

	Apelin concentration				
	0 nM	1 nM	10 nM	100 nM	1000 nM
Frequency ( $\text{min}^{-1}$ )	2.33 ± 0.16	1.90 ± 0.14	1.85 ± 0.15	2.36 ± 0.27	2.51 ± 0.18
mean ± sem	(n=119)	(n=28)	(n=27)	(n=33)	(n=31)
(n neurons)					

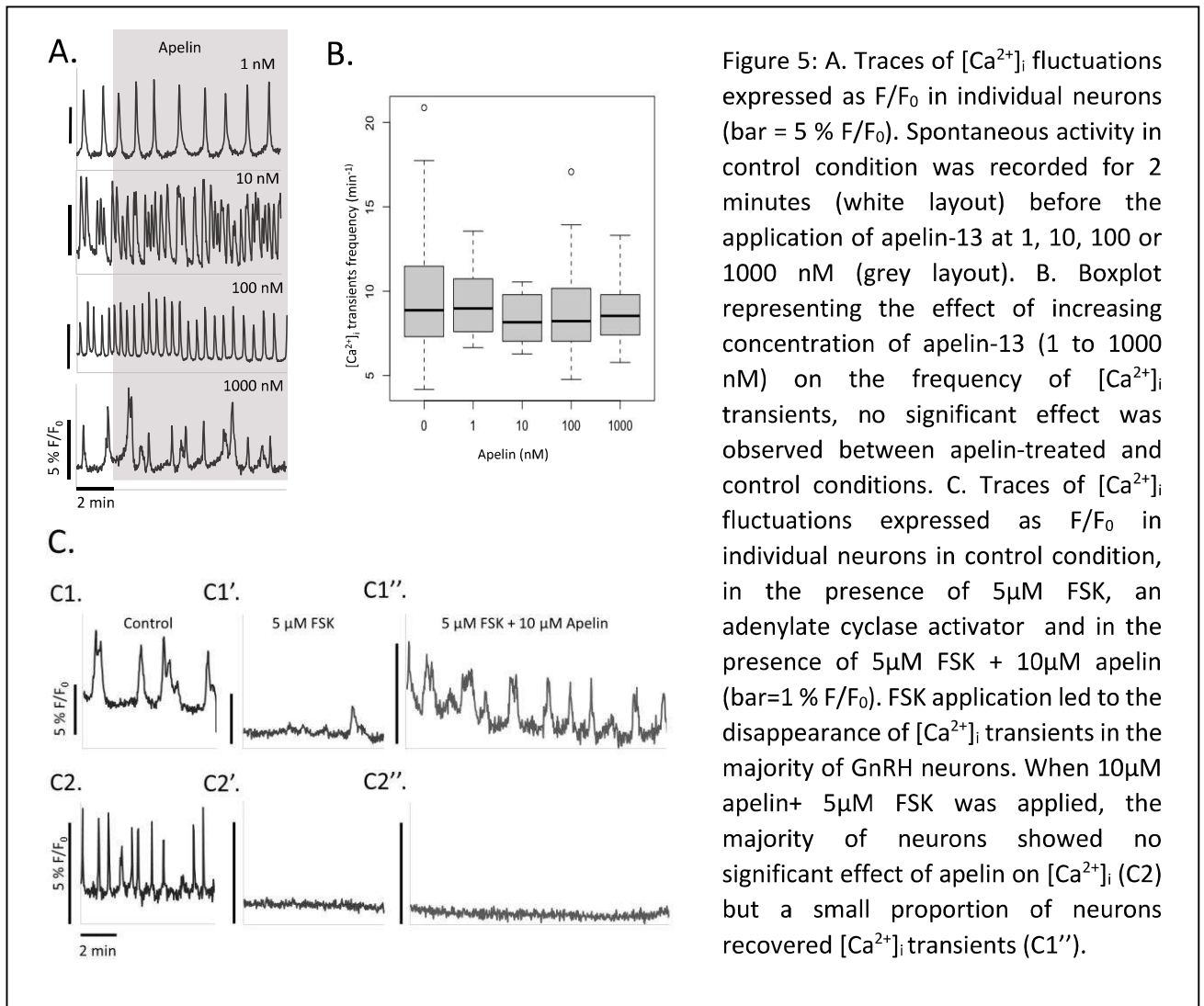


Figure 5: A. Traces of  $[Ca^{2+}]_i$  fluctuations expressed as  $F/F_0$  in individual neurons (bar = 5%  $F/F_0$ ). Spontaneous activity in control condition was recorded for 2 minutes (white layout) before the application of apelin-13 at 1, 10, 100 or 1000 nM (grey layout). B. Boxplot representing the effect of increasing concentration of apelin-13 (1 to 1000 nM) on the frequency of  $[Ca^{2+}]_i$  transients, no significant effect was observed between apelin-treated and control conditions. C. Traces of  $[Ca^{2+}]_i$  fluctuations expressed as  $F/F_0$  in individual neurons in control condition, in the presence of 5µM FSK, an adenylate cyclase activator and in the presence of 5µM FSK + 10µM apelin (bar=1%  $F/F_0$ ). FSK application led to the disappearance of  $[Ca^{2+}]_i$  transients in the majority of GnRH neurons. When 10µM apelin+ 5µM FSK was applied, the majority of neurons showed no significant effect of apelin on  $[Ca^{2+}]_i$  (C2) but a small proportion of neurons recovered  $[Ca^{2+}]_i$  transients (C1'').

### 3.3.2 Set 2: effect of 10 nM apelin on $[Ca^{2+}]_i$ transients in FSK-stimulated GnRH neurons

In order to detect an inhibitory effect of apelin mediated through the coupling of APJ receptor to  $G\alpha_i$  we used 5 µM FSK to stimulate the production of cAMP (N = 5 embryos, n = 145 neurons). Pretreatment with 5 µM FSK for 30 min suppressed  $[Ca^{2+}]_i$  transients (figure 5 C1', C2'). In the presence of 10 µM apelin + 5 µM FSK we observed two different types of responses: apelin restored  $[Ca^{2+}]_i$  transients from FSK inhibition (figure 5 C1'') and apelin had no effect on  $[Ca^{2+}]_i$  transients observed in the presence of FSK alone (figure 5 C2''). The restoration of  $[Ca^{2+}]_i$  transients was observed in  $18.2 \pm 3.8\%$  of the recorded GnRH neurons.

## 4. Discussion

1 This is the first study to examine the potential influence of apelin-13 on the main neuronal  
2 populations mastering reproductive activity namely GnRH and KP neurons. In line with previous results  
3 (Sandal et al., 2015, Taheri et al., 2002), we observed for the first time some apelin fibers in close  
4 proximity with both GnRH neurons and KNDy neurons raising the possibility of a direct action of this  
5 neurohormone on these two populations. As experimental procedures were previously set and  
6 validated in our labs for both chronic apelin infusion (Duparc et al., 2011) and culture of GnRH neurons  
7 (Constantin et al., 2009) in the mouse, we investigated the potential impact of apelin on these two  
8 populations in this model. In contrast with previous data using acute i.c.v injections of apelin (Sandal  
9 et al., 2015, Taheri et al., 2002), using a chronic infusion of apelin we failed to observe a significant  
10 variation of LH levels in treated rodents. The somewhat rapid and transient effect (LH-inhibition  
11 induced by apelin occurred from 30 min after i.c.v infusion and lasted for 45 min) induced by a single  
12 i.c.v injection of apelin reported previously (Sandal et al., 2015, Taheri et al., 2002) suggests that the  
13 discrepancy between previous works and our present data may stem from an APJ receptor  
14 desensitization at the membrane level.  
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25 The lack of effect of our apelin long term treatment on LH levels is in line with the absence of  
26 variation of the expression of genes coding for KP, neurokinin and dynorphin in the mediobasal  
27 hypothalamus despite the presence of apelin terminals onto KP neurons of the ARC. Of note the  
28 presence of APJ receptors on KNDy neurons has not been documented yet but APJ receptor transcripts  
29 have been reported in the rat ARC and are widely expressed by POMC neurons (56%) as well as by  
30 uncharacterized neurons (Reaux-Le Goazigo et al., 2011). As KP and POMC neurons originate from the  
31 same cell lineage (Sanz et al., 2015), it may be that a few KP neurons also express APJ receptors but  
32 this remains to be assessed. We also cannot exclude that apelin terminals observed in the present  
33 study in close proximity with KNDy neurons are in fact apposed to glial processes and act on neurons  
34 through glial activation as APJ receptor is expressed by neurons and also by oligodendrocytes and  
35 astrocytes (Choe et al., 2000). Message transduction of apelin signal to KP neurons may involve local  
36 activation of nitric oxide synthase (NOS) as previously shown both centrally (Duparc et al., 2011) and  
37 peripherally (Mughal et al., 2018). If the interaction between apelin terminals and KP neurons is direct  
38 this may remain true as the coexpression of KP and NOS has been recently demonstrated at the level  
39 of the ARC in sheep (Bedenbaugh et al., 2018). Our results suggest that apelin does not affect  
40 chronically the expression of these three genes coexpressed within KNDy neurons but we cannot rule  
41 out a modulation of the release of the three encoded peptides by an action at the membrane level. It  
42 may also be that apelin affects the plastic processes displayed by ARC neurons among which KNDy  
43 neurons at the level of dendritic terminals to master GnRH release as apelin-13 was demonstrated to  
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have anti-apoptotic effects and to promote the expression of proteins involved in tight junctions such as ZO-1, claudin-5 and occludin therefore improving neurological functions (Xu et al., 2018).

Results obtained here therefore suggest a possible action of apelin at the membrane level involving transient local regulation of neurosecretion rather than modulation of gene expression at the nuclear level. Of note, in contrast with previous data from Sandal and coworkers (Sandal et al., 2015) no statistical variation of testes weight was noted between groups after a two-week treatment. This may stem from the developmental stage of the animals. Results for weight and weight gain appear to be concurring with previous data obtained in mice showing an increased body weight gain between days 6 and 8 following i.c.v infusion of apelin-13 (1µg/day) but no more difference between treated and control animals at day 10 of apelin infusion (Valle et al., 2008).

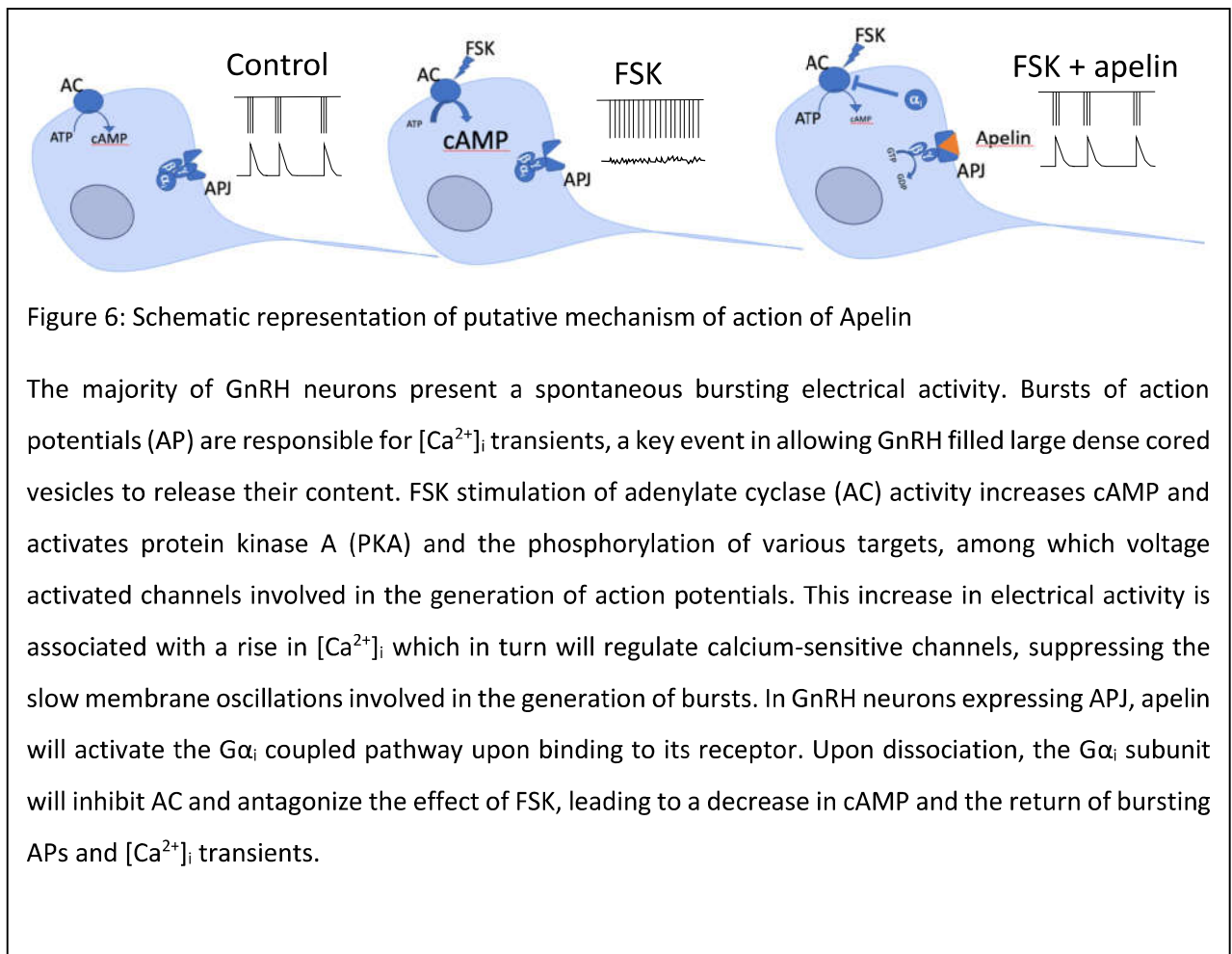
As we found apelin buttons on GnRH neurons and as apelin was reported to affect GT1-7 cells, immortalized murine cell line that express GnRH (Sisli et al., 2020), we then studied activity of GnRH neurons cultured from embryo nasal explants following apelin application using intracellular calcium imaging. We chose to monitor intracellular calcium levels and their response to apelin for two main reasons. The first one is that intracellular calcium plays a major role in GnRH secretion by inducing the process of GnRH containing-large dense core vesicles (LDCV) fusion with neuron's plasma membrane. The second reason is related to apelin signaling: apelin binding to APJ receptor induces activation of  $G\alpha_{q/11}$  or  $G\alpha_{i/o}$  and thus can modify intracellular calcium dynamics (Masri et al., 2006, Szokodi et al., 2002). When recorded *in vitro* or *in vivo*, a proportion of GnRH neurons exhibited  $[Ca^{2+}]_i$  transients lasting several seconds (Constantin et al., 2009, Constantin et al., 2012). Recording simultaneously GnRH neurons electrical activity and intracellular calcium in neurons showed a robust correlation between bursts of action potentials (APs) and  $[Ca^{2+}]_i$  transients (Lee et al., 2010) whereas GnRH neurons presenting APs without a bursting pattern did not display  $[Ca^{2+}]_i$  transients. Electrophysiological studies have revealed a great heterogeneity of electrical activity in GnRH neurons with a proportion of GnRH neurons presenting a sustained bursting electrical activity (Lee et al., 2010, Nunemaker et al., 2003, Pinet-Charvet et al., 2020). Mathematical simulation using a stochastic Hodgkin-Huxley-like model of electrical activity based on parameter values estimated in *ex vivo* experiments can reproduce the different modes of electrical activity observed in GnRH neurons: non-bursting, irregular bursting or parabolic bursting (Moran et al., 2016) and showed that the duration of  $[Ca^{2+}]_i$  transients was strongly correlated with the duration of bursts.  $[Ca^{2+}]_i$  transients involve  $Ca^{2+}$  entry through voltage-activated T- and L-type channels as well as  $Ca^{2+}$  release from inositol-1,4,5-triphosphate (IP3)-dependent internal stores (Jasoni et al., 2007) suggesting that activation of GPCR could play a role in shaping electrical activity and bursting patterns. The restoration of  $[Ca^{2+}]_i$  transients

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by apelin through  $G\alpha_i$  suggested that in  $G\alpha[Ca^{2+}]_i$  transients cAMP-stimulated GnRH neurons, apelin could restore bursting and probably GnRH release.

When apelin binds APJ,  $G\alpha_{q/11}$  or  $G\alpha_{i/o}$  can be recruited according to the cellular environment. For example, in Chinese hamster ovary (CHO) cell line, APJ signals through  $G\alpha_{i/o}$  leading to protein kinase B (Akt) activation and a decrease in cAMP production. Whereas the vasodilatory effects of apelin on smooth muscle cells are mediated by the activation of the phospholipase C  $\beta$ - protein kinase C (PLC $\beta$ -PKC) cascade following  $G\alpha_{q/11}$  recruitment leading to a rise in intracellular calcium and nitric oxide synthase activation (Masri et al., 2006, Tatemoto et al., 2001). In the present study, we investigated  $G\alpha_{q/11}$  or  $G\alpha_{i/o}$  involvement by two different protocols. To investigate  $G\alpha_{q/11}$  mediated effect of apelin, we tested the effect of increasing concentrations of apelin (1 to 1000 nM) on GnRH neurons. We did not see any change in the frequency or duration or shape of  $[Ca^{2+}]_i$  transients between control and apelin conditions. Therefore, these results do not support the involvement of  $G\alpha_{q/11}$  upon apelin binding to APJ in GnRH neurons. To investigate  $G\alpha_{i/o}$  signaling we first stimulated cAMP production using 5  $\mu$ M FSK and then applied 10  $\mu$ M apelin (Fig. 6). Over 90% of recorded GnRH neurons responded to FSK by the suppression of  $[Ca^{2+}]_i$  transients, probably due to the cAMP-dependent stimulation of electrical activity through cAMP-dependent activation of protein kinase A (PKA) and phosphorylation of voltage-gated channels (Constantin et al., 2008). The increase in the frequency of action potential raises basal calcium concentration. This rise in basal  $[Ca^{2+}]_i$  modulates the slow inward calcium current ( $I_s$ ) and the calcium activated potassium current ( $I_{KCa}$ ), both involved in the slow membrane oscillations that is essential for initiating bursts. The loss of bursting behavior induced the loss of burst-dependent  $[Ca^{2+}]_i$  transients. In the presence of apelin and FSK, 18% of recorded neurons responded by the reappearance of  $[Ca^{2+}]_i$  transients (Fig. 6). Such inhibitory effect could be due to  $G\alpha_i$  inhibition of adenylate cyclase activity or through  $G\beta\gamma$  subunits -mediated inward potassium current (GIRK family) activation such as in the case of Y1R mediated inhibitory effect of NPY on GnRH neurons (Klenke et al., 2010). However, we know that adenylate cyclase inhibition is not sufficient to decrease GnRH neurons activity and calcium transient frequency (Constantin and Wray, 2008), in contrast to the  $G\beta\gamma$  activation of GIRK current. Thus, since apelin has no effect on basal calcium transient frequency, we deem it more

likely that apelin effect on GnRH neurons involves  $G\alpha_i$  inhibition of adenylate cyclase rather than  $G\beta\gamma$  activation of GIRK current.



The majority of GnRH neurons present a spontaneous bursting electrical activity. Bursts of action potentials (AP) are responsible for  $[Ca^{2+}]_i$  transients, a key event in allowing GnRH filled large dense cored vesicles to release their content. FSK stimulation of adenylate cyclase (AC) activity increases cAMP and activates protein kinase A (PKA) and the phosphorylation of various targets, among which voltage activated channels involved in the generation of action potentials. This increase in electrical activity is associated with a rise in  $[Ca^{2+}]_i$  which in turn will regulate calcium-sensitive channels, suppressing the slow membrane oscillations involved in the generation of bursts. In GnRH neurons expressing APJ, apelin will activate the  $G\alpha_i$  coupled pathway upon binding to its receptor. Upon dissociation, the  $G\alpha_i$  subunit will inhibit AC and antagonize the effect of FSK, leading to a decrease in cAMP and the return of bursting APs and  $[Ca^{2+}]_i$  transients.

The restoration of  $[Ca^{2+}]_i$  transients by apelin in a subset of GnRH neurons suggests that apelin may restore bursting in these neurons and perhaps GnRH secretion. We hypothesize that this effect was mediated through a decrease in membrane potential through  $G\alpha_i$  activation leading to the reappearance of bursting. Extrapolation of these results in vivo is by no means simple. Firstly, studies *in vivo* have shown that secretion can occur in the absence of action potential generation (d'Anglemont de Tassigny et al., 2008, Glanowska et al., 2015). Secondly, chemogenetic inhibition of distal GnRH neuron terminals in the median eminence through  $G\alpha_i$  signalling activation failed to completely suppress LH pulsatile release (Iremonger et al., 2017). Authors suggested that  $G\alpha_i$  activation was not sufficient to inhibit  $G\alpha_{q/11}$ -mediated effects of kisspeptin signalling

## 5. Conclusions

1 Taken together, our results suggest that, in contrast with the acute central effect of apelin on LH  
2 levels, chronic central apelin infusion does not affect LH secretion levels nor KNDy neurons gene  
3 expression in rodents. As both KNDy and GnRH neurons display apelin appositions, these data suggest  
4 that transient modulation of LH secretion under apelin influence pass possibly through membrane  
5 effects at level of KNDy neurons as well as at the level of the OVLT through a modulation of the activity  
6 of a subset of GnRH neurons. Future studies will be necessary to assess the importance of nitric oxide  
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## Highlights

- Apelin terminals are present both on KNDy and GnRH neurons in male rats and mice
- Apelin chronic infusion does not decrease LH blood levels in male mice
- Apelin chronic infusion does not affect gene expression in KNDy neurons
- Apelin acute treatment modifies electrical activity in 18% of GnRH neurons

**Table 1: Primer sequences**

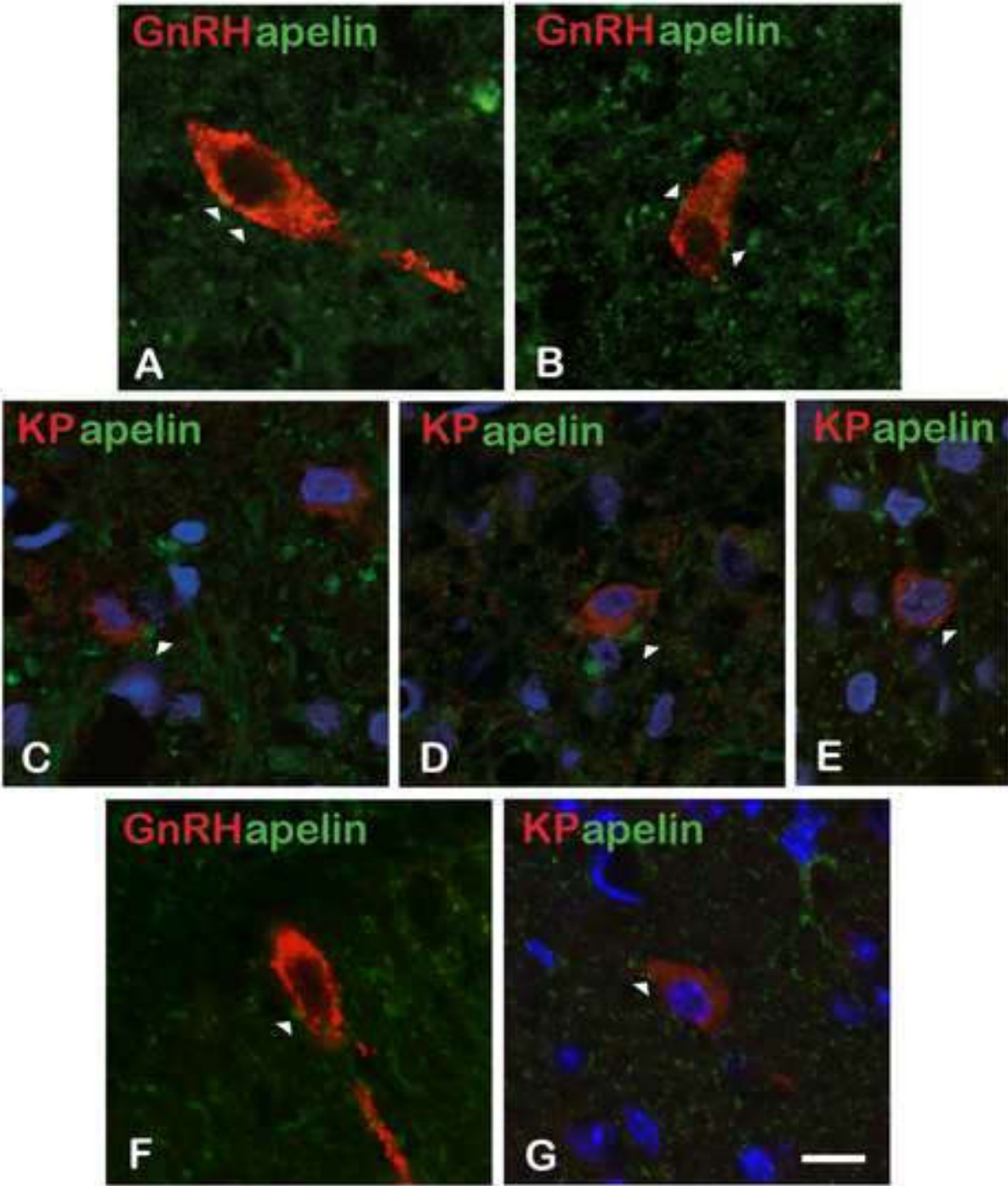
<b>Gene-Primer</b>	<b>NCBI reference</b>	<b>Oligo Sequence (5'3')</b>	<b>Reference</b>
mGADPH Fwd	XM_011241214.1	ACCACAGTCCATGCCATCAC	(Xu et al., 2017)
mGADPH Rev		TCCACCACCCTGTTG CTGTA	
mKP Fwd	XM_006497292.2	AGCTGCTGCTTCTCCTCTGT	(Cravo et al., 2011)
mKP Rev		GATTCCTTTTCCCAGGCATT	
mNKB Fwd	NM_001199971.1	TTCCACAGAAACGTGACATGC	(Popa et al., 2013)
mNKB Rev		GGGGGTGTTCTCTTCAACCAC	
mPDyn Fwd	NM_018863	GAGGTTGCTTTGGAAGAAGGC	(Popa et al., 2013)
mPDyn Rev		TTTCCTCTGGGACGCTGGTAA	

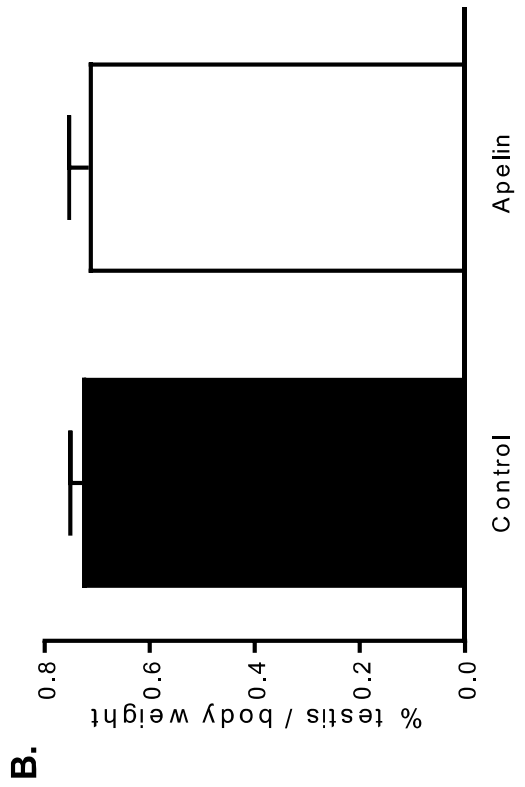
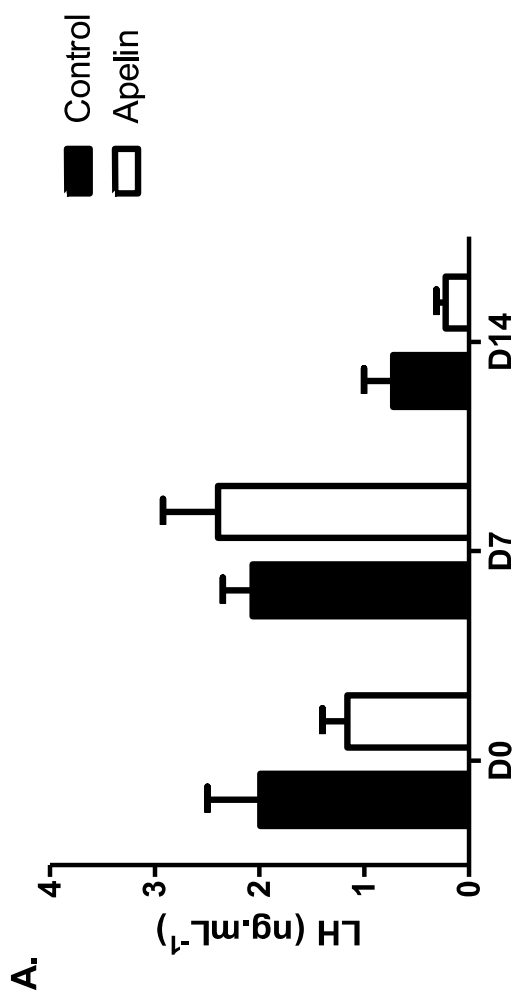
**Table 2: Effect of increasing apelin concentrations on  $[Ca^{2+}]_i$  transients frequency in GnRH neurons**

	Apelin concentration				
	0 nM	1 nM	10 nM	100 nM	1000 nM
Frequency ( $\text{min}^{-1}$ )	$2.33 \pm 0.16$	$1.90 \pm 0.14$	$1.85 \pm 0.15$	$2.36 \pm 0.27$	$2.51 \pm 0.18$
mean $\pm$ sem	(n=119)	(n=28)	(n=27)	(n=33)	(n=31)
(n neurons)					

Figure 1

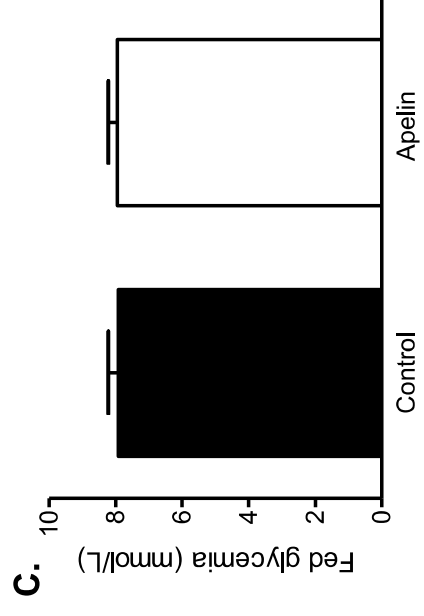
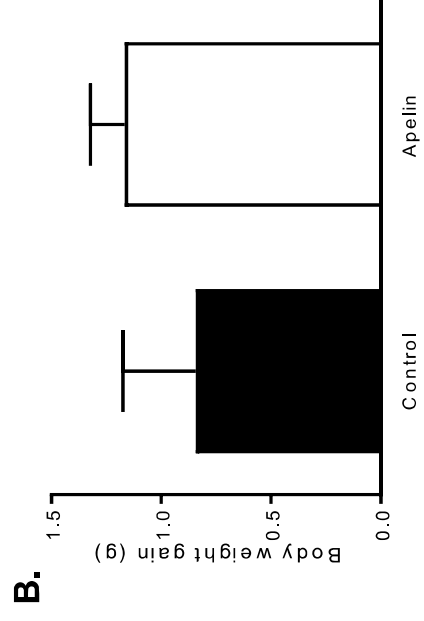
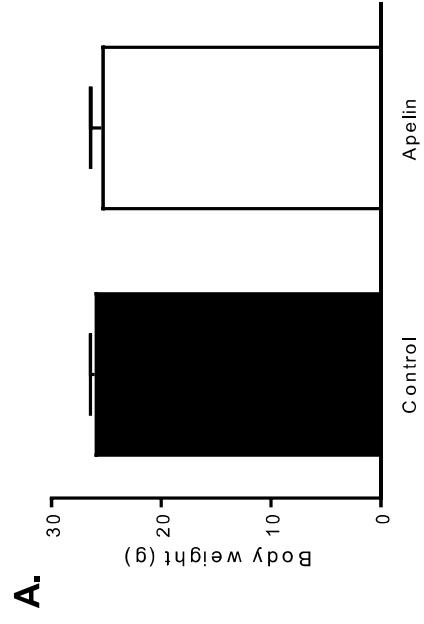
[Click here to access/download;Figure;Fig1souris.tif](#)



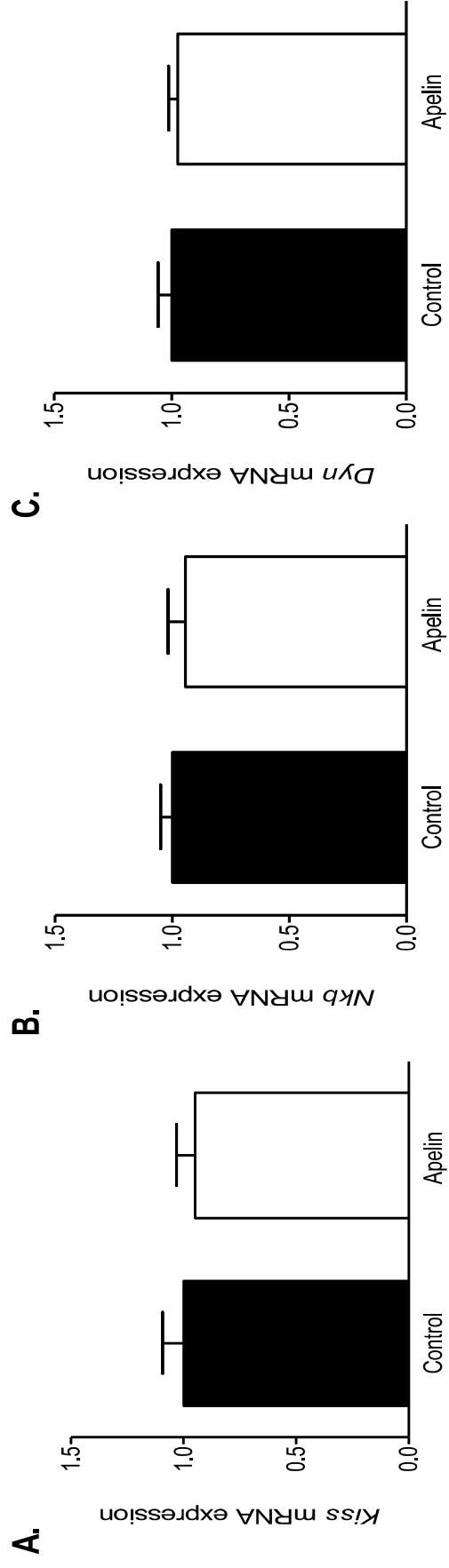


**FIGURE 2**

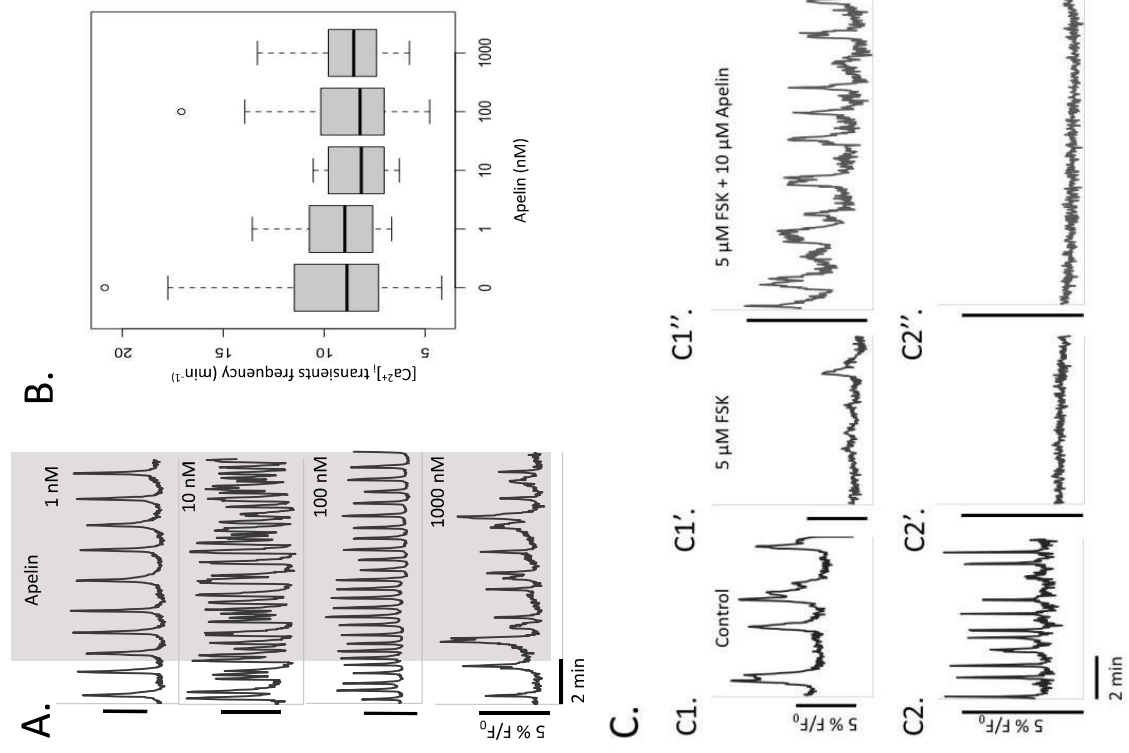




**FIGURE 3**



**FIGURE 4**



**FIGURE 5**

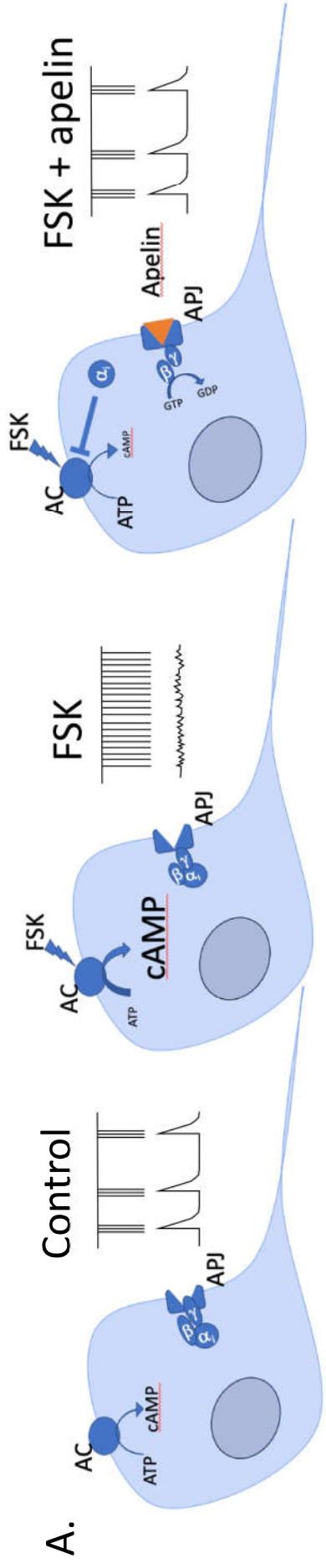


Figure 6

Credit Author Statement

Anne Abot: Investigation, Resources, Data Curation; Vincent Robert: Resources, Investigation; Renaud Fleurot: Resources; Hugues Dardente: Validation, Writing-Original Draft, Writing-Review and Editing; Vincent Hellier: Resources; Pascal Froment: Investigation; Anne Duittoz: Methodology, Validation, Formal Analysis, Writing-Original Draft, Writing-Review & Editing; Claude Knauf: Methodology, Validation, Writing-Original Draft, Writing-Review & Editing; Laurence Dufourny: Supervision, Methodology, Resources, Investigation, Visualization, Writing-Original Draft, Writing-Review & Editing.