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Original Article

Physically disconnected non-diffusible cell-to-cell communication between neuroblastoma SH-SY5Y and DRG primary sensory neurons

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Abstract: Background: Cell-cell communication occurs via a variety of mechanisms, including long distances (hormonal), short distances (paracrine and synaptic) or direct coupling via gap junctions, antigen presentation, or ligand-receptor interactions. We evaluated the possibility of neuro-hormonal independent, non-diffusible, physically disconnected pathways for cell-cell communication using dorsal root ganglion (DRG) neurons. Methods: We assessed intracellular calcium ($[Ca^{2+}]$) in primary culture DRG neurons that express ATP-sensitive P2X3, capsaicin-sensitive TRPV1 receptors modulated by estradiol. Physically disconnected (dish-in-dish system; inner chamber enclosed) mouse DRG were cultured for 12 hours near: a) media alone (control 1), b) mouse DRG (control 2), c) human neuroblastoma SHSY-5Y cells (cancer intervention), or d) mouse DRG treated with KCl (apoptosis intervention). Results: Chemosensitive receptors $[Ca^{2+}]_i$ signaling did not differ between control 1 and 2. ATP (10 μM) and capsaicin (100nM) increased $[Ca^{2+}]_i$ transients to 425.86 ± 49.5 nM, and 399.21 ± 44.5 nM, respectively. 17β-estradiol (100 nM) exposure reduced ATP (171.17 ± 48.9 nM) and capsaicin (175.01±34.8 nM) $[Ca^{2+}]_i$ transients. The presence of cancer cells reduced ATP- and capsaicin-induced $[Ca^{2+}]_i$ by >50% ($p<0.05$) and abolished the 17β-estradiol effect. By contrast, apoptotic DRG cells increased initial ATP-induced $[Ca^{2+}]_i$, flux four fold and abolished subsequent $[Ca^{2+}]_i$, responses to ATP stimulation ($p<0.001$). Capsaicin (100nM) induced $[Ca^{2+}]_i$ responses were totally abolished. Conclusion: The local presence of apoptotic DRG or human neuroblastoma cells induced differing abnormal ATP and capsaicin-mediated $[Ca^{2+}]_i$ fluxes in normal DRG. These findings support physically disconnected, non-diffusible cell-to-cell signaling. Further studies are needed to delineate the mechanism(s) of and model(s) of communication.

Keywords: Cell-cell communication, TRPV1, P2X3, DRG, SH-SY5Y

Introduction

Despite remarkable advances in biomedical sciences and medical therapeutics in recent decades, the anticipated improvements in patient outcomes have not been realized owing to limited success in translating and widely diffusing scientific advances into clinical practice [1]. The promise of translational approaches can best be achieved by embracing the interdependence of basic science, clinical discovery, and patient-oriented research (clinical trials, biobehavioral, community engagement, policy, etc.) and by testing new paradigms that might open previously unseen doors to accelerate improved patient outcomes [2]. One such door is the conventional conception of cellular communication, critical to clinical intervention outcomes, as driven mainly by direct coupling via gap junctions, antigen presentation, ligand-receptor interactions, or mediating diffusible factors. Likewise, cell-cell communication can occur over very short (paracrine and synaptic) or very long distances (hormonal). These modes of communication, however, all require either physical contact between cells or contact with mediating, diffusible factors. By contrast, while self organization in biology has been viewed to be driven by diffusible signal mediated cell-cell communication, self organization of dynamic collective systems events such as murmuration in flocks of birds may occur in part, through a higher level of non-linear communication [3]. We hypothesized that the non-linear mode of

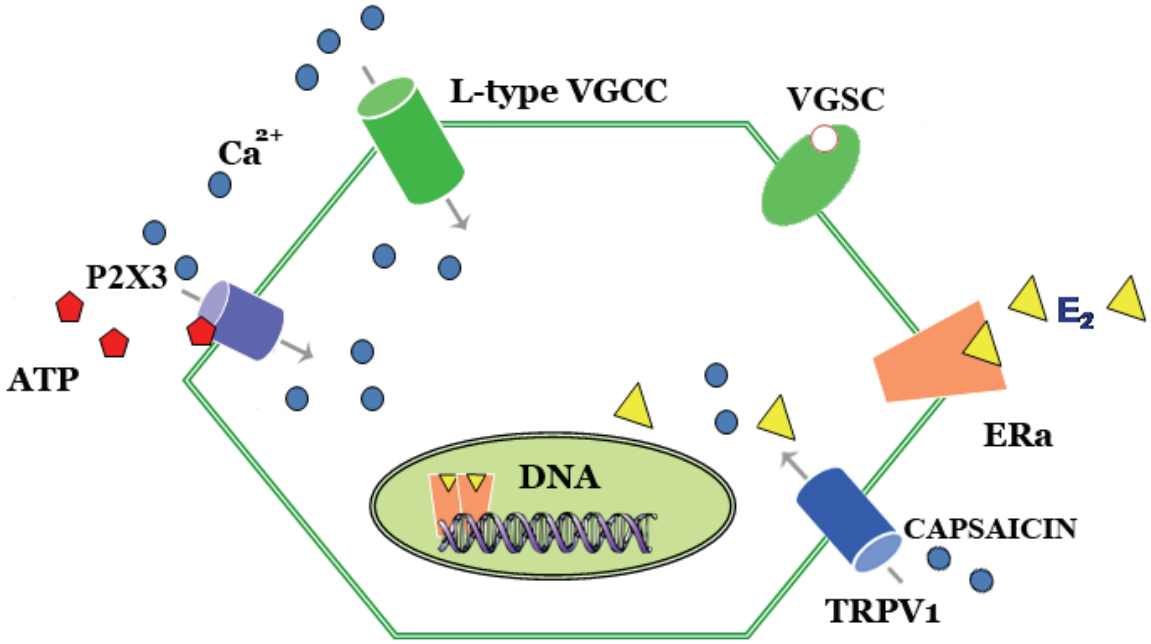

Figure 1. Proposed mechanism of estradiol effect on ATP-induced $[Ca^{2+}]_i$ signaling in primary sensory neurons. ATP acts on P2X3 receptor resulting in activation of the L-type voltage-gated calcium channel (VGCC) stimulating an increase in $[Ca^{2+}]_i$. Subsequent activation of voltage-gated sodium channels (VGSC) will lead to pain sensation. Capsaicin acting on nociceptive TRPV1 receptors directly induces $[Ca^{2+}]_i$. Both pathways are attenuated by membrane-associated estrogen receptor-α (ERα). ERα agonist 17β-estradiol attenuated increases P2X3 and TRPV1-mediated $[Ca^{2+}]_i$.

self organization principles should be active for cells as well, and therefore cells are also capable of non-linear cell-cell communication independent of diffusible factors, neural pathways, or other traditionally recognized pathways. Because neurons and neuroendocrine cells are important mediators of the body's intercellular signaling system, we further hypothesized that neurons would be most likely to show a robust responses to non-neural, non-diffusible signaling in physically disconnected cells if such signaling does in fact occur.

Dorsal root ganglia (DRG) neurons in primary culture are highly sensitive cells and have been a useful model system for investigating sensory physiology and putative nociceptive signaling [4]. These sensory neurons can be activated and/or modulated by the activation of chemosensitive receptors such as ATP-sensitive P2X3 and capsaicin-sensitive TRPV1 on peripheral nerve terminals. The TRPV1 receptor is expressed in several areas of the nervous system, but it is most highly expressed in small diameter (<25 μm) and medium (<40 μm) size DRG. TRPV1 and P2X3 expressing neurons are nociceptors [5], suggesting P2X3/TRPV1 receptors expression and activity, and their activation by exogenous stimuli (e.g. ATP and capsaicin) might be considered as markers for a specific subtype of sensory neurons. In addition, P2X3/TRPV1 receptor expression has been shown to be modulated by 17β-estradiol supporting gender driven influences on pain reception [5, 6] (**Figure 1**).

Accordingly, we devised a "dish-in-dish" cell culture system to establish a framework for investigating whether physically disconnected cells could influence the behavior of other local cells in the absence of diffusible factors or other physical connection. We assessed invoked intracellular calcium currents ($[Ca^{2+}]_i$) in cultured DRG cells in response to ATP and capsaicin in the presence or absence of estradiol, as this model of chemosensitive receptors $[Ca^{2+}]_i$ signaling has been well characterized [6-8].

We posited that the local presence of dying cells and/or aberrantly behaving, e.g. cancer cells, were good candidates to express a form of communication that might alter the behavior

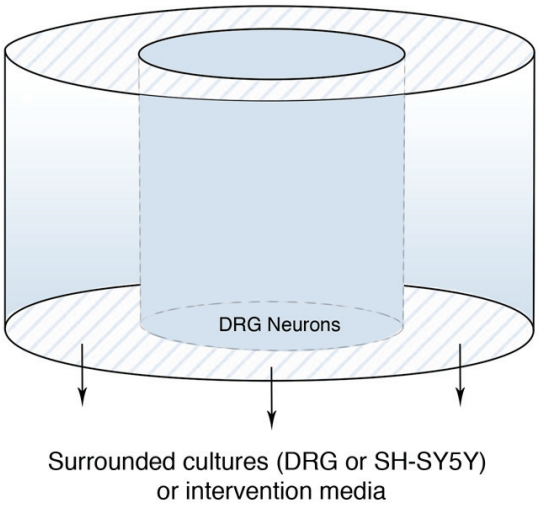

Figure 2. Experimental "dish in dish" system (patent pending). DRG sensory neurons were plated and cultured in the "center" dish. Other DRG neurons, SH-SY5Y neuroblastoma cells cultured under experimental condition or intervention media containing no cells were physically disconnected from the neurons in the center dish.

of local, but normal cells. If physically disconnected neuro-hormonal independent, non-diffusible pathways do exist, then such influences should be detectable within cell signaling pathways. Thus, we assessed $[Ca^{2+}]_i$ cell signaling responses in highly sensitive DRG neurons to well characterized chemosensitive receptors modulators in the presence of varying local, but physically isolated cells. Specifically, we investigated the $[Ca^{2+}]_i$ cell signaling responses in normal physically isolated DRG neurons in primary culture in the local presence of media and no cells (control condition 1), the local presence of other normal DRG cells (control 2), and the local presence two distinct cell populations: human neuroblastoma SHSY-5Y cells (intervention 1) or DRG cells after KCl induced apoptosis (intervention 2).

Materials and methods

"Dish-in-dish" culture system

To examine the possibility that physically disconnected cell populations with the same proximity could influence one another, we designed a "dish-in-dish" cell culture system (**Figure 2**, patent pending) involving an outer "surround" cell culture chamber and an inner "center" cell culture chamber, which can be manipulated independently. The diameter of the inner chamber is 15 mm and the outer chamber is 60 mm. The distance between the inner and outer chamber is 1.5 mm. Both chambers were covered to prevent transfer of vapor or airborne droplets. This arrangement allowed for adjacent culture of cells without the potential for diffusible cell signaling.

Physical environment

We considered the possibility that if physically disconnected neuro-hormonal independent, non-diffusible cell-cell communication pathways do exist the presence of a substantial research activity with other cell studies in the area might influence our findings. Therefore, we performed all experiments in a new building with a near empty 30,000 sq. ft. basic science floor that had no other active research labs. In addition, recordings were made in Faraday cage to protect neurons from electromagnetic influence.

Primary culture of DRG neurons

DRG tissues were obtained from C57BL/6J (Jackson Laboratory; 20 g). Upon arrival mice were housed in microisolator caging and maintained on a 12 hours light/dark cycle in a temperature-controlled environment with access to food and water *ad libitum* for two weeks. All studies were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Charles R. Drew University of Medicine and Science and the NIH Guide for the Care and Use of Laboratory Animals. Lumbosacral adult DRGs (level L1-S1) were collected under sterile technique and placed in ice-cold medium Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich St. Louis, MO). Adhering fat and connective tissue were removed and each DRG was minced with scissors and placed immediately in a medium consisting of 5 ml of DMEM containing 0.5 mg/ml of trypsin (Sigma, type III), 1 mg/ml of collagenase (Sigma, type IA) and 0.1 mg/ml of DNAase (Sigma, type III) and kept at 37°C for 30 minutes with agitation. After dissociation of the cell ganglia, soybean trypsin inhibitor (Sigma, type III) was used to terminate cell dissociation. Cell suspensions were centrifuged for one minute at 1000 rpm and the cell pellet was resuspended in DMEM supplemented with 5% fetal bovine serum, 2 mM glutamine-penicil-

lin-streptomycin mixture, 1 μg/ml DNAase and 5 ng/ml NGF (Sigma). Cells were placed on Matrigel® (Invitrogen, Carlsbad, CA)- coated 15-mm coverslips (Collaborative Research Co., Bedford, PA) and kept at 37°C in 5% CO_2 incubator for 24 hrs, given fresh media and maintained in primary culture until used for experimental procedures.

Human neuroblastoma (SH-SY5Y) cell culture

The human SH-SY5Y neuroblastoma cells (ATCC CRL-2266) were cultured in a medium consisting of a 1:1 mixture of ATCC-formulated Eagle's Minimum Essential Medium and Ham's F-12 medium containing 10% heat-inactivated FBS, 4mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL amphotericin B in 5% (v/v) CO_2 and balanced moist air at 37°C.

"Dish-in-dish" culture of SH-SY5Y cells with dorsal root ganglion cells

To assess a potential communicating effect of cancer in the "surround" environment on cell behavior SH-SY5Y cells were plated in the outer "surround" culture chamber containing 10% FBS, 4mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL amphotericin B in DMEM for 12 hours. Mouse DRG neuron cells were seeded in the inner "center" culture chamber in medium consisting of 5% FBS, 2 mM glutamine-penicillin-streptomycin mixture, 1 μg/ml DNAase and 5 ng/ml NGF for 24 hours at 5% CO_2 and 37°C. Thus, the two cell populations were physically disconnected while being co-"dish-in-dish" cultured in adjacent culture dish for 12 hours prior to assessing $[Ca^{2+}]_i$ response to chemical stimulation.

"Dish-in-dish" culture of KCl exposed dorsal root ganglion cells with dorsal root ganglion cells

To assess a potential communicating effect of local cell death in the "surround" environment on cell behavior in the "center", we again used "dish-in-dish" culture system (**Figure 2**): normal mouse DRG were plated in the outer "surround" culture chamber comprising 5% FBS, 2 mM glutamine-penicillin-streptomycin mixture, 1 μg/ml DNAase and 5 ng/ml NGF for 24 hours at 5% CO_2 and 37°C for 12 hours with KCl (50 mM) added to the DRG in the surround to induce apoptosis and cell death. Mouse DRG neuron cells were seeded in the inner "center" culture chamber in medium consisting of 5% FBS, 2 mM glutamine-penicillin-streptomycin mixture, 1 μg/ml DNAase and 5 ng/ml NGF for 24 hours at 5% CO_2 and 37°C. The two cell populations were co-"dish-in-dish" cultured in adjacent culture dish for 12 hours after which we assessed $[Ca^{2+}]_i$ response to chemical stimulation.

Control conditions

"Dish-in-Dish" culture of normal control dorsal root ganglion cells with dorsal root ganglion cells

To establish a control condition for assessing the effect of non-diffusible cell-cell communication, using the "dish-in-dish" culture system, we plated normal mouse DRG in the outer "surround" culture chamber with medium comprising 5% FBS, 2 mM glutamine-penicillin-streptomycin mixture, 1 μg/ml DNAase and 5 ng/ml NGF for 12 hrs at 5% CO_2 and 37°C. Mouse DRG neuron cells were seeded in the inner "center" chamber with medium comprising 5% FBS, 2 mM glutamine-penicillin-streptomycin mixture, 1 μg/ml DNAase and 5 ng/ml NGF for 24hrs at 5% CO_2 and 37°C. Thus, cells were co-cultured for 12 hours prior to assessing $[Ca^{2+}]_i$ response to chemical stimulation.

"Dish-in-dish" culture of normal control dorsal root ganglion cells with intervention media containing no cells

To establish additional control conditions for assessing the effect of non-diffusible cell-cell communication two additional controls were performed. "Dish-in-dish" culture of normal control DRG cells in the inner "center" culture chamber for 24 hours with: 1) dorsal root ganglion cell media only in the outer "surround" culture chamber or 2) human SH-SY5Y neuroblastoma cell media only in the outer "surround" culture chamber for 12 hours.

$[Ca^{2+}]_i$ fluorescence imaging

Ca^{2+} fluorescence imaging was carried out as previously described [7]. DRG neurons were loaded with fluorescent dye 5 mM Fura-2 AM (Invitrogen, Carlsbad, CA) for 45 min. at 37°C in HBSS supplemented with 20 mM HEPES, pH

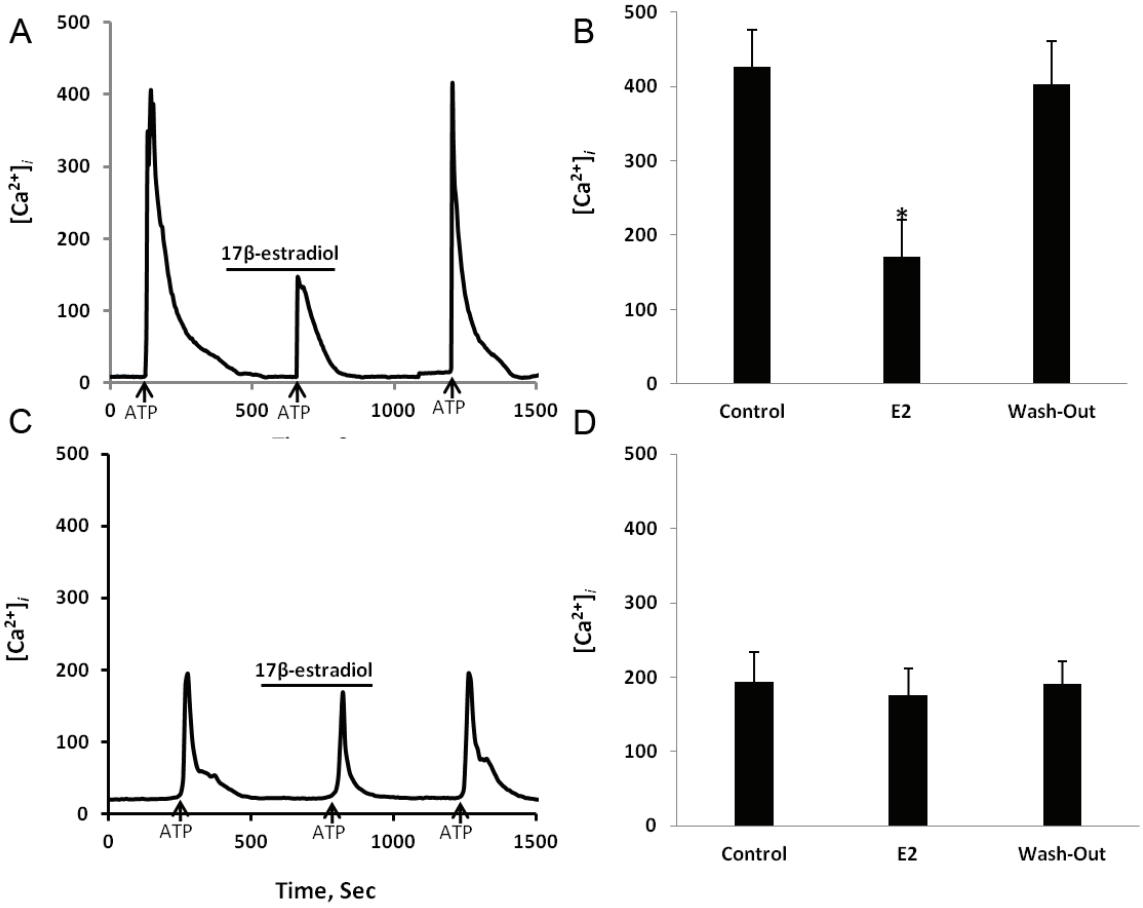

Figure 3. 17β-estradiol inhibits ATP-induced calcium signaling in DRG neurons but not in the presence of physically isolated SH-SY5Y neuroblasoma cells. A: Typical ATP (10 μM)-induced $[Ca^{2+}]_i$ response (first ATP stimulation) and attenuated response after 5 min pretreatment with E2 (100 nM) (second ATP stimulation). ATP-induced $[Ca^{2+}]_i$ response rapidly attenuated in dorsal root ganglion cells. After wash-out with experimental medium, ATP response on $[Ca^{2+}]_i$ returned to initial (control) amplitude of stimulation (third ATP stimulation). B: Summary of ATP-induced $[Ca^{2+}]_i$ influxes in control state, in the presence of E2, and after wash-out of the E2 effect. E2 significantly decreased $[Ca^{2+}]_i$ response to ATP. Values are expressed as mean ± SEM (n=7). * indicates statistically significant difference from control, $P<0.05$. C: Response of DRG neurons after 12 hours incubation near but physically-disconnected from SH-SY5Y neuroblastoma cells. Initial response to ATP is markedly attenuated compared to panel A (first ATP stimulation). There was a slight non significant effect of 17β-estradiol (100 nM) on ATP (10 M)-induced $[Ca^{2+}]_i$ (second ATP stimulation) and a consistent reduced $[Ca^{2+}]_i$ response to ATP after washout (third ATP stimulation). D: Summary of ATP-induced $[Ca^{2+}]_i$ influxes in the presence of local physically-disconnected SH-SY5Y cells, in the presence of E2, and after wash-out of the E2 effect. E2 shows no effect on $[Ca^{2+}]_i$ response to ATP. Values are expressed as mean ± SEM (n=4).

7.4. The coverslips were mounted in a RC-26 recording chamber P-4 (Warner Instruments, Hamden, CT) and placed on a stage of Olympus IX51 inverted microscope (Olympus America, Center Valley, PA). Observations were made at room temperature (20-23°C) with 20X UApo/340 objective. Neurons were bathed and perfused with HBSS buffer using gravity at a rate of 1-2 ml/min. Fluorescence intensity at 505 nm with excitation at 334 nm and 380 nm were captured as digital images (sampling rates of 0.1-2 s). Regions of interest were identified within the soma from which quantitative measurements were made by re-analysis of stored image sequences using Slidebook® Digital Microscopy software. $[Ca^{2+}]_i$ was determined by ratiometric method of Fura-2 fluorescence from calibration of series of buffered Ca^{2+} standards. E2 was applied acutely for five minutes onto the experimental chamber. Repeated application of DRG chemosensitive receptor modulators achieved by superfusion

in a rapid mixing chamber into individual neurons for specific intervals (100-500 ms). We calculated actual $[Ca^{2+}]_i$ in select areas in each neurons with the formula:

$$[Ca^{2+}]_i = k_d \times (R - R_{min})/(R_{max} - R) \times \beta$$

Where K_d is the indicator's dissociation constant of the fluoroprobe; R is ratio of fluorescence intensity at two different wavelengths (340/380 nm for fura-2); R_{max} and R_{min} are the ratio at Fura-2 with a saturated Ca^{2+} and free Ca^{2+}. β is the ratio of the denominators of the minimum and maximum conditions.

Statistical analysis

The amplitude of $[Ca^{2+}]_i$ response represents the difference between baseline concentration and the transient peak response to drug stimulation. Differences in response to DRG chemosensitive receptor modulators were assessed by comparing $[Ca^{2+}]_i$ increases during the first stimulation with the second. All of the data are expressed as the mean ± SEM. Statistical analysis was performed using Statistical Package for the Social Sciences 18.0 (SPSS, Chicago, IL, USA). To assess the significance among different groups, data were analyzed with one-way ANOVA followed by Schéffe post hoc test. A $p < 0.05$ was considered statistically significant.

Results

Effect of estradiol on ATP-induced $[Ca^{2+}]_i$ in DRG neurons in the presence of physically isolated local DRG cells (control setting 1)

Consistent with prior studies [7], we observed that a brief 10 second application of ATP (10μM) by fast superfusion produced equal $[Ca^{2+}]_i$ spikes in center and surround DRG neurons. After a 5-min washout with HBSS, additional stimulation with ATP (10μM) induced subsequent $[Ca^{2+}]_i$ transients. Pretreatment with purinergic receptor antagonist PPADS (5 μM) blocked the ATP-induced $[Ca^{2+}]_i$ transients. Similarly, ATP stimulation in a Ca^{2+}-free media in the presence of the Ca^{2+} chelator, BAPTA (10 mM), eliminated $[Ca^{2+}]_i$ spikes indicating the necessity for P2X3 receptors and extracellular Ca^{2+} (data not shown). 17β-estradiol (E2) (100 nM) by itself had no effect on basal $[Ca^{2+}]_i$, but potently attenuated ATP-induced $[Ca^{2+}]_i$ transients. The effect of E2 was reversible. After the initial ATP response, five minute incubation with E2 reduced ATP-induced $[Ca^{2+}]_i$ transient from 425.86 ± 49.5 nM to 171.17 ± 48.9 nM, n=5, $p < 0.05$) (**Figure 3A** and **3B**). Similarly, the estrogen receptor antagonist ICI 182,780 (1 μM) blocked the 17β-estradiol inhibitory effect on ATP-induced $[Ca^{2+}]_i$ transients.

Brief application of TRPV1 agonist capsaicin (3 second 100 nM) by fast superfusion produced $[Ca^{2+}]_i$ spikes which were almost completely blocked by 100 nM capsazepine, a TRPV1-selective antagonist. Since the effect of capsaicin was non-reversible we applied the estradiol first assuming its non-reversibility on TRPV1 receptors. The E2 by itself had no effect on basal $[Ca^{2+}]_i$, but E2 (100 nM) attenuated the peak of capsaicin-induced $[Ca^{2+}]_i$ transients from 399.21 ± 44.5 nM to 175.01 ± 34.8 nM (n=5, p<0.05) (**Figure 4A** and **4B**).

Effect of 17-β estradiol inhibition of ATP/capsaicin-induced calcium signaling in DRG neurons in the presence of local physically isolated media (control setting 2)

We compared ATP/capsaicin-induced $[Ca^{2+}]_i$ spikes from DRG neurons in the inner "center" culture dish in the presence of only DRG media or SH-SY5Y media in the outer "surround" culture dish (see Material and Methods). In these experimental settings we did not detect any significant difference in DRG neuron response to ATP (10 μM), capsaicin (100 nM) or 17β-estradiol (100 nM) application compared to control setting 1 conditions above with normal DRG in the outer "surround" culture dish (data not shown).

Effect of estradiol inhibition of ATP/capsaicin-induced calcium signaling in DRG neurons in the presence of local physically isolated SH-SY5Y neuroblastoma cells (cancer intervention)

We compared ATP/capsaicin-induced $[Ca^{2+}]_i$ spikes from inner "center" culture dish DRG in the presence of neuroblastoma SH-SY5Y cells cultured in the outer "surround" culture dish (see Material and Methods). Both ATP (10 μM) and capsaicin (100 nM)- induced $[Ca^{2+}]_i$ responses were significantly reduced (2 fold; p<0.05) compared to dish-in-dish cultures where DRG cells occupied the surround (193.45 ± 40.16 nM, and 145.90 ± 28.84 nM respectively, n=4, vs. 425.86 ± 49.5 nM, and 399.21

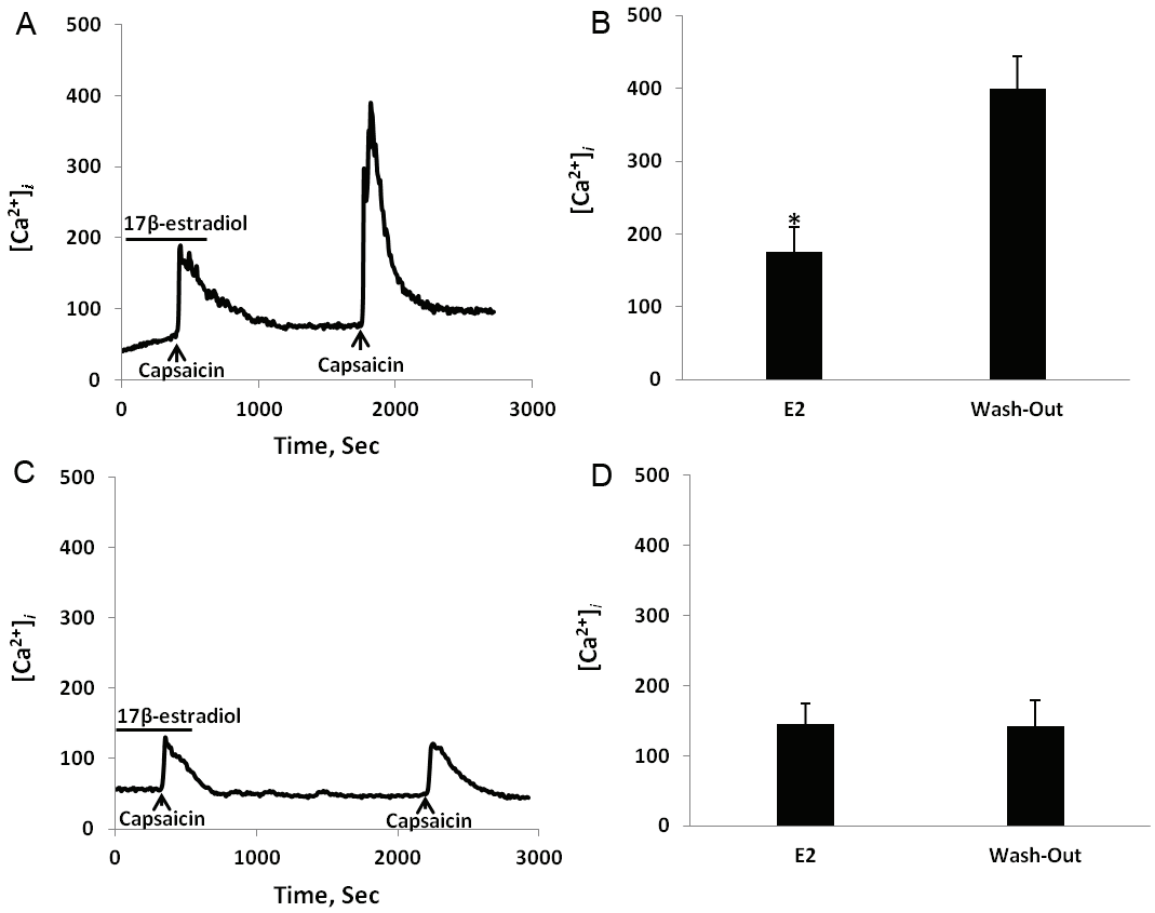

Figure 4. Effect of 17β-estradiol on capsaicin-induced $[Ca^{2+}]_i$ increase in control and in the local presence of physically disconnected SH-SY5Y cells. A: Pre-incubation for 5 min with 17β-estradiol (100 nM) attenuates capsaicin (300 nM)- induced $[Ca^{2+}]_i$ response (first stimulation) compared with controlled capsaicin response (second stimulation). B: Summary of capsaicin- induced $[Ca^{2+}]_i$ increase in the presence of E2 and after wash-out with experimental medium. Values are expressed as mean ± SEM (n=8). * indicates statistically significant difference from control, $P<0.05$. C: Effect of 17β-estradiol (100 nM) on capsaicin (300 nM)-induced $[Ca^{2+}]_i$ increase was abolished after 12 hours incubation near but physically-disconnected from human neuroblastoma SH-SY5Y cells. D: Summary of capsaicin- induced $[Ca^{2+}]_i$ increase in the presence of E2 and after wash-out with experimental medium in sensory neurons cultured in the local presence of physically-disconnected SH-SY5Y cells. Values are expressed as mean ± SEM (n=4).

± 44.5 nM respectively in control setting). The effect of E2 on ATP-induced P2X3-mediated $[Ca^{2+}]_i$ observed under control conditions was abolished with neuroblastoma SH-SY5Y cells in the surround (**Figure 3C** and **3D**). Moreover, E2 similarly abolished capsaicin-induced TRPV1-mediated $[Ca^{2+}]_i$ flux (**Figure 4C** and **4D**).

Effect of ATP/capsaicin-induced calcium signaling in DRG neurons in the presence of local physically isolated DRG neurons exposed to KCl (apoptosis intervention)

In another set of experiments, we compared ATP-induced $[Ca^{2+}]_i$ spikes from inner "center" culture dish DRG in the presence of DRG neurons exposed to KCl (50 mM >80% apoptosis 12 hours following KCl exposure) in the outer "surround" culture dish (see Material and Methods). In control, ATP (10 μM) induced equal $[Ca^{2+}]_i$ responses 387.63 ±17.3 nM (**Figure 5A**) but failed to produce a typical $[Ca^{2+}]_i$ influx in DRG cells incubated in the local presence of physically-separated dying DRG cells (**Figure 5B**, n=4). There was a 4 fold increase in $[Ca^{2+}]_i$ concentration during the first exposure to ATP stimulation ($p<0.05$), followed by no increase in $[Ca^{2+}]_i$ with further ATP stimulation. Brief application of TRPV1 agonist capsaicin (100 nM) by fast superfusion produced equal $[Ca^{2+}]_i$ spikes 355.83 ± 57.23 nM (**Figure 5C**) under control conditions but this effect was

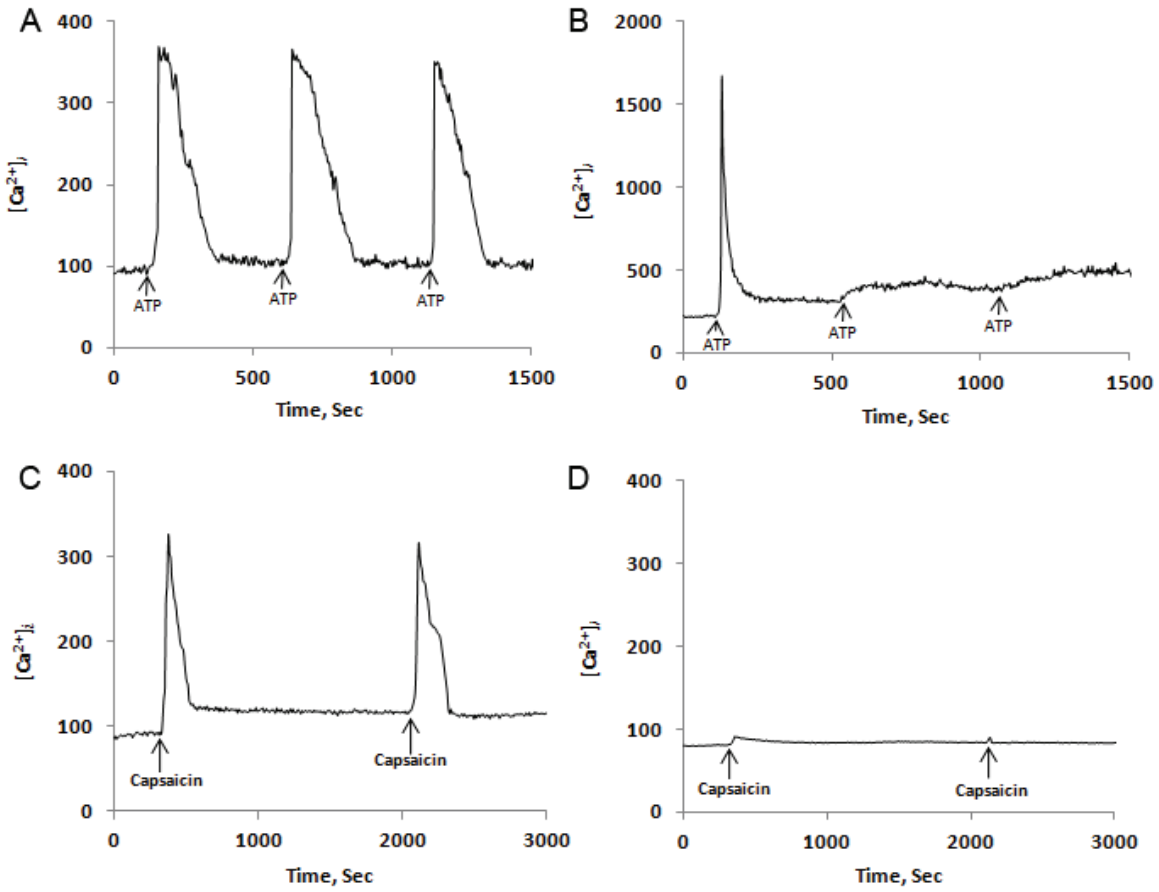

Figure 5. Pattern of multiple ATP-and capsaicin stimulations in isolated DRG neurons in the local presence of physically-disconnected DRGs treated with KCl to induce apoptosis and cell death. A: Typical indication of equal $[Ca^{2+}]_i$ responses to repeated ATP (10 μM) stimulation (indicated by arrow) with 10 min interval under control condition. B: ATP-induced $[Ca^{2+}]_i$ response in DRG neurons co-cultured near physically-disconnected DRG neurons (see Materials and Methods) treated with KCl (50 mM) for 12 hours. Under this experimental conditions the first ATP stimulation produces 3-4 times higher response in $[Ca^{2+}]_i$ increase but subsequent stimulations failed to induce any significant response compared to control. C: Typical indication of equal $[Ca^{2+}]_i$ response to repeat capsaicin (300 nM) stimulation (indicated by arrow) under control condition. D: Elimination of $[Ca^{2+}]_i$ to initial and repeat capsaicin stimulation in DRG neurons co-cultured near physically-disconnected DRG neurons treated with KCl (50 mM) for 12 hours.

completely blocked under experimental conditions in sensory neurons exposed to local DRG neurons with KCl-induced apoptosis (**Figure 5D**).

Discussion

In order to provide proximity and separation between evaluated, normal DRG cells and either cancerous, dying, or other normal (control) neuronal cells, we devised a novel "dish-in-dish" cell culture system, wherein an outer chamber surrounds an inner chamber providing a physical barrier. This system was specifically designed to allow us to assess neuro-hormonal independent, non-diffusible, physically disconnected pathways for cell-cell communication. In the current study calcium transients were analyzed in the inner "center" DRG neurons following 12 hours of co-localization to local, physically disconnected cell culture with either neuroblastoma cells, apoptotic DRG cells, media from neuroblstoma or DRG cells, or control DRG cells in the surround chamber. Accordingly, we were able to compare, in a controlled way, the influence of the outer chamber surround cell population on inner chamber DRG calcium signaling in response to well described DRG chemosensitive receptor stimuli. Direct application of ATP and capsaicin to activate DRG cells in the inner chamber in control settings demonstrated increased $[Ca^{2+}]_i$ in DRG in

response to ATP as well as capsaicin in the control setting and attenuation of ATP and capsaicin increases in $[Ca^{2+}]_i$ by 17β-estradiol consistent with prior studies [6]. By contrast, the local presence of cancer cells or apoptotic cells in the outer chamber significantly altered inner chamber DRG $[Ca^{2+}]_i$ responses to ATP and capsaicin, as well as 17β-estradiol in different ways.

In the local presence of cancer cells ATP and capsaicin stimulated $[Ca^{2+}]_i$ was markedly attenuated and the effect of 17β-estradiol was abolished. The same media minus the cells however, did not alter inner chamber DRG $[Ca^{2+}]_i$ responses indicating the local presence of cells rather than the media was responsible for this effect.

In the local presence of apoptotic DRG cells a different response was noted. The initial ATP stimulated increase in $[Ca^{2+}]_i$ was dramatically exaggerated, while subsequent exposure to ATP led to no response in $[Ca^{2+}]_i$. In addition, neither initial nor subsequent exposure to capsaicin induced any response in inner chamber DRG $[Ca^{2+}]_i$. The exaggerated increase in $[Ca^{2+}]_i$ concentration during the first exposure to ATP stimulation in normal DRG nearby DRG cells undergoing apoptosis is similar to that seen in murine neurons following sodium fluoride toxicity which was reported to increase intercellular Ca^{2+} concentration leading to apoptosis [9]. While further studies are needed to better understand the observed $[Ca^{2+}]_i$ changes and their implications. Our findings demonstrate that apoptotic and cancerous cells are capable of exerting a non-diffusible, non-neuronal influence over distance on nearby, but physically disconnected cells.

The findings here are the first to our knowledge to support physically disconnected, non-diffusible cell-to-cell signaling. This influence may or may not represent a biologically significant form of cell-cell communication, but the magnitude of the influence in the current experimental model on $[Ca^{2+}]_i$ signaling indicates the likelihood that such a mode of communication does occur and may have biologic/physiologic significance. Our findings are consistent with reports of non-linear communication in higher level forms of being such as groups of whole organisms [3]. Other nontraditional models of communication such as "quorum sensing" appear to be directed by certain signaling molecules in bacteria [10], while in animal groups it may be mediated in part through indirect pheromonal signals [11], or vibratory sensations or other [12]. However, aspects of collective behavioral of groups of animals may still be mediated through distance-dependent and direction-dependent social interactions, facilitated by poorly defined non-linear or nonlocal forms of communication [13, 14]. Our findings are also consistent with the hypothesis of Ventegodt et al. suggesting the communication of biological information at the subcellular, cellular, and supracellular levels may occur through "collective connectivity" in excitable neuronal cells [15]. How such interactions may occur among single cells remains speculative.

We have previously shown visceral nociception and nociceptor sensitization are regulated by ATP-sensitive P2X3 and capsaicin-sensitive TRPV1 receptors and modulated by 17β-estradiol [5, 16]. The present findings suggest non-visceral nociception and nociceptor sensitization may also be modulated by P2X3 and capsaicin. On the other hand, our study does not identify the mechanism(s) of action by which the physically disconnected, non-diffusible cell-to-cell signaling changes observed in DRG receptor mediated $[Ca^{2+}]_i$ fluxes are mediated, e.g. gene expression, epigenetic modification or other. Changes in $[Ca^{2+}]_i$ fluxes could be mediated through modification of ion and calcium related gene expression (i.e. g proteins) as reported by Xiao et al. who identified marked changes following DRG axotomy with respect to the expression of over 170 genes including neuropeptides, receptors, ion channels, and signal transduction molecules [17].

Other forms of physically disconnected cell-cell communication have been described such as post radiation therapy "bystander effect", but this is represents diffusible cell-cell interactions, whereby irradiated cells induce effects on local non-irradiated cells via gap junction communication or paracrine diffusible cellular factors [18, 19]. By contrast, the response to radiation effecting tissues or cells on more distant tissues have been termed "abscopal effects" and is characterized by cellular changes commonly believed to be mediated through neuronal pathways or systemic diffusible factors [2]. In addition, Liu et al reported cancer cells can alter intracellular signaling in endo-

thelial cells via circulating RNA transcripts in microvesicular bodies [20]. Astrocytes have been reported to communicate with neurons and with each other through a gap-junction-coupled syncytium cell-to-cell communication via propagating Ca^{2+}-waves based on cytoplasm-to-cytoplasm transport of inositol trisphosphate (IP3) through gap junctions. It is postulated that this intracellular route is able to support the propagation of long-distance regenerative Ca^{2+} waves via nonlinear coupling in the gap junctions, but is still restricted to physically connected cells [21]. Our results differ from these in that our system eliminated the availability of any potential pathways for neural or diffusible factor mediated cell-cell communication.

Limitations of our study are that although the inner chamber is enclosed we still cannot exclude the possibility of volatile communication via aromatic compounds. Activation of phospholipase can potentially serve as biochemical messengers across nearby cellular colonies. In addition, other potent signal transduction gases including NO and CO produced by nNOS and Heme oxygenases can be carried in air from one compartment to other where through formation of second messenger (e.g. cGMP) can alter $[Ca^{2+}]_i$ and cell function in the cells residing in the neighboring compartment. Finally, hydrogen sulfide (H_2S) is another recognized signal transduction gas produced by cystathionine c-lyase or cystathionase, cystathionine b-synthase and 3-mercaptopyruvate sulphurtransferase. H_2S activates ATP-sensitive potassium channels and large-conductance Ca^{2+}-activated potassium channels, and can induce changes in mitogen-activated protein kinase, cell cycle-related kinase, cell death-related gene and ion channels [22, 23]. In addition, we did not explore a dose/distance relationship between the inner and outer chambers, or vary the number of local cancer or apoptotic cells. Also, we only looked at the effects of two different cell types/conditions on the inner DRG cells and we did not examine other cell types in the inner chamber. However, we felt these additional permutations while important were beyond the scope of this initial study, which was to examine if physically distinct non-diffusible cell-cell communication could be identified.

In summary, we show that the local presence of human neuroblastoma SH-SY5Y cells or DRG undergoing KCl induced apoptosis alter both the direct and 17β-estradiol regulated effect of ATP and capsaicin induced P2X3 and TRPV1 receptor mediated $[Ca^{2+}]_i$ in mouse DRG neurons through what appears to be a non-local form of cell-to-cell communication. The inner enclosure suggests aerosolized communication of aromatic hydrocarbons is unlikely, though not absolutely excluded. Further studies are needed to confirm our findings and to explore the exact mechanism of action.

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