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Short communication

Colocalization of calretinin and calbindin-D_{28k} with oxytocin and vasopressin in rat supraoptic nucleus neurons: A quantitative study

Seiji Miyata^{a,1}, Arshad M. Khan^b, Glenn I. Hatton^{a,*}^a Department of Neuroscience, University of California, Riverside, CA 92521, USA^b Division of Biomedical Sciences, University of California, Riverside, CA 92521, USA

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Abstract

Recent electrophysiological experiments, in which purified calbindin-D_{28k} (calbindin) and calretinin antibodies were diffused into these neurons, showed that Ca²⁺-dependent membrane potentials and firing patterns were profoundly and predictably affected by Ca²⁺-binding proteins (CaBPs). The present study used quantitative analyses of a dual-labeling immunofluorescence method to investigate the colocalization of the CaBPs, calbindin and calretinin in oxytocin (OT)- and (VP)-containing neurons of the supraoptic nucleus. Analyses of tissue immunostained with two different dilutions of each CaBP antibody used, revealed that 84% and 72% of the OT neurons were positive for calbindin immunoreactivity (-ir) at the higher and lower antibody concentrations, respectively. 52% and 50% of OT neurons were positive for calretinin-ir; thus, many OT neurons express both calbindin and calretinin. In contrast, only 25% and 18% of VP neurons showed calbindin-ir, and they were virtually devoid of calretinin-ir. These results provide evidence that CaBP expression in OT neurons is both greater and more diverse than in VP neurons, and are consistent with the hypothesis that Ca²⁺ buffering capacity contributes to the control of intrinsic firing patterns. © 1998 Elsevier Science B.V.

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The EF-hand family of Ca²⁺-binding proteins (CaBPs) participates in many physiological processes with free Ca²⁺. Both immunohistochemical [6,12] and in situ hybridization studies [11] have revealed that the regional distribution of these proteins is limited to discrete regions of the central nervous system. Indeed, the localization of the CaBPs calbindin, calretinin and parvalbumin have been examined immunohistochemically in the rat thalamus where some regions were shown to contain both calbindin and calretinin, but other regions had only one type of CaBP, either calbindin or calretinin [3].

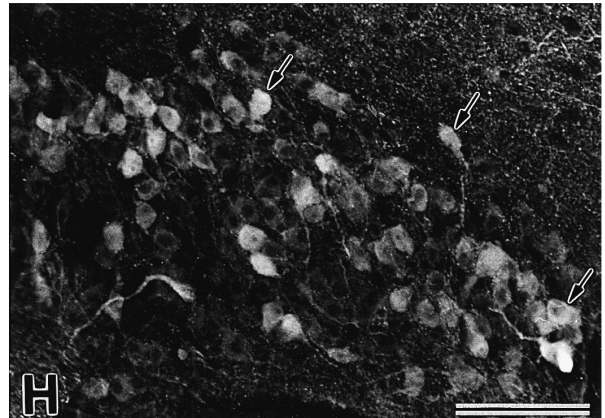
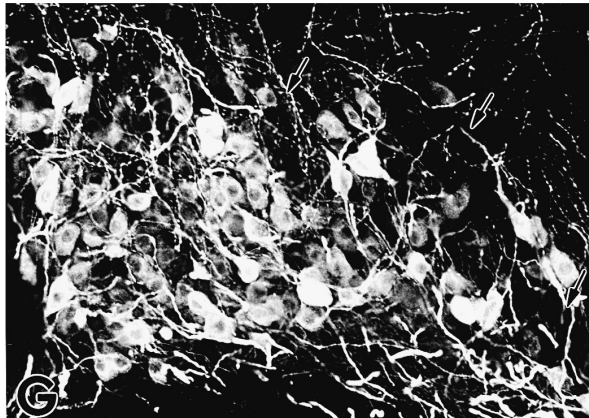
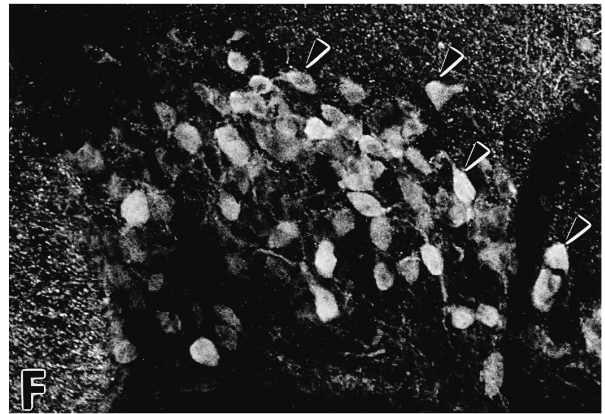
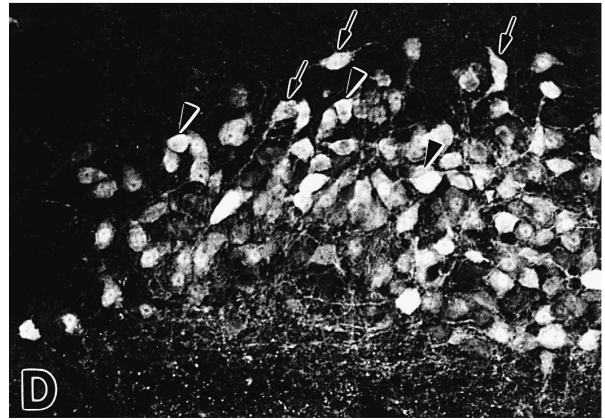
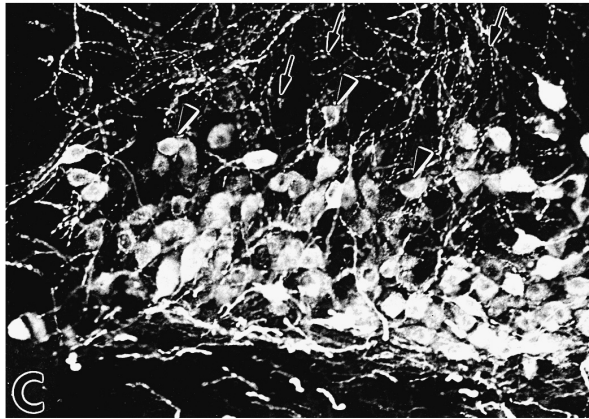
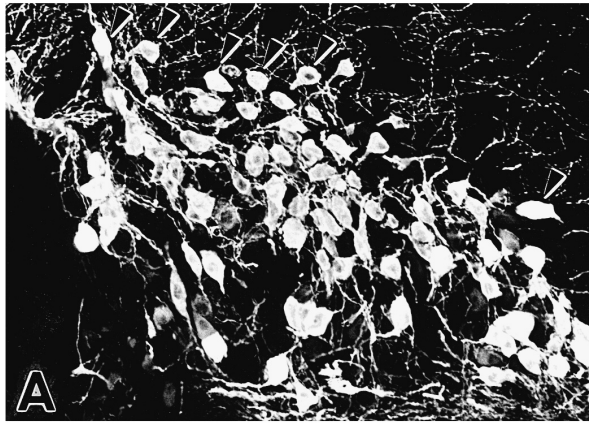
Parvalbumin is colocalized with GABAergic neurons in the cerebral cortex [7], and has also been found to colocalize with glycine in reticular neurons [2]. Calbindin and calretinin also colocalize with GABA in some neurons [19]. To date, although little is known about the possible

additional roles of CaBPs in neurons and glia, they are primarily considered to function as Ca²⁺ buffering proteins. Injection of high concentrations of both calbindin and parvalbumin have been shown to reduce the increase in free Ca²⁺ concentration produced by brief depolarizations in dorsal root ganglion neurons [8]. A study involving transfection of the calbindin gene into pyramidal neurons has demonstrated that while calbindin does not alter evoked neurotransmitter release, it suppresses post-tetanic potentiation at excitatory synapses [9].

In oxytocin (OT) and vasopressin (VP) magnocellular neurons in the hypothalamus, the role of Ca²⁺ in some of these physiological factors has been examined in our recent work [15–17]. When physiologically activated, VP neurons typically display depolarizing afterpotentials (DAPs) and phasic firing patterns. Most activated OT neurons, on the other hand, are characterized by patterns of continuous firing and an absence of DAPs [5]. A series of dual-label immunofluorescence studies demonstrated colocalization in the SON of calbindin and OT, calbindin and VP, and calretinin and either OT or VP [3,4]. The present investigation was carried out to clarify the differences in

* Corresponding author. Fax: +1-909-787-2966; E-mail: ghatton@pop.ucr.edu

¹ Present address: Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan.



colocalization for calbindin (or calretinin) between OT and VP neurons of the rat SON by quantitative analysis, using a dual immunofluorescence method.

A total of 27 male Sprague–Dawley rats were used. All experimental protocols were carried out in accordance with the National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of California, Riverside.

Rats were deeply anesthetized with intraperitoneal injections of a lethal dose of pentobarbital (50 mg/kg body weight) and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer. The hypothalamus was removed from the brain and post-fixed in the same fixative for 24 h at 4°C, and sectioned coronally (20 μ m thick) using a Vibratome. Sections were first incubated with a polyclonal antibody against calbindin-D_{28k} (dilution 1:1250 or 1:5000) or calretinin (1:100 or 1:400, Chemicon, Temecula, CA) for 24 h at 4°C, then with biotinylated anti-rabbit IgG (1:200), reacted with Texas Red Streptavidin, (TRS, 1:50), and incubated with a monoclonal antibody against OT-neurophysin (PS 38, 1:20) or VP-neurophysin (PS 41, 1:20). Finally, sections were incubated with fluorescein isothiocyanate labeled goat anti-mouse IgG adsorbed with rat serum (1:50).

Analyses were carried out on photomicrographs of coronal sections through the middle portion (largest coronal profiles) of the SON. Micrographs of three sections were taken for each animal, a mean number of cells from these three sections was computed. The photographed sections had been immunostained first for either calbindin or calretinin and secondly for either OT or VP. Immunoreactive neurons were identified under epifluorescence, and their locations marked on the photomicrograph taken of the same field of view. Calbindin- or calretinin-positive neurons were identified when neurons of the appropriate size and shape demonstrated a clear increased immunoreactivity in comparison with the background level. The percentages of calbindin- (or calretinin-) positive neurons were expressed as means (\pm S.E.M.) from four groups of rats: calbindin (1:1250, $n = 8$ or 1:5000, $n = 7$) and calretinin (1:100, $n = 6$ or 1:400, $n = 6$). Percentage values were compared by ANOVA ($p < 0.05$), and where overall ANOVA was significant, multiple comparisons were performed using the Newman–Keuls test.

By comparing two micrographs showing the identical field of SON neurons, one micrograph showing OT-ir neurons (Fig. 1A) and the other calbindin-positive neurons

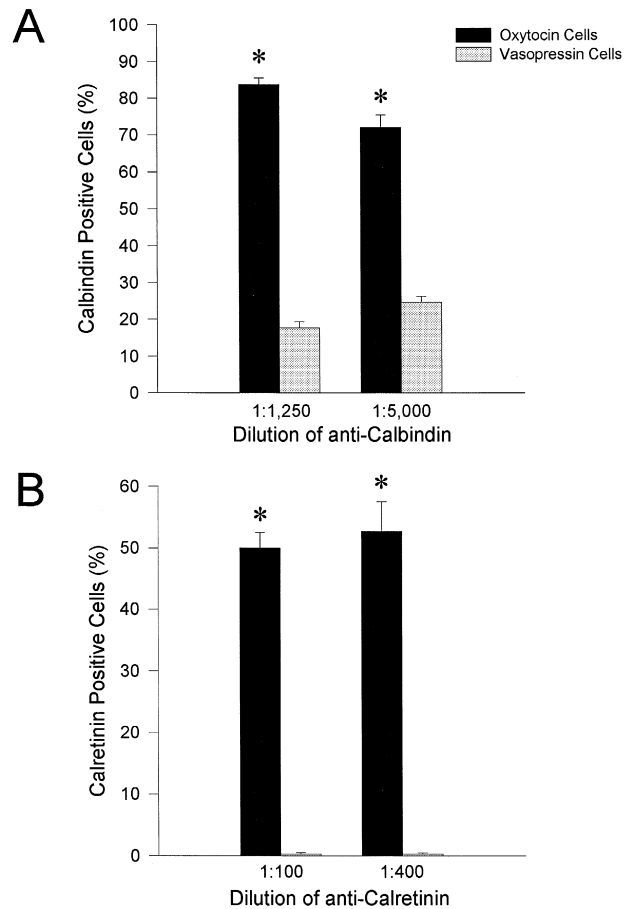


Fig. 2. Quantitative analysis of calbindin (A) and calretinin (B) positive neurons (expressed as a percentage of total OT-positive or VP-positive neurons) in either OT or VP neurons using two different antibody dilutions. (A) The percentages of calbindin positive neurons are significantly greater in OT neurons as compared to that in VP neurons ($* p < 0.0001$, $n = 8$). Differences between antibody dilutions were also significant at $p < 0.01$ (1:1250, $n = 8$; 1:5000, $n = 7$). (B). Around 50% of OT neurons are calretinin-positive, but almost all VP neurons were calretinin-negative. The difference in calretinin-positivity between OT and VP neurons is significant ($* p < 0.003$); $n = 6$. For both A and B, bars are mean percentages (\pm S.E.M.).

(Fig. 1B), it is apparent that the distribution of calbindin-ir corresponds closely to that of OT. While most OT neurons were calbindin-positive, however, some calbindin-positive neurons did not show OT-ir. In contrast to the frequent colocalization of calbindin-ir in OT neurons, the colocalization of calbindin-ir with VP-ir (Fig. 1C and D) was far less extensive. Calretinin-ir in SON neurons colocalized with OT-ir in a distinct subpopulation of OT neurons, with

Fig. 1. Fluorescence photomicrographs of coronal sections showing dual immunostaining for either OT or VP and calbindin (or calretinin). (A and B) FITC-labeled OT-ir (A) and TRS-labeled calbindin-ir (B) in the same section of the SON. (C and D) FITC-labeled VP-ir (C) and TRS-labeled calbindin-ir (D). Most VP neurons are calbindin-negative (arrows), but a minority of these neurons are calbindin-positive (arrowheads). (E and F) FITC-labeled OT-ir (E) and TRS-labeled calretinin-ir (F), showing colocalization of OT and calretinin-ir. (G and H) FITC-labeled VP-ir (G) and TRS-labeled calretinin-ir (H). VP neurons are negative for calretinin-ir (arrows). Arrowheads indicate some of the neurons that are positive for both OT or VP and calbindin or calretinin. Arrows represent OT and VP neurons that are negative to calbindin or calretinin. Scale bar = 100 μ m.

many OT neurons being calretinin-negative (Fig. 1E and F). In striking contradistinction, there was virtually no colocalization of calretinin- and VP-ir (Fig. 1G and H).

To evaluate the degree of colocalization of calbindin- (or calretinin-) positive staining with either OT- or VP-ir, quantitative analyses were performed. For statistical tests, dilution factors were between-groups comparisons, and colocalization factors were within-groups comparisons. The percentages of neurons found to express both calbindin-ir and OT-ir were high (83.7 ± 1.8 and 72.0 ± 3.4 at dilutions of 1:1250 and 1:5000, respectively, Fig. 2A). However, the percentages of neurons found to express both calbindin-ir and VP-ir (17.7 ± 1.7 and 24.6 ± 1.6 at dilutions of 1:1250 and 1:5000, respectively) were significantly lower ($p < 0.0001$). Differences between antibody dilutions were also significant at $p < 0.01$. When percentages of calretinin-positive neurons were obtained, it was observed that calretinin-ir in OT-ir neurons was 50.0 ± 2.5 and 52.0 ± 4.8 at dilutions of 1:100 and 1:400, respectively (Fig. 2B). Moreover, the percentages of neurons colocalizing calretinin-ir and VP-ir were nearly equal to zero (0.3 ± 0.2 and 0.3 ± 0.2 at dilutions of 1:100 and 1:400, respectively). For calretinin, the differences between antibody dilutions were not significant ($p > 0.6$). Note that, compared to Fig. 2A, the scale on the ordinate of Fig. 2B is expanded.

The neurohypophysial hormones, OT and VP, differ in their primary structure by only two amino acids but nevertheless have very different physiological functions. However, OT and VP neurons have similar morphological characteristics (same shape, size, and ultrastructural features). Differences in intracellular signal transduction mechanisms between OT and VP neurons have also not been found. The present experiments demonstrate that a higher percentage of OT neurons express CaBPs (both calbindin and calretinin) than is seen in VP neurons. These results are consistent with previous dual labeling immunofluorescence studies, although those previous experiments were not designed to produce quantitative results and comparisons [3,4]. Electrophysiological experiments indicate that putative VP neurons display phasic firing patterns and DAPs following action potentials [5,16,17]. In contrast, most OT neurons display continuous firing patterns and no DAPs [1,14]. Until recently there was little evidence implicating the precise mechanism underlying the difference in these firing patterns between OT and VP neurons [15]. Also, our electrophysiological studies have shown that injection of either a Ca^{2+} chelator or a CaBP changes phasic firing patterns into continuous ones [15]. Members of the EF-hand family of CaBPs, including both calbindin and calretinin, have the ability to modulate intracellular free Ca^{2+} concentration [13,18]. These results are consistent with the quantitative data of the present study and with the idea that OT neurons generally have a greater capability of buffering intracellular Ca^{2+} . Together these results further support the hypothesis that the abundance of

CaBPs in OT neurons, which allows for a high degree of Ca^{2+} buffering action, largely accounts for the differential intrinsic firing patterns of these two cell types [10].

Another probable function of CaBPs is that of protecting neurons against the damaging effects of excess Ca^{2+} influx during prolonged periods of intense neuronal activity. OT neurons have been observed to display continuous firing patterns, and to produce high frequency bursts of action potentials. These bursts would be expected to be accompanied by large increases of intracellular Ca^{2+} , so it may be that CaBPs in OT neurons may participate in protecting these cells from the neurotoxic effects of excess intracellular Ca^{2+} . It is probable, therefore, that abundant CaBPs in OT neurons allow the repeated high frequency bursts to occur during suckling without concomitant neurotoxicity for excess Ca^{2+} .

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References

- [1] R.D. Andrew, F.E. Dudek, Burst discharge in mammalian neuroendocrine cells involves an intrinsic regenerative mechanism, *Science* 221 (1983) 1050–1052.
- [2] E. Aoki, R. Semba, A. Seto-Oshima, C.W. Heizmann, S. Kashiwamata, Co-existence of parvalbumin and glycine in the rat brainstem, *Brain Res.* 525 (1990) 140–143.
- [3] R. Arai, D.M. Jacobowitz, S. Deura, Heterogeneity in calbindin- $\text{D}_{28\text{k}}$ expression in oxytocin-containing magnocellular neurons of the rat hypothalamus, *Histochemistry* 101 (1994) 9–12.
- [4] R. Arai, D.M. Jacobowitz, I. Nagatsu, Calretinin is differentially localized in magnocellular oxytocin neurons of the rat hypothalamus: A double-labeling immunofluorescence study, *Brain Res.* 735 (1996) 154–158.
- [5] W.E. Armstrong, B.N. Smith, M. Tian, Electrophysiological characteristics of immunohistochemically identified rat oxytocin and vasopressin neurones in vitro, *J. Physiol.* 475 (1994) 115–128.
- [6] K.G. Baimbridge, J.J. Miller, C.O. Parkes, Calcium-binding protein distribution in the rat brain, *Brain Res.* 239 (1982) 519–525.
- [7] M.R. Celio, Parvalbumin in most gamma-aminobutyric acid-containing neurons of the rat cerebral cortex, *Science* 231 (1986) 995–997.
- [8] P.S. Chard, D. Bleakman, S. Christakos, C.S. Fullmer, R.J. Miller, Calcium buffering properties of calbindin- $\text{D}_{28\text{k}}$ and parvalbumin in rat sensory neurones, *J. Physiol.* 472 (1993) 341–357.
- [9] P.S. Chard, J. Jordan, C.J. Marcuccilli, R.J. Miller, J.M. Leiden, R.P. Roos, G.D. Ghadge, Regulation of excitatory transmission at hippocampal synapses by calbindin- $\text{D}_{28\text{k}}$, *Proc. Natl. Acad. Sci. U.S.A.* 92 (1995) 5144–5148.
- [10] P. Cobbett, K.G. Smithson, G.I. Hatton, Immunoreactivity to vasopressin- but not oxytocin-associated neurophysin antiserum in phasic neurons of rat hypothalamic paraventricular nucleus, *Brain Res.* 362 (1986) 7–16.

- [11] G.D. Frantz, A.J. Tobin, Cellular distribution of calbindin-D_{28k} mRNAs in the adult mouse brain, *J. Neurosci. Res.* 37 (1994) 287–302.
- [12] L.M. Garcia-Segura, D. Baetens, J. Roth, A.W. Norman, L. Orci, Immunohistochemical mapping of calcium-binding protein immunoreactivity in the rat central nervous system, *Brain Res.* 296 (1984) 75–86.
- [13] V.L. Leathers, S. Linse, S. Forsen, A.W. Norman, Calbindin-D_{28k}, a 1,25-dihydroxyvitamin D₃-induced calcium-binding protein, binds five or six Ca²⁺ ions with high affinity, *J. Biol. Chem.* 265 (1990) 9838–9841.
- [14] P. Legendre, D.A. Poulain, Intrinsic mechanisms involved in the electrophysiological properties of the vasopressin-releasing neurons of the hypothalamus, *Prog. Neurobiol.* 38 (1992) 1–17.
- [15] Z. Li, C. Decavel, G.I. Hatton, Calbindin-D_{28k}: Role in determining intrinsically generated firing patterns in rat supraoptic neurones, *J. Physiol.* 488 (1995) 601–608.
- [16] Z. Li, G.I. Hatton, Oscillatory bursting of phasically firing rat supraoptic neurones in low-Ca²⁺ medium: Na⁺ influx, cytosolic Ca²⁺ and gap junctions, *J. Physiol.* 496 (1996) 379–394.
- [17] Z. Li, G.I. Hatton, Ca²⁺ release from internal stores: role in generating depolarizing after-potentials in rat supraoptic neurones, *J. Physiol.* 498 (1997) 339–350.
- [18] K. Ren, M.A. Ruda, A comparative study of the calcium-binding proteins calbindin-D_{28k}, calretinin, calmodulin and parvalbumin in the rat spinal cord, *Brain Res. Rev.* 19 (1994) 163–179.
- [19] J.H. Rogers, Immunohistochemical markers in rat cortex: co-localization of calretinin and calbindin-D_{28k} with neuropeptides and GABA, *Brain Res.* 587 (1992) 147–157.