PACAP MODULATION OF CALCIUM ION ACTIVITY IN DEVELOPING GRANULE CELLS OF THE MOUSE OLFATORY BULB

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Running Title: PACAP in Mouse OB

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Abstract

Ca\(^{2+}\) activity in the CNS is critical for the establishment of developing neuronal circuitry prior to and during early sensory input. In developing olfactory bulb (OB), the neuromodulators that enhance network activity are largely unknown. Here we provide evidence that PACAP-specific PAC1 receptors expressed in P2-P5 mouse OB are functional and enhance network activity as measured by increases in calcium in genetically identified granule cells. We used confocal Ca\(^{2+}\) imaging of OB slices from Dlx2-tdTomato mice to visualize GABAergic granule cells. To address whether the PACAP-induced Ca\(^{2+}\) oscillations were direct or indirect effects of PAC1R activation, we used antagonists for the GABA receptors (GABARs) and/or glutamate receptors (GluRs) in the presence and absence of PACAP. Combined block of GABARs and GluRs yielded a 68% decrease in the numbers of PACAP responsive cells suggesting that 34% of OB neurons are directly activated by PACAP. Similarly, immunocytochemistry using 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 which activate GCs. The appearance of PACAP-induced Ca\(^{2+}\) activity in immature GCs suggests a role for PACAP in GC maturation. To conclude, we find that PACAP has both direct and indirect effects on neonatal OB GABAergic cells and may enhance network activity by promoting glutamate and GABA release. Furthermore, the numbers of PACAP responsive granule cells significantly increased between postnatal day...
two and five suggesting PACAP-induced Ca\textsuperscript{2+} activity contributes to neonatal OB development.

Key Words

Olfaction, development, granule cell layer, PACAP
Introduction

Pituitary Adenylate Cyclase Activating Peptide (PACAP) is a potential candidate for neurogenesis therapy following injury or neurodegenerative disease due to its anti-apoptotic properties (Arimura, 1998; Atlasz et al., 2010; Bourgault et al., 2009; Chen et al., 2006; Cowan and Roskams, 2002; Dejda et al., 2005; Dejda et al., 2008; Delgado et al., 2003; Di et al., 2012; Doursout et al., 2013; Hansel et al., 2001; Kanekar et al., 2010; Makela et al., 2010; Mulder et al., 1999; Nakamachi et al., 2008; Ohtaki et al., 2006; Ohtaki et al., 2008; Ruan et al., 2012; Sherwood et al., 2007; Shioda et al., 2006; Szabadfi et al., 2012; Szabadfi et al., 2014; Vaudry et al., 2009; Yang, 2008; Brown et al., 2014).

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; Dejda et al., 2008; Delgado et al., 2003; Ravni et al., 2006; Sherwood et al., 2007; Vaudry et al., 2002). 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 (Raineiki 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; Kiss et al., 2006; Markhotina et al., 2007; Silveira et al., 2002; Szabadfi et al., 2012; Szabadfi et al., 2014; Denes et al., 2014), cerebellum (Allais et al., 2007; Botia et al., 2007; Falluel-Morel et al., 2007; Falluel-Morel et al., 2008; Jozwiak-Bebenista et al., 2007; Mei et al., 2004; Vaudry et al., 1998; Vaudry et al., 1999; Vaudry et al., 2000b; Vaudry et al., 2000a; Vaudry et al., 2002; Vaudry et al., 2003; Zhokhov et al., 2008), hippocampus (Ago et al., 2011; Costa et al., 2009; Di et al., 2003; Kambe and Miyata, 2012; Liu et al., 2003; Macdonald et al., 2005; Taylor et al., 2014), and suprachiasmatic nucleus (SCN) (Dziema and Obrietan, 2002; Irwin and Allen, 2010; Kopp et al., 1999; Kopp et al., 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 (sec) to rapid (msec) increases in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ). Following 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 dominate 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-
Within the olfactory system, the constant generation of new neurons is required to maintain normal function of both olfactory epithelium and the olfactory bulb (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 Brain Altas, 2013; Jaworski and Proctor, 2000; Shioda et al., 1997). Several studies have investigated the physiological effects of PACAP and PAC1Rs in the olfactory epithelium (Han and Lucero, 2005; Han and Lucero, 2006; Hansel et al., 2001; Hegg et al., 2003b; Hegg et al., 2003a). However, little is known about the physiological effects of PACAP in the OB (Olianas and Onali, 1999). Here we provide the first physiological recordings of PACAP-induced \([Ca^{2+}]_i\) 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 \(\mu\)m) granule cells (GCs), and the other 3% are the large (12–18 \(\mu\)m), 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 (Batista-Brito et al., 2008; Jaworski and Proctor, 2000; Allen Brain
Altas, 2013; Shioda et al., 1997).

In the following experiments, we examined the effects of PACAP on cells
in the GCL of P2 to 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 \([\text{Ca}^{2+}]\) 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 \([\text{Ca}^{2+}]\) in
the developing granule neurons within the olfactory bulb 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 GAD65-tdTomato). The changes in the population of
PACAP-responding cells within the 4 days evaluated provide clues about the first
week of PACAP activity in the developing OB.

Understanding the role of PACAP in modulating \([\text{Ca}^{2+}]\) in developing
neurons may provide important insight for mechanisms mediating the maturation
required for establishing sensory maps and neuronal circuits. In the future, this
understanding may be critical for promoting therapeutic integration of neuroblasts during early development, following injury, or in neurodegenerative disease.

Materials and Methods

Animals

All animal procedures were approved by the University of Utah Institutional Animal Care and Use Committee and were conducted under the guidelines of the NIH Guide for Care and Use of Laboratory Animals.

To visually identify immature and mature GABAergic GCs that originated as Dlx2 precursors from the SVZ, we used mice expressing the red tdTomato gene in cells that express Dlx2. Specifically, the defined Cre recombinase (IRES-CRE linked) transgenic C57/BL6 mice for Dlx2-IRES-CRE (gift from Dr. M. Capecchi, Salt Lake City, UT) was crossed with mice containing the Rosa-CAG-tdTomato gene (JAX stock #007914, Jackson Laboratory). The Dlx2-IRES-Cre mouse line was generated by inserting an IRES-Cre-FRT-neo-FRT cassette in the 3’ untranslated region of the Dlx2 gene. The targeting vector was prepared with 129S6/SvEvTac mouse genomic DNA previously isolated from the RPCI-22 BAC library (BACPAC Resources). The vector was electroporated in 129R1 embryonic stem (ES) cells, and successfully targeted clones were confirmed by Southern blotting. ES cells were injected in the blastocysts of C57Bl6 recipients to generate chimera founders. Positive offspring were bred to FLP delete mice to remove the neo selection marker, and the line was bred to homozygosity.

Automated genotyping service for this strain is available from Transnetyx (probes: “Dlx2-1 WT” and “Dlx2-1 KO”).
Additionally, the defined Cre recombinase (IRES-CRE linked) transgenic C57/BL6 mice for GAD65-IRES-CRE [JAX Stock #0101802; The Jackson Laboratory (Taniguchi et al., 2011)] and PCdh21 [The GENSAT Project; Stock #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-tdTomato gene (ROSA26-CAG promoter). The pups were checked for specific expression using 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 the OB

Live coronal slices of the OB (400 µM thick) were prepared between 11 AM to 12:30 PM [due to circadian changes in PACAP (Granados-Fuentes et al., 2006)] from P2–P5 mice (Hegg et al., 2003c). Briefly, following decapitation, the jaws, skin, and eyes were removed, the whole skull was embedded in carrot and sliced using a vibratome. 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 cerebral spinal 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 Ca\(^{2+}\) ACSF containing the membrane permeant Ca\(^{2+}\) 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 37ºC for an hour. The slices were used between 1 to 4 hours after cutting.

Confocal Calcium Imaging

Fluo-4 loaded slices were submerged in a Warner RC27 chamber. The gassed ACSF solution continuously flowed over the slices (1.5–3.0 ml/min). Test solutions were applied using 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-tdTomato). Time series experiments were performed collecting 256 x 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 to the edge of the mitral cell layer (MCL).

Immunocytochemistry

Immediately following decapitation, whole skulls of P2-P5 were prepared by removing skin, jaws, and eyes then fixed in 4% paraformaldehyde overnight at 4ºC. The heads were sequentially equilibrated with 15% and 30% sucrose prior to embedding in OCT compound and were stored at –80ºC. The 12 µm thick frozen sections were captured on glass slides using 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 minutes at 96°C, then cooled to room temperature and rinsed 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 ARP59945 (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 hour at room temperature. The slides were washed 2 x 5 minutes in PBS, once for 5 minutes in water with DAPI, and cover slipped. The tissue sections were imaged on a Zeiss Axio Imager microscope using Axiovision 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 hours.) 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⁺ (HK) solution. HK (95 mM NaCl, 50 mM KCl, 1 mM MgCl₂, 2 mM
CaCl₂, 10 mM HEPES, 10 mM Glucose, and 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 experiment day and kept on ice.

The dose response curve of PACAP included 1 nM, 10 nM, 20 nM, 40 nM, 60 nM, and 100 nM PACAP-27 with increasing washing times between each PACAP application to give the slices time to recover (>10 minutes after 10 nM, >20 minutes after 20 nM, >60 minutes after 40 nM, and >80 minute after 60 nM). To determine the possibility of partial desensitization between 40 nM and 60 nM, the order was reversed in a subset of slices.

An antagonist cocktail containing 5 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 SR95531 (GABAₐ; Tocris), 100 µM LY341495 (mGluR₁, ₂, ₃,₄,₅,₇,₈; Tocris), and 10 µM CGP52432 (GABAₐ; Tocris). The effectiveness of the antagonists was confirmed by blocking 50 mM L-glutamate or 50 µM GABA induced [Ca²⁺]ᵢ responses in our slice preparation (data not shown). PPADS (P2Yₑ and A₂ₐ; Tocris) was also originally included in the cocktail, but omitted for the present data due to its significant side effect of decreasing resting [Ca²⁺]ᵢ.
levels. In addition, Doengi et al. (2008) suggested that PPADS has no effect on [Ca^{2+}]_i responses in the OB. The expression of purinergic receptors in the OB is primarily in the astrocytes and olfactory ensheathing cells [personal observations using SR101 astrocyte-labeling dye (Doengi et al., 2008; Doengi et al., 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 Brain Atlas, 2013; Thyssen et al., 2013; Doengi et al., 2008).

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 to baseline (%ΔF/F). The BSA control was used to identify cells with unstable baselines (125/1502 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 curve (120 recorded seconds of response), and amplitudes (Fig. 1). We considered measuring response durations, but these were difficult to measure at higher PACAP concentrations due to some post-PACAP repetitive oscillations lasting tens of minutes. To correct for the lag time between initiating the loop injection and maximal stimulus delivery to the cells, the time between the start of loop injection to start of HK responses (average of 14.4 ± 1.4 secs; n=48) was subtracted from each PACAP trace. To obtain the area under curve (Ca^{2+} flux), Origin 6.0 was used to measure and subtract a baseline from the
data. After baseline subtraction, the area under the curve (AUC) of $\Delta F$ from the start of the response to 120 secs was calculated using GraphPad Prism 5.

All cells that were counted as PACAP responding cells met the following three conditions: Firstly, the PACAP-induced [Ca$^{2+}$]$_i$ activity showed an amplitude increase of >5% above the baseline noise and a duration of >50 secs. Secondly, the PACAP response began at or after the average latency for HK. Thirdly, the PACAP response started within the range of the HK duration (100 - 120 secs), which is the approximate duration the antagonists would be on the tissue. For the experiments involving antagonists, which might block PACAP responses in individual cells, one more condition was met; the HK was applied before and after each PACAP plus/minus 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 to 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.
counts were done blind to treatment and averaged across slices. Only one slice was used per pup. The total number of cells analyzed and n (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-ANOVA) 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\(^{2+}\)] 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 predominate response was a sustained increase in [Ca\(^{2+}\)] that reached a peak within seconds and then slowly returned to baseline over the course of minutes (796/1413 cells, 56%; Fig. 2C). The second most common response to PACAP was a single transient [Ca\(^{2+}\)] increase) that returned to baseline within the ~120 secs duration of the HK response (305/1413 cells, 22%; Fig. 2, D and E). Of the remaining 22% of PACAP responsive cells, the majority developed a slow “saw tooth” post-PACAP [Ca\(^{2+}\)] oscillation (0.01–0.04 Hz, 246/1413 cells, 17%; Fig. 2A) and a few cells developed a fast “saw tooth” oscillation (0.05–0.1 Hz, 66/1413 cells, 5%; Fig. 2B). Kopp et al. (1999) found somewhat similar
percentages for PACAP responses in cultured SCNs: single transients (20%), [Ca\textsuperscript{2+}] oscillations (15%) and “sustained oscillations” (called biphasic response with initial [Ca\textsuperscript{2+}], followed by plateau phase; 65%) (Kopp et al., 1999). PACAP-induced [Ca\textsuperscript{2+}] oscillations lasted between 5 to 20-plus minutes (Fig. 2, A and B).

Although the responses to PACAP were heterogeneous with cells in close proximity showing very different response profiles 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, the developmental time course, and identified responding cell types within the GCL region of OB.

**PACAP Concentration-Response Curve**

Dickson et al. (2006) tested the dose response curve of PACAP, VIP, and their antagonists using the cultured CHO cell line by measuring both the intracellular cAMP and Ca\textsuperscript{2+} concentrations (Dickson et al., 2006). They reported an EC\textsubscript{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 to 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 (Jaworski and Proctor, 2000; Allen Brain Altas, 2013). For the EC\textsubscript{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 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 healthy neurons in the GCL. For a slice to be deemed “healthy” we looked for approximately 80% of the visible cells to respond to HK. Thus, the dose response curve was based on the normalized percentage of PACAP responsive cells relative to the number of HK responsive cells recorded within 10 minutes of each PACAP application. We found that the percentages of PACAP responsive cells significantly increased with concentration. Although the curve did not saturate, we did not use concentrations higher than 100 nM PACAP because the responses to 100 nM PACAP did not desensitize and were not blocked with PAC1R antagonists (personal observations).

Dickson et al. (2006), who used cultured CHO cells, did EC\textsubscript{50} curves for a variety of PACAPs, VIPs, and their antagonists/agonists (Dickson et al., 2006). Interestingly, even though the CHO-cells PACAP [Ca\textsuperscript{2+}]\textsubscript{IC\textsubscript{50}} was 3–5 nM, blocking about 80% of the 30 nM PACAP [Ca\textsuperscript{2+}]\textsubscript{IC\textsubscript{50}}, activity required 10 µM M65 [a specific PAC1R antagonist from sand flies, (Yu et al., 2008)]. We found that a cocktail of 1 µM M65 and 150 nM PACAP 6-38 [a truncated version of PACAP which competitively binds the receptor (Robberecht et al., 1992)] was able to
block an average of 84 ± 4% 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).

Kinetics 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 using 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 to P5 (data not shown). Thus, the slices were pooled together regardless of age between P2 to P5 for the kinetic analyses.

We measured the kinetics and amplitude of the PACAP-induced [Ca^{2+}]_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 to 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 Ca\textsuperscript{2+} signaling, the area under curve or Ca\textsuperscript{2+} flux (ΔF-seconds) was measured for the first 120 secs after PACAP reached the slice (as determined by HK application). Figure 4D shows a significant increase in Ca\textsuperscript{2+} flux with increasing PACAP concentrations with 100 nM PACAP having the highest Ca\textsuperscript{2+} 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; 51/124 cells) and 20 nM (only 48% oscillating cells; n=19; 134/277 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 Versus Indirect PACAP Effects**

The observation of a larger response amplitude at 40 nM as compared to 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 [Ca\(^{2+}\)]\(_i\) were causing PAC1 receptor expressing mitral and granule cells 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 the Methods. The first cocktail (Glu/GABA/Na) was designed to block all neurotransmission and any other possible pathways that may elevate the [Ca\(^{2+}\)]\(_i\) in interneurons. To ensure that neurotransmission was blocked prior to PAC1 receptor activation during co-application of the cocktail and PACAP, we examined the time course of control application of the antagonists. Out of 119 cells (n=3) only 10 post-PACAP cells had calcium activity preceding control application of Glu/GABA/Na antagonists. Application of the Glu/GABA/Na cocktail eliminated all calcium activity in all 10 cells within 12.5 ± 4.0 secs (see example cell in Fig. 5A).

The Glu/GABA/Na cocktail contains antagonists for blocking NMDA, AMPA, mGluR (all types), GABA\(_A\), and GABA\(_B\) receptors. 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 using the same approach as for PACAP at different concentrations. Only the latency of the PACAP response was not significantly changed by the co-application of the antagonist cocktail (36.3 ± 3.9 secs pre and 36.9 ± 5.4 secs during antagonist + PACAP, Fig. 6A). Amplitude (p = 0.01, paired t-test), time to half-peak (p < 0.01, paired t-test), and the area under curve (p < 0.001, paired t-test) were significantly reduced in the presence of the antagonist cocktail, suggesting involvement of GluRs and GABARs activation in the [Ca^{2+}]_i response to PACAP even within cells directly activated by PACAP (Fig. 6B-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 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 glutamate receptors (GluRs) versus GABA receptors (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\textsubscript{A} and GABA\textsubscript{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 middle bar). GABAR-only antagonists reduced PACAP responses to 54 ± 11% of the PACAP control (129 cells, n = 4; Fig. 5 right bar). 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 to P5. It is known that the interneuron precursors (neuroblasts) take about 3 days to migrate from their origin in the subventricular zone (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).

In order 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-tdTomato (using the ROSA26-
CAG promoter) transgenic mice line to provide gene-specific red cells. The ROSA26-CAG promoter was used to label IRES-CRE-linked *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 olfactory bulb, 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 lifespan. The *Dlx2*-tdTomato is expected to label the majority of GCs residing in the GCL (Potter et al., 2009). Figure 7A-E shows the expression of *Dlx2*-tdTomato (red) in 12 µm sections of OB from P2 and P4 mice. PAC1R-specific antibody labeling (green) co-localizes with a subset of *Dlx2* expressing cells in the GCL and labels PCdh21-tdTomato+ mitral cells in the MCL (Fig. 7F-H). Compared to total cell counts using DAPI nuclear staining, 89% (1945; n = 2 pups) of GCL cells are *Dlx2*+ and 21 ± 1% (3160 *Dlx2*+ cells; n = 3 pups) are co-labeled with PAC1R antibodies at P2, while in the P4 OB, the average percentage of cells co-labeled with both *Dlx2*+ and PAC1R antibody increases to 34 ± 1% (2979 *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 mice 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-week old mice, about 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 67 (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 both subtypes enter the OB at similar developmental stages (Plachez and Puche, 2012; De et al., 2007; Kelsch et al., 2007; Merkle et al., 2007). The Dlx2 and GAD65 transgenic lines were used for the identification of GABAergic GCs during the PACAP versus 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 \([\text{Ca}^{2+}]_i\) 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 internal \([\text{Ca}^{2+}]_i\) in response to non-synaptically released GABA as they migrate over long distances to their destinations (Dzhala et al., 2010; Tong et al., 2009; Darcy and Isaacson, 2009; Mejia-Gervacio et al., 2011; Wang and Kriegstein, 2008). GABA binds to ionotropic GABA\(_A\) receptors and the cell will either depolarize or hyperpolarize, depending on intracellular Cl\(^-\) concentration. Immature neurons maintain high intracellular Cl\(^-\) levels via activity of highly expressed Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporter 1 (NKCC1) transporting Cl\(^-\) into the cell (Dzhala et al., 2010; Takayama and Inoue, 2004; Glykys et al., 2009; Wang et al., 2005). Thus GABA\(_A\) receptor activation of migrating neuroblasts and
immature neurons causes Cl\(^-\) to exit the cell. The resulting Cl\(^-\) efflux depolarizes the cell and opens voltage-gated Ca\(^{2+}\) channels (Glykys et al., 2009). In mature neurons, NKCC1 is down regulated and internal Cl\(^-\) is kept low with the increased expression of K\(^+\)-Cl\(^-\) cotransporter 2 (KCC2), so opening of GABAA channels hyperpolarizes the cell. Thus immature neurons can be physiologically distinguished from mature neurons by [Ca\(^{2+}\)]\(_i\) responses to GABA (Wang and Kriegstein, 2008). Figure 9A shows an example of a functionally immature cell responding to 50 µM GABA with an increase in [Ca\(^{2+}\)]\(_i\).

Based on Ca\(^{2+}\) responses to sequential application of GABA and PACAP (Fig. 9B), 3 groups of cells were identified in the GCL of neonatal OB. The first group is the “GABA-only” GCs, which respond to GABA and not PACAP (Fig. 9C). The GABA-only GCs may or may not become PAC1R\(^+\) when mature. The ‘GABA+PACAP’ group in Fig. 9C includes the GCs that respond to both GABA and PACAP with increases in [Ca\(^{2+}\)]\(_i\), implying that they are immature cells with either expression of PAC1Rs on the plasma membrane or some early connections formed. The “PACAP-only” group in Fig. 9C includes the presumably mature GCs that respond to PACAP but do not increase [Ca\(^{2+}\)]\(_i\) in response to GABA. Both transient increases and repetitive oscillations in [Ca\(^{2+}\)]\(_i\) activity are seen for PACAP-responsive cells in the GABA+PACAP and PACAP-only groups (Fig. 9C).

Age-related Increases in the Percentage of PACAP Responsive Granule Cells

Figure 10A shows the calculated percentage of all GABA and/or PACAP responsive cells labeled red for Dlx2. The graph shows a shift in the percentage
of immature GABA-only cells at P2 (n = 8 mice; 867 cells) to mature PACAP-only
cells at P5 (n = 5 mice; 967 cells). This shift suggests that GCs express
functional PAC1 receptors during their maturation process. The non-red cell
counts in the images taken from Dlx2-tdTomato mice made up 6% (165/2708
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
unlabeled population. The other 3–4% of unlabeled responsive cells might be
other non-Blanes interneurons in the GCL. (The glial cells such as astrocytes
are not a factor in the analysis, please 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 non-red cells (GAD65⁻), which we assume
to be GAD67 cells since all of the interneurons in the GCL are either GAD65⁺ or
GAD67⁺ or both GAD65⁺ and GAD67⁺ (Batista-Brito et al., 2008; Lemasson 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. Out 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
GABA 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 PAC1 receptors are functional during development of GABAergic neurons.

Discussion

In the studies described above, we found that PACAP is capable of eliciting [Ca^{2+}] responses in cells at various stages of maturation in the GCL of the olfactory bulb in P2–P5 mice. We found that the PACAP-induced [Ca^{2+}] changes were heterogeneous and dependent on both direct activation of PAC1 receptors 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 immunocytochemistry. 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 $[\text{Ca}^{2+}]_i$ oscillations during the first postnatal week of development.

The OB interneuron response to PACAP in acute slices shows that the majority of the $[\text{Ca}^{2+}]_i$ activity occurs through network activation of both the glutamate and GABA receptors on non-PAC1$^+$ GCs, providing a wide variety of $[\text{Ca}^{2+}]_i$ activity responses ranging from single transients, slow to fast saw tooth 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 glutamate and/or GABA receptors (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 $[\text{Ca}^{2+}]_i$ signal are blocked supports the idea that PAC1R activation initiates a $[\text{Ca}^{2+}]_i$ 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 $\text{Ca}^{2+}$ kinetics and intensities during 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 $\text{Ca}^{2+}$ 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 ones.

Dose Dependence and Block of PACAP Responses

The reported EC₅₀ for PACAP responses ranges between 0.25 and 3 nM in cell culture systems, and increases to 30–50 nM PACAP in acute slices (Jozwiak-Bebenista et al., 2007; Kambe and Miyata, 2012; Nicot and Dicicco-Bloom, 2001; Dickson et al., 2006). 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; Kopp et al., 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, Jozwiak-Bebenista et al. (2007) measured a PACAP EC₅₀ ~0.25 nM for both primary cultured neuron and astrocyte cells. They also tested acute cerebral cortical slices, which are PAC1R⁺ rich compared to the majority of the CNS apart from the OB and obtained an EC₅₀~30 nM (Jozwiak-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 using only PACAP 6-38 (PACAP 38 with first 6 peptides deleted). Other studies used between 1 to 10 nM PACAP in the cultures and showed block using only PACAP 6-38 (Kambe and Miyata, 2012; Shioda et al., 2006; Vaudry et al., 2002). The doses of PACAP 6-38 were reported to be 600 to 1000 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 due to 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 (Kambe and Miyata, 2012; Pugh et al., 2010; Doan et al., 2012a; Doan et al., 2012b). The role of GluRs and/or GABARs on the PACAP-induced Ca^{2+} oscillations was individual and independent. The 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\(^{2+}\) responses are significantly reduced in terms of amplitude, time to half-peak, and total Ca\(^{2+}\) 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\(_{50}\) of PACAP in cultured glial cells (astrocytes) is almost exactly the same as for cultured neuronal cells. However, when we used SR-101 (a red dye labeling astrocytes) on slices, we find astrocytes to be star-like shaped cells appearing as “background” and having little to no [Ca\(^{2+}\)] response to 40 nM PACAP, suggesting that the astrocytes in OB slices have a different physiology than in cultures and/or higher EC\(_{50}\) than the surrounding OB interneurons. The concentration of GABA used in our experiments was 50 µM. Doengi et al. (2009) used [Ca\(^{2+}\)] signaling to determine the GABA EC\(_{50}\) of 35 µM in neurons and 100 µM in astrocytes with a few astrocytes responding to 20 µM GABA (Doengi et al., 2009). Accordingly, our 50 µM GABA may excite a small number of astrocytes. However, the unique star-like shape of astrocytes with typically weak [Ca\(^{2+}\)] intensity is discernible from the smaller, rounder, stronger [Ca\(^{2+}\)] intensity GCs.

**Cell Types and Development in GCL**

For the age range (P2 to P5) studied, about 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 Dlx-2 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 respond to PACAP by P5 (Fig. 10A). The relatively rapid changes in maturation over the 3-day period may be affected by the migration of SVZ 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 to P7 (Lemasson et al., 2005). This may mean 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 Dlx2+ GCs may be the GAD67 cells, the results for which (Fig. 10C) are similar to the results for Dlx2+ 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 GAD65s 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 about 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). In addition, Lopez-Bendito et al. (2004) showed an increase in GABA production in GAD65+ cells [indicating functional maturation between E18 (48%) and P21 (70%) (Lopez-Bendito et al., 2004)]. Since we see no GAD65+ cells shift from immature excitatory GABA responsive cells to mature GCs that only show excitatory responses to PACAP between P2 and P5, the significant maturation of GAD65+ cells is expected to occur in the second to third postnatal week.

**Conclusion**

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 [Ca2+]i 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 PAC1 receptors 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 modulating the $[Ca^{2+}]_i$ 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 both 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 to not be during the first neonatal week.

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**Figure Captions**

**Fig. 1:** The PACAP induced $[\text{Ca}^{2+}]_i$ transient was analyzed for latency, time to half-peak, amplitude, and net $\text{Ca}^{2+}$ flux (area under curve for the first 120 seconds of PACAP responses). All of the PACAP response latencies were measured relative to the average latency of elevated potassium (HK) responses.

**Fig. 2:** PACAP-induced $\text{Ca}^{2+}$ responses in neonatal OB are heterogeneous. A time series of confocal images from a Fluo4-loaded slice of P4 mouse OB shows the changes in $[\text{Ca}^{2+}]_i$ in response to 40 nM PACAP. The traces in (A-D) reflect the $\%\Delta F/F$ changes over time for the regions of interest (ROIs) marked A-D. The dashed lines match the images to the time points on the traces. The PACAP-induced calcium oscillations of 1413 cells (from 37 pups) were categorized into four main groups: (A) Slow sawtooth oscillation, 17% of cells; (B) Fast sawtooth oscillation, 5% of cells; (C) Sustained response, 56% of cells; (D) Single transient (returns to baseline within 100 seconds), 22% of cells. (E) The HK (50 mM $K^+$) is used to confirm the vitality of the cells and (F) 0.1% BSA, is the PACAP vehicle control. Scale bar is 20 µm.

**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) A 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) The percentage of cells responding to 40 nM PACAP is reduced by $84 \pm 4\%$ in the presence of PAC1R antagonists (1 µM M65...
and 150 nM PACAP 6-38). Numbers near data points indicate number of pups, then the number of PACAP-responsive cells analyzed.

**Fig. 4:** The kinetic and fluorescence intensity responses to increasing PACAP concentrations are dose-dependent. (A) The Ca\(^{2+}\) response latency significantly decreased between 10 nM and 100 nM PACAP\(^{27}\). (B) The time to half-peak showed no significant change. (C) Amplitude did not follow a classic dose-response curve, with 40 nM PACAP having a significantly larger amplitude than 100 nM PACAP. (D) The Ca\(^{2+}\) flux measured during the first 120 secs increased significantly with increased PACAP concentrations. Numbers near the data points indicate number of pups, then number of cells analyzed; One-Way Kruskal-Wallis ANOVA (KW-ANOVA).

**Fig. 5:** PACAP directly and indirectly activates GC neurons. (A) The time course of the Glu/GABA/Na antagonists is similar to HK and much faster than activation of PAC1 responses. The open box is the latency to HK response; the solid boxes indicate the wash on and off of antagonists and HK. The cell shown was indirectly activated by PACAP. (B) The bar graph shows the average % of PACAP responding cells in each antagonist treatment relative to PACAP control. Blocking neurotransmission and receptors, especially glutamatergic receptors significantly reduces the average percentages of PACAP responsive cells compared to matched vehicle controls. The numbers above the graph indicate pups and number of cells responding in PACAP control.

**Fig. 6:** The cells directly activated by PACAP have faster smaller responses compared to 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. The antagonists significantly reduced the (B) time to half-peak (63% of control), (C) amplitude (71% of control), and (D) area under curve (66% of control) (paired t-test). Numbers above the bar indicate number of pups, then number of cells analyzed.

**Fig. 7:** PAC1R$^+$ expression is enriched throughout the GCL and MCL of the OB. OB from P2 (A-C) and P4 (D-F) Rosa tdTomato IRES-CRE-Dlx2 (red) was stained with anti-PAC1R antibodies (green). The black and white pictures are the red or green channels, showing the labeling separately. The region indicated by the yellow box in P4 is shown at higher magnification. Scale bars are 100 µm for P2 and P4. (G) The scale bars in the zoom-ins of the magnified P4 are 20 µm. (H) A P4 tdTomato IRES-CRE-PCdh21 (red) OB tissue section was stained with anti-PAC1R antibodies (green). The arrows indicate PCdh21$^+$ mitral cells expressing PAC1 receptors. The blue nuclear stain is the DAPI. Scale bar is 50 µm. Abbreviations: glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL).

**Fig. 8:** Model for functional identification of immature neurons. Migrating or immature neurons have elevated [Cl$^-$]i due to high expression of the Na$^+$-K$^+$-Cl$^-$ cotransporter 1 (NKCC1). When GABA opens the GABA$\alpha$R, the resulting Cl$^-$ efflux depolarizes the cell. The GABA-mediated depolarization opens the voltage-gated Ca$^{2+}$ channels and results in calcium influx. Thus immature Dlx2$^+$ GCs show GABA-mediated Ca$^{2+}$ responses while mature Dlx2$^+$ GCs do not.
**Fig. 9: Physiological identification of immature and mature GCs.** (A) Image of the Fluo-4 loaded (green) Dlx2\(^+\) (red) cell responding to 50 µM GABA. Scale bars in images are 10 µm and 100 µm. (B) HK was tested at the start and end of each experiment to confirm the cells are healthy throughout. BSA and GABA were tested before PACAP to avoid false positives due to possible post-PACAP activity. Each set of runs takes about 20 minutes. (C) Sample recordings for each of three developmental stages of GCs with either PACAP-induced single transients (left column) or \([\text{Ca}^{2+}]_i\) oscillations (right column). GABA only group = immature, PACAP + GABA = maturing, and PACAP-only = mature Dlx2\(^+\) cells.

**Fig. 10: A subtype of GCs, GAD67\(^-\), has a developmental switch from immature to mature between P2 to P5 that parallels increases in PACAP responses.** (A) The percentage of total Dlx2-labeled GCs significantly switch from predominately migrating neuroblasts at P2 and P3 to predominately mature GCs at P5 (p < 0.03). The percentage of PACAP responsive cells also increases. (B) The percentage of total GAD65-labeled GCs remains fairly constant between P2 to P5. (C) The percentage of total unlabeled GCs in GAD65 data (GAD67) switch from predominately migrating neuroblasts at P2 to predominately mature GCs at P5 (p < 0.05 to 0.01). Stimuli were 40 nM PACAP and 50 µM GABA.
Figure 1

- **Amplitude (of first peak)**
- **Time to ½ Peak**
- **PACAP Latency**
- **Area Under Curve (120 secs)**
- **(120 seconds duration)**

- **Start**
- **Ave HK Latency**
Figure 2

At 60 secs

At 94 secs

At 100 secs

A

17% of cells

B

5% of cells

C

56% of cells

D

22% of cells

E

F

500% ΔF/F

100 secs
Figure 3

A

1st 40 nM PACAP

2nd 40 nM PACAP

Intensity (%ΔF/F)

Time (secs)

B

C

# PACAP-responsive cells/

#HK-responsive cells

[ PACAP 27] nM

0.0 0.1 0.2 0.3 0.4 0.5


5;258

5 pups;

278 cells

* p < 0.05

Percentage of PACAP 27

responsive cells

40 nM PACAP 27

control

40 nM PACAP 27

in 1 uM M65 + 150 nM

PACAP 6-38 antagonists

0 20 40 60 80 100
Figure 4

A. Latency

Time (seconds)

<table>
<thead>
<tr>
<th>[PACAP 27] nM</th>
<th>10</th>
<th>15.8</th>
<th>25.1</th>
<th>39.8</th>
<th>63.1</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.39</td>
<td>10;100</td>
<td>13;345</td>
<td>13;380</td>
<td>4;348</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p<0.0001 KW-ANOVA

B. Time to 1/2 Peak

Time (seconds)

<table>
<thead>
<tr>
<th>[PACAP 27] nM</th>
<th>10</th>
<th>15.8</th>
<th>25.1</th>
<th>39.8</th>
<th>63.1</th>
<th>100</th>
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<td>13;345</td>
<td>13;380</td>
<td>4;348</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p=0.06 KW-ANOVA

C. Amplitude

Intensity (%ΔF/F)

<table>
<thead>
<tr>
<th>[PACAP 27] nM</th>
<th>10</th>
<th>15.8</th>
<th>25.1</th>
<th>39.8</th>
<th>63.1</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.39</td>
<td>10;100</td>
<td>13;345</td>
<td>13;380</td>
<td>4;348</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p=0.003 KW-ANOVA

D. Area under curve

Ca^2+ Flux (ΔF-secs; up to 120 seconds)

<table>
<thead>
<tr>
<th>[PACAP 27] nM</th>
<th>10</th>
<th>15.8</th>
<th>25.1</th>
<th>39.8</th>
<th>63.1</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>2;33</td>
<td>4;142</td>
<td>5;186</td>
<td>5;265</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p<0.0001 KW-ANOVA
Figure 5

(A) Graph showing the effects of different intervals on cell activity. PACAP, Glu/GABA/Na blockers, and HK are indicated with arrows.

(B) Bar graph showing the percentage of 40 nM PACAP-responsive cells not blocked in each treatment condition. The data are presented as follows:
- 40 nM PACAP in Glu/GABA/Na blockers: 34 ± 6%
- 40 nM PACAP in Glu blockers: 26 ± 3%
- 40 nM PACAP in GABA blockers: 54 ± 11%
Figure 6

(A) Latency: 4.46 seconds.

(B) Time to 1/2 Peak: 4.46 seconds.

(C) Amplitude: Intensity (%ΔF/F) 150, 4.46.

(D) Area under curve: Ca²⁺ Flux (ΔF/sec; up to 120 seconds) 8000, 4.46.

40 nM PACAP

40 nM PACAP in Glu/GABA/Na blockers

** = p ≤ 0.01  *** = p = 0.0008
Figure 7

Overlap of PAC1R+ with dt-Tomato IRES-CRE-Dlx2

Red channel (dt-Tomato IRES-CRE-Dlx2)

Green channel (PAC1R+)

P2

A GCL MCL EPL GL

D GCL MCL EPL GL

G

B

E

H GCL MCL EPL GL

C

F

Figure 7
\[ \text{NKCC1} \rightarrow [\text{Cl}^-]_i \rightarrow [\text{Ca}^{+2}]_i \]

**Figure 8**
Series of runs in a set:
1—50 mM HK
2—0.01% BSA control
3—50 μM GABA + 0.01% BSA
4—40 nM PACAP 27 + 0.01% BSA
5—50 mM HK

Completed in ~20 minutes

- blue = PACAP
- pink = GABA

Figure 9
Figure 10

A

Ratio of all PACAP and GABA responding Dlx2 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>8</td>
<td>867 cells</td>
</tr>
<tr>
<td>P3</td>
<td>3</td>
<td>306 cells</td>
</tr>
<tr>
<td>P4</td>
<td>4</td>
<td>568 cells</td>
</tr>
<tr>
<td>P5</td>
<td>5</td>
<td>967 cells</td>
</tr>
</tbody>
</table>

- GABA-only
- GABA and PACAP
- PACAP-only

*

B

% of all PACAP and GABA responding GAD65 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>3</td>
<td>400 cells</td>
</tr>
<tr>
<td>P3</td>
<td>9</td>
<td>1296 cells</td>
</tr>
<tr>
<td>P4</td>
<td>4</td>
<td>323 cells</td>
</tr>
<tr>
<td>P5</td>
<td>8</td>
<td>965 cells</td>
</tr>
</tbody>
</table>

- GABA-only
- GABA and PACAP
- PACAP-only

**

*

C

% of all PACAP and GABA responding non-GAD65 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>3</td>
<td>41 cells</td>
</tr>
<tr>
<td>P3</td>
<td>9</td>
<td>105 cells</td>
</tr>
<tr>
<td>P4</td>
<td>4</td>
<td>45 cells</td>
</tr>
<tr>
<td>P5</td>
<td>8</td>
<td>67 cells</td>
</tr>
</tbody>
</table>

- GABA-only
- GABA and PACAP
- PACAP-only

**

*

* *