Differential $\text{Ca}^{2+}$ signaling characteristics of inhibitory and excitatory myenteric motor neurons in culture

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GUT MOTILITY IS MAINLY CONTROLLED by the myenteric plexus. This plexus comprises several types of neurons, such as sensory, inter-, and motor neurons. Each of these is required to exert the autonomous reflex activity within the gut and to efficiently control other intestinal functions. Primary cultures have proven to be a suitable model for the in vitro study of these neurons, especially because they retain the capacity of forming networks and synaptic connections (8, 22, 37, 38). In these cultures, the electrical stimulation of neuronal processes induced a synaptically mediated rise in intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) (35). Direct application of excitatory neurotransmitters, shown to be endogenously present in fibers and neuronal cell bodies, mimicked these transient changes (34).

The neuronal diversity of the plexus is also reflected in cultured conditions (8, 22), but it remains hard to assess what the original function was of each and every individual neuron. Until now, heterogeneity of cultures has not been addressed sufficiently, and the physiological differences that might accompany neuronal function have not been taken into account. If, therefore, $[\text{Ca}^{2+}]_i$ signaling could be studied in a subset of neurons with an identical function, it might yield valuable physiological information.

Retrograde labeling techniques have been widely used to label neurons projecting to and from specific sites (1, 14). Brookes and Costa (1) used 1,1-didodecyl-3,3',3'-tetramethyl indocarbocyanine (DiI), a dye that selectively labels neurons by migration through lipid double layers, to identify the neurons supplying specific loci in the muscle layer of the guinea pig small intestine. The visualization of distinct groups of neurons allowed subsequent definition of chemical content and morphology (3, 30–33). DiI labeling, in combination with immunohistochemistry, was also used to identify other types of neurons in the intestinal tract of several different species (2, 12, 18, 20, 21, 23, 24, 26, 27, 40). Electrophysiological recordings from DiI-labeled neurons did not reveal any differences in membrane properties and excitability caused by the DiI labeling (12, 19).

It was our aim to study the $[\text{Ca}^{2+}]_i$ signaling in a functionally identified group of neurons. Therefore, optical $[\text{Ca}^{2+}]_i$ imaging techniques were applied that, if feasible, would enable us to record simultaneously from DiI-labeled and nonlabeled neurons. Since we wanted to combine two optical techniques, we chose to use primary cultures, because the optical monitoring of multiple neurons in intact contractile tissue is still in its infancy. The combination of these techniques might provide a method to simultaneously study functionally identified and other neurons, possibly revealing physiologically relevant differences.

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**EXPERIMENTAL PROCEDURES**

**Dil labeling of neurons.** Guinea pigs of either sex were killed by concussion and exsanguination from the cervical vessels. The *Principles of Laboratory Animal Care* were followed as well as the specific national laws of the Ministerie van Landbouw, Belgium. The small intestine was removed under sterile conditions, opened along the serosal border, and pinned flat in a Sylgard-bottomed dissection dish. The mucosa was removed, and small glass beads with Dil were placed on the circular muscle layer (1). The small intestine preparations were cultured in an organ-typical medium (Ham's F-12) supplemented with 2% FBS and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin, 1 μg/ml amphotericin B, and 50 μg/ml gentamycin). Nifedipine (10⁻⁶ M) was added to prevent muscle contraction. During the incubation (24–48 h), the Dil retrogradely migrated through the axon into the cell body of the motor neurons. Dil did not label any neurons when applied to muscle strips that were removed from and then replaced on the myenteric plexus. Furthermore, no clear labeling was observed when Dil was placed on the remnants of connective tissue between mucosa and muscle layer (data not shown). When Dil was applied to the mucosa, a different pattern of labeling was observed; the cells had distinctly larger somas than the neurons labeled from the circular muscle layer (data not shown). This suggested that labeling of fibers that ran through the muscle to the uppermost layers, such as mucosa and submucous plexus, was limited.

**Cell cultures.** Primary cultures were prepared according to a previously described method (8, 9). In brief, after 2 days of incubation, the circular muscle, together with the excess Dil, was removed from the small intestine preparations by digestion. The longitudinal muscle and myenteric plexus was segmented and digested in an enzymatic solution containing protease (1 mg/ml) and collagenase (1.25 mg/ml). Following a 30-min incubation at 37°C, the suspension was placed on ice and the enzymatic reaction was stopped by centrifugation (1,600 rpm). Ganglia were picked up under a binocular microscope and placed in culture dishes (NUNC), in which they adhered to the cover-glass bottom. The neurons started growing in network-like structures reminiscent of the ganglionic plexus. The culture medium was Medium 199 enriched with 10% FBS and 50 ng/ml 7s nerve growth factor. Antibiotics and the enzymatic reaction were stopped by centrifugation (1,600 rpm). Ganglia were picked up under a binocular microscope and placed in culture dishes (NUNC), in which they adhered to the cover-glass bottom. The neurons started growing in network-like structures reminiscent of the ganglionic plexus. The culture medium was Medium 199 enriched with 10% FBS and 50 ng/ml 7s nerve growth factor. Antibiotics were added (identical concentrations as in the organ-typical culture medium), and glucose concentration was elevated to 30 mM. The [Ca²⁺] in the medium was adjusted to 2.5 mM by adding CaCl₂. The culture chambers were kept in an incubator at 37°C and continuously gassed with 5 CO₂:95% O₂. The medium was changed every 2 days. Addition of 10 μM arabinos-C-furanoside prevented the proliferation of dividing cells.

**Experimental medium.** Experiments were performed at room temperature in a modified Krebs solution containing (in mM) 150 NaCl, 6 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, with pH adjusted to 7.38 with NaOH (5 M). The zero-Ca²⁺ solution contained 2.5 mM MgCl₂ and 2 mM EDTA. In the high-K⁺ medium, the K⁺ concentration was elevated to 75 mM. Osmolarity was adjusted by lowering the Na⁺ concentration to 81 mM.

**[Ca²⁺]ᵢ measurements.** [Ca²⁺]ᵢ, changes were measured using the Ca²⁺ indicator Indo-1. After rinsing, the neurons were loaded (30–45 min) in a modified Krebs solution containing 5 μM Indo-1-AM and 2.5 μM Pluronic F-127 (25% wt/wt). Indo-1-AM is cleaved by esterases present in the soma. The Ca²⁺-bound form emits light at 405 nm, whereas the emission of the free Indo-1 lies close to 480 nm. The ratio of these two signals is directly proportional to the [Ca²⁺]ᵢ. Experiments were performed on an MRC 1024 Bio-Rad confocal microscope equipped with an Ar⁺ ion laser. Sample frequency varied between 1.25 and 1.75 Hz, depending on the number of regions of interest selected. Three neurotransmitters, ACh, substance P (SP), and serotonin (5-HT), were used. A sufficiently long recovery period occurred in between successive applications of several neurotransmitters. If both a rise at 405 nm and a drop at 480 nm were observed, the neuron was judged positive to the particular agonist. The proportions of positive and negative neurons were compared using a χ² test. The baseline of the signals and the amplitude of the responses were analyzed on a personal computer.

**Immunocytochemistry.** Cultured cells were fixed in a freshly prepared 2%/0.2% paraformaldehyde/picric acid solution, washed in PO₄⁻ buffer, and processed for permeabilization and blocking of nonspecific binding sites. A rabbit antibody against choline acetyltransferase (ChAT) was used to label cholinergic neurons. After subsequent incubation in an FITC-labeled secondary antibody against rabbit IgG, the neurons could be visualized with a Nikon microscope equipped with a fluorescence unit. Filtrcure Y-2EC (EX BP540–580, DM RK595, EM BA 600–660) was used for DI (excitation 547 nm; emission 565 nm) and Filtrcure B-2A (EX BP470–490, DM 505, EM BA 510/20) for FITC. The nitrergic neurons were identified with an NADPH diaphorase reaction. During an incubation (2 h, 37°C) in the presence of NADPH (1 mg/ml), tetrazolium blue (0.2 mg/ml), and 0.5% Triton X-100, NADPH diaphorase-positive cells were stained blue and could be visualized under a conventional light microscope.

**Chemicals.** Culture Medium 199, Ham's F-12, FBS, and antibiotics were from Gibco (Merelbeke, Belgium); 7s nerve growth factor was from Alomone Labs (Jerusalem, Israel); protease, collagenase, nifedipine, 5-HT, ACh, and SP were from Sigma (Bornem, Belgium); primary antibody to ChAT was provided by Dr. Michael Schemann (Hanover, Germany); secondary antibodies were from Jackson Labs (West Grove, PA); Indo-1-AM, DiI, and Pluronic F-127 were from Molecular Probes (Leiden, The Netherlands).

**RESULTS**

**Culturing of Dil-labeled neurons.** Dil-labeled neurons were readily detectable in the myenteric plexus of the guinea pig small intestine (Fig. 1). These segments were used to prepare myenteric neuron cultures. After 2–3 days in culture, the neurons formed network-like structures, reminiscent of the myenteric plexus. In this network, the Dil-labeled neurons were easily discerned and the number of cells per ganglion as well as the staining patterns were similar compared with the myenteric ganglia in situ. The motor neurons did not have a specific location within the ganglia, either in situ or in culture. Within the cell, the Dil was equally distributed in the cytosol of the neurons, whereas nuclei were never stained. High-quality Dil stainings allowed us to assess the morphological features of the neurons. All of these neurons were small Dogiel type I-like neurons. As with Dil labeling in tissue, the dye also remained in the neurons in culture. Even after 7 days, no blurred edges due to Dil diffusion could be observed (Fig. 2).

**[Ca²⁺]ᵢ signaling in Dil-labeled neurons.** The Indo-1-AM loading of the cultured neurons was not affected by the presence of DiI. No significant differences in
initial Indo-1 emission ratio could be observed for Dil-labeled and nonlabeled neurons (0.21 ± 0.02 vs. 0.24 ± 0.02; n = 20). Apparently, putative negative side effects such as color and chemical quenching were absent. The high-K+ Krebs depolarization, inducing a \([Ca^{2+}]_i\) rise in the neurons due to the opening of voltage-operated \(Ca^{2+}\) channels, was used to distinguish between neurons and other cells (35). No significant differences were observed between the amplitude (0.49 ± 0.07 vs. 0.53 ± 0.04; n = 20) and shape of the \(K^+\)-induced rise in \([Ca^{2+}]_i\) in Dil-labeled neurons and the nonlabeled neurons.

ACh is an important neurotransmitter in the myenteric plexus, controlling fast synaptic events between neurons. Candidate neurotransmitters for slow synaptic neurotransmission are SP and 5-HT (5, 6, 11, 17, 36, 39). Therefore, the effects of ACh, SP, and 5-HT on the \([Ca^{2+}]_i\) signaling in the functionally identified neurons were studied. Each neurotransmitter induced a transient \([Ca^{2+}]_i\) rise in a subset of the Dil-labeled neurons (Figs. 3 and 4). The application of \(10^{-5}\) M ACh resulted in a \([Ca^{2+}]_i\) rise in 66.7% of the Dil-labeled neurons (n = 42). In the nonlabeled neurons, which were considered controls, the number of responding cells (NRC) to ACh (\(10^{-5}\) M) was 67.9% (n = 84; not significant). When SP (\(10^{-5}\) M) was administered, 39 out of 69 Dil-labeled neurons displayed a \([Ca^{2+}]_i\) rise, which accounted for 56.5%. In the control population, consisting of 200 cells, an NRC of 55.0% was observed (not significant). The application of 5-HT (\(10^{-5}\)M) induced \([Ca^{2+}]_i\) signaling in the majority of myenteric neurons in culture. In the Dil-labeled population (n = 70), the NRC was as high as 84.3%, whereas in the control population this was 91.1% (n = 180; not significant). The shape of the responses did not differ between the control and Dil-labeled population for each of the neurotransmitters.

**Immunocytochemistry of Dil-labeled motor neurons.** In the guinea pig small intestine, the myenteric neurons can be subdivided in two groups according to their chemical content. Either they contain ChAT, the main enzyme in the synthesis of ACh, or they express nitric oxide synthase, responsible for the production of nitric oxide (4). Distinction between the two populations can be made by an immunohistochemical staining for ChAT or an NADPH-diaphorase reaction. The former labels the excitatory cholinergic neurons, whereas the latter identifies the nitric oxide-producing nitrergic subpopulation (Fig. 5). Twelve out of forty-five Dil-labeled motor neurons were found to be nitrergic (26.6%), and the other 33 neurons were cholinergic (73.3%).

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**Fig. 1.** 1,1-Didodecyl-3,3,3',3'-tetramethyl indocarbocyanine (Dil) labeling of circular muscle motor neurons (arrowheads) in the guinea pig ileum. Bar, 100 μm.

**Fig. 2.** Dil-labeled motor neurons in culture. A: confocal image of cultured myenteric neurons loaded with the \(Ca^{2+}\) indicator Indo-1 (recorded at 480 nm). B: confocal image of the Dil-labeled motor neurons recorded at the same x-y-z site as A. Dil-labeled neurons also contained Indo-1 (arrows). Bar, 50 μm.
The neurotransmitter data (Fig. 6) demonstrated that the cholinergic and nitrergic DiI-labeled motor neurons did not differ with respect to their response to 5-HT (±80%). Sixty-nine percent of the cholinergic neurons (n = 29) responded to ACh, whereas none of the six inhibitory neurons displayed an ACh-induced 
\(\text{[Ca}^{2+}\text{]}_i\) rise \( (P < 0.01)\). Another significant difference was found in SP-induced 
\(\text{[Ca}^{2+}\text{]}_i\) signaling. Cholinergic DiI-labeled neurons \( (n = 26)\) showed significantly more responses to SP than the nitrergic ones \( (69.2\text{ vs. }12.5\%; \; P < 0.01)\). Similarly, the DiI-labeled subpopulation, which was positive for 5-HT and negative for SP, was significantly larger for the nitrergic neurons \( (85.7\text{ vs. }29.6\%; \; P = 0.02)\). The 5-HT+/SP+ subgroup was also larger for the cholinergic neurons \( (59.4\text{ vs. }14.3\%; \; P = 0.02)\).

Subpopulations of DiI-labeled neurons. The DiI-labeled motor neurons could be classified in several subgroups based on the response displayed to each of the neurotransmitters. The combination of the ACh and 5-HT data showed that all DiI-labeled motor neurons responding to ACh also responded to 5-HT. The DiI-labeled subgroup responding neither to ACh nor to 5-HT \( (\text{ACh}^-/\text{5-HT}^-)\) was significantly larger than in the control population \( (11.4\text{ vs. }1.8\%; \; P = 0.04)\). Similarly, the combination of ACh and SP data revealed that the ACh−/SP− subgroup was larger for the DiI-labeled than for control neurons \( (20.9\text{ vs. }8.8\%; \; P = 0.06)\). Few neurons \( (2\% \text{ of the DiI-labeled and } 6\% \text{ of the controls})\) did not have a response either to SP or to 5-HT.

In a limited number of experiments, three neurotransmitters could be studied subsequently. This revealed seven distinct subpopulations both for DiI-labeled motor neurons \( (n = 29)\) and control neurons \( (n = 46)\) (Table 1). Three main populations with a similar distribution in both DiI-labeled and control populations could be defined. The ACh+/5-HT+/SP+ subpopulation was the largest group, accounting for 55.2% of the DiI-labeled and 56.5% of the control neurons. About 20% of the neurons had responses to both 5-HT and ACh without having a SP response, and a third group only had responses to 5-HT \( (13.8\text{ and }10.9\% \text{ for DiI-labeled and control neurons, respectively})\).

DISCUSSION

This study reports the successful combination of retrograde labeling and \(\text{[Ca}^{2+}\text{]}_i\) measurements, allowing physiological experiments on functionally identified neurons in culture. We showed that \(\text{[Ca}^{2+}\text{]}_i\) signaling was not affected by DiI labeling. In addition, there were no qualitative differences in responses to three excitatory neurotransmitters when DiI-labeled and nonlabeled neurons were compared. However, subsequent immunochemical processing revealed that there was a difference between cholinergic and nitrergic motor neurons in response to SP and ACh, but not to 5-HT. The combination of neurotransmitter data suggested the existence of neuronal subpopulations for both DiI-labeled and control neurons.

Retrograde labeling is an effective means to identify motor neurons in the myenteric plexus of the guinea pig \( (1, 2, 24)\). We chose to use cultures to avoid the

![Fig. 3](http://ajpgi.physiology.org/) Effect of excitatory neurotransmitters on the intracellular Ca\(^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)) of DiI-labeled motor neurons. The ratio of the 2 Indo-1 emission wavelengths was plotted against time. Arrows represent the addition of 75 mM K\(^+\) (10 s), which activated voltage-dependent Ca\(^{2+}\) entry. Substance P (SP; \(10^{-5}\) M), serotonin (5-HT; \(10^{-5}\) M), and ACh (\(10^{-5}\) M) were applied as indicated by the horizontal bars.

![Fig. 4](http://ajpgi.physiology.org/) The number of neurons responding to different excitatory neurotransmitters. The absolute number of cells tested for each group is shown on each bar. No significant \( (P < 0.05)\) differences between DiI-labeled and non-DiI-labeled neurons were observed.
technical difficulties that were inherent in optical measurements in intact tissues. Furthermore, the optical technique had the advantage that both labeled and nonlabeled cells could be monitored simultaneously. Our results showed that this DiI labeling did not alter the efficacy of neuron culturing. The DiI staining was preserved in culture, and apparently there was no loss, or certainly no preferential loss, of DiI-labeled neurons. The number of DiI-labeled neurons in culture was rather low. This was due to the fact that on average three DiI crystals were applied per square centimeter of circular muscle, which was only \( \approx 0.05\% \) of the total surface. Since the innervation density of the circular muscle layer is not quite known, it was impossible to estimate the proportion of labeled motor neurons. We did not thoroughly examine the morphology of the DiI-labeled neurons. However, the finding that almost all neurons projecting to the circular muscle of the small intestine had Dogiel type I morphology (1) seemed to be preserved in culture.

The results of this study demonstrated that the combination of DiI-labeling and Indo-1-AM loading had little impact on the vital functions of the neurons. Both labeled and nonlabeled neurons were equally loaded with Indo-1, implying identical membrane properties and esterase activity. Moreover, there was no significant difference in the amplitude of the \([\text{Ca}^{2+}]_i\) rise between the two groups, suggesting that the membrane channels involved in the \([\text{Ca}^{2+}]_i\) signaling were still functional. Similarly, electrophysiological recordings from DiI-labeled neurons in the guinea pig duodenum and colon did not reveal any differences in membrane properties and excitability caused by the DiI labeling (12, 19).

Cultured myenteric neurons still express functional receptors for various neurotransmitters and neuroactive compounds (7, 13, 28, 29, 34). We previously reported that electrical stimulation of neuronal processes

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**Table 1. Subpopulations of DiI-labeled and nonlabeled neurons in culture**

<table>
<thead>
<tr>
<th>5-HT</th>
<th>SP</th>
<th>ACh</th>
<th>Di-I-Labeled, % (n = 29)</th>
<th>Non-Di-I-Labeled, % (n = 46)</th>
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<tr>
<td>+</td>
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<td>+</td>
<td>55.2</td>
<td>56.5</td>
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<td>+</td>
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<td>-</td>
<td>3.4</td>
<td>6.5</td>
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<td>+</td>
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<td>+</td>
<td>20.7</td>
<td>21.7</td>
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<td>+</td>
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<td>-</td>
<td>13.8</td>
<td>10.9</td>
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<td>0.0</td>
<td>2.2</td>
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Neurons were classified on the basis of the presence (+) or the absence (−) of an intracellular \([\text{Ca}^{2+}]_i\) concentration response induced by serotonin (5-HT), substance P (SP), and acetylcholine (ACh).
induced a \([Ca^{2+}]_i\) rise in a subset of cultured neurons (35). This was mimicked by ACh, SP, and 5-HT (34, 35). These neurotransmitters also induced a \([Ca^{2+}]_i\) rise in a subgroup of Dil-labeled motor neurons, and the number of neurons responding to each of them did not differ between Dil-labeled and control neurons. This suggested that Dil-labeled neurons were still as vital as other neurons. Putative differences between the two populations might be blurred because the Dil technique only labels a small proportion of motor neurons. Therefore, a large number of unstained neurons might have been motor neurons as well. Nevertheless, the use of immunochemical techniques and the combination of the single neurotransmitter data revealed that functional differences might be reflected in a characteristic receptor expression in cultured motor neurons.

The majority of the Dil-labeled motor neurons (73.3%) in culture were cholinergic. This finding was in line with the immunohistochemical stainings of motor neurons in whole mount preparations, showing that cholinergic motor neurons outnumbered the nitricergic ones (3). Although the response to 5-HT was a common feature of both cholinergic and nitricergic motor neurons, a significant difference was observed with respect to their SP and ACh response. Significantly fewer nitricergic neurons displayed a \([Ca^{2+}]_i\) rise induced by SP and ACh, suggesting that inhibitory motor neurons were not activated by these neurotransmitters. Costa et al. (4) distinguished neurons into functional classes based on their neurotransmitter or peptide content. They showed that part of the descending interneurons contained 5-HT, whereas SP was present in a subset of orally projecting interneurons. Recently LePard et al. (15) showed that fast synaptic transmission in the descending inhibitory drive also involved nonnicotinic receptors. This finding was confirmed by Johnson et al. (10), who showed that there was indeed noncholinergic fast synaptic transmission to inhibitory motor neurons.

The absence of ACh responses in nitricergic motor neurons might reflect the fact that nicotinic receptors are less important in the descending inhibitory pathway. To meet the general concept of propulsion, implying a contraction orally and relaxation anally, excitatory motor neurons should have neurokinin (NK) and/or ACh receptors. This agreed with our results, in that SP indeed induced a \([Ca^{2+}]_i\) rise in the majority of the cholinergic motor neurons. On the other hand, some authors reported NK1 receptors to be predominantly present on nitric oxide synthase-immunoreactive neurons in the myenteric plexus of the guinea pig small intestine (16, 25). Our data apparently contrasted with the latter finding. However, the nitric oxide synthase-immunoreactive neurons in the latter studies were not functionally identified. These neurons might have been predominantly inhibitory interneurons or neurons projecting to the longitudinal muscle.

The combination of the data obtained from each single neurotransmitter allowed the classification of the neurons into several classes. Three major classes of neurons could be identified, one of which, the 5-HT\(^+\)/ACh\(^-\)/SP\(^-\) subgroup, were likely to be inhibitory neurons. It is hard, yet not impossible, to link the other two main subpopulations with a specific function. We might speculate that one of the groups might comprise orally projecting neurons and the other consists of circumferentially orientated neurons. Further experiments are needed to assess the functional significance of these differences in \([Ca^{2+}]_i\) signaling between subpopulations of motor neurons and other neurons in culture.

It is clear that extrapolation of these data to the in vivo situation should be done with caution. Although cultures are valuable for studying myenteric neurons, altered receptor expression might have turned them into phenotypes not representative of the in vivo situation. Nevertheless, the data provide sufficient proof that both optical techniques can be combined and can reveal differential receptor expression in a functionally isolated group of neurons. Furthermore, they might be a good lead for similar investigations on motor neurons in the in situ myenteric plexus.

In conclusion, the combination of retrograde labeling and culturing of myenteric motor neurons provides us with a technique to perform \([Ca^{2+}]_i\) signaling measurements in a functionally identified group of neurons. The AM loading of the \([Ca^{2+}]_i\) indicator has the advantage that multiple neurons can be studied simultaneously. We demonstrated that this technique allows identification of differential receptor expression in functionally identified neurons in culture. Application of this technique to whole mount preparations has the potential to yield physiologically relevant information.

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