PACAP modulation of calcium ion activity in developing granule cells of the neonatal mouse olfactory bulb

Mavis Irwin,1 Ann Greig,1 Petr Tvrdík,2,3 and Mary T. Lucero1,3,4
1Department of Physiology, University of Utah School of Medicine, Salt Lake City, Utah; 2Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, Utah; 3Interdepartmental Neuroscience Program, University of Utah School of Medicine, Salt Lake City, Utah; and 4Department of Neuroscience and Physiology, American University of the Caribbean, Cupecoy, Sint Maarten, Netherlands Antilles

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Irwin M, Greig A, Tvrđík P, Lucero MT. PACAP modulation of calcium ion activity in developing granule cells of the neonatal mouse olfactory bulb. J Neurophysiol 113: 1234–1248, 2015. First published December 4, 2014; doi:10.1152/jn.00594.2014.—Ca2+ fluctuations in the olfactory bulb (OB) are under strict control throughout development. Interneurons expressing PACAP receptors (PAC1Rs) may regulate OB function by controlling extracellular calcium (Ca2+) ions. We used a PAC1R antagonist to assess the role of endogenous PACAP receptor activation. Pharmacological activation of PAC1Rs in primary culture of OB neurons resulted in a two- to fivefold increase in Ca2+ activity in the majority of PACAP-responsive neurons. In vivo, PACAP modulation of Ca2+ activity in immature granule cells (GCs) was assessed by calcium imaging of OB slices from Dlx2-tdTomato mice to visualize GABAergic GCs. Combined block of GABA A- and GABA B-receptors (GABARs) and/or glutamate receptors (GluRs) in the presence and absence of PACAP increased the percentage of OB neurons that exhibited Ca2+ activity. Immunocytochemistry using an anti-PAC1 antibody showed that 34% of OB neurons express PAC1R. Blocking either GluRs or GABARs alone indirectly showed that PACAP stimulates release of both glutamate and GABA. Blocking both receptors together directly activated PACAP-induced Ca2+ oscillations in OB neurons. Blocking PACAP receptors alone showed that PACAP-responsive GCs significantly increased between postnatal days (P)2 and P5, suggesting that PACAP-induced Ca2+ activity contributes to OB development.

PITUITARY ADENYLATE CYCLASE-activating peptide (PACAP) is a potential candidate for neurogenesis therapy after injury or neurodegenerative disease because of its antiapoptotic properties (Arimura 1998; Atlasz et al. 2010; Bourgault et al. 2009; Brown et al. 2014; Chen et al. 2006; Cowan and Roskams 2002; Dejda et al. 2005, 2008; Delgado et al. 2003; Di Michele et al. 2012; Doursout et al. 2013; Hansel et al. 2001; Kanekar et al. 2002; Dejda et al. 2005, 2008; mulcero@asucl.edu). PACAP was originally discovered in 1989 by Miyata et al. and was described as a neuropeptide that stimulates cAMP formation in the pituitary cells, hence the current name (Miyata et al. 1989). The functions of PACAP in the CNS are largely neurotrophic during development and neuroprotective or neuromodulatory during adulthood (Arimura 1998; Delgado et al. 2003; Ravni et al. 2006; Sherwood et al. 2007). Thus, despite its relatively recent discovery, PACAP is now considered to be one of the most important regulators of biological functions (Vaudry et al. 2000c). Our interest in the physiological functions of PACAP and its specific G protein-coupled receptors (PAC1Rs) developed partly from the noteworthy problem of PACAP-knockout mice often dying before weaning. The high mortality of PACAP knockouts suggests that PACAP is required for normal development (Raineki et al. 2010; Sherwood et al. 2007).

PACAP and PAC1R expression are highest in the CNS regions that continue to regenerate throughout adulthood such as the dentate gyrus and olfactory system (Hansel et al. 2001; Mulder et al. 1999) as well as in sensory integration areas including the retina (Atlasz et al. 2010; Delwig et al. 2013; Denes et al. 2014; Kiss et al. 2006; Markhotina et al. 2007; Silveira et al. 2002; Szabados et al. 2012, 2014), cerebellum (Allais et al. 2007; Botia et al. 2007; Falluel-Morel et al. 2007, 2008; Jozwiak-Bebenista et al. 2007; Mei et al. 2004; Vaudry et al. 1998, 1999, 2000a, 2006a, 2006b, 2002, 2003; Zhokhov et al. 2008), hippocampus (Ago et al. 2011; Costa et al. 2009; Di Mauro et al. 2003; Kambe and Miyata 2012; Liu et al. 2003; Macdonald et al. 2005; Taylor et al. 2014), and suprachiasmatic nucleus (SCN) (Dziemia and Obrietan 2002; Irwin and Allen 2010; Kopp et al. 1999, 2001; Michel et al. 2006; Webb et al. 2013). Ca2+ imaging studies in SCN showed that the initial PACAP-induced response is variable, ranging from slow (s) to rapid (ms) increases in intracellular Ca2+ concentration ([Ca2+]i). After the initial response, durations of post-PACAP Ca2+ fluctuations are also variable and can persist from minutes to hours (Kopp et al. 1999). It appears that the variability in PACAP response profiles mainly depends on the dominant type of PAC1R splice variant being activated (Ago et al. 2011; Blechman and Levkowitz 2013; Nicot and Dicicco-Bloom 2001; Yan et al. 2013; Zhou et al. 2002). Either way, it is generally agreed that PACAP has a role in directly or indirectly inducing long-term changes in Ca2+ activities (Baxter et al. 2011; Dziemia and Obrietan 2002; Kopp et al. 1999, 2001; Michel et al. 2006; Tanaka et al. 2007).
Within the olfactory system, the constant generation of new neurons is required to maintain normal function of both olfactory epithelium and the olfactory bulb (OB) (Breton-Provencher et al. 2009). PACAP and PAC1Rs are found throughout the olfactory system, with high mRNA expression in the olfactory epithelium, OB, and anterior olfactory nucleus (Allen Institute for Brain Science 2013; Jaworski and Proctor 2000; Shiода et al. 1997). Several studies have investigated the physiological effects of PACAP and PAC1Rs in the olfactory epithelium (Han and Lucero 2005, 2006; Hansel et al. 2001; Hegg et al. 2003a, 2003b). However, little is known about the physiological effects of PACAP in the OB (Oliasan and Onali 1999). Here we provide the first physiological recordings of PACAP-induced [Ca$^{2+}$] responses in genetically identified neurons of the neonatal mouse OB.

The mouse OB has several discrete layers containing at least seven different types of interneurons and two classes of projection neurons, mitral cells and tufted cells (Batista-Brito et al. 2008). We limited our study of PACAP to the granule cell layer (GCL). The GCL has two main types of GABAergic interneurons, 97% of which are the small (4–8 μm) granule cells (GCs) and 3% of which are the large (12–18 μm), ovoid-soma Blanes cells (Potter et al. 2009). The GCL has one of the highest expression levels of PAC1R mRNA in the CNS. Although the GCL region has little to no PACAP gene expression, the GCs form reciprocal dendrodendritic synapses with the mitral cells and receive input from the anterior olfactory nucleus; both are areas of enriched PACAP gene expression (Allen Institute for Brain Science 2013; Batista-Brito et al. 2008; Jaworski and Proctor 2000; Shiода et al. 1997).

In the following experiments, we examined the effects of PACAP on cells in the GCL of postnatal day (P)2–P5 mice, the time period when the majority of GCs migrate into the OB from the subventricular zone (SVZ) (Batista-Brito et al. 2008; Lemasson et al. 2005).

We hypothesized that the PACAP activation of GCs triggers developmentally important [Ca$^{2+}$] oscillations and participates in the establishment of OB circuitry within the first postnatal week. In the first experiments, we asked whether PACAP directly or indirectly modulates [Ca$^{2+}$] in the developing granule neurons within the OB by evaluating the kinetics of PACAP responses in the presence and absence of neurotransmission. In the second study, we used functionally (based on excitatory GABA responses) and genetically identified cell types to determine the developmental time course of PACAP responsiveness in GCL neurons in neonatal mouse OB. We evaluated both the GC cells (labeled Dlx2-tdTomato) and the GAD65 GC subtype group (labeled Dlx2-1 KO). Additionally, the defined Cre recombinase (IRESCRE-linked) transgenic C57/BL6 mice for GAD65-IRESCRE [JAX stock no. 0101802, Jackson Laboratory (Taniguchi et al. 2011)] and Pcdh21 [The GENSAT Project; stock no. 030952-UCD; Mutant Mouse Regional Resource Centers (Nagai et al. 2005)] were used. To specifically label the IRES-CRE linked genes as red, mice from each line were crossed with mice containing the Rosa-ttdTomato gene (ROSA26-CAG promoter). The pups were checked for specific expression with ultraviolet light. Any pups with nonspecific expression, which appeared red from head to toe, were not used. The specific expression of Dlx2 is primarily in the head region. GAD65, GAD67, and Pcdh21 had expression mainly in the head but in patterns on the body, too.

**Live slices of OB.** Live coronal slices of the OB (400 μm thick) were prepared between 11 AM and 12:30 PM because of circadian changes in PACAP (Granados-Fuentes et al. 2006) from P2–P5 mice (Hegg et al. 2003b). Briefly, after decapitation the jaws, skin, and eyes were removed and the whole skull was embedded in carrot fluid (ACSF) (in mM: 125 NaCl, 3 KCl, 1.3 MgSO4·7H2O, 1.25 NaH2PO4·H2O, 25 NaH2CO3, 25 d-glucose, and 0.2 mM CaCl2·2H2O bubbled with 95% O2-5% CO2). Slices were then placed in low-Ca2 + artificial cerebrospinal fluid (ACSF) (in mM: 125 NaCl, 3 KCl, 1.3 MgSO4·7H2O, 1.25 NaH2PO4·H2O, 25 NaH2CO3, 25 d-glucose, and 0.2 mM CaCl2·2H2O bubbled with 95% O2-5% CO2 pH 7.4). The slices were cut in ice-cold low-Na+ high-Mg2+ bicarbonate buffer (in mM: 220 sucrose, 3 KCl, 10 MgSO4·7H2O, 1.25 NaH2PO4·H2O, 25 NaH2CO3, 25 d-glucose, and 0.2 mM CaCl2·2H2O bubbled with 95% O2-5% CO2). Slices were then placed in low-Ca2+ artificial cerebrospinal fluid (ACSF) (in mM: 125 NaCl, 3 KCl, 1.3 MgSO4·7H2O, 1.25 NaH2PO4·H2O, 25 NaH2CO3, 25 d-glucose, and 0.2 mM CaCl2·2H2O bubbled with 95% O2-5% CO2 pH 7.4). The slices recovered for at least 40 min at room temperature before loading with 1.3 mM Ca2+ ACSF containing membrane-permeant Ca2+-sensitive dye [37 μM fluo-4 AM (Invitrogen, Carlsbad, CA), which is prepared with 20% Pluronic F-127 (Calbiochem, San Diego, CA)] and 100 μM probenecid (Sigma-Aldrich, St. Louis, MO) at 37°C for an hour. The slices were used between 1 and 4 h after cutting.

**Confocal calcium imaging.** Fluoro-4-loaded slices were submerged in a Warner CR27 chamber. The gassed ACSF solution continuously flowed over the slices (1.5–3.0 ml/min). Test solutions were applied with a small-volume loop injector (500 μl) in line with the bath flow. A Zeiss LSM 510 Version 3.0 SP3 confocal laser scanning system was used for data collection and analysis (488-nm excitation filter for fluo-4 and 568-nm excitation filter for Rosa-ttdTomato). Time series experiments were performed collecting 256 × 256-pixel images at 1.27 Hz. Imaging studies were performed at 50–100 μm below the...
surface of the slice to avoid damaged cells. A z stack of images, 11–13 μm apart, was collected at the end of each experiment to confirm that the recording site was located between the middle of the GCL and the edge of the mitral cell layer (MCL).

**Immunocytochemistry.** Immediately after decapitation, whole skulls of P2–P5 mice were prepared by removing skin, jaws, and eyes and then fixed in 4% paraformaldehyde overnight at 4°C. The heads were sequentially equilibrated with 15% and 30% sucrose prior to embedding in OCT compound and were stored at −80°C. The 12-μm-thick frozen sections were captured on glass slides with a cryostat microtome and stored at −20°C. The sections were postfixed for 5 min in 4% paraformaldehyde-0.1 M PBS and rinsed thoroughly with PBS-0.2% Tween 20. Antigen retrieval was performed by incubating the slides in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 2 min at 96°C and then cooling to room temperature and rinsing several times in PBS. Nonspecific binding was blocked with 2% horse serum in PBS for 30 min at room temperature. Sections were incubated with a rabbit anti-Pac1 antibody [kind gift of Dr. Seiji Shioda or ARPS95945 (Aviva Systems Biology, San Diego, CA); both 1:200 (Matsuno et al. 2008)] overnight at 4°C. After the primary antibody was removed, the sections were rinsed several times with PBS and incubated with a secondary antibody (goat anti-rabbit Alexa 488, Jackson ImmunoResearch Laboratory, West Grove, PA; 1:300) for 1 h at room temperature. The slides were washed twice for 5 min in PBS and once for 5 min in water with DAPI and coverslipped. The tissue sections were imaged on a Zeiss Axio Imager microscope with Axiosvision software. Zero primary controls were run for all mouse lines and ages.

**Chemicals and drugs.** PACAP is normally produced as a 38- or a 27-amino acid polypeptide, with PACAP 27 being more stable in solution and therefore used in these studies. The powder form of PACAP 27 (Phoenix Pharmaceuticals, Burlingame, CA) was freshly dissolved in ACSF with 0.01% BSA (Sigma) kept on ice and discarded at end of each experiment day (~4 h). An ACSF loop of 0.01% BSA alone was used as a control. BSA was added to all other experimental solutions except the elevated-K⁺ solution (HK). HK (in mM: 95 NaCl, 50 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose, with 100 μM probenecid) was applied to monitor the health of the slice and identify cells with voltage-gated Ca²⁺ channels. The PAC1R-specific antagonists M65 (gift from Dr. Ethan Lerner, Harvard University; Bachem, Torrance, CA) and PACAP 6-38 (Phoenix Pharmaceuticals) were stored at −40°C until day of use. The 50 μM GABA (Tocris, Minneapolis, MN) solution was also made fresh every preparation (data not shown). PPADS (P2Y₁ and A₂A; Tocris) was also originally included in the cocktail, but was omitted for the present study because of its significant side effect of decreasing resting [Ca²⁺], levels. In addition, Doengi et al. (2008) suggested that PPADS has no effect on [Ca²⁺], responses in the OB. The expression of purinergic receptors in the OB is primarily in the astrocytes and olfactory ensheathing cells [personal observations with SR101 astrocyte-labeling dye (Doengi et al. 2008, 2009; Rieger et al. 2007)]. The interneurons in the OB, especially in juvenile mice, are believed to have little to no expression of purinergic receptors (Allen Institute for Brain Science 2013; Doengi et al. 2008; Thyessen et al. 2013).

**Analysis.** PACAP-, GABA-, and HK-responsive cells within the red Dlx2- and GAD65-tdTomato-expressing cell population in the GCL zone were identified by measuring changes in fluorescence intensity compared with baseline (%ΔF/F). The BSA control was used to identify cells with unstable baselines (125/1,502 cells; 8.3%, n = 32), which were removed from further analysis. The numbers of responsive cells were analyzed by placing regions of interest (ROIs) on each PACAP-induced responsive cell and measuring the latencies, time to half-peak, area under the curve (120 s of response recorded), and amplitudes (Fig. 1). We considered measuring response durations, but these were difficult to measure at higher PACAP concentrations because of some post-PACAP repetitive oscillations lasting tens of minutes. For this reason, we measured the time between initiation of the loop injection and maximal stimulus delivery to the cells, the time between the start of loop injection and the start of HK responses (average of 14.4 ± 1.4 s; n = 48) was subtracted from each PACAP trace. To obtain the area under the curve (Ca²⁺ flux), Origin 6.0 was used to measure and subtract a baseline from the data. After baseline subtraction, the area under the curve of 3F from the start of the response to 120 s was calculated with GraphPad Prism 5.

All cells that were counted as PACAP-responding cells met the following three conditions: First, the PACAP-induced [Ca²⁺], activity showed an amplitude increase of >5% above the baseline noise and a duration of >50 s. Second, the PACAP response began at or after the average latency for HK. Third, the PACAP response started within the range of the HK duration (100–120 s), which is the approximate duration that the antagonists would be on the tissue. For the experiments involving antagonists, which might block PACAP responses in between 40 nM and 60 nM, the order was reversed in a subset of slices.

An antagonist cocktail containing five receptor/channel blockers was designed to allow recording of PACAP responses in the absence of synaptic transmission. The antagonist cocktail included 1 μM TTX (Na⁺ channels; Sigma), 50 μM APV (NMDA; Tocris), 50 μM DNQX (AMPA/kainate; Tocris), 10 μM S95531 (GABA₆; Tocris), 100 μM LY341495 [metabotropic glutamate receptor (mGluR)₂; Tocris], and 10 μM CGP52432 (GABA₆; Tocris). The effectiveness of the antagonists was confirmed by blocking 50 mM L-glutamate- or 50 μM GABA-induced [Ca²⁺], responses in our slice preparation (data not shown). PPADS (P2Y₁ and A₂A; Tocris) was also originally included in the cocktail, but was omitted for the present data because of its significant side effect of decreasing resting [Ca²⁺], levels.

**Fig. 1.** The pituitary adenylate cyclase-activating peptide (PACAP)-induced intracellular Ca²⁺ concentration ([Ca²⁺]) transient was analyzed for latency, time to half-peak, amplitude, and net Ca²⁺ flux (area under curve for first 120 s of PACAP responses). All of the PACAP response latencies were measured relative to the average latency of elevated potassium (HK) responses.
individual cells, one more condition was met: The HK was applied before and after each PACAP antagonist treatment. Only the PACAP-activated cells that showed HK responses at the start and end of the series of runs were evaluated for PACAP responsiveness in the antagonists.

For counting the total number of PACAP-, GABA-, control BSA-, and HK-responsive cells, the series of runs from each slice was exported from the LSM files (510 LSM version 3.0 SP3) into ImageJ (http://rsbweb.nih.gov/ij/) as TIFF image sequence files of 200–500 images. The first 20–40 images in the sequence were summed and used as a baseline for subtracting from the remainder of the sequence to yield a picture of fluorescence changes (responding cells) occurring after the baseline time range. The baseline-subtracted images showing responsive cells were superimposed on an image showing the red tdTomato-labeled cells. Responsive cell counts were categorized into red- and non-red-labeled groups for each test substance. The counts were done blind to treatment and averaged across slices. Only one slice was used per pup. The total number of cells analyzed and the number of pups are reported for each experiment with the exception of the data in Fig. 2, which provides the percentages of the total cell count. Student’s t-tests were run on data with two independent variables (Figs. 3, 6, and 10) and were considered significant if the P value was <0.05. Since Gaussian fits of PACAP dose-response data revealed that the distribution was skewed, one-way Kruskal-Wallis ANOVAs (KW-ANOVAs) were used to test for differences among doses and were considered significant if the post hoc Dunn’s test P value was <0.05.

RESULTS

PACAP responses are heterogeneous. Application of 40 nM PACAP routinely elicited increases in [Ca\(^{2+}\)]\(_i\), in HK-responsive cells in slices of P4 mouse OB (Fig. 2, A–E). The PACAP-induced responses showed variation in latency, duration, and oscillation profile. The predominant response was a sustained increase in [Ca\(^{2+}\)]\(_i\), that reached a peak within seconds and then slowly returned to baseline over the course of minutes (796/1,413 cells, 56%; Fig. 2C). The second most common response to PACAP was a single transient [Ca\(^{2+}\)]\(_i\), increase that returned to baseline within the ~120-s duration of the HK response (305/1,413 cells, 22%; Fig. 2, D and E). Of the remaining 22% of PACAP-responsive cells, the majority developed a slow “sawtooth” post-PACAP [Ca\(^{2+}\)]\(_i\), oscillation (0.01–0.04 Hz, 246/1,413 cells, 17%; Fig. 2A) and a few cells developed a fast “sawtooth” oscillation (0.05–0.1 Hz, 66/1,413 cells, 5%; Fig. 2B). Kopp et al. (1999) found somewhat similar percentages for PACAP responses in cultured SCNs: single transients (20%), [Ca\(^{2+}\)]\(_i\) oscillations (15%), and “sustained oscillations” (called biphasic response with initial [Ca\(^{2+}\)]\(_i\), followed by plateau phase; 65%). PACAP-induced [Ca\(^{2+}\)]\(_i\) oscillations lasted between 5 and 20+ min (Fig. 2, A and B). Although the responses to PACAP were heterogeneous, with cells in close proximity showing response profiles very different from each other (Fig. 2), repeated PACAP application produced similar responses within the same cell (Fig. 3A). To determine the basis for the heterogeneity of the responses to PACAP, we examined the PACAP dose dependence, the kinetics, and the developmental time course and identified responding cell types within the GCL of OB.

PACAP concentration-response curve. Dickson et al. (2006) tested the dose-response curve of PACAP, VIP, and their antagonists in the cultured CHO cell line by measuring both the intracellular cAMP and Ca\(^{2+}\) concentrations. They reported an EC\(_{50}\) of 10 nM for PACAP 27. Because it is difficult to extrapolate from cell culture to in situ, we examined the dose dependence of PACAP 27 on slices from P2–P5 pups. Unlike most regions of the rodent CNS, PACAP and PAC1R were shown to be spatially enriched in the OB at P0 and maintained within the OB throughout development. The GCL in particular has high expression of PAC1Rs. The MCL was shown as a mixture of PACAP and PAC1R expression (Allen Institute for Brain Science 2013; Jaworski and Proctor 2000). For the EC\(_{50}\) curve (Fig. 3B), concentrations of PACAP were tested at 0, 1, 10, 20, 40, 60, and 100 nM. To minimize desensitization to repeated PACAP applications, the minimum washing/recovery time was empirically determined for each concentration (see MATERIALS AND METHODS). Every slice had a maximum of four PACAP exposures with appropriate washing times between applications.

The dose-response curve is presented as the ratio of PACAP-responsive cells/slice divided by HK-responsive cells/slice. By normalizing to the number of HK-responsive cells, we are counting the number of PACAP-sensitive cells among the...
EC₅₀ curves for a variety of PACAPs, VIPs, and their antagonists/agonists. Interestingly, even though the CHO cell receptor (Robberecht et al. 1992) was able to block an average of 84% of 40 nM PACAP responses (278 cells, n = 5; P < 0.05, paired t-test). This confirms that the majority of the 40 nM PACAP responses in slices are blocked by the well-characterized PAC1R antagonists (Fig. 3C).

Kineti cs of PACAP responses. To see whether there are any age-related changes in the kinetics of the PACAP effects, the slices were age-grouped as P2, P3, P4, and P5. The slices were treated with one of the two series of PACAP concentrations. The 10–20–40–60 nM PACAP series was tested on slices from 13 pups; the 20–60–40–100 nM PACAP series was tested on slices from 5 pups. No significant age-dependent or series-dependent kinetic changes were found between P2 and P5 (data not shown). Thus the slices were pooled together regardless of age between P2 and P5 for the kinetic analyses.

We measured the kinetics and amplitude of the PACAP-induced [Ca²⁺]ᵢ activity as outlined in Fig. 1. We found that, on average, the latency for PACAP responses significantly decreased with increasing concentrations (KW-ANOVA) (P < 0.0001; Fig. 4A). In addition, the time to half-peak also shortened between 40 nM and 100 nM but was not statistically significant (KW-ANOVA; Fig. 4B).

The dose-dependent changes in fluorescence intensity amplitude with PACAP application did not follow a classic dose-response curve. Figure 4C shows that the average amplitude of PACAP responses increased significantly with concentrations up to 40 nM PACAP (KW-ANOVA; P < 0.05). Both 60 nM and 100 nM PACAP concentrations elicited lower average PACAP response amplitudes.

To address whether the lower average response amplitude at 100 nM PACAP affected the Ca²⁺ signaling, area under the curve or Ca²⁺ flux (ΔF-seconds) was measured for the first 120 s after PACAP reached the slice (as determined by HK application). Figure 4D shows a significant increase in Ca²⁺ flux with increasing PACAP concentrations, with 100 nM PACAP having the highest Ca²⁺ flux (P < 0.0001, KW-ANOVA).

Increasing the concentration of PACAP shifted the percentages of PACAP response profiles from mostly single transient responses at 10 nM (only 41% oscillating cells; n = 15; 134/327 cells) to mostly oscillatory responses at 40 nM (72% oscillating cells; n = 20; 461/640 cells). The percentage of oscillatory to single transient responses did not further increase for the PACAP concentrations above 40 nM [65% oscillating cells at 60 nM (n = 20; 522/802 cells) and 70% oscillating cells at 100 nM (n = 8; 287/409 cells)].

Direct vs. indirect PACAP effects. The observation of a larger response amplitude at 40 nM compared with 60 nM PACAP, as well as the ability to effectively block 40 nM PACAP, suggested that 40 nM was the ideal concentration for physiological experiments done on live acute OB slices. Therefore we used 40 nM PACAP for evaluating PACAP responses in the remainder of the studies. Because the dose-response curve for PACAP did not appear to saturate at 100 nM PACAP, we questioned whether PACAP-induced increases in

Fig. 3. PACAP elicits dose-dependent increases in the numbers of activated cells. A: repeated application of 40 nM PACAP yields similar responses after a 60-min wash. B: log scale plot of the number of PACAP-responsive cells divided by the number of HK-responsive cells shows that the increases in cell numbers do not saturate with a relatively high PACAP concentration of 100 nM. C: % of cells responding to 40 nM PACAP is reduced by 84 ± 4% in the presence of PAC1R antagonists (1 μM M65 and 150 nM PACAP 6-38). Numbers near data points indicate number of pups followed by number of PACAP-responsive cells analyzed.
were causing PAC1R-expressing mitral cells and GCs to release glutamate and GABA, respectively, which in turn increased the number of responsive cells. To tease out the cells that were directly activated by PACAP from those responding to PACAP-mediated release of glutamate or GABA, we applied 40 nM PACAP in the presence and absence of a series of cocktails of neurotransmitter receptor antagonists described in MATERIALS AND METHODS. The first cocktail (Glu-GABA-Na) was designed to block all neurotransmission and any other possible pathways that may elevate the [Ca\(^{2+}\)], in interneurons.

To ensure that neurotransmission was blocked prior to PAC1R activation during coapplication of the cocktail and PACAP, we examined the time course of control application of the antagonists. Of 119 cells (n = 3) only 10 post-PACAP cells had Ca\(^{2+}\) activity preceding control application of Glu-GABA-Na antagonists. Application of the Glu-GABA-Na cocktail eliminated all Ca\(^{2+}\) activity in all 10 cells within 12.5 ± 4.0 s (see example cell in Fig. 5A).

The Glu-GABA-Na cocktail contains antagonists for blocking NMDA receptor, AMPA receptor, mGluR (all types),...
GABA_A receptor (GABA_A R), and GABA_B R. TTX was included to reduce occasional spontaneous action potentials. We found that on average, the Glu-GABA-Na cocktail reduced the percentage of PACAP-responding cells to 34 ± 6% of the PACAP control done on the same slice (143 cells; n = 4; Fig. 5B). Overall, 46 of the 143 cells that responded to the PACAP control also responded to PACAP + Glu-GABA-Na antagonists and were therefore directly activated by PACAP. All [Ca^{2+}]_i activities (oscillations or sustained responses) in the indirectly PACAP-activated cells were completely blocked by the antagonist cocktail (Fig. 5A). We evaluated the kinetics and amplitude of the 46 directly activated cells with the same approach as for PACAP at different concentrations. Only the latency of the PACAP response was not significantly changed by the coapplication of the antagonist cocktail (36.3 ± 3.9 s before and 36.9 ± 5.4 s during antagonist + PACAP; Fig. 6A). Amplitude (P = 0.01, paired t-test), time to half-peak (P < 0.01, paired t-test), and area under the curve (P < 0.001, paired t-test) were significantly reduced in the presence of the antagonist cocktail, suggesting involvement of GluR and GABAR activation in the [Ca^{2+}]_i response to PACAP even within cells directly activated by PACAP (Fig. 6, B–D).

Further analysis of directly activated cells revealed that the [Ca^{2+}]_i response profiles of 22 of the 46 cells (48%) that responded in both PACAP and PACAP/Glu-GABA-Na antagonists changed enough to be reassigned to new categories. The single transient response cells did not change into something more complex (4/4 cells remained single transients), but 22 of the 42 cells with complex [Ca^{2+}]_i responses switched to slower or no oscillations in the presence of PACAP and blockers. For the remaining 20 cells, the PACAP oscillation responses were similar in the presence and absence of the antagonist cocktail. Collectively, the data show that the Glu-GABA-Na antagonist cocktail reduces the oscillatory activity of roughly half of the directly PACAP-activated cells and completely blocks [Ca^{2+}]_i oscillations in the indirectly activated cells.

To examine the independent effects of the activated GluRs versus GABARs in the presence of PACAP, the antagonist cocktail described above was separated into two groups. The groups were GluR-only antagonists (NMDA, AMPA, and mGluRs) and GABAR-only antagonists (GABA_A and GABA_B). The antagonist for sodium channels (TTX) was not included in the individually grouped cocktails because we wanted to test whether blocking one or another group had consequences on the occasional global spontaneous action potentials (none was seen within the duration time of blockage; data not shown). Addition of GluR antagonists reduced the number of PACAP-responding cells to 26 ± 3% of PACAP control (118 cells, n = 3; Fig. 5B, center). GABAR-only antagonists reduced PACAP responses to 54 ± 11% of the PACAP control (129 cells, n = 4; Fig. 5B, right). These data indicate that both GluRs and GABARs contribute to the PACAP-initiated [Ca^{2+}]_i oscillations but GluRs are the main contributors.

Role of PACAP in maturation of Dlx2- and GAD65 tdTomato-expressing cells in GCL. The connection of GCs to the OB network depends on the maturity of the cells. In the next section, we look at the rapidly developing OB between P2 and P5. It is known that the interneuron precursors (neuroblasts) take ~3 days to migrate from their origin in the SVZ to the OB, which not surprisingly correlates with the rapidly growing OB increasing from 25% of adult interneurons at P0 to 55% by the end of the first week (Lemasson et al. 2005).

To specifically identify PACAP-responsive cells in the GCL, we primarily used two lines of IRES-CRE-linked transgenic mice, Dlx2-CRE and GAD65-CRE, which were crossed with the Rosa-ttdTomato (using the ROSA26-CAG promoter) transgenic mouse line to provide gene-specific red cells. The ROSA26-CAG promoter was used to label IRES-CRE-linked transgenic mouse line to provide gene-specific red cells. The ROSA26-CAG promoter was used to label IRES-CRE-linked

Fig. 6. Cells directly activated by PACAP have faster smaller responses compared with indirectly activated cells. A: only the latency is unchanged between 40 nM PACAP-only controls and 40 nM PACAP in Glu-GABA-Na antagonists done on the same slice. B–D: the antagonists significantly reduced the time to half-peak (63% of control; B), amplitude (71% of control; C), and area under curve (66% of control; D) (paired t-test). Numbers above the bars indicate number of pups and number of cells analyzed.
Dlx2, an essential migrating gene, at the neuroblast stage (Batista-Brito et al. 2008). The SVZ-born Dlx2-labeled neuroblasts, which have migrated to the OB, become both types of GABAergic GCs (GAD65 and GAD67) as well as periglomerular cells (Plachez and Puche 2012). Although Dlx2 is switched off in mature cells, the red fluorescence remains throughout the cell life span. The Dlx2-tdTomato is expected to label the majority of GCs residing in the GCL (Potter et al. 2009). Figure 7, A–E, show the expression of Dlx2-tdTomato (red) in 12-μm sections of OB from P2 and P4 mice. PAC1R-specific antibody labeling (green) colocalizes with a subset of Dlx2-expressing cells in the GCL and labels PCdh21-tdTomato mitral cells in the MCL (Fig. 7, F–H). Compared with total cell counts using DAPI nuclear staining, 89% (1,945; n/2 pups) of GCL cells are Dlx2+ and 21 ± 1% (3,160 Dlx2+ cells; n = 3 pups) are colabeled with PAC1R antibodies at P2, while in the P4 OB the average percentage of cells colabeled with both Dlx2+ and PAC1R antibody increases to 34 ± 1% (2,979 Dlx2+ cells; n = 4 pups). The 34 ± 1% of PAC1R+ cells in the immunocytochemistry (ICC) at P4 correlates nicely with the percentage of cells that responded directly to PACAP in the presence of Glu-GABA-Na antagonists (34 ± 6%; ages P4–P6; Fig. 5B).

The second transgenic mouse line was the GABAergic GAD65-tdTomato, in which the ROSA26-CAG promoter was used to label the IRES-CRE-linked GAD65 gene (not shown). In 8-wk-old mice, ~50% of the OB GABAergic GCL cells express GAD65 (Parrish-Aungst et al. 2007). The other 50% are generally the larger GAD67-expressing cells. However, 20% of the GABAergic GCL cells are capable of expressing both GAD65 and GAD67 (Parrish-Aungst et al. 2007). GAD65 and GAD67 protein expression is not turned on until the cells exit the migratory pathway, but these subtypes are predetermined while still neuroblasts in the SVZ. The similar timings of protein expression suggest that these subtypes enter the OB at similar developmental stages (De Marchis et al. 2007; Kelsch et al. 2007; Merkle et al. 2007; Plachez and Puche 2012). The Dlx2 and GAD65 transgenic lines were used for the identification of GABAergic GCs during the PACAP vs. GABA experiments.

Using GABA responses to identify immature GCs. To help discern whether PACAP is activating mature or immature GCs, we used GABA to elevate the [Ca2+]i in immature cells. Figure 8 is a diagram showing how GABA acts as an excitatory neurotransmitter in migrating neuroblasts or immature neurons. In neonatal OBs, the immature neuroblast cells elevate
suggestions that GCs express functional PAC1Rs during their maturation process. The nonred cell counts in the images taken from Dlx2-tdTomato mice made up 6% (165/2,708 cells; n = 20 mice) of the GABA- and/or PACAP-responsive OB GCL cells across all ages evaluated. The Blanes cells, which originate in the OB and do not migrate from the SVZ (Plachez and Puche 2012), may account for 2–3% of the unlabelled population. The other 3–4% of unlabelled responsive cells might be other non-Blanes interneurons in the GCL. (The glial cells such as astrocytes are not a factor in the analysis; see DISCUSSION.)

Figure 10B shows the calculated percentages of all GAD65+ GABA- and/or PACAP-responsive cells. The graph shows no significant changes for all three groups within the age range studied. Figure 10C shows the comparable percentages of the GAD65-tdTomato nonred cells (GAD65+), which we assume to be GAD67 cells since all of the immature neurons in the GCL are either GAD65+ or GAD67+ or both GAD65+ and GAD67+ (Batista-Brito et al. 2008; Leman et al. 2005; Parrish-Aungst et al. 2007). As with the Dlx2-labeled cells, the GAD65− cells shifted from mostly GABA-only cells to PACAP-only cells between P2 and P5. Of all of the GABA- and/or PACAP-responsive GAD65− cells, those responding only to GABA (immature) made up an average of 50 ± 3% at P2 (41 cells, n = 3) and immature cells significantly decreased to 24 ± 6% by P5 (67 cells, n = 8; unpaired t-test, P < 0.005). Those cells responding to both BACAP and PACAP (maturing) significantly decreased from an average of 30 ± 2% at P2 to 11 ± 3% by P5 (P = 0.001). Conversely, although an average of only 20 ± 5% of the GAD65+ cells responded to PACAP at P2, their numbers significantly increased to 53 ± 9% by P5 (P = 0.01). Thus the appearance of PACAP responses during GCL maturation observed in the P2–P5 GAD65− cells is similar to that observed in Dlx2-tdTomato cells, suggesting that PAC1Rs are functional during development of GABAergic neurons.

**DISCUSSION**

In the studies described above, we found that PACAP is capable of eliciting [Ca2+]i responses in cells at various stages of maturation in the GCL of the OB in P2–P5 mice. We found that the PACAP-induced [Ca2+]i changes were heterogeneous and dependent both on direct activation of PAC1Rs and on subsequent recruitment of additional cells through release of glutamate and GABA. The percentage of functional PAC1R-expressing cells in the GCL that were directly activated by PACAP increased over the P2–P5 time window and matched the percentage of PAC1R+ cells identified by ICC. By utilizing mouse lines that express the red tdTomato in specific cell types, we determined that the Dlx2+ cells that migrate into the OB via the rostral migratory stream are capable of responding to PACAP before they transition to mature GCs. The further subdivision of the migrating GC population into GAD65 and GAD67 subtypes revealed the surprising observation that the time-dependent increase in mature PAC1R-expressing cells observed in the Dlx2 studies occurs as a result of maturation in the GAD67 GCs but not the GAD65 GCs. This observation supports our hypothesis that PACAP activation of immature GCs initiates developmentally important [Ca2+]i oscillations during the first postnatal week of development.

The OB interneuron response to PACAP in acute slices shows that the majority of the [Ca2+]i activity occurs through
network activation of both the gluRs and GABARs on non-PAC1+ GCs, providing a wide variety of [Ca\(^{2+}\)]i activity responses ranging from single transients to slow to fast sawtooth oscillations and sustained oscillations (Fig. 2). Possible factors contributing to the variety of responses are the dose of PACAP (Figs. 3 and 4) and the PACAP-induced activation of gluRs and/or GABARs (Figs. 5 and 6). The time to half-peak is constant across the concentrations tested but was shown to decrease in Glu-GABA-Na antagonists. The faster time to half-peak when neurotransmitter contributions to the [Ca\(^{2+}\)]i signal are blocked supports the idea that PAC1R activation initiates a [Ca\(^{2+}\)]i response, but it is the PACAP-mediated GABA and glutamate release that augments the signal in both the directly and indirectly activated cells. Collectively, these findings suggest that PACAP may be an important modulator of glutamate and GABA release in developing mouse OB.

The factor appearing to have no significant effect on PACAP-induced Ca\(^{2+}\) kinetics and intensities during the first neonatal week is age; the P2, P3, P4, and P5 age groups show similar responses in terms of latency, time to half-peak, amplitude, and Ca\(^{2+}\) flux (data not shown). However, the percentage of PACAP-responding cells does rapidly increase during the first neonatal week (Fig. 10). The day-to-day shift from immature GABA-only neuroblasts at P2 to mature PACAP-only GCs at P5 appears to be driven by the maturation of the GAD67 subtype of GCs. The increase in PACAP-responsive cells correlates with the ICC-measured increase in PAC1R+ between P2 and P4.

Despite having the same rate of development as GAD65 cells upon entering the OB, the GAD67 cell subtype develops complicated connections to PAC1R+ projection neurons such as mitral cells (Plachez and Puche 2012). It is still unclear when, if actually ever, the GAD65 subtype GCs shift from mostly immature, PACAP-unresponsive cells to mature PACAP-responding cells.

Dose dependence and block of PACAP responses. The reported EC\(_{50}\) for PACAP responses ranges between 0.25 and 3 nM in cell culture systems and increases to 30–50 nM PACAP in acute slices (Dickson et al. 2006; Joziwak-Bebenista et al. 2007; Kambe and Miyata 2012; Nicot and Dicecco-Bloom 2001). In vitro experiments used as much as 100 nM PACAP (one used 200 nM) to obtain maximal neuroprotective effects (Basille-Dugay et al. 2013; Dziema and Obrietan 2002; Kopp et al. 1999, 2001; Masmoudi et al. 2003; Pugh et al. 2010; Scharf et al. 2008; Vaudry et al. 2002). At least one lab also used extremely high PACAP doses in slices (Sun et al. 2003). In contrast to the studies in cell lines, Joziwak-Bebenista et al. (2007) measured a PACAP EC\(_{50}\) of ~0.25 nM for both primary cultured neuron and astrocyte cells. They also tested acute cerebral cortical slices, which are PAC1R+ rich compared with the majority of the CNS apart from the OB and obtained an EC\(_{50}\) of ~30 nM (Joziwak-Bebenista et al. 2007). At least two different studies (Masmoudi et al. 2003; Vaudry et al. 2002) confirmed that responses to 100 nM PACAP in cultured cells are blocked by using only PACAP 6-38 (PACAP 38 with first 6 peptides deleted). Other studies used between 1 and 10 nM PACAP in the cultures and showed block with only PACAP 6-38 (PACAP 38 with first 6 peptides deleted). The doses of PACAP 6-38 were reported to be 600–1,000 nM. The dose-response curve for our OB slices suggested that 40 nM PACAP is ideal for our [Ca\(^{2+}\)]i imaging approach because response amplitudes decreased at higher PACAP concentrations, possibly because of desensitization during the PACAP application. Unlike the previous cell culture studies, we were unable to completely block 40 nM PACAP-induced [Ca\(^{2+}\)]i activity when using as much as 1 μM PACAP 6-38 (data not shown), perhaps because we study our cells in slices. Costa et al. (2009) used hippocampus slices, and they were able to block 0.5 nM PACAP with 500 nM PACAP 6-38. However, at 10 nM PACAP responses were not blocked by 500 nM PACAP 6-38 (Costa et al. 2009).

Direct vs. indirect PACAP-induced Ca\(^{2+}\) transients and oscillations. PACAP binding to PAC1Rs is capable of activating numerous pathways that increase [Ca\(^{2+}\)]i, including Ca\(^{2+}\)-dependent synaptic release and Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Doan et al. 2012a, 2012b; Kambe and Miyata 2012; Pugh et al. 2010). The role of GluRs and/or GABARs in the PACAP-induced Ca\(^{2+}\) oscillations was individual and independent. The
GABA and PACAP

uniqueness of each oscillation profile likely depends on the network connections, cell maturity (GABA-induced excitation discussed below), and expression of the GluRs and/or GABARs. Figure 6 showed that when antagonists block Glu/GABA input the PACAP-induced Ca\textsuperscript{2+} responses are significantly reduced in terms of amplitude, time to half-peak, and total Ca\textsuperscript{2+} flux. The lower effectiveness of GABAR antagonists in reducing indirect PACAP responses suggested that the majority of PAC1R-negative cells are either mature GCs or immature GCs with GluRs.

Astrocytes are not a factor in data analysis. Astrocytes are PACAP and GABA excitable (Hansson et al. 2009; Jozwiak-Bebenista et al. 2007). The EC\textsubscript{50} of PACAP in cultured glial cells (astrocytes) is almost exactly the same as for cultured neuronal cells. However, when we use SR-101 (a red dye labeling astrocytes) on slices, we find astrocytes to be starlike-shaped cells appearing as “background” and having little to no [Ca\textsuperscript{2+}]i response to 40 nM PACAP, suggesting that the astrocytes in OB slices have a different physiology than in cultures and/or higher EC\textsubscript{50} than the surrounding OB interneurons. The concentration of GABA used in our experiments was 50 μM. Doengi et al. (2009) used [Ca\textsuperscript{2+}], signaling to determine the GABA EC\textsubscript{50} of 35 μM in neurons and 100 μM in astrocytes, with a few astrocytes responding to 20 μM GABA. Accordingly, our 50 μM GABA may excite a small number of astrocytes. However, the unique starlike shape of astrocytes with typically weak [Ca\textsuperscript{2+}]i intensity is discernible from the smaller, rounder, stronger-[Ca\textsuperscript{2+}]i intensity GCs.

Cell types and development in GCL. For the age range (P2–P5) studied, ~97–98% of the OB GCL is composed of GCs and the other 2–3% is made of primarily large, rotund, GABAergic Blanes cells. [The percentage of the Blanes cells to other types of OB cells diminishes down to 1–2% when the OB reaches adult size (Batista-Brito et al. 2008).] Thus approximately all of the Dlx2-labeled cells in the GCL are GCs.

The majority of Dlx2-labeled GCs shifted from immature cells that only responded to GABA at P2 to mature GCs that only responded to PACAP by P5 (Fig. 10A). The relatively rapid changes in maturation over the 3-day period may be affected by the migration of PACAP progenitors covering the distance to the OB in a matter of 3 days [in rats; this may happen in less time for mice, which are smaller and faster developing (Yang 2008)]. The survivorship of newly generated cells in the GCL may change with age, but the data in the work done by Lemasson et al. (2005) suggested that the density of surviving cells was unchanged between P3 and P7. This may mean that the survival of cells in the age range studied may have little to no factor in the shift. Dlx2 labels both GAD65 and GAD67, so GAD65 was evaluated as a subtype of GCs. Interestingly, GAD65-labeled GCs showed no significant shift from GABA-only to PACAP-only responsive cells (Fig. 10B). The significant shift from GABA-only to PACAP-only for Dlx\textsuperscript{2+} GCs may be the GAD67 cells, the results for which (Fig. 10C) are similar to the results for Dlx\textsuperscript{2+} GCs (Fig. 10A). The PAC1R+ GAD67 cells in general may need to mature within the first week, as they are multiglomerular neurons connecting to a broader complex network within the GCL, while GAD65 neurons have uniglomerular dendritic projections (Kiyokage et al. 2010; Parrish-Aungst et al. 2007). In the GAD65 cells, the ratio of GABA- to PACAP-responding cells may change outside the age range studied. The hypothesis for any future evaluation is that the change among GAD65 cells may occur somewhere between P7 and P14. Supporting this hypothesis are other studies suggesting that the OB reaches 80% of the average adulthood number of cells at P7 (98% for P14) (Lemasson et al. 2005; Mirich et al. 2002). Furthermore, norepinephrine modifies the OB response in neonatal pups, and the supply of this neurotransmitter from the locus coeruleus is reduced at P10 (Raineki et al. 2010). Supporting this hypothesis are other studies suggesting that the OB reaches 80% of the average adulthood number of cells at P7 (98% for P14) (Lemasson et al. 2005; Mirich et al. 2002). Furthermore, norepinephrine modifies the OB response in neonatal pups, and the supply of this neurotransmitter from the locus coeruleus is reduced at P10 (Raineki et al. 2010). In addition, Lopez-Bendito et al. (2004) showed an increase in GABA production in GAD65\textsuperscript{+} cells [indicating functional maturation between E18 (48%) and P21 (70%)]. Since we see no GAD65\textsuperscript{+} cells shift from immature excitatory GABA-responsive cells to mature GCs that only show excitatory responses to PACAP.

Fig. 10. A subtype of GCs, GAD67\textsuperscript{−}, has a developmental switch from immature to mature between P2 and P5 that parallels increases in PACAP responses. A: % of total Dlx2-labeled GCs significantly switches from predominantly migrating neuroblasts at P2 and P3 to predominantly mature GCs at P5 (P < 0.03); % of PACAP-responsive cells also increases. B: % of total GAD65-labeled GCs remains fairly constant between P2 and P5. C: % of total unlabelled GCs in GAD65 data (GAD67) switches from predominantly migrating neuroblasts at P2 to predominantly mature GCs at P5 (P < 0.05 to 0.01). Stimuli were 40 nM PACAP and 50 μM GABA.
between P2 and P5, the significant maturation of GAD65\(^+\) cells is expected to occur in the second to third postnatal week.

**Conclusions.** Cells that migrate into the OB during the first postnatal week initially have high excitatory responses to GABA and low responses to PACAP. However, even by P2 there are cells that are in all stages of functional maturation in terms of responding to both PACAP and GABA or only responding to PACAP. Our studies indirectly show that the cells mediating the shift in maturation profiles are the GAD65\(^-\) cells, which we assume are GAD67\(^+\) since that is the only other GAD-expressing interneuron in the OB. PACAP seems to initiate long-lasting \([\text{Ca}^{2+}]\) activity in the developing OB that is mainly amplified by glutamatergic pathways, but there is also a significant contribution from GABA. While it is still unclear as to the developmental role of PACAP in postnatal development, these studies clearly show that PAC1Rs become functional while the cells are still in the maturation process and remain functional after cells mature. The GCL network of GluRs and/or GABARs has an important role in modifying the \([\text{Ca}^{2+}]\) activity in both immature and mature GCs. The suggestions for future works are to better understand why GAD67 and GAD65 appear to have a majority shift from immature to mature cells at seemingly different time windows even through the subtypes develop and function during a similar time window. It is not as clear when most GAD65 cells will mature as it is as for GAD67 cells, but the GAD65 window appears not to be during the first neonatal week.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: M.I. and M.T.L. conception and design of research; M.I., A.G., and M.T.L. performed experiments; M.I., A.G., P.T., and M.T.L. analyzed data; M.I., A.G., and M.T.L. interpreted results of experiments; M.I., A.G., and M.T.L. prepared figures; M.I. and M.T.L. drafted manuscript; M.I., A.G., P.T., and M.T.L. edited and revised manuscript; M.I., A.G., and M.T.L. approved final version of manuscript.

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