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Differential Modulation of N-Type Calcium Channels by μ -Opioid Receptors in Oxytocinergic Versus Vasopressinergic Neurohypophysial Terminals

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Abstract

Opioids modulate the electrical activity of magnocellular neurons (MCN) and inhibit neuropeptide release at their terminals in the neurohypophysis. We have previously shown that μ -opioid receptor (MOR) activation induces a stronger inhibition of oxytocin (OT) than vasopressin (AVP) release from isolated MCN terminals. This higher sensitivity of OT release is due, at least in part, to the selective targeting of R-type calcium channels. We now describe the underlying basis for AVP's weaker inhibition by MOR activation and provide a more complete explanation of the complicated effects on neuropeptide release. We found that N-type calcium channels in AVP terminals are differentially modulated by MOR; enhanced at lower concentrations but increasingly inhibited at higher concentrations of agonists. On the other hand, N-type calcium channels in OT terminals were always inhibited. The response pattern in co-labeled terminals was analogous to that observed in AVP-containing terminals. Changes in intracellular calcium concentration and neuropeptide release corroborated these results as they showed a similar pattern of enhancement and inhibition in AVP terminals contrasting with solely inhibitory responses in OT terminals to MOR agonists. We established that fast translocation of Ca^{2+} channels to the plasma membrane was not mediating current increments and thus, changes in channel kinetic properties are most likely involved. Finally, we reveal a distinct Ca-channel β -subunit expression between each type of nerve endings that could explain some of the differences in responses to MOR activation. These results help advance our understanding of the complex modulatory mechanisms utilized by MORs in regulating presynaptic neuropeptide release.

The release of oxytocin and vasopressin from presynaptic nerve endings regulates several physiological functions; OT being primarily important in processes related to parturition (Russell et al., 2003) and AVP controlling diuresis and homeostasis (Bourque, 1998; Sharif-Naeini et al., 2008). Neuroendocrine function in the Hypothalamic Neurohypophysial System (HNS) is modulated by opioids, either acting at the MCN cell body level (Soldo and Moises, 1998; Brown et al., 2000) or at their nerve endings in the neurohypophysis (NH) (Rusin et al., 1997; Ortiz-Miranda et al., 2003). Interruption of oxytocin release by the administration of opiate drugs disrupts parturition (Leng et al., 1987, 1988; Bicknell et al., 1988b) and inhibits the milk ejection reflex during lactation (Clarke et al., 1979; Hartman et al., 1987; Bicknell et al., 1988a). Water and salt balance are also affected by central or peripherally administered opioids acting on MCN cell bodies (Slizgi and Ludens, 1982; Hartman et al., 1986; Oiso et al., 1988; Pumford et al., 1993; Paronis et al., 1995).

Even though direct effects of opioids on neuropeptide release from isolated nerve terminals had been reported by a number of studies (Summy-Long et al., 1984; Zhao et al., 1988; Dayanithi et al., 1992; Kato et al., 1992), the effects of μ -opioid receptor activation at the MCN's nerve endings had been more controversial (Nordmann et al., 1986). Previous work from our laboratory have shown that neuropeptide release and calcium currents from isolated terminals (Ortiz-Miranda et al., 2003) are unequivocally inhibited by the μ -opioid agonist, DAMGO ([D-Ala², MePhe⁴, Glyol⁵] enkephalin), in a dose-dependent manner. However, compared to AVP, oxytocin release shows a much higher sensitivity to inhibition, both in isolated terminals (Ortiz-Miranda et al., 2003) as well as in the intact system (Lemos et al., 2002).

As direct regulators of neurotransmitter release, presynaptic voltage-gated Ca^{2+} channels are a frequent target for opioids in the central and peripheral nervous systems (Grudt and Williams, 1995). In the rat neurohypophysis, OT and AVP release is mediated by three out of four different subtypes of calcium channels depending on the type of peptide expressed by the terminal (Wang et al., 1997, 1999). N-type and L-type calcium channels seem to contribute to the release of AVP and OT equally. On the other hand, the R-type appears to contribute exclusively to OT release (Wang et al., 1999), while the P/Q-type only mediates AVP release (Wang et al., 1997). We have previously shown that R-type channels are particularly sensitive to inhibition by DAMGO (Ortiz-Miranda et al., 2005), providing a reasonable explanation for OT's preferential inhibition by μ -opioid agonists. Here we demonstrate that N-type channels are also important contributors to the disparity in sensitivity to MOR agonists shown by OT and AVP release. AVP's lack of sensitivity seems to be, in great part, mediated by the N-type Ca-channel's opposite responses at low versus high concentrations of MOR agonists.

Materials and Methods

Ethical approval

Rats were sacrificed as described below, using a method that causes minimum distress or discomfort, and is consistent with recommendations provided by the Panel on Euthanasia of the American Veterinary Medical Association. Rats were maintained and used in accordance with the Guide for the Care and Use of Laboratory Animals, created by the Institute of

Animal Resources, National Research Council, DHHS, NIH and the protocol was approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Isolation of nerve endings

Male Sprague–Dawley rats (Taconic Farms, Germantown, NY) weighing 200–250 g were sedated using CO₂ and immediately decapitated (Cazalis et al., 1987). Following removal of the anterior and intermediate lobes of the pituitary, the neural lobe was dissociated in a buffer at 37°C that contained (in mM): sucrose, 270; EGTA, 0.01; HEPES-Tris (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid-tris(hydroxymethyl)aminomethane), 10, buffered at pH 7.3; 295–300 mOsm/L. For electrophysiological, [Ca²⁺]_i measurements and immunolabeling experiments, the homogenate was placed directly into plastic Petri dishes and washed initially with a Low (3 μM free Ca²⁺) Locke's saline containing (in mM): NaCl 140; KCl 5; CaCl₂ 1.9; EGTA (ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid) 2; HEPES 10; Glucose 10; MgCl₂ 1.2; pH 7.3; 295–300 mOsmol/L, and slowly changed to a Normal (2.2mM CaCl₂; no EGTA) Locke's to remove any floating debris. For peptide release measurements, the dissociated neural lobe was centrifuged at 100g for 1 min, with the supernatant spun at 2,400g for 3 min. The resulting pellet, containing highly purified terminals, was resuspended in Normal Locke's saline and loaded onto filters (Acrodisc, LC13, 0.45 μm, Gelman Scientific, Ann Arbor, MI) where they were perfused for 1 h with Locke's buffer.

Peptide hormone release experiments

After 1 h of perfusion with Locke's saline, the terminals were perfused for another 10 min with 50mM K⁺ in the presence or absence of the drug being tested. The solutions containing elevated K⁺, to stimulate active release from the terminals, were prepared by replacing an equimolar amount of NaCl with 50mM KCl. High K⁺ solution contained (in mM): NaCl 90; KCl 55; MgCl₂ 1.2; CaCl₂ 2.2; glucose 10; HEPES-Tris 10; pH 7.2; at 37°C, and the osmolarity of the buffer was 295–300 mOsm/L. The solutions containing opioids were prepared just before they were needed. The eluate containing the released AVP and OT from the terminals was collected in 200 μl fractions and was assayed by radioimmunoassay using specific antibodies (kindly supplied by Dr. R.J. Bicknell, Babraham Institute, Cambridge, UK). AVP and OT antisera were raised in sheep (the AVP antiserum was raised by Dr. Bicknell; the OT antisera by Drs. Sheldric and Flint), and the final antibody dilution was 1:100,000 for AVP and 1:25,000 for OT. The cross-reactivity of OT antiserum with AVP was 0.015%, whereas that of the AVP antiserum with OT was 0.001%. The sensitivities of the assays were 0.5 pg (AVP) and 1pg (OT). The inter- and intra-assay coefficients of variation were 5–7% for AVP and 7–9% for OT.

Electrophysiological experiments

Using an inverted microscope the terminals were visually identified by their characteristic appearance, spherical shape, lack of nuclei, and relative small size (5–10μm in diameter). For the experiments in which Ca²⁺ currents were recorded, the terminals stayed in the 2.2mM Ca²⁺-Locke's solution described above. For the experiments where barium currents

were recorded, a solution containing BaCl₂ was perfused into the recording chamber right before the beginning of the recording period. This bath solution contained (in mM): BaCl₂ 5; NaCl 145; KCl 5; HEPES 10; Glucose 15; MgCl₂ 1; pH 7.3. The terminals were patched in the perforated (Rae et al., 1991), whole-terminal configuration (Hamill et al., 1981) using freshly prepared amphotericin B (240µg/ml) dissolved in DMSO (dimethyl sulfoxide) and added to a pipette solution containing (in mM): Cs-glutamate 135; HEPES 10; Glucose 5; CaCl₂ 2; MgCl₂ 1; Tetraethylammonium (TEA) 20; pH 7.2. In some experiments Tetrodotoxin (TTX) 1µM was added to the bath. Pipettes were made of thin borosilicate glass (Drummond Scientific Co., Broomall, PA), using a vertical puller (David Kopf Instruments, Tujunga, CA) and fire-polished (Narashige, Tokyo, Japan) to display resistances in the range of 3–6MΩ. Series resistance was partially compensated using the patch clamp compensation circuit and was always below 15MΩ. Terminals were held at –80mV and depolarized to various test potentials every 30 sec using an Axopatch 200B patch interfaced via TL-1 DMA Interface (Axon Instruments, Foster City, CA) with a personal computer. Currents were low pass filtered at 2 kHz using an eight pole Bessel filter (Frequency Devices Inc., Haverhill, MA) and digitized at 10 kHz. pClamp software was used for data acquisition and analysis. Curve fitting was done with Originsoftware.

Current inactivation during voltage steps were obtained from a first order exponential fit (Chebyshev method) of the first 150 msec after peak currents were reached using the equation,

$$I = Ae^{(-t/\tau)} + C \quad (1)$$

where A is the maximal amplitude of the component, t is the time t at which the current had decreased to 1/e of its initial amplitude, and C is the steady-state current level. Current–voltage curves were fitted using the equation

$$I = \frac{G_{max} (V - E_{rev})}{1 + \exp^{(V_{0.5} - V)/k}} \quad (2)$$

where G is the maximum conductance, E_{rev} is the apparent reversal potential, V is the test potential, V_{0.5} is the potential at half activation, and k is the slope factor. Activation curves were fitted with a Boltzmann function of the type,

$$\frac{G}{G_{max}} = 1 + \left[1 + \exp^{(V - V_{0.5}/k)} \right] \quad (3)$$

where k is the slope factor or the potential sensitivity for an e-fold change in G/G_{max}, V is the test potential, and V_{0.5} is the voltage for half maximal values.

Identification of neuropeptide content on isolated individual terminals

At the end of each electrophysiological experiment, terminals were aspirated into the recording pipette. The pipette tip containing the terminal was broken against the bottom of a microcentrifuge tube and frozen until assayed for its neuropeptide content. Before assaying, 0.5% Triton-X 100 was added to each vial to maximize the amount of free peptide. The AVP or OT content of each terminal was determined by a specific enzyme-linked

immunosorbant assay (ELISA; Assay Designs, Inc., Ann Arbor, MI). The sensitivity of the AVP and OT ELISA was previously determined (Custer et al., 2007) as 0.25 and 1 pg/well, respectively. When testing for only one peptide, the entire volume in each vial was assayed for AVP or OT. When both peptides were assayed from the same terminal, the content was divided so as to increase the probability to detect both peptides according to each assay's sensitivity.

Intracellular Ca^{2+} measurements

Dissociated nerve terminals were incubated with 2.5 μM Fura-2 AM in a low calcium Locke's saline for 45 min at 37°C. Terminals were thoroughly washed with normal Locke's saline and their resting global cytosolic $[\text{Ca}^{2+}]_i$ determined. This was performed according to the method of Grynkiewicz et al. (1985), with an assumed Ca^{2+} -Fura K_d of 200 nM, as previously described by Becker et al. (1989). Resting values for global cytosolic $[\text{Ca}^{2+}]_i$ in the presence and absence of extracellular Ca^{2+} were 68.5 ± 22.3 nM ($n = 7$) and 46.2 ± 7.5 nM ($n = 8$), respectively, and these values were not significantly different ($P = 0.4$). High K^+ solutions with and without toxins or DAMGO were delivered via a SF-77B microperfusion system (Warner Instruments, Hamden, CT) and gravity driven. Fluorescence images were viewed with a Nikon Diaphot TMD inverted microscope, using a quartz Plan-Neofluar 100 $\times$ -oil immersion lens (Carl Zeiss Inc., Thornwood, NY) adapted with a 71000A Fura-2 filter cube (Chroma Technology, Corp., Rockingham, VT) and interfaced to a SenSys CCD camera (Photometrics Ltd., Tucson, AR). Terminals were excited using a Xenon arc lamp within a Lambda DG4 high-speed filter changer (Sutter Instruments Incorporated, Novato, CA) with the appropriate filters (340 and 380 nm wavelength) in place. Intraterminal emission of the Fura-2 indicator was gathered at 510 nm wavelength. Fluorescent images were acquired and processed with Axon Imaging Workbench 2.1 software (Molecular Devices, Foster City, CA).

Immunohistochemistry

Neuropeptide identification—Once all $[\text{Ca}^{2+}]_i$ measurements were concluded, terminals were fixed in place and processed as described below. The time exposure to both primary and secondary antibodies was 2 and 1.5 h, respectively, at room temperature. Dishes were exposed to a 1% Triton X-100 PB solution containing 10% donkey serum, an affinity purified goat anti-Neurophysin-II (AVP) at 2 $\mu\text{g}/\text{ml}$ (Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse monoclonal anti-Neurophysin-I (OT) (P38) kindly provided by Dr. Harold Gainer (National Institute of Health, Bethesda, MD; described in Ben-Barak et al. (1985)). Fluorescently tagged secondary antibodies, Alexa Fluor 488 donkey anti-mouse and Alexa Fluor 594 donkey anti-goat (Molecular Probes, Carlsbad, CA), 10% donkey serum in a 1% Triton X-100 PB solution were used to visualize the presence of each neuropeptide. Images were obtained as described before for the Fura-2 experiments with the microscope adapted to an 83500 filter cube (Chroma Technology, Corp.) and excited to the respective wavelengths. Images were photographed at 10 msec exposures for bright fields and 800 ms exposures for fluorescence.

Cav2.2 subunit and neuropeptide identification—The homogenate containing dissociated terminals was aliquoted equally into four 35 mm polypropylene glass-bottom

microwell dishes (MatTek Corporation, Ashland, MA). Two dishes were incubated with low concentrations of DAMGO and two were kept in Normal Locke's for up to 2 h. The tissue was fixed overnight in 4% paraformaldehyde–phosphate buffer solution (pH 7) and washed repetitively in phosphate buffer (PB) afterwards. The remaining dishes were exposed to a 1% Triton X-100 PB solution containing 10% donkey serum, an affinity purified rabbit anti-Cav2.2 (α_{1B}) at 8.5 $\mu\text{g/ml}$ (Alomone Labs Ltd., Jerusalem, Israel), an affinity purified goat anti-Neurophysin-II (AVP) at 2 $\mu\text{g/ml}$ (Santa Cruz Biotechnology), and a mouse monoclonal anti-Neurophysin-I (OT) (P38) kindly provided by Dr. Harold Gainer (NIH). All dishes were incubated overnight, washed with PB and exposed to fluorescently tagged secondary antibodies (Molecular Probes, Carlsbad, CA) for 3–5 h at room temperature to visualize each labeled peptide. The mixture of secondary antibodies included Alexa Fluor 350 donkey anti-goat IgG, Alexa Fluor 488 donkey anti-mouse IgG and, Alexa Fluor donkey anti-rabbit IgG, 10% donkey serum in a 1% Triton X-100 PB solution. After a final series of washes the dishes were mounted with Prolong (Molecular Probes) and sealed with a glass coverslip to increase the fluorescent life of the secondary antibodies. Images were obtained with an Axiovert 200M Inverted microscope (Carl Zeiss Inc., Thornwood, NY) interfaced to a Zeiss AxioCam MRm camera fitted to a 63 $\times$ Plan/Apochromat oil immersion lens, with a numerical aperture of 1.4. Axiovert 40 (V.4.6.3.0) software was used for acquisition and image analysis. Images of one or more terminals were obtained in stacks at every 0.27 μm along the length of the terminals. The length was set as the point where the fluorescent signal was no longer visible from both, the top and bottom ends of terminals. Images were deconvoluted using a high stringency fast iterative algorithm and corrected for fluorescence cross-talk between stacks. Fluorescence levels in control dishes were at or below non-specific levels for all three wavelengths (not shown).

N-type channel distribution was estimated from the Cav2.2 (α_{1B}) immunofluorescent signal. The amount of fluorescence within 0.45 μm of the outer terminal's boundary (established from the DIC images) was obtained by calculating the difference in fluorescent signal between two tightly fitted circles: one at the terminal outer perimeter and another circle 0.45 μm inside of the first one. This distance is our highest resolution limit and was corroborated in independent experiments using non-permeabilized terminals exposed to antibodies against extracellular epitopes of other membrane-associated proteins (i.e., μ -opioid receptors and L-type channels)(data not shown). This approach has also, been used by other groups to estimate amount of antigen labeling associated with a cell's plasma membrane and calcium channels (see Chiu et al., 2002; Pietrzykowski et al., 2004). Percentage values were calculated for individual terminals and means $\pm$ errors obtained for each group/treatment. Intensity correlation coefficient-based (ICCB) analyses were performed to establish the level of colocalization between the α_{1B} and β subunit signals. Pearson's coefficients (PC) were estimated for each condition.

β subunit and neuropeptide identification—To identify each individual beta subunit, the homogenate containing dissociated terminals was aliquoted equally into 35 mm polypropylene glass-bottom microwell dishes (MatTek Corporation, Ashland, MA). The tissue was fixed overnight in 4% paraformaldehyde–phosphate buffer solution (pH 7) and washed repetitively in phosphate buffer (PB) afterwards. To identify the presence of Cav β_{2a} ,

two dishes were exposed to a 1% Triton X-100 PB solution containing 10% donkey serum, an affinity purified rabbit anti-Cav2.2 (α_{1B}) at 8.5 $\mu\text{g/ml}$ (Alomone Labs Ltd.), a mouse monoclonal anti-Cav β_2 at 5 $\mu\text{g/ml}$ (Clone N8b/1; UC Davis/NINDS/NIMH NeuroMab facility, NIH grant U24NS050606 Davis, CA) and, either an affinity purified goat anti-Neurophysin-II (AVP) at 2 $\mu\text{g/ml}$ (Santa Cruz Biotechnology) or an affinity purified goat anti-Neurophysin-I (OT) at 4 $\mu\text{g/ml}$ (Santa Cruz Biotechnology). To identify the presence of Cav β_{1b} , two dishes were exposed to an affinity purified rabbit anti-Cav2.2 (α_{1B}) at 8.5 $\mu\text{g/ml}$ (Alomone Labs Ltd.), a mouse monoclonal anti-Cav β_1 at 5 $\mu\text{g/ml}$ (Clone N7/18; UC Davis/NINDS/NIMH NeuroMab facility, NIH grant U24NS050606 Davis, CA) and, either an affinity purified goat anti-Neurophysin-II (AVP) at 2 $\mu\text{g/ml}$ (Santa Cruz Biotechnology) or an affinity purified goat anti-Neurophysin-I (OT) at 4 $\mu\text{g/ml}$ (Santa Cruz Biotechnology). To identify the presence of Cav β_4 , two dishes were exposed to an affinity purified rabbit anti-Cav2.2 (α_{1B}) at 8.5 $\mu\text{g/ml}$ (Alomone Labs Ltd.), a mouse monoclonal anti-Cav β_4 at 5 $\mu\text{g/ml}$ (Clone N10/7; UC Davis/NINDS/NIMH NeuroMab facility, NIH grant U24NS050606 Davis, CA) and, either an affinity purified goat anti-Neurophysin-II (AVP) at 2 $\mu\text{g/ml}$ (Santa Cruz Biotechnology) or an affinity purified goat anti-Neurophysin-I (OT) at 4 $\mu\text{g/ml}$ (Santa Cruz Biotechnology). To identify the presence of Cav β_3 , two dishes were exposed an affinity purified goat anti-Cav2.2 (α_{1B}) at 2 $\mu\text{g/ml}$ (Santa Cruz Biotechnology), an affinity isolated rabbit anti-Cav β_3 at 1.5 $\mu\text{g/ml}$ (Sigma–Aldrich, Inc, MO) and, either a mouse monoclonal anti-Neurophysin-II (AVP) (P41) or mouse monoclonal anti-Neurophysin-I (OT) (P38) kindly provided by Dr. Harold Gainer (NIH). All dishes were incubated overnight, washed with PB and exposed to fluorescently tagged secondary antibodies (Molecular Probes) for 3–5 h at room temperature. The mixture of secondary antibodies included Alexa Fluor 350 donkey anti-goat IgG, Alexa Fluor 488 donkey anti-mouse IgG and, Alexa Fluor donkey anti-rabbit IgG, 10% donkey serum in a 1% Triton X-100 PB solution. Dishes were mounted with Prolong (Molecular Probes) and sealed with a glass coverslip to increase the fluorescent life of the secondary antibodies. Images were obtained and processed as described above. Fluorescence levels in control dishes without exposure to primary antibodies were at or below non-specific levels for all three wavelengths (not shown). Colocalization analysis of the α_{1B} and β subunit fluorescent signals was done using JACoP (Bolte and Cordelieres, 2006) under ImageJ software. Pearson's coefficients (PC) were obtained using an ICCB (intensity correlation coefficient-based) approach.

Statistical analysis

Data are given as means $\pm$ SEM. The effects of DAMGO on calcium currents, $[\text{Ca}^{2+}]_i$, and peptide release were analyzed using a one-way ANOVA test followed by a Dunnett's test to determined significant differences between means (Sigma Stat; Jandel Scientific, San Rafael, CA). Statistical significance for all analyses was established as $P < 0.05$.

Source of chemicals

All chemicals, including DAMGO, nicardipine and ω -conotoxin MVIIC were obtained from RBI/Sigma Chemical Co. (St. Louis, MO). The ω -conotoxins GVIA and MVIIA were obtained from Molecular Probes Inc. (Eugene, OR) and Alomone Labs Co. (Jerusalem, Israel). SNX 230 and SNX 482 were supplied by the Neurex Corporation (Menlo Park, CA).

Nicardipine was first dissolved in DMSO at 1% of the final concentration and further diluted in Locke's buffer at 0.1–0.01% before used. DAMGO and all toxins stocks were prepared in distilled water, separated in aliquots and stored at -20°C . The final working dilutions were made in Locke's buffer and used immediately, with the exception of SNX 482 that was diluted 1 h before it was used, to allow for an optimal blocking conformation of the toxin.

Results

N-type calcium currents are differentially modulated in OT versus AVP NH terminals

In order to test the effects of μ -opioid agonists on N-type channel activity, individual isolated terminals showing stable calcium currents were exposed to highly specific toxins able to block three out of the four possible current subtypes present in NH terminals (Fig. 1). The N-type current was isolated by inhibiting P/Q-type, L-type and R-type currents using 100–250 nM SNX230, 1–2 μM nicardipine, and 25 nM SNX482, respectively.

Figure 1A–C shows responses to DAMGO from a terminal that was positive for OT (OT+). The isolated N-type current comprised 38% of the total initial current. Peak currents were inhibited dose-dependently, with a 7% decrease by 100 nM DAMGO and a 29% by 300 nM DAMGO. The time-course of the current inhibited by DAMGO is displayed in Figure 1B. We used single exponential fits of the raw data to derive time constants of inactivation for the toxin's N-type isolated component as well as from the DAMGO-inhibited current component. Across all OT+ terminals, the opioid-inhibited current shows similar inactivation kinetics (38 ± 2 msec) as the toxin-isolated N-type current (44 ± 2 msec; $P > 0.05$; $n = 5$). Figure 1C shows the current–voltage relationship in control, isolated N-type, and 300 nM DAMGO conditions corresponding to the isolated OT+ terminal in Figure 1A,B. Inhibition by DAMGO was observed at all test voltages, with lower sensitivity to inhibition at negative potentials and without relief of inhibition at positive potentials. Steady-state activation curves obtained from all OT+ terminals showed a small shift of 5.4 mV in the voltage-dependence of activation toward more positive potentials upon exposure to DAMGO. $V_{0.5}$ values were estimated to be -7.8 ± 0.9 mV, $k = 5.5 \pm 0.8$ without DAMGO and -2.4 ± 0.6 mV, $k = 7.6 \pm 1.8$ in the presence of the opioid (Fig. 1D). Current inhibition by toxins and DAMGO was reversible, although required extensive washes (data not shown, but see, e.g., Fig. 3A). Mean inhibitory values for isolated N-type currents in all OT+ terminals were $6.8 \pm 7.3\%$ at 10 nM, $14 \pm 6\%$ at 100 nM and $42 \pm 5\%$ at 300 nM DAMGO (Fig. 3C).

Vasopressinergic terminals exhibited a different N-type modulation profile by DAMGO. N-type channel responses to DAMGO from a positively identified AVP (AVP+) terminal are shown in Figure 2A. In this terminal, 42% of the total initial current remained after exposure to the same concentrations of the toxin mix described above. When the terminal was exposed to 100 nM DAMGO in the continuous presence of these toxins, the N-type current was increased by 43%. Although variable in magnitude, all AVP+ terminals exhibited current increments induced by DAMGO. Addition of 1.5 μM GVIA, a highly specific N-type toxin, completely blocked the potentiated, isolated current, corroborating its identity. The time-course and magnitude of the current increased by DAMGO is shown in Figure 2B. Time constants of inactivation obtained from the enhanced current component showed

slower inactivation kinetics (65 ± 6.4 msec) than the control N-type current (41 ± 4.2 msec; $P < 0.05$; $n = 7$). The corresponding current–voltage relationship for this AVP+ terminal at all conditions tested is shown in Figure 2C. Current enhancements in 100 nM DAMGO were obtained at all test potentials, with higher increments at more positive potentials. The steady-state activation curve from the enhanced current shifted 6.6 mV per e-fold change toward more negative potentials when compared to control N-type currents. $V_{0.5}$ values were estimated to be -8.4 ± 1.1 mV, $k = 5.3 \pm 0.7$ without DAMGO and -14.4 ± 0.6 mV, $k = 8.2 \pm 2.2$ in the presence of the opioid (Fig. 2D). This shift in voltage was observed in all AVP+ terminals ($n = 7$). As was observed with the inhibitory responses in OT+ terminals, current enhancements were reversible after washing with bath solution for 5–10 min (Fig. 3A). Mean values for N-type currents in all AVP+ terminals increased by $18 \pm 8\%$ and $67 \pm 9\%$ in the presence of 10 nM and 100 nM DAMGO, but decreased by $20 \pm \%$ at 300 nM DAMGO (Fig. 3C).

Approximately 5% of NH terminals express both types of neuropeptides (Custer et al., 2007; see also Kiyama and Emson, 1990; Mezey and Kiss, 1991). N-type currents from one of these colabeled terminals are shown in Figure 3B. Even though this kind of terminal expressed all four types of calcium conductances (not shown), 45% of the total initial current remained after exposure to the toxin mix isolating the N-type component. Exposure to low concentrations of DAMGO (15 nM) induced a robust 30% increase of the N-type current. However, increasing DAMGO's concentration to 115 nM caused a smaller 16% enhancement, thus following a similar response pattern as AVP+ terminals; that is, net current enhancements followed by inhibition at higher agonist concentrations. Similar responses were observed in two more colabeled terminals. Hence, current responses to μ -opioid agonist in colabeled terminals portray a dominant phenotype characteristic of terminals containing exclusively AVP+.

Modulation of intracellular calcium

Ca^{2+} influx measured with the calcium indicator Fura-2 was used as another independent method to test for differential behavior of Ca^{2+} influx through N-type channels in OT versus AVP terminals. If the N-type current is modulated differentially in each kind of peptidergic terminal, corresponding differences in $[\text{Ca}^{2+}]_i$ should also be detected. High K^+ (50 mM) - induced changes in $[\text{Ca}^{2+}]_i$ were monitored in control conditions, in the presence of a toxin mix to isolate the N-type component, and in conjunction with increasing concentrations of DAMGO (10–500 nM). The toxin mixture was constant across experiments, with 25 nM SNX482, 100–150 nM SNX230 (or AgaIVA at equally effective concentrations), and 1–3 μM nifedipine to block R-, Q-, and L-type calcium channels, respectively. Three independent experiments were performed using this approach; two of them were followed with peptide immunolabeling to identify each terminal's content. The terminals were fixed in place after completing all $[\text{Ca}^{2+}]_i$ measurements and processed for immunolabeling as described in the methods section. Bright field images were obtained at the beginning of the experiment and cross-referenced with those taken during and at the end of the experiment to correct for any shift in the field at each experimental step. Figure 4 shows examples of one of those experiments. Out of nine individual nerve endings (Fig. 4A), three contained OT, four contained AVP, and two did not label for either peptide (assumed to be damaged during

the isolation process). Only clearly labeled terminals were used in the analysis. High K^+ induced changes in $[Ca^{2+}]_i$ for two strongly labeled terminals, #9 (OT) and #6 (AVP) are shown in Figure 4B,C. Consecutive images on the top part of Figure 4B, illustrate the response of terminal #6 to high K^+ stimulation in the presence of the toxin mix and 10 nM DAMGO. The increased $[Ca^{2+}]_i$ is distinctively higher in this terminal than in terminal #9 (bottom part) following exposure to DAMGO. Quantification of the $[Ca^{2+}]_i$ responses (see Materials and Methods Section) for both types of terminals in the absence and presence of DAMGO at various concentrations provides a clear, distinctive pattern of enhancement versus inhibition of Ca^{2+} influx (Fig. 4C). Terminal #6 (AVP+), showed a 16% increase in the N-type dependent calcium influx when exposed to 10 nM DAMGO. This increment was decreased to only 3% above the toxin isolated level at 100 nM DAMGO and becomes inhibited by 14% with 500 nM DAMGO. To confirm the identity of the Ca^{2+} influx source, 800 nM MVIIA, a specific N-type channel blocker, was added to the dish. The final bar shows the remaining level of $[Ca^{2+}]_i$ after this application. Increments in $[Ca^{2+}]_i$ were absent in terminal #9 (OT+) and only inhibitory responses to DAMGO were obtained. A 4% decrease in $[Ca^{2+}]_i$ from the previous isolated level was obtained in 10 nM DAMGO followed by 22% and 33% decreases when exposed to 100 and 500 nM DAMGO, respectively. The final bar shows the remaining level of $[Ca^{2+}]_i$ after 800 nM MVIIA was added to the dish.

Data from all three independent experiments measuring $[Ca^{2+}]_i$ were pooled together in Figure 4D. In all, 23 Fura-2 loaded terminals were studied; 13 were positively identified as AVP or showed enhancements in $[Ca^{2+}]_i$ at low concentrations of DAMGO, 6 were OT positive or showed only inhibitory responses and 4 did not respond at all (and assumed to not be viable). The response patterns to DAMGO, although variable in magnitude, held true for both OT and AVP terminals. At low 10 nM DAMGO, AVP terminals showed a significant $21 \pm 3.7\%$ increase in $[Ca^{2+}]_i$ compared to the previous isolated N-type dependent level. Enhancement was more pronounced ($27 \pm 6.4\%$) at 100 nM DAMGO but decreased beyond this point. At 300 nM DAMGO, $[Ca^{2+}]_i$ was $15 \pm 4.6\%$ higher than the toxin isolated level and at 500 nM DAMGO there were no longer significant differences ($5.5 \pm 6.1\%$) in $[Ca^{2+}]_i$ when compared to the initial toxin isolated level. However, close to half of AVP terminals showed moderate net inhibitions in $[Ca^{2+}]_i$ at high concentrations of DAMGO (see terminal #6 in Fig. 4C). In OT+ terminals, $[Ca^{2+}]_i$ levels were $1.5 \pm 4.4\%$ below the control N-type values at 10 nM DAMGO. At 100 and 300 nM DAMGO, intracellular Ca^{2+} values had diminished by $4.1 \pm 3.9\%$ and $7 \pm 2.3\%$, respectively. At 500 nM DAMGO, $[Ca^{2+}]_i$ was significantly inhibited ($16 \pm 2.1\%$) from control levels. Within all OT+ terminals, no significant enhancements in N-type dependent Ca^{2+} influx occur at either low or high concentrations of DAMGO.

Modulation of neuropeptide release

Since neuropeptide release is tightly controlled by Ca^{2+} influx, we expected a similar pattern of inhibition and enhancement of OT and AVP release by DAMGO. Figure 5A,B shows high K^+ -elicited, N-type dependent neuropeptide release in the absence and presence of DAMGO. Vasopressin release was significantly decreased by $51 \pm 1.7\%$ once terminals were exposed to the toxin mix able to block L-, P/Q-, and R-type calcium channels (Fig.

5A), thus, this amount of peptide release should solely depend on the activation of N-type calcium channels during high K^+ depolarization. Exposure to 10 nM DAMGO in the continuous presence of the toxin mix induced a significant $27 \pm 1\%$ increase in AVP release. However, subsequent stimulation with a higher concentration (100 nM) of DAMGO induced a $45 \pm 2.1\%$ reduction in release. All effects by DAMGO and toxins were transient in nature as AVP release recovers to initial levels once all drugs were washed away. Figure 5B shows oxytocin levels, measured from the same samples used to quantify AVP. Oxytocin release was decreased by $65 \pm 9\%$ in the presence of the toxin mix. When this N-type-dependent component was tested with either low or high opioid agonist concentrations, all responses were inhibitory. Exposure to 10 and 100 nM DAMGO reduced OT release by $60 \pm 7\%$ and $64 \pm 2\%$, respectively.

Thus, to summarize the work presented up to this point, the changes in N-type currents, $[Ca^{2+}]_i$, and neuropeptide release displayed a consistent, distinct modulatory profile by DAMGO, characteristic of each terminal type.

N-type channel immunohistochemical distribution in NH terminals

We tried to determine whether mobilization and incorporation of N-type channels into the plasma membrane accounted for MOR current enhancement in AVP+ terminals. For these experiments, isolated terminals were equally distributed into two dishes; one exposed to a low concentration of DAMGO and one kept as a control (exposed only to Locke's saline) for a similar amount of time. Both dishes were immediately fixed and exposed to antibodies selective for the N-type α_{1B} ($Ca_v2.2$) subunit as well as antibodies against Neurophysin I (OT) and Neurophysin II (AVP) proteins. A total of 102 terminals were analyzed (see Materials and Methods Section and Fig. 6A) in four separate experiments. The distribution of α_{1B} immunoreactivity in OT+ and AVP+ terminals shows a distinctive, punctated pattern, both at the membrane as well as in the terminal's cytoplasm (see red labeling in Figs. 7 and 8). α_{1B} -dependent immunoreactivity is significantly higher ($P < 0.05$) in AVP+ terminals ($2.76 \times 10^5 \pm 2.38 \times 10^4$ gfu/ μm^2) than in neighboring OT+ terminals ($1.84 \times 10^5 \pm 3.66 \times 10^4$ gfu/ μm^2 ; Fig. 6B). However, when the immunoreactivity associated with the membrane was estimated (Fig. 6C; Control), no significant differences between terminal type was obtained. In AVP+ terminals, $29 \pm 4\%$ of the total α_{1B} fluorescence localized to the membrane area. Similar values ($28 \pm 5\%$) were obtained for OT+ terminals. Thus, N-type channels inside AVP+ terminals may mobilize to the membrane following exposure to DAMGO. To test this possibility, immunostaining of control terminals was compared to terminals exposed to 25 nM DAMGO prior to fixation. α_{1B} fluorescence associated with the membrane did not differ ($P > 0.05$) from control values (Fig. 6C). At this agonist concentration OT+ terminals exhibited $31 \pm 1\%$ of the total fluorescent signal to be associated to the membrane area versus $26 \pm 2\%$ in AVP+ terminals. Higher concentrations of DAMGO (100 nM) and longer incubation times—up to 2 h—did not significantly change α_{1B} fluorescence in the membrane area compared to control values. Thus, we could not detect any mobilization of N-type channels to the membrane in AVP+ terminals as a result of μ -opioid stimulation.

β_2 is present only in vasopressinergic NH terminals

As we described previously, the N-type current component increased by DAMGO showed slower inactivation kinetics than the current component lost during inhibition (Figs. 2B vs. 1B). Similar current enhancement such as the one induced by DAMGO in AVP+ terminals have been described in other systems. For example, activation of muscarinic receptors can lead to N-type current augmentation in sympathetic neurons and heterologous expression systems (Liu and Rittenhouse, 2003; Heneghan et al., 2009; Mitra-Ganguli et al., 2009). The crucial determinant for N-type current enhancement appears to be the presence of a palmitoylated β_{2a} subunit in the channel protein complex. However, expression levels of β_{2a} in brain tissue have been reported to be very low or undetectable (Scott et al., 1996; Ludwig et al., 1997; Vance et al., 1998). Nevertheless, using immunolabeling techniques, we tried to determine if β_{2a} was indeed present and, in particular, associated with the β_{1B} subunit in AVP terminals. Two examples of the expression pattern for both calcium channel subunits in each type of peptidergic terminal can be observed in Figure 7. Positive immunoreactivity against α_{1B} (red) and β_{2a} (green) was detected in 92% of AVP+ (blue) terminals ($n = 42$; Fig. 7A). The fluorescence pattern for β_{2a} was punctate and noticeable in the terminal's cytoplasm although, by far, the majority of the labeling was contained closer to the membrane area. Two dimensional profiles of the fluorescent signals taken at the white dotted line in each image shows that α_{1B} immunoreactivity seems to be closely associated – although not exclusively with β_{2a} immunoreactivity. However, colabeling analysis (Pearson's coefficient = 0.36 ± 0.09) could not provide clear evidence for a partial colocalization between both signals. Surprisingly, OT+ terminals did not express β_{2a} immunoreactivity (95%; $n = 41$; Fig. 7B). While a small number of OT+ terminals (5%) showed a slight amount of β_{2a} immunoreactivity, they were colabeled terminals and thus, dependent on the presence of AVP. Two distinct OT+ (blue) terminals and one not labeled (presumably AVP+) are shown in both images. All three terminals expressed α_{1B} , but only the OT-negative terminal showed β_{2a} immunoreactivity. These results could explain why only AVP+ terminals show N-type current enhancements when exposed to μ -opioid agonists.

To provide a more complete description of the subunit composition of calcium channels in NH terminals we determined which other β subunits, were present in OT+ and AVP+ terminals using immunofluorescence techniques (Fig. 8). Not surprisingly, both types of terminals show a substantial proportion of β_3 immunoreactivity: 86% of AVP+ terminals ($n = 21$) and 90% of OT+ terminals ($n = 21$) expressed β_3 immunoreactivity (third column in Fig. 8). Pearson's coefficients were estimated to be 0.62 ± 1.0 for AVP+ and 0.45 ± 0.13 for OT+ terminals, demonstrating a partial level of colocalization between both signals. The percentage of β_4 expression in AVP+ terminals was 91% ($n = 23$) and 73% OT+ terminals ($n = 18$; second column, in Fig. 8). OT+ terminals showed a low level of colocalization (PC = 0.41 ± 0.04), and in most instances, the β_4 -dependent signal in AVP+ terminals was not associated with β_{1B} immunoreactivity (PC = 0.17 ± 0.02). Seventy-four percent of AVP+ terminals ($n = 15$) and only 23% ($n = 17$) of OT+ terminals showed β_{1b} immunoreactivity (first column, Fig. 8). Labeling of this subunit was very low and sparingly associated with α_{1B} immunoreactivity in AVP+ terminals (PC = 0.13 ± 0.06). In OT+ terminals the signal was too low and PC values were not determined. Thus, even though colocalization of α and

β subunits was hard to assess in our system, in general we can still conclude that AVP+ terminals showed a higher variability by expressing all four β subunit types, while OT+ terminals expressed a smaller repertoire with only two main subunits. This difference could entail a higher functional variability in AVP+ terminals and a more limited one for those containing OT.

Discussion

Secretion of OT and AVP is highly regulated by endogenous mechanisms targeting MCN's excitability at the cell body level in the hypothalamus as well as at their nerve endings in the Neurohypophysis (Renaud et al., 1992; Brown et al., 2000). Since neurosecretory endings of MCNs are localized anatomically outside the blood–brain barrier, local regulation of neuropeptide release by exogenous/synthetic drugs, such as opiates, becomes an important modulatory component that can adjust and fine-tune the amount of neuropeptide released from these presynaptic nerve terminals.

Even though both neuropeptides can be expressed and released from single, individual MCN nerve endings, each neuropeptide has specific mechanisms and agents regulating its particular release. MOR activation is one of such mechanisms; even though both types of peptidergic terminals are affected by the MOR agonist DAMGO, AVP+ terminals exhibit a response pattern distinctive from OT+ terminals. Differences are particularly evident at low agonist concentrations, where OT release is inhibited but AVP release is not (Ortiz-Miranda et al., 2003). Higher concentrations of MOR agonists are eventually able to inhibit AVP release, but there is a conspicuous right hand shift in sensitivity, clearly distinguishing it from OT release. We previously demonstrated that one of three Ca^{2+} channels controlling OT release—the R-type channel—is highly susceptible to block by MOR agonists (Ortiz-Miranda et al., 2005), thus, providing a reasonable explanation for OT's preferential inhibition. The additional block of N-type channels that we demonstrate here, although smaller in extent, enhances the net inhibitory effects on OT release. In AVP+ terminals however, MOR activation cannot use either of these two inhibitory options. R-type channels do not control AVP release (Wang et al., 1999) and instead, as we demonstrated here, low concentrations of DAMGO enhance rather than inhibit N-type Ca^{2+} channels (Figs. 2 and 3). Thus, the shift in MOR-dependent inhibition observed in the response curves for AVP release (Ortiz-Miranda et al., 2003) can be explained by the combined responses of these two Ca^{2+} channels in each type of peptidergic nerve ending. Moreover, in light of the enhancements in N-type dependent current, $[\text{Ca}^{2+}]_i$, and AVP release observed at low agonist concentrations, we hypothesize that at least one of the other two Ca^{2+} channels controlling AVP release must be inhibited as no net enhancements in AVP release was observed in the response curves when all other channels were active (Ortiz-Miranda et al., 2003).

Relative differences in μ -opioid receptor affinity between the release experiments, Ca^{2+} imaging, and electrophysiology were obtained in this work. However, even though there is variability in DAMGO's effects across techniques, the effects of this μ -opioid selective agonist were always concentration-dependent and followed a similar characteristic pattern of enhancements vs. inhibitions within each of the techniques used. In addition, more

variability is expected in general terms, in electrophysiological and $[Ca^{2+}]_i$ measurements than in the OT release experiments since the latter measures the response of a population of terminals rather than that of individual terminals. Terminal size is another variable between techniques as small terminals ($\sim 5\mu m$) dominate the responses in neuropeptide release experiments while the size of those terminals used in calcium imaging or electrophysiology are above this range. Furthermore, the differences across techniques may be due to a number of other reasons; such as different divalent cation concentrations, or differences in the state of the terminals caused by patch-clamping procedures, or by membrane potential gradients. It is known that higher monovalent cation (e.g., Na^+) concentrations, such as those used for the electrophysiological experiments, decrease the affinity of the receptor for agonists like DAMGO (Fowler and Fraser, 1994). The membrane potential in isolated terminals can fluctuate, especially during release studies, yet in patch-clamped terminals the potential is under voltage-clamp control. Furthermore, even though the perforated-patch configuration is more physiologically intact than when using conventional whole-cell methods, it still perturbs the cytoplasm of the terminal. These caveats are not true for neuropeptide release or $[Ca^{2+}]_i$ measurements, however.

High concentrations of μ -opioid agonists induce inhibition of peptide release by suppressing Ca^{2+} currents and a rise in $[Ca^{2+}]_i$ in NH terminals. These inhibitory responses are currently under study and our data indicates a mechanism that is voltage-independent (Velázquez-Marrero et al., 2010). The presence of $G_{\alpha q/11}$ in NH terminals (Velázquez-Marrero et al., 2008), provides a potential candidate to test as a mediator of these inhibitory effects. Finally, even though far from conclusive, the relatively small shift to more depolarized potentials in the steady-state activation curves together with minor changes in inactivation time constants of the inhibited N-type current are consistent with such a mechanism. The role of kinases and/or other activators of soluble intracellular second messenger pathways are currently being explored as targets of MOR activation.

In AVP+ terminals, a mechanism like the one we just proposed to mediate inhibitory responses is obviously different from the one mediating the increments in current. We hypothesize that low agonist concentrations activate a pathway, available only to AVP+ terminals, that is overpowered at higher concentrations by the activation of another pathway mediating only inhibitory responses. Thus, currents would initially increase and gradually decrease when enough inhibition is attained by either other $Ca_v2.2$ channels associated to other β subunits or the same $Ca_v2.2-\beta_{2a}$ channel targeted by the inhibitory pathway. We cannot differentiate between these two alternatives at this point.

Interestingly, colabeled terminals producing both types of neuropeptides seemed to follow the behavior of AVP+ terminals as if the mechanism used dominates the response pattern to MOR opioids (Fig. 3). Because these terminals express all channel subtypes based on electrophysiological and immunolabeling data, they should also expressed the β_{2a} subunit as part of their subunit complement. Thus, their response pattern to MOR activation is consistent with our hypothesis that the presence of β_{2a} could be enough to confer these group of N-type channels the capacity to be enhanced by DAMGO. It is tempting to speculate that the small proportion of OT released by the $\sim 5\%$ of colabeled terminals would

not be decreased by MOR agonists as N-type and R-type currents are modulated in opposite ways.

In rat brain, β_{2a} is found at very low levels compared to all other β subunits ($-\beta_3$, $-\beta_4$, and $-\beta_{1b}$) (Vance et al., 1998). Colocalization attempts using immunoreactive probes against β_{2a} and α_1 subunits (Ludwig et al., 1997) or immunoprecipitation of α_{1B} with β_{2a} (Scott et al., 1996) yielded undetected or extremely low levels for this subunit. However, the hypothalamic area was not shown or mentioned in those studies and it is unknown if the neurohypophysis was included in them. We conclusively see β_{2a} expression in AVP terminals and in some instances it seems to be closely associated to α_{1B} (Fig. 7A). However, a good proportion of the α_{1B} immunoreactivity is not associated with β_{2a} , and PC colabeling estimates were not conclusive. α_{1B} immunoreactivity was mostly associated to with other β subunits, such as β_3 and in different amounts to β_4 and β_{1b} (Fig. 8). Part of the difficulty in obtaining a PC value above 0.5 in these terminals could be the fact that the α_{1B} -dependent signal could be potentially associated with multiple β 's in different proportions. In OT+ terminals almost all the α_{1B} immunoreactivity is only associated with β_3 and β_4 immunoreactivity, however, PC values were still below 0.5 in both instances. In spite of this we hypothesize that Ca^{2+} channels in OT+ terminals are solely inhibited as a consequence of a less variable β subunit complement, while the expression of a more variable β subunit set in AVP terminals allows their Ca^{2+} channels to be up or down regulated upon stimulation by MOR.

The changes in channel kinetics and voltage-dependent properties that N-type channels portray in AVP terminals provide preliminary evidence suggesting β_{2a} as a possible mediator of MOR-dependent increments in currents. Compared to all other β subunits, β_{2a} 's presence is characterized by a retardation in current inactivation (Bogdanov et al., 2000; Stephens et al., 2000). Our data shows a significant reduction (~ 1.6 -fold slower) in current inactivation, consistent with a functional expression of β_{2a} . Furthermore, Ca^{2+} channels containing β_{2a} activate at more negative potentials compared to all other β subunits. In our study, steady-state activation curves obtained for the MOR-dependent increased N-type current shows that they are indeed activated at slightly more negative potentials, compared to the total isolated N-type current. It is well documented that β_{2a} subunits retard inactivation by tethering the I–II linker (or N-terminus) of α_{1B} subunits and interfering with G protein modulation at this site (reviewed in Dolphin (2003a) and Tedford and Zamponi (2006)). Thus, the presence of a β_{2a} subunit can prompt channels into a state where they are more easily activated (Herlitze et al., 2001).

β subunits in voltage-dependent Ca^{2+} channels not only regulate channel kinetic properties but also facilitate the assembly and movement of the multi-subunit channel complex to the plasma membrane (Chien et al., 1995; Brice and Dolphin, 1999; Gao et al., 1999; reviewed in Birnbaumer et al., 1998; Dolphin, 2003b). The expression of $Ca_v\alpha$ subunits in heterologous expression systems reaches a steady level at 24 h and depends on the presence of $Ca_v\beta$ subunits, thus, called *chaperones* (Brice and Dolphin, 1999). A more rapid enhancement of ionic currents can occur within a few minutes, via translocation of channels to plasma membranes by PKC activation (Strong et al., 1987), insulin-like growth factor-1 (Kanzaki et al., 1999), epidermal growth factor (Bezzarides et al., 2004), or PI3K

phosphorylation of Cav β subunits (Viard et al., 2004). In the later work, phosphorylation of β_{2a} induces larger current increments than any other Cav β subunit. Given that AVP+ terminals express significantly higher amounts of α_{1B} than OT+ terminals and that most of this immunoreactivity is contained in the cytoplasm of AVP+ terminals, we tested the possibility that MOR activation could stimulate trafficking of channels and increase channel expression at the plasma membrane. However, we were not able to detect significant differences in membrane distribution of α_{1B} immunoreactivity by DAMGO (Fig. 6C). Longer incubation times (up to 2 h) and higher concentrations of DAMGO (up to 100 nM) yielded similar results (Fig. 6). Thus, translocation of Ca v 2.2 to the plasma membrane by low levels of MOR agonists is not mediating N-type currents enhancements in AVP+ NH terminals.

In conclusion, we found that N-type Ca $^{2+}$ channels in OT terminals are consistently inhibited while AVP terminals are differentially modulated by MOR: potentiated at lower concentrations but increasingly inhibited at higher concentrations of agonist. Changes in [Ca $^{2+}$] $_i$ and neuropeptide release followed a similar pattern of enhancement and inhibition in each type of terminal. We also presented evidence indicating that current enhancement is not a consequence of an upregulation of channel trafficking to the plasma membrane. Finally, we showed that β_{2a} is present only in AVP terminals and that the enhanced, MOR-dependent current shows some of the characteristic kinetic properties exhibited by N-type channels expressing β_{2a} as part of their channel structural complex. The mechanisms and pathways used by each type of terminal to control channel activity, [Ca $^{2+}$] $_i$ and thus, the net amount of neuropeptide released from these presynaptic endings not only help us understand the physiology of the HNS and how it responds to opiates, but they also represent potential strategies and/or methods used by other neurons in the CNS to regulate their synaptic output.

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Fig. 1.

DAMGO inhibits the isolated N-type current in an OT-containing terminal. A: N-type currents were isolated pharmacologically from Control Ca²⁺ currents (1) by selectively blocking all other Ca²⁺ current components with 175nMMSNX230, 2 μ MNicardipine, and 25 nMMSNX482. Voltage-gated Na⁺ currents were not blocked in this terminal. The isolated N-type current (2) was inhibited 7% by 100nMDAMGO (3) and 29% by 300nMDAMGO(4). The peptide content of this terminal was identified as oxytocinergic (OT+). B: N-type current inhibited by 300nMDAMGO, obtained by subtracting traces (2-4) before and after opioid exposure. First order exponential fits (Equation 1) gave time constants for inactivation of 40.1 msec for the isolated N-type component (2) and 35.8 msec for the DAMGO-inhibited current component (2-4). C: Current-voltage relationship generated by holding the terminal at -80 mV and stepping to test potentials (-50 to 50mV) every 30 sec in the same conditions as shown in (A). Dotted lines were obtained by fitting each individual data set to Equation (2). D: Steady-state activation curves (Equation 3) were obtained in the absence and presence of DAMGO. A small shift of 6.1mV in V_{0.5} toward more negative potentials was observed upon exposure to DAMGO. n=5.

Fig. 2.

Isolated N-type currents are enhanced by DAMGO in an AVP-containing terminal. A: Control Ca^{2+} currents (1) were inhibited as previously described (see Fig. 1) to isolate the N-type current (2). TTX (μM) was also added to block I_{Na^+} . Exposure to 100nMDAMGO (3) induced an increase in Ca^{2+} current blocked by 1.5 μM GVIA (4). This terminal was assayed with an ELISA, and found to be vasopressinergic (AVP+). B: Opioid-dependent current component was obtained by subtracting current traces (3–2) before and after opioid exposure. First order exponential fits were obtained as described in Figure 1. Time constants for inactivation were 40.3 msec for the isolated N-type component (3) and 81.8 msec for the DAMGO enhanced current component (3–2). C: Current-voltage curve generated by holding the terminal at -80 mV and stepping to test potentials (-50 to 50 mV) every 30 sec in identical conditions as shown in (A) for the current traces. Dotted lines were obtained by fitting each individual data set to Equation (1). D: Steady-state activation curves (Equation 3) were obtained in the absence and presence of DAMGO. A small shift of 5.4 mV in $V_{0.5}$ toward more positive potentials was observed upon exposure to DAMGO. $n=7$.

Fig. 3.

DAMGO affects N-type currents in AVP+ and AVP+/OT+ terminals in a similar way. N-type mediated Ca^{2+} currents were isolated pharmacologically by selectively blocking all other current components as previously described. Currents were recorded in the presence of 5mM $BaCl_2$ using identical stimulation protocols as those described in previous figures. A: Barium currents in an AVP+ terminal. The isolated N-type current component (2) comprised 29.5% of the total initial current (1). A 40.2% current increase was induced by 100nM DAMGO (3). Toxin and opioid effects are fully reversible (103% of initial current) after all agents are washed out (4). TTX (μ M) was present to block I_{Na+} . B: Terminal containing both AVP and OT. Fifty-five percent of the total initial current was blocked by the toxin mix (2). Exposure to low concentrations of DAMGO (15 nM) induced a 30% increase of the isolated N-type component (3). A higher concentration of DAMGO (115 nM) only caused a 16.5% current increment (4). I_{Na+} was not blocked in this terminal. $n=3$. C: Summary of DAMGO effects on isolated N-type dependent currents in OT+ and AVP+ terminals. Vasopressinergic N-type currents increased $18.3 \pm 8.3\%$ and $66.7 \pm 9.4\%$ in the presence of 10 and 100nM DAMGO and decreased $20 \pm 5.4\%$ by 300 nM DAMGO. N-type currents in oxytocinergic terminals decreased by $6.8 \pm 7.3\%$ by 10nM DAMGO, 14 ± 6.4 at 100 nM DAMGO, and $41.7 \pm 4.6\%$ by 300 nM DAMGO. Significance was determined for each agonist concentration and its corresponding N-type isolated level and expressed as $*P < 0.05$ and $**P < 0.01$, SEM, $n=12$.

Fig. 4.

DAMGO's ability to either increase or inhibit N-type dependent changes in $[Ca^{2+}]_i$ depends on the peptide content of the terminal. A: Fura-2 loaded terminals from a representative experiment showing bright field (BF) images (top, left) containing individual terminals identified subsequently as OT+ (green; bottom, left) and AVP+ (red; bottom, right) by using antibodies against Neurophysin I and Neurophysin II, respectively. B: Bar graph distribution of $[Ca^{2+}]_i$ changes for T6 & T9. Baseline $[Ca^{2+}]_i$ measurements were subtracted from peak responses in all experimental conditions and values expressed as percent changes from each terminal's initial $[Ca^{2+}]_i$ response to high K^+ stimulation (1). The N-type dependent component (2) remaining after exposure to the toxin mix increases 15.8% in T6 (AVP+) and decreases 4.1% in T9 (OT+) when stimulated with high K^+ in the presence of 10nM DAMGO (3). At 100nM DAMGO, $[Ca^{2+}]_i$ decreased 21.9% in OT+, an increase of 3% in AVP+ terminals (4). At 500nMDAMGO both terminals showed a decrease of 13.7% in the AVP+ and 33.9% for OT+ terminals when compared to control values (5). High K^+ challenge in the presence of 800 nM MVIIA is shown in (6). Eleven percent of the initial $[Ca^{2+}]_i$ signal remained in the AVP+ terminal while 10.3% remained in the OT+ terminal. C: Consecutive images, 10 sec apart, of terminals 6 (AVP+) and 9 (OT+) loaded with Fura-2 AM. Both terminals were challenged simultaneously with 50mM High K^+ for 20 sec in the presence of 10nM DAMGO. Pseudo-color scale bar indicates $[Ca^{2+}]_i$; Scale bar=1.5 μ m. D: Bar graph summarizing all the changes in $[Ca^{2+}]_i$ induced by DAMGO across all experiments. Baseline $[Ca^{2+}]_i$ measurements were subtracted from peak responses in all experimental conditions for all isolated terminals (n=3 independent experiments) and expressed as percentage of the initial control response in high K^+ . Terminals were segregated into two groups based on their peptide content and/or their responses to low concentrations of DAMGO. Means were calculated for every condition within each group and presented as percentages $\pm$ SEM. In the AVP group, the toxin mix decreased the calcium signal to 39.6 $\pm$ 3.8% of control values. In the presence of 10, 100, 300, and 500nMDAMGO, calcium levels were 60.9 $\pm$ 3.7%, 66.4 $\pm$ 6.4%, 54.4 $\pm$ 4.6, and 45.1 $\pm$ 6.2% of control values, respectively. In the OT group the toxin mix decreased the calcium signal to 36.3 $\pm$ 4.3% of control values. In the presence of 10, 100, 300, and 500nMDAMGO calcium levels were 34.9 $\pm$ 4.4%, 32.2 $\pm$ 3.9%, 29.2 $\pm$ 2.3%, and 20 $\pm$ 2.1% from control values, respectively.

Significance was estimated between each agonist concentration and its corresponding N-type isolated level and expressed as $*P < 0.05$ and $**P < 0.01$.

Fig. 5.

N-type dependent neuropeptide release is differentially modulated by DAMGO. Histograms summarizing the high K^+ -elicited neuropeptide release measured in the absence and presence of DAMGO and specific Ca^{2+} channel blockers. Bars represent the area calculated for each release peak, baseline subtracted, for all conditions tested. Notice that both neuropeptides were measured from the same experimental samples. A: AVP release was measured in the absence (first bar) and presence of 2 μ M Nicardipine, 100 nM MVIIC, and 10 nM SNX482 to segregate the N-type dependent release component (second bar). AVP release decreases from 926 ± 46 to 452 ± 25 pg with the toxin treatment. Exposure to 10 nM DAMGO caused an increase in AVP release (573 ± 22 pg) but a decrease (246 ± 16 pg) when exposed to 100 nM DAMGO. Last bar shows AVP levels recovering to 942 ± 46 pg after both toxins and DAMGO are washed away. B: OT release was measured in the same conditions as described in (A). In the presence of the toxin mix, OT release was reduced to 320 ± 33 pg when compared to the initial control values of $1,028 \pm 66$ pg. Exposure to 10 and 100 nM DAMGO caused a reduction in the N-type dependent OT release to 125 ± 29 and to 116 ± 8 pg respectively. Last bar shows complete recovery to control values ($1,224 \pm 176$ pg). Significance was estimated for each agonist concentration and its corresponding N-type isolated level and expressed as * $P < 0.05$ and ** $P < 0.01$, SEM, $n = 4$ for AVP; $n = 3$ for OT.

Fig. 6.

Immunocytochemical localization of N-type channels in AVP+ versus OT+ terminals is not altered by DAMGO. A: Bright field image of a terminal (left) showing the boundaries used to estimate α_{1B} (Cav_{2.2}) immunofluorescence associated with the membrane area (right). Total fluorescence inside circle #2 was subtracted from that inside circle #1 and expressed as percentage of total fluorescence (C). B: Mean total signal intensity (SI) values per square micron for α_{1B} immunofluorescence in OT terminals are 23% less than in AVP immunolabeled terminals ($P < 0.05$; $n=102$ terminals; four separate experiments). All terminals were exposed simultaneously to all three antibodies; that is, anti- α_{1B} , anti-Neurophysin I and anti-Neurophysin II. All secondary antibodies were also presented concurrently. C: α_{1B} -dependent fluorescence associated with each terminal's membrane area with and without exposure to 25 nM DAMGO. Terminals were exposed to DAMGO for 30 min and immediately fixed. Control terminals were washed with Locke's saline during the same period of time before fixation. Antibodies were presented concurrently and from the same exact aliquots. α_{1B} -dependent fluorescence associated with each terminal's membrane area was not significantly different between OT and AVP containing terminals ($P > 0.05$) in control conditions. α_{1B} -dependent fluorescence associated with each terminal's membrane area in the presence of DAMGO was also not significantly different from control values for either terminal type ($P > 0.05$; $n=49$ terminals). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 7.

β_{2a} immunoreactivity is present in AVP+, but not in OT+ terminals. Isolated terminals were divided into two groups and each one was exposed simultaneously to a mixture of three antibodies; anti- α_{1B} (red), anti- β_{2a} (green) and either anti-Neurophysin I or anti-Neurophysin II (both in blue). A mix of secondary antibodies (Alexa 350, 488, and 594) were presented concurrently and from the same aliquots. A: Two vasopressinergic terminals showing the presence of both α_{1B} and β_{2a} immunoreactivity. A two-dimensional profile of all three immunofluorescence signals (in gray fluorescent units (gfu) vs. μ m) was taken along the white dotted line (inserts)). The orange dot represents an area where both α_{1B} and β_{2a} are closely expressed (also visible in the profile). Similar results were observed in 42 AVP+ terminals. B: Fluorescence images of two oxytocinergic (blue) and one vasopressinergic (not labeled) terminals. Bright field image of all three terminals is inserted on top left. All three terminals showed α_{1B} immunoreactivity, however, neither of the OT-containing terminals showed β_{2a} immunoreactivity. Yet, the terminal that does not contain OT (and is thus assumed to be AVP+) does show the presence of β_{2a} . A two-dimensional profile of all three immunofluorescence signals (gfu/ μ m) taken along the white dotted line is shown as an insert (left). Similar results were observed on 41 OT+ terminals (see right panel). Scale bar=1 μ m.

Fig. 8.

β_{1b} , β_4 , and β_3 immunoreactivity in AVP+ and OT+ terminals. Isolated terminals were plated into individual Petri dishes and exposed simultaneously to a mix of three antibodies per dish. To identify the presence of β_{1b} , terminals were exposed to anti- α_{1B} (red), anti- β_{1b} (green) and either anti-Neurophysin I or anti-Neurophysin II (both in blue). To identify the presence of β_4 , terminals were exposed to anti- α_{1B} (red), anti- β_4 (green) and either anti-Neurophysin I or anti-Neurophysin II (both in blue). To identify β_3 , a mix of anti- α_{1B} (blue), anti- β_3 (red) and either anti-Neurophysin I or anti-Neurophysin II (both in green) was used. Secondary antibodies (Alexa 350, 488, and 594) were presented concurrently and from the same aliquots. Bright field images are shown on the top left and a two-dimensional profile of all three immunofluorescence signals (in $\text{gf}/\mu\text{m}$) was taken along the white dotted line and shown on the bottom, left of each image. Similar results were observed in 56 OT+ and 59 AVP+ terminals. gf =gray fluorescent units.