Expression and Pharmacology of Endogenous Cav Channels in SH-SY5Y Human Neuroblastoma Cells

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Abstract

SH-SY5Y human neuroblastoma cells provide a useful in vitro model to study the mechanisms underlying neurotransmission and nociception. These cells are derived from human sympathetic neuronal tissue and thus, express a number of the Cav channels that are essential for control of calcium signaling events, such as muscle contraction, gene expression, and neurotransmitter and hormone release. Dysfunction of Cav channels is related to a variety of heart, circulatory and neurological diseases, including arrhythmias, hypertension, some forms of epilepsy, migraine and other chronic diseases such as cancer, diabetes, ischemic brain injury and neuropathic pain [1,2]. The Cav1 α subunit contains the voltage sensor and gating machinery and is the binding site for most auxiliary subunits expressed in native systems vs whole cells in the binding assays and functional assays, suggesting auxiliary subunits expressed endogenously in native systems can strongly influence Cav2.2 channels pharmacology. These results may have implications for strategies used to identify therapeutic leads at Cav1.2 channels.

Introduction

Voltage-gated Ca\(^{2+}\) channels (Cav) are membrane proteins essential for the control of calcium signaling events, such as muscle contraction, gene expression, and neurotransmitter and hormone release. Dysfunction of Cav channels is related to a variety of heart, circulatory and neurological diseases, including arrhythmias, hypertension, some forms of epilepsy, migraine and other chronic diseases such as cancer, diabetes, ischemic brain injury and neuropathic pain [1,2]. The Cav1 α subunit contains the voltage sensor and gating machinery and is the binding site for most auxiliary subunits expressed in native systems vs whole cells in the binding assays and functional assays, suggesting auxiliary subunits expressed endogenously in native systems can strongly influence Cav2.2 channels pharmacology. These results may have implications for strategies used to identify therapeutic leads at Cav1.2 channels.

Table 1 (for reviews see: [3,8,9]). Of these, Cav2.2 has been of particular interest as a therapeutic target given the central role it plays in mediating neurotransmitter release in nociceptive pathways such as presynaptic nerve terminals and dendrites [10]. Cavα subunits are co-expressed in native systems together with two or three auxiliary subunits (β, γδ and γ7), which undergo alternative splicing (for review see: [9]) and dramatically influence Cav channel function, intracellular trafficking and posttranslational modifications [11]. Indeed, when expressed alone in recombinant system, the α1B subunit, for example, encodes a voltage-dependent calcium channel with kinetic properties different from those of native Cav2.2 channels [9,12]. In contrast, when co-expressed with auxiliary β and γδ, increased current amplitudes are observed and the kinetics of activation and inactivation are closer to those of native channels [12].

Cell-based systems are desirable in the field of high-throughput screening assays due to their similarity to in vivo environment. SH-SY5Y human neuroblastoma cells are derived from human sympathetic neuronal tissue. This cell line maintains in culture many of the properties of nerve cells, providing a useful model for the characterisation of molecules affecting human neuronal function, including endogenously expressed Cav channels [13–15]. In particular, SH-SY5Y cells have been an attractive model system for the study of Cav2.2 function [13]. Although heterologous expression models provide control of subunit expression, native systems provide potentially more complex models which, when characterized, can help to determine the pharmacology of drugs in a native context and the physiology and pathophysiology...
of endogenously expressed receptors and channels. However, little is known about the CaV subtypes and auxiliary subunits endogenously expressed in SH-SY5Y cells, limiting the interpretation of pharmacological data. Here we report a detailed characterisation of endogenously expressed CaV channels expressed in SH-SY5Y cells using PCR and pharmacological approaches, with particular emphasis on the nociceptive target CaV2.2.

Results

SH-SY5Y Cells Endogenously Express Multiple CaV Subtypes, CaV2.2 Isoforms and Auxiliary Subunits

We assessed expression of mRNA transcripts for CaV subtypes and auxiliary αδ, β and γ subunits isoforms in SH-SY5Y cells by performing RT-PCR using specific primers (Fig. 1A–D). Bands with the predicted sizes were detected for CaV1.3, CaV2.2, and CaV3.1, while CaV1.1, 1.2, 1.4, 2.1, 2.3, 3.2 and 3.3 were not detected (Fig. 1A). In addition, bands of expected sizes for (Table 2) β1, β3, α2δ1, γ1, γ4, and γ7 auxiliary subunits (Fig. 1C–D) were also identified. Since splice variants can be generated by alternative RNA processing, which can influence function and pharmacology [16], we also investigated the expression of some human splice variants [16,17]. PCR bands with the predicted sizes for CaV1.3 isoforms 1 and 2; full length CaV2.2, α2δ1 (Gene bank accession number M91472.1), shorter α2δ1 variant, α1B2 (Gene bank accession number M91473.1) [17]; and Δ1 (but not Δ2) [16,18] were detected for the first time in the SH-SY5Y cells (Fig. 1A, Table 1).

The best annealing temperature for each gene analysed (see Table 1–2) was determined using a gradient PCR protocol in rounds of control experiments prior to testing each CaV gene-specific primer. Target-specific primers for the housekeeping gene GAPDH were designed as previously described [19] and GAPDH was detected in all PCRs, indicating amplifications were cDNA specific. PCR master mix using random primers without cDNA was used as negative gDNA control in all PCRs. Specificity of primers was demonstrated in a range of control experiments (data not shown), including detection of CaV2.2 plasmid but not other CaV subtypes by CaV2.2 primers; and absence of CaV2.2 in HEK cells. β1 and α2δ1 primers were positive for β1 and α2δ1 plasmids, while the same primers were negative for β2δ1 and α2δ3 plasmids (data not shown), indicating primers were selective for β1 and α2δ1 auxiliary subunits. The identity of each of these PCR products, including γ1, γ4, γ5 and γ7, was confirmed by sequencing analysis (data not shown).

Displacement of 125I-GVIA Binding from SH-SY5Y Cell Membranes

GVIA is a highly selective CaV2.2 blocker [20] and 125I-GVIA binding assays have been well established using rat brain membranes [21–23]. We performed binding assays and confirmed SH-SY5Y cells contain 125I-GVIA binding sites which can be fully displaced by CaV2.2 selective inhibitors CVID, GVIA and MVIIA. Affinities of α-conotoxins for human and rat CaV2.2 channels were next compared using these assays. CVID, GVIA and MVIIA each fully displaced 125I-GVIA binding to crude rat brain membranes with similar affinities (pIC50 ± SEM values; CVID 10.53 ± 0.15, GVIA 10.43 ± 0.16, and MVIIA 10.19 ± 0.04) (Fig. 2A, Table 3), consistent with earlier studies [21]. Intriguingly, the affinity of GVIA (pIC50 10.55 ± 0.15) to displace 125I-GVIA binding to SH-SY5Y membranes was similar to that shown in rat brain, while both CVID and MVIIA had significantly higher affinity for the human cell membranes (pIC50 of 11.51 ± 0.12 and 11.29 ± 0.23, respectively) than for rat brain membranes (Fig. 2A, B and D, Table 3). In addition, the affinities of these α-conotoxins dramatically decreased when determined on the intact SH-SY5Y cells instead of membranes, with GVIA affinity shifted ~10-fold, and CVID and MVIIA affinity shifted ~100-fold (see Fig. 2C, Table 3).

Pharmacology of the Endogenously Expressed CaV Channels

To investigate if the CaV channels endogenously expressed in SH-SY5Y cells were functional, and to further study the pharmacology of these channels, we assessed KCl-evoked Ca2+ responses using a fluorescent high-throughput Ca2+ imaging assay.

Table 1. Primers used to identify CaV channels α subunits in SH-SY5Y cells.

| Subtype | Accession Number | Primer Forward/Reverse | Size (bp) | Annealing T (°C) |
|---------|------------------|------------------------|----------|-----------------|
| CaV1.1 | NM_000069.2      | CGCATCTGCAATGCGCACTCTGGTTTA/AGACAACTGTGCAAGTGGAGGC 623 (?) ND |
| CaV1.2 | [19]             | CTGCGAGGTGATGAGGAGGAGG/GCGGATGCTGTGAGGTCGCTGTGTGTTG 502 58 [19] |
| CaV1.3 | EU 363339.1      | ACCCGGCGCTTGAATGCTCTC/CTCCGACTAGTGCAGTTAGTGGTCGCG 541 68 |
| CaV1.3 | NM_00128840.1    | GTCTGCTGAAGAAGATGTGTTTGC/TCGTGCTGAAGAAGATGTGTTTGC 343 68 |
| CaV1.4 | NM_005183.2      | AGGACAGGCAGAGAGTAGATGA/AAGACAGGCAGAGAGTAGATGA 899 (?) ND |
| CaV2.1 | FJ040507.1       | AGGACAGAGGAGGAGGAGGAGG/GCTGCTGAAGAAGATGTGTTTGC 365 (?) ND |
| CaV2.2 | NM_000718.2      | GGAAGAAGCTGGAGCAGCTGGCTGGAAG/AGATGAGGAGGAGGAGGAG 754 60 |
| α1B1   | Bp 22+33        | AGGACAGGAGGAGGAGGAGGAGG/GCAGAGAGGAGGAGGAGGAGGAGG 900 58 |
| α1B2   | Bp 23+33 [16]   | CGAGCAGAGGAGGAGGAGGAGG/GCTGCTGAAGAAGATGTGTTTGC 900 60 |
| α1B3   | Bp 21+31        | CAGGGAAGGAGGAGGAGGAGGAGG/GCTGCTGAAGAAGATGTGTTTGC 700 (65) |
| α1B4   | Bp 24+33 [16]   | CAGGGAAGGAGGAGGAGGAGGAGG/GCTGCTGAAGAAGATGTGTTTGC 1300 (7) ND |
| CaV3.1 | BC110995.1      | GCTGCTGAGGACAGGAGGAGGAGG/GCTGCTGAAGAAGATGTGTTTGC 397 60 |
| CaV3.2 | NM_021098.2     | CCGGAGCCCTCTCCAGGACAG/AGGAGGAGGAGGAGGAGGAGGAGG 433 60 |
| CaV3.3 | AF393229.1      | AGATTGAGGAGGAGGAGGAGGAGG/GCTGCTGAAGAAGATGTGTTTGC 526 60 |

(?) ND: isofrom not detected, unknown annealing temperature.

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on the FLIPTetra (Fluorescent plate image reader, Molecular Devices, Sunnyvale, CA) ([Fig. 3A–D]). CaCl2 (5 mM) was added to the KCl stimulation solution in all experiments to maximize the Ca^{2+} influx signal. Co-addition of 90 mM KCl and 5 mM CaCl2 evoked a large transient response indicating increase in intracellular Ca^{2+} ([Fig. 3A–B]). Concentration-response curves for KCl-mediated stimulation showed activation of Cav responses with an EC_{50} of 17.3 mM (pEC_{50} 1.88±0.06, Hill slope of 2.5) ([Fig. 3A, Table 4]), similar to previously described values [24–26]. To assess the contribution of each Cav channel expressed in SH-SY5Y cells to the KCl-evoked Ca^{2+} responses, we determined concentration-response curves for KCl/Ca^{2+} stimulation in the presence of subtype-specific inhibitors. The Cav1 (L-type) inhibitor nifedipine was used at a concentration (10 μM) that does not affect responses of other Cav subtypes (N, R, P/Q or T-type) [27], to isolate non-L-type responses. The KCl concentration-response curve was shifted...
Table 2. Primers used to identify Ca₃ channel auxiliary subunits in SH-SY5Y cells.

| Subunit | Accession Number | Primer Forward/Reverse | Size (bp) | Annealing T (°C) |
|---------|-----------------|------------------------|-----------|-----------------|
| β₁      | NM_000723.3     | ATGCAGGATCCAGGGGAG/CAGCGCAGTAGCGGGCCTTATT | 331       | 60              |
| β₂      | NM_000724.3     | TGGCTGGCAAACGCTGCTGT/ATGAGCTGCTGCGCTGCTTGT | 909       | ND              |
| β₃      | NM_000725.2     | GACGAGTCTGAAGGAGCACA/ATGCGGACGGGGGCAGAGGA | 594       | 65              |
| β₄      | NM_001005747.2  | TGAAGACTGCGAGGCTGCTGGC/TTGACGGGCTGCTGCTGCT | 731       | ND              |
| δ₁      | NM_000722.2     | TGCTGATGGGCCCCCTCCGCT/CCAGGCGCACCAGGGCTTTAG | 252       | 60              |
| δ₂      | NM_00140505.1   | AGCTGAGACCAGGGGACACT/CTCAGCAAGTCTACGACTCGCGG | 878       | 60              |
| δ₃      | NM_018398.2     | GACGAGAAGGCTGCTGCTGCTTGA/CCAGGCGCGCTAAGCACGTGAA | 132       | 65              |
| δ₄      | NM_172364.4     | TGGCTGGCGCTTGTGAC/GGCCCTCTCGGCAGGCTTAC | 328       | ND              |
| γ₁      | NM_000727.3     | TGGCTGGCGCTTGTGAC/GGCCCTCTCGGCAGGCTTAC | 367       | 60              |
| γ₂      | NM_006078.3     | TGGCTGGCGCTTGTGAC/GGCCCTCTCGGCAGGCTTAC | 439       | ND              |
| γ₃      | NM_006539.3     | TGGCTGGCGCTTGTGAC/GGCCCTCTCGGCAGGCTTAC | 840       | ND              |
| γ₄      | NM_014405.3     | TGGCTGGCGCTTGTGAC/GGCCCTCTCGGCAGGCTTAC | 909       | 62–64           |
| γ₅      | NM_145811.2     | TGGCTGGCGCTTGTGAC/GGCCCTCTCGGCAGGCTTAC | 257       | 64              |
| γ₆      | NM_145814.1     | TGGCTGGCGCTTGTGAC/GGCCCTCTCGGCAGGCTTAC | 334       | ND              |
| γ₇      | NM_031896.4     | TGGCTGGCGCTTGTGAC/GGCCCTCTCGGCAGGCTTAC | 910       | 65              |
| γ₈      | NM_031895.5     | TGGCTGGCGCTTGTGAC/GGCCCTCTCGGCAGGCTTAC | 987       | ND              |

(?) ND: Isoform not detected, unknown annealing temperature.

Discussion

Ca₃.2 channels play a key role in regulating nociception. Inhibition of Ca₃.2 at the spinal cord produces analgesia in animal models of pain [23,36] and in humans [37], with direct (e.g., Prialt) and indirect (e.g., gabapentin) inhibitors among some of the

to the right in the presence of nifedipine (EC₅₀ of 20.4 mM, pEC₅₀ 1.69±0.12, Hill slope of 2.9) (Fig. 3A Table 4). Conversely, the Ca₂.2 [N-Type] inhibitor α-conotoxin CVID was used at a concentration that does not affect responses of other Ca₃.8 (up to 3 μM) [21,28], to isolate non-N-type responses. Compared to responses in the presence of nifedipine, the KCl concentration-response curve was shifted to the left in the presence of CVID (EC₅₀ of 18.6 μM, pEC₅₀ 1.86±0.10, Hill slope of 3.5) (Fig. 3A, Table 4). These differences can be accounted for by the electrophysiological properties of each Ca₃ channel subtype identified, since L-type requires a larger depolarization than N-type to be activated [28], and the control KCl responses is a result identified, since L-type requires a larger depolarization than N-type responses in native and recombinant systems, when Ca₂.2 was co-expressed with β and δ subunits [22–24]. In contrast, GVIÁ potency at Ca₂.2 expressed in SH-SY5Y cells (IC₅₀ of 0.15 μM; IC₅₀ 6.8±0.072) was consistently lower than previously described for heterologous expressed rat [24,26] and human [25] α₁b co-expressed with δ₁ and β₂, but similar to data obtained using native expression systems such as dissociated rat DRG cells [30] and chicken synaptosomes [31].

A small portion (5–15%) of the KCl-evoked responses was insensitive to block by co-application of 10 μM nifedipine and 3 μM CVID (Fig.3C–D). To pharmacologically characterize these remaining responses, we assessed the effects of Ca₂.1 and Ca₂.3 subtype-specific inhibitors, as well as of compounds with activity at Ca₃.3, on the Ca²⁺ responses evoked by 90 mM KCl/5 mM CaCl₂ in the presence of both CVID and nifedipine. The Ca₂.1 blockers α-agatoxin IVA (data not shown) and α-agatoxin TK did not significantly affect KCl-evoked Ca²⁺ responses at concentrations up to 10 μM (Fig. 4A, Table 4). The Ca₂.3 antagonist SNX 482 also had no significant inhibitory effect at concentrations up to 10 μM (Fig. 4A and C, Table 4). On the other hand, mibebradil (30 μM), a benzimidazolyl-substituted tetraline reported to inhibit Ca₃.3 responses in different systems with weak affinity [32,33] fully inhibited these remaining responses with an IC₅₀ of 3 μM (pIC₅₀ 5.3±0.035) (Fig. 4A and D, Table 4). Similar IC₅₀ values for mibebradil block of T-type responses in native systems have been previously reported (see [32–35]). In addition, another Ca₃.3 inhibitor, the antipsychotic pimozide, also fully inhibited the remaining responses with an IC₅₀ of 1.3 μM (pIC₅₀ 5.2±0.097) (Fig. 4A, Table 4), similar to previously reported literature values [34]. These findings are in agreement with our PCR (Fig. 1A-B) which detected mRNA transcripts for Ca₃.1, but neither Ca₂.1 nor Ca₂.3 was identified.
most recently developed analgesics [18]. Neuroblastoma cells, including the sympathetically derived human neuroblastoma cell line SH-SY5Y, provide excellent model systems to study Cav2.2 channels in a native context [14,15]. However little is known about the Cavα and auxiliary subunits expressed, limiting interpretation of pharmacological data from these cells. To address this limitation, we have characterized the expression and pharmacology of Cav channels in SH-SY5Y cells and investigated mechanisms likely to influence the pharmacology of ω-conotoxins at Cav2.2 channels.

Previous electrophysiological studies have identified L- and N-currents from high voltage activated channels Cav1 and Cav2.2 in SH-SY5Y cells, but not low voltage activated T-type currents from Cav3 channels [13–15,38]. In contrast, we detected mRNA transcripts for the N-type (Cav2.2), two L-type (Cav1.3 isoform 1 and 2) and one T-type isoform (Cav3.1). In addition, we also detected mRNA transcripts for Cav2.2 splice variants, including α1B2 (74 amino acid shorter) [16,17,39] and the splice α1BΔ1 (382 amino acid shorter) [16].

Functional Cavα responses elicited by addition of KCl/CaCl2 were assessed using a fluorescent high-throughput Ca2+ imaging assay on the FLIPRTetra. KCl has been used extensively to activate Cavα channels, influx of Ca2+ and a resultant increase in intracellular fluorescence. While the change in fluorescence elicited by addition of KCl at the concentrations used here is approximately linear, accumulation of intracellular Ca2+ is saturable and fits a sigmoidal concentration-response curve because a change in membrane potential leads to a finite change in channel open probability and thus Ca2+ influx.

Cav2.2 channels expressed in SH-SY5Y cells were functional and generated KCl activated responses that were inhibited by ω-conotoxins CVID, GVIA and MVIIA in the presence of saturating concentrations of nifedipine. As expected, when L-type responses were isolated by addition of saturating concentrations of CVID, nifedipine concentration-dependently blocked KCl responses. However, a small response remained (5–15%) in the presence of

![Figure 2. Displacement of 125I-GVIA from SH-SY5Y whole cell and membranes by ω-conotoxins.](image-url)
a combination of Ca v2.2 and Ca v1 inhibitors. This resistant response was completely abolished by the Ca v3 inhibitors mibefradil and pimozide. While mibefradil and pimozide are not specific inhibitors of T-type currents and also inhibit L-type channels [32, 41], inhibition of the residual Ca²⁺ response was also observed in the presence of saturating concentrations of nifedipine.

Figure 3. Cav2.2 and Cav1 channels endogenously expressed in SH-SY5Y cells are functional. Data obtained from fluorescent Ca²⁺ imaging assays of KCl-evoked Ca²⁺ responses in SH-SY5Y cells. (A) Cav1 and Cav2.2 activation in the presence of CVID (open ball) and nifedipine (filled ball), respectively, shifted control KCl-evoked Ca²⁺ responses (quadrilateral) significantly in SH-SY5Y cells (p < 0.05). (B) Time course of Ca²⁺ responses is shown for control KCl 90 mM (black), KCl in the presence of nifedipine (blue) and KCl in the presence of CVID (green). (C) Concentration-response curve for nifedipine inhibition of Cav1 responses (D) Concentration-response curves for CVID, GVIA and MVIIA inhibition of Cav2.2 responses. The responses were normalized using controls: positive KCl and negative PSS buffer; and plotted across increasing concentrations of antagonists (E) Comparison of ω-conotoxins CVID, GVIA and MVIIA potencies (IC₅₀/Kᵣ ± SEM of n = 3–4 replicates for each experiment, n = 3 experiments) in displacing ¹²⁵I-GVIA from SH-SY5Y whole cell and SH-SY5Y cell membranes with the functional assays data. doi:10.1371/journal.pone.0059293.g003
suggesting that activity of these compounds at L-type channels did not contribute to inhibition of residual response. Based on our observations that this resistant response was not blocked by inhibitors of L-type (nifedipine), N-type (CVID), R-type (SNX 482) or P/Q-type channels (ω-agatoxin), but was completely abolished by compounds with known activity at T-type channels, it seems plausible that this response may be mediated by Ca_{3.1}, which mRNA expression was detected in SH-SY5Y. Alternatively, it is known that the Δ1 splice variant, which mRNA expression was detected in SH-SY5Y cells, is significantly more resistant to the blockade by MVIIA and GVIA [42]. While inhibition by CVID of the Ca_{2.2} splice variants detected in SH-SY5Y cells has not been characterised, it is possible that, akin to inhibition of Na_{v} channels by the ω-conotoxin GIIIA, complete current inhibition by CVID cannot be achieved for these splice variants. Alternatively, the response remaining in the presence of nifedipine and CVID could represent another undefined resistant current, or an artifact of the KCl/Ca^{2+} activation buffer used in this study.

Development of non-electrophysiological HTS Ca_{v} channel assays has been hampered by some of the properties of this channel, including their low voltage threshold for activation and inactivation and rapid inactivation kinetics. However, although T-type currents inactive rapidly, fluorescence Ca^{2+} assays detect accumulation of intracellular Ca^{2+} rather than currents, and are thus not subject to the same temporal resolution constraints. In addition, compared to heterologous systems, SH-SY5Y cells have a relatively hyperpolarised resting membrane potential [43], which would be conducive to channels being present in the resting state. Accordingly, Ca^{2+} assays at Ca_{3} channels using the FLiPR have been successfully developed [40] and it is clearly conceivable that functional responses of Ca_{3.1} expressed in SH-SY5Y cells could be elicited using KCl/Ca^{2+} stimulation. In addition to functional characterization, we also confirmed Ca_{2.2} expression at the protein level using ^{125}\text{I}-GVIA binding assays. The ω-conotoxins CVID, GVIA and MVIIA each fully displaced ^{125}\text{I}-GVIA binding to SH-SY5Y cell membranes with high affinity. Interestingly, while the affinity of GVIA was not significantly different between species, CVID and MVIIA affinities were ~10-fold higher in human SH-SY5Y membranes compared to rat brain membranes. These results support the findings that MVIIA and CVID interacts with Ca_{2.2} human channels through a different pharmacophore, as compared with GVIA [44].

Variation in the affinity of ω-conotoxins between species is likely influenced by Ca_{v} splice variants, with differences in toxin sensitivity, time course and voltage-dependence of inactivation, single channels conductance, gating behavior and sensitivity to G-protein-mediated modulation reported for splice isoforms endogenously expressed in neuronal cells of rat, mouse, rabbit and humans [16,17,39,42,45–48] (for review see: [46]). In pain, the Ca_{2.2} splice variant 37a replaces the usual variant 37b in a specific subset of nociceptive neurons, and thus may represent a potential therapeutic target [42,46,49]. However, this variant has to date only been described in rat dorsal root ganglion neurons, and is not known to be present in human tissue.

Additional human splice variants include two α_{1B} isoforms that have long or short C-termini [17], and two human forms that lack large parts of the domain II-III linker region, including the synaptic protein interaction site. These splice variants, termed Δ1 and Δ2, have been previously isolated from IMR32 human neuroblastoma cell line and human brain cDNA libraries [16]. We have identified mRNA transcripts for the full length α_{1B1}, α_{1B2} (74 amino acid shorter) [16,17,39] and the splice variant Δ1 (382 amino acid shorter) [16] in SH-SY5Y cells. The α_{1B1} is an axonal/synaptic isoform, while α_{1B2} is restricted to neuronal soma and

| Ca_{v} Activator/Inhibitor | Ca^{2+} Stimulation EC_{50} (mM) | Ca^{2+} Inhibition IC_{50} (μM) |
|---------------------------|---------------------------------|---------------------------------|
| KCl                       | 17.28 ± 3.41                    | –                               |
| KCl+CVID                  | 18.61 ± 3.22                    | –                               |
| KCl+NIFEDIPINE            | 20.35 ± 3.17                    | –                               |
| CVID                      | –                               | 0.16 ± 0.025                    |
| GVIA                      | –                               | 0.15 ± 0.009                    |
| MVIIA                     | –                               | 0.024 ± 0.005                   |
| NIFEDIPINE                | –                               | 0.23 ± 0.046                    |
| MIBEFRADIL                | –                               | 3.0 ± 0.031                     |
| PIMOZIDE                  | –                               | 1.3 ± 0.097                     |
| ω-AGATOXIN TK             | –                               | NDR                             |
| SNX 482                   | –                               | NDR                             |

NDR: Non-detectable response.

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dendrites [39,50], however, apart from differential susceptibility to G\(\alpha_i\)/G\(\alpha_o\)-versus G\(\alpha_q\)-mediated inhibition, little is known regarding its biophysical and pharmacological properties. On the other hand, the D\(1\) splice variant has lost part of the synaptic protein interaction (synprint) site and is thus unlikely to play a role in fast synaptic transmission, with shifts in the voltage dependence of steady-state inactivation and a more rapid recovery from inactivation compared to full length a\(1\)B1 [16]. Importantly and clinically relevant, D\(1\) variant was significantly more resistant to the blockade by MVIIA and GVIA; however the degree of effect varied for each toxin [16]. Thus, expression of the D\(1\) variant in SH-SY5Y cells may contribute to the reduced v-conotoxin affinity observed. While expression of these splice variants in SH-SY5Y cells was detected using gene specific primers, which have been extensively validated in the literature [17], further confirmation of expression at the protein level is warranted.

Cav channel auxiliary subunits can also influence the pharmacology of Cav inhibitors, with v-conotoxins displaying reduced affinity in the presence of the a\(2\)d subunit [11,22–24,27,51]. Specifically v-conotoxins GVIA, MVIIA and CVID had reduced affinity when a\(2\)d1 subunit was co-expressed with the Cav a\(1\)B 

a\(2\)d up-regulation has been associated with chronic pain and epilepsy, with gabapentin and pregalin binding to a\(2\)d reducing Ca\(2+\) trafficking and the symptoms of pain [11]. The a\(2\)d7–9, b\(1\), b\(2\) and b\(3\), b\(4\), c\(1\), c\(4–5\) and c\(7\) subunits were detected in SH-SY5Y cells and potentially contribute to the differences in v-conotoxins potency in whole cell vs. membrane assays.

The a\(1\) subunit was originally identified in skeletal muscle in complex with Cav1 channels [52], but effects of this subunit on the v-conotoxins affinity at Cav2.2 have not been determined. In contrast, co-expression of the a\(2\) subunit almost abolished the functional expression of Cav2.2 in either Xenopus oocytes or COS-7 cells [53,54]. The neuronal a\(2\) subunit is associated with epileptic and ataxic phenotypes of stargazer mouse [55], but was not detected in SH-SY5Y cells. The c\(1\) and c\(7\) subunits represent a distinct subdivision of the c subunit family of proteins identified by structural and sequence homology to stargazing. The c\(5\) subunit may be a regulatory subunit of Cav3.1 channels (for review see: [57]). These subunits may also potentially contribute to differences in v-conotoxins binding affinities observed in whole cell vs. membrane assays.

While auxiliary subunits affect v-conotoxin affinity in functional studies, this quaternary complex is likely to be disrupted upon preparation of homogenized membranes for the binding assays [58]. To examine this possibility, we studied the ability of GVIA to displace 125I-GVIA from whole SH-SY5Y cells compared to homogenized membranes. Interestingly, v-conotoxins CVID, MVIIA and GVIA had higher affinity to displace 125I-GVIA from the homogenized membranes compared to the whole cells,

**Figure 4. Characterization of resistant Ca\(2+\) responses in SH-SY5Y cells.** Data obtained from fluorescent Ca\(2+\) imaging of KCl-evoked Ca\(2+\) responses in SH-SY5Y cells. (A) Concentration-response curves for mibefradil, pimozide, \(\omega\)-agatoxin TK and SNX 482 in inhibiting resistant KCl-evoked Ca\(2+\) responses in SH-SY5Y cells, pretreated with CVID (3 \(\mu\)M) plus nifedipine (10 \(\mu\)M) (B–D) Time course of transient Ca\(2+\) responses activated by 90 mM KCl/5 mM CaCl\(2\) in the presence of CVID (3 \(\mu\)M) and nifedipine (10 \(\mu\)M) and following the addition of agatoxin TK, SNX-482 and mibefradil.

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an effect that was most pronounced for CVID and MVIIA (−100-fold) compared to GVIA (−10-fold). We have previously reported a similar trend for both CVID and MVIIA in hetrologous expression system with and without the αδ subunit [22]. Potency estimates obtained with the functional assays were significantly lower than estimates obtained in whole cell radioligand binding assays. The relatively high level of Ca2+ in the physiological saline used traditionally for functional assays compared to binding assays could contribute to these differences, since Ca2+ non-competitively inhibits ω-conotoxin binding [21]. However, our whole cell data was also obtained by incubating ω-conotoxins in a Ca2+-free physiological saline solution and the origin of these differences is unclear. Interestingly, this effect was most marked for GVIA, intermediate for CVID and insignificant for MVIIA.

In summary, we have characterized functional Ca channels expressed in SH-SY5Y human neuroblastoma cell line. Our studies have shown expression of different Ca,α splice variants, in conjunction with auxiliary subunits in a native context, can modulate the pharmacology of Ca,2.2 channel inhibitors. SH-SY5Y cell line provides a useful model for the investigation of a similar trend for both CVID and MVIIA in heterologous modulate the pharmacology of Cav2.2 channel inhibitors. SH-SY5Y cell line provides a useful model for the investigation of a similar trend for both CVID and MVIIA in heterologous

Materials and Methods

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Ca channel subtype and auxiliary subunits mRNA expression profiles were investigated in SH-SY5Y cells using standard RT-PCR and specific primers. The primers were designed using The Basic Local Alignment Search Tool (BLAST) [60,61], or otherwise specified as, previously described in the literature. Primer sequences, Gene Bank reference numbers, predicted PCR product sizes, and optimum annealing temperatures are shown in Table 1. The primers used to identify Ca,α subtypes and auxiliary subunits were designed so that all splice variants of specific isoforms would be amplified. On the other hand, primers to amplify Ca,2.2 splice variants isoform were designed to be specific to each isoform. PCR conditions to detect splice variants were set as previously described [16,17], with gradient PCR performed for all sets of primers, allowing the identification of optimal annealing temperatures. Different sets of primers were used to identify the full length and isoforms Δ1 and Δ2 (see table 1) [16]. These primers were designed based on the region of the domain II-III linker of Ca,2.2 channels, as previously described [16]. Primers used to identify the full length α1B1 and short α1B2 isoforms were designed based on the C-terminus region [17]. Data is representative of at least three independent experiments.

SH-SY5Y cells (1×10^6) were harvested and total RNA isolated using Trizol® Reagent (Invitrogen, Carlsbad, CA). The isolated RNA was subsequently treated with RNase-free DNase to remove any genomic DNA contamination. RNA concentration was determined by absorbance measurements at 260 nm and its purity/integrity was accessed by analyzing the ratio 260/280 nm with a Nanodrop® (Thermo Scientific). Synthesis of first strand cDNA was performed using 1 μg of the extracted RNA and the Omniscript Reverse Transcription Kit (Qiagen), according to the manufacturer’s instructions. cDNA amplifications were performed using Taq Polymerase (New England Biolabs, US). The reaction mix (total 25 μL) included (μL): 1 cDNA (100 ng), 0.125 of the enzyme, 0.5 reverse and 0.5 forward primers (10 μM), 0.5 dNTPs (10 mM), 2.5 Thermopol reaction buffer (10×) and nuclease free water. RT-PCR was carried through as an initial denaturation step at 95°C for 3 min followed by 35 cycles of the steps: 95°C for 30 s, optimal annealing temperature as previously determined (Table 1) for 60 s, 68°C extension for 60 s, plus an extra 5 min elongation step at 68°C. PCR products were analyzed by 1% agarose gel and predicted sizes estimated by comparison with DNA molecular weight makers (50 and 100 bp ladder, New England Biolabs). Target-specific primers for the housekeeping gene GAPDH were designed as previously described [19]. PCR master mix using random primers without cDNA was used as negative qDNA control in all PCRs. Specificity of primers was demonstrated in a range of control experiments (data not shown), including detection of Ca,2.2 plasmid but no other Ca,α subtypes by Ca,2.2 primers; and absence of detectable levels of Ca,2.2 in HEK cells. β1 and αδ,1 primers were positive for β1 and αδ,1 plasmids, while the same primers were negative for β2,4 and αδ,2,3 (data not shown), indicating primers were selective for β1 and αδ,1 auxiliary subunits. In addition, identity of PCR products was further confirmed by sequencing analysis (data not shown). Figures 1A-D is representative of the average of 3–10 independent experiments.

Sequencing

PCR amplicons were first separated on agarose gels and bands of expected sizes identified. PCR products were purified using the Wizard SV Gel and PCR clean-up system (Promega), and a sample of each purified PCR product was sent for sequencing at the Australian Genome Research Facility. cDNA sequences of human Ca,α subtypes and auxiliary subunits were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Entrez/) and BLASTn [62] was used for confirmation of the identity of human Ca,α subtypes and auxiliary subunits.

Cell Culture

The human neuroblastoma SH-SY5Y cells (Victor Diaz, Goettingen, Germany) were cultured and routinely maintained at 37°C and 5% CO2 in RPMI 1640 antibiotic-free medium (Invitrogen) supplemented with 10% heat-inactivated FBS and 2 mM GlutaMAX™ (Invitrogen). Tryptsin/EDTA was used to detach the cells from the T-75 or T175 flasks and cells were split in a ratio of 1:5–1:10 every 3–4 days or when ~80% confluent.

Membrane Preparation for the Radioligand Binding Assay

Radioligand binding assays were performed using rat brain or SH-SY5Y cell membranes prepared as described by Wagner, et al., 1988 [63] with slight modification. For rat brain membranes, male Wistar rats weighing 175–250 g were sacrificed by cervical dislocation and the whole brain was rapidly removed and dissected on ice. At 4°C, tissue was re-suspended in 50 mM HEPES, pH 7.4 (50 mg wet weight tissue/ml buffer), homogenized using a Brinkmann Polytron homogenizer and centrifuged for 15 min at 40,000×g. The pellet was re-suspended in 50 mM HEPES and 10 mM EDTA at pH 7.4, incubated on ice for 30 min and centrifuged at 40,000×g for 10 min. The pellet was then re-suspended in 50 mM HEPES pH 7.4 containing 10% glycerol, aliquots were made and kept at −80°C prior to use. Bicinchoninic acid (BCA) assay reagent (Pierce Rockford, IL) was used for protein quantification.

SH-SY5Y cell membranes were harvested using trypsin/EDTA, washed once with DPBS, and centrifuged for 4 min at 500 ×g. After centrifugation, the supernatant was discarded and the pellet
re-suspended in 10 ml binding assay buffer at pH 7.2 containing (mM): 20 HEPES, 75 NaCl, 0.2 EDTA, 0.2 EGTA and complete protease inhibitor (Roche Diagnostics, AU) and sonicated. The homogenates were then centrifuged for 30 min at 40,000 x g and 4 °C. The supernatant was discarded and the pellet dissolved in aliquots of binding assay buffer containing 10% glycerol stored at −80 °C prior to use. BCA was used for protein quantification.

Whole Cell Preparation for the Radioligand Binding Assay
Whole cells were prepared as described for SH-SY5Y cell membranes with the following modifications: after cells were harvested and centrifuged, the supernatant was discarded and the pellet re-suspended in sufficient volume of binding buffer to plate 50 µL/well in triplicates in 96 well plates. Specific o-conotoxins binding was determined using the same concentration of protein as used for SH-SY5Y cell membranes (20 µg/50 µL), corresponds to 600,000 cells per well.

Radioligand Binding Assay
Tyr22-[125I]-GVIA, was prepared using IODOGEN, as previously described by Ahmad [64], purified using reverse phase HPLC and stored at 4 °C for use within 3 weeks. On the day of the assay, membranes were thawed on ice and reconstituted to 10 µg/50 µL (rat) or 10–20 µg/50 µL (SH-SY5Y) in binding assay buffer containing 2% complete protease inhibitor and 0.1% bovine serum albumin. Stock [125I]-GVIA was diluted to 20000 cpm/50 µL or 30 pM. For displacement studies, [125I]-GVIA was incubated with rat brain or SH-SY5Y membranes or whole cells and varying concentrations of the competing ligand in triplicates in 96 well plate formats. Specific o-conotoxins binding was determined using analysis of variance (ANOVA) or student’s t-test, with statistical significance defined as p<0.05, unless otherwise stated.

Intracellular Ca2+ Response Measurement Using the FLIPR
SH-SY5Y cells were seeded onto 96-well or 384-well flat, clear bottom, black-walled imaging plates (Corning, Lowell, MA, US) at a density of 160,000 or 40,000 cells/well, respectively, resulting in 90–95% confluent monolayer after 40 h. On the day of the Ca2+ imaging assays, cells were loaded for 30 min in the dark at 37 °C with 5 µM Fluo-4 acetoxyxymester (Fluo-4-AM), in physiological salt solution (PSS composition: NaCl 140 mM, glucose 11.5 mM, KCl 5.9 mM, MgCl2 1.4 mM, NaH2PO4 1.2 mM, NaHCO3 5 mM, CaCl2 1.8 mM, HEPES 10 mM, pH 7.4) containing in addition 0.3% BSA and 10 µM nifedipine. After the incubation period, the cells were washed once with 100 µL assay buffer (no Fluo-4-AM or BSA), and replaced with 100 µL of the same buffer. Plates were then transferred to the FLIPR TETRA (Molecular Devices, Sunnyvale, CA) fluorescent plate image reader, camera gain and intensity were adjusted for each plate to yield between 800–1000 arbitrary fluorescence units (AFU) baseline fluorescence, and Ca2+ responses measured using a cooled CCD camera with excitation at 470–495 nM, and emission at 515–575 nM. Ten baseline fluorescence readings were taken prior to the addition of antagonists, and then fluorescent readings every 2 s for 300 s before 90 mM KCl/5 mM CaCl2 buffer was added and fluorescence readings again recorded each second for further 300 s. To ensure full inhibition of Ca2+ responses, the cells were pre-incubated for 40 min with 10 µM nifedipine. To ensure full inhibition of Ca2,2 responses, the cells were pre-incubated for 10 min with 1–3 µM CVID.

Statistical Analysis
Concentration-response curves were determined following nonlinear regression analysis using a 4-parameter Hill equation, with variable Hill slope fit to the functional assays data and one site fit to the radioligand binding assays; and normalized using GraphPad Prism (Version 5.00, San Diego, California). Negative and positive controls (PSS buffer and KCl 90 mM ±5 mM CaCl2, respectively) were used to normalize functional data. All data is presented as mean ± SEM of 6–10 independent experiments performed in triplicate, unless otherwise stated. Statistical significance was determined using analysis of variance (ANOVA) or student’s t-test, with statistical significance defined as p<0.05, unless otherwise stated.

Author Contributions
Conceived and designed the experiments: IV RJL. Performed the experiments: SRS IV LR. Analyzed the data: SRS IV LR RJL. Contributed reagents/materials/analysis tools: RJL. Wrote the paper: SRS IV LR RJL.
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