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Regulation of axonal and dendritic growth by the extracellular calcium-sensing receptor (CaSR)

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Abstract

The extracellular calcium-sensing receptor (CaSR) monitors the systemic extracellular free ionized calcium level ($[Ca^{2+}]_o$) in organs involved in systemic $[Ca^{2+}]_o$ homeostasis. However, the CaSR is also expressed in the nervous system where its role is unknown. Here we find high levels of the CaSR in perinatal mouse sympathetic neurons when their axons are innervating and branching extensively in their targets. Manipulating CaSR function in these neurons by varying $[Ca^{2+}]_o$, using CaSR agonists and antagonists or expressing a dominant-negative CaSR markedly affects neurite growth *in vitro*. Sympathetic neurons lacking the CaSR have smaller neurite arbors *in vitro*, and sympathetic innervation density is reduced in CaSR-deficient mice *in vivo*. Hippocampal pyramidal neurons, which also express the CaSR, have smaller dendrites when transfected with dominant-negative CaSR in postnatal organotypic cultures. Our findings reveal a crucial role for the CaSR in regulating the growth of neural processes in the peripheral and central nervous systems.

The growth, guidance and branching of neural processes in the developing nervous system is controlled by numerous locally acting and diffusible signalling proteins that bind specific receptors on the growing tips of these processes 1,2. While changes in cytoplasmic Ca^{2+} participate in transducing many of these growth and guidance signals, changes within the narrow physiological range of extracellular Ca^{2+} have not been thought to play a direct role in regulating growth cone motility 3. The level of extracellular Ca^{2+} is monitored by the CaSR, and in accordance with its crucial regulatory function in maintaining $[Ca^{2+}]_o$ within very narrow physiological limits 4, it is conspicuously expressed in all structures and organs involved in systemic calcium homeostasis, namely, the parathyroid glands, kidneys, bone and gut. It is also expressed in several other tissues and in multiple sites within the adult brain, including the subfornical organ, olfactory bulbs, striatum, cerebellum, basal ganglia and hippocampus, where its functions are unclear 5,6.

To investigate whether CaSR has a role in neuronal development, we screened for the expression of CaSR transcripts in several experimentally tractable populations of neurons in the peripheral nervous system of fetal mice. We found significant CaSR expression in the superior cervical ganglion (SCG), a population of sympathetic neurons that is extensively

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AUTHOR CONTRIBUTIONS

T.N.V. and G.W.O. conducted the experiments on sympathetic neurons, H.G. conducted the experiments on hippocampal neurons, C.H.K. generated the CaSR mutant mice, D.R. and A.M.D. supervised the project.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

used for investigating various aspects of neuronal development and is known to depend on the neurotrophin nerve growth factor (NGF) for survival and target field innervation *in vivo* 7-10. Here we demonstrate a surprising and previously unrecognised role for CaSR in enhancing sympathetic axon growth and branching at a stage in development when sympathetic neurons are innervating and branching extensively in their target fields, and also demonstrate a role for CaSR in regulating the growth and complexity of pyramidal dendrites in the postnatal hippocampus.

RESULTS

CaSR is expressed in developing sympathetic neurons

The developmental profile of CaSR mRNA expression in the SCG (Fig. 1a) revealed a relatively low level of expression at E16 followed by a marked increase from E17 to a peak at embryonic day 18 (E18). The peak was followed by a decrease in the immediate perinatal period and a slower decline to low levels over later postnatal stages. Western blot analysis revealed similar developmental changes in endogenous CaSR protein over the period E16 to P1 (Supplementary Fig. 1). To determine which SCG cells express CaSR, we localized CaSR protein in dissociated SCG cultures established at the peak of CaSR expression by immunocytochemistry using two different affinity-purified antibodies that recognise specific epitopes in the extracellular and cytoplasmic domains of the CaSR protein, respectively. In double-labelled preparations in which neurons were positively identified with anti- β -III tubulin antibodies, both CaSR antibodies stained the cell bodies and entire neurite arbors of all neurons and also stained some of the non-neuronal cells (Fig. 1b and Supplementary Fig. 2).

$[Ca^{2+}]_o$ influences neurite growth from sympathetic neurons

The high level of CaSR expression just before and after birth encompasses a period of development when many sympathetic axons are branching extensively in their distal targets 7. For this reason we investigated whether modulating the function of CaSR at this stage of development would affect sympathetic neurite growth. We incubated low-density, dissociated cultures of E18 SCG neurons in defined, serum-free medium with several different concentrations of Ca^{2+}_o spanning the CaSR response range. Because of the *in vivo* requirement of these neurons for NGF at this stage of development 7, we supplemented all cultures with this neurotrophin. Initial experiments revealed a very marked and consistent effect of $[Ca^{2+}]_o$ on neurite growth, however, to rule out any potential effects of lowering $[Ca^{2+}]_o$ on neuronal viability, we included a broad-spectrum, irreversible caspase inhibitor (Boc-D-FMK) in all experiments to ensure maximum neuronal survival in all experimental groups.

The sensitivity of CaSR to changes in $[Ca^{2+}]_o$ varies somewhat according to cell type and the signalling events or physiological responses quantified within the range 0.5 to 3 mM with the EC_{50} or IC_{50} ranging between ~1 mM and ~1.7 mM 11,12. In the case of cultured E18 SCG neurons, quantification of total neurite length (Fig. 2a) and Sholl analysis (Fig. 2b), which provides a graphic representation of neurite length and branching with distance from the cell body 13, revealed that neurite arbor size and complexity were affected by varying $[Ca^{2+}]_o$ within the range 0.7 to 2.3 mM, concentrations at which the CaSR is known to be either minimally (i.e., 0.7 mM) or maximally active (i.e., 2.3 mM) in native tissue 14. The similar sizes of the neurite arbors of neurons grown with 1.4 mM and 2.3 mM $[Ca^{2+}]_o$ suggests that the influence of $[Ca^{2+}]_o$ on neurite growth is effectively saturating at these concentrations. The great majority of neurons in these cultures were surviving at 24 hours when their neurite arbors were analysed (~80% of those plated) and there was no significant difference in neuronal survival over the $[Ca^{2+}]_o$ range studied in these caspase inhibitor

treated cultures (Supplementary Fig. 3a). The neurite arbors of typical E18 SCG neurons grown in medium containing 0.7 mM and 2.3 mM $[Ca^{2+}]_o$ are illustrated in Figure 2c. It is possible that 0.7 mM $[Ca^{2+}]_o$ exerts mild CaSR activation in SCG neurons, as has been shown in other cell systems 5,12,14, because the neurite arbors of SCG neurons of wild type E18 mice grown with 0.7 mM $[Ca^{2+}]_o$ were significantly larger than those of SCG neurons from *Casr*^{-/-} littermates (Supplementary Fig. 4). Taken together, these findings demonstrate that varying $[Ca^{2+}]_o$ over the CaSR sensitivity range influences the magnitude of neurite growth from late fetal SCG neurons.

The effect of $[Ca^{2+}]_o$ is restricted to a brief developmental window

The developmental peak in CaSR expression just before birth (Fig. 1a) raised the possibility that the influence of $[Ca^{2+}]_o$ on neurite growth might be restricted to a developmental window related to CaSR expression. To examine this possibility, we set up dissociated cultures of SCG neurons at stages before, during and after the peak of CaSR mRNA expression in medium containing either 0.7 or 2.3 mM $[Ca^{2+}]_o$. Analysis of neurite arbors after 24 hours of incubation revealed large, highly significant differences in neurite length (Fig. 3a) at these two concentrations of Ca^{2+}_o in E18 and P0 cultures, but no significant differences in E16 and P1 cultures. Likewise, the Sholl plots were markedly different for E18 and P0 neurons grown with 0.7 or 2.3 mM $[Ca^{2+}]_o$ and overlapping for E16 and P1 neurons (Fig. 3b). These findings suggest that changes in $[Ca^{2+}]_o$ over the response range of CaSR markedly influences the growth of neurites from sympathetic neurons during a brief developmental window in the immediate perinatal period when CaSR expression peaks in these neurons. Axon extension commences very early in sympathetic neuron development, and the earliest sympathetic axons reach some distal targets between E12 and E13 7. By E16.5 sympathetic axons are ramifying within distal targets, and by P0.5 more extensively branched axonal networks are evident in these targets 8. Thus, the window of development over which CaSR promotes sympathetic axon growth and branching corresponds to a time when sympathetic axons are ramifying and branching extensively in their distal targets, but not to the period of initial axonal outgrowth or to the early stages of target field innervation.

The CaSR mediates the effect of $[Ca^{2+}]_o$ on neurite growth

The above findings implicate the CaSR in mediating the developmentally restricted effects of $[Ca^{2+}]_o$ on neurite growth from cultured SCG neurons. To provide direct proof for the involvement of the CaSR, we used three additional experimental approaches: pharmacological modulation of CaSR function using positive and negative allosteric modulators of CaSR, expression of wild type and dominant-negative CaSR protein and comparative studies of neurite growth from neurons obtained from wild type and CaSR-deficient mice. A CaSR agonist (the calcimimetic NPS R-467 12) significantly increased the size of the neurite arbors of E18 SCG neurons grown in medium containing 0.7 mM $[Ca^{2+}]_o$ (Fig. 4a), but did not affect neurite growth from E18 CaSR-deficient SCG neurons (Supplementary Fig. 5), confirming its specificity. On the other hand, a CaSR antagonist (the calcilytic NPS 89636 15) significantly reduced the size of the neurite arbors of E18 SCG neurons grown in medium containing 2.3 mM $[Ca^{2+}]_o$ (Fig. 4b). Neither calcimimetic nor calcilytic affected neuronal survival in these experiments (Supplementary Figs. 3b and 3c). The neurite arbors of E18 SCG neurons transfected with a plasmid expressing a CaSR with an arginine-to-glutamine substitution (R185Q) that exerts a dominant-negative effect on CaSR function 16 were significantly smaller than the neurite arbors of control-transfected neurons in medium containing 2.3 mM $[Ca^{2+}]_o$ (Fig. 5a). To determine if the reduction in neurite growth by the dominant-negative CaSR was a non-specific effect of expressing this protein in neurons, we transfected P1 SCG neurons with the dominant-negative CaSR plasmid because neurite growth from these neurons is unaffected by varying $[Ca^{2+}]_o$ over the sensitivity range of the CaSR (Fig. 3b). There were no significant differences in the

overall neurite length and Sholl profiles of dominant-negative CaSR-transfected and control-transfected P1 SCG neurons grown in medium containing 2.3 mM $[Ca^{2+}]_o$ (Fig. 5b), ruling out a non-specific detrimental effect of this protein on neurite growth on E18 neurons. The lack of effect of the dominant-negative CaSR on neurite growth from P1 SCG neurons was most likely due to the lack of functionally significant levels of CaSR at this stage of development (Fig. 1a, Supplementary Fig. 1) because transfecting these neurons with a plasmid that expresses wild type CaSR conferred responsiveness to 2.3 mM $[Ca^{2+}]_o$ (Fig. 5c). Overexpressing wild type CaSR in E18 neurons that already express functionally significant levels of CaSR did not further increase the neurite arbor size of neurons grown in medium containing 2.3 mM $[Ca^{2+}]_o$ (Figs. 5d). Neither dominant-negative CaSR nor overexpression of wild type CaSR affected neuronal survival in these experiments (Supplementary Figs. 3d and 3e). Finally, the neurite arbors of E18 SCG neurons from *Casr*^{-/-} mice were significantly smaller than those of wild type littermates grown in medium containing 2.3 mM $[Ca^{2+}]_o$ (Figs. 6a and 6b). The length and Sholl plots of E18 SCG neurons from *Casr*^{+/-} mice were consistently smaller though not significantly different from those of wild type littermates (Fig. 6b), suggesting a possible small gene dosage effect. Neurons from all three genotypes survived equally well in culture (Supplementary Figs. 3f). Furthermore, E18 SCG neurons from *Casr*^{+/+} and *Casr*^{-/-} mice displayed identical survival dose responses to NGF (Supplementary Fig. 6), indicating that the CaSR plays no role in promoting SCG neuron survival. Taken together, these findings demonstrate that activation of CaSR by $[Ca^{2+}]_o$ enhances neurite growth, but not survival, from cultured SCG neurons during a narrow developmental window in the immediate perinatal period. Because the neurites in short-term SCG cultures, such as those used in our study, are exclusively axons rather than dendrites, it is reasonable to assume that CaSR positively regulates axonal growth and branching during this period of development.

CaSR-deficient mice have decreased sympathetic innervation

To determine if our *in vitro* demonstration of the involvement of the CaSR in regulating axonal growth from developing sympathetic neurons is physiologically relevant for sympathetic innervation *in vivo*, we compared the sympathetic innervation density of the iris in P1 in wild type mice and littermates that are homozygous and heterozygous for a null mutation in the *Casr* gene. The iris receives a dense innervation from the SCG by axons that can be easily identified and quantified by immunostaining for tyrosine hydroxylase. Representative fields of tyrosine hydroxylase-expressing sympathetic fibres of *Casr*^{+/+}, *Casr*^{+/-} and *Casr*^{-/-} iris reveal a clear decrease in innervation density in *Casr*^{-/-} mice compared with *Casr*^{+/+} mice (Fig. 6c). Quantification of tyrosine hydroxylase staining in multiple sections from all three genotypes revealed a marked, highly significant reduction in the sympathetic innervation density of *Casr*^{-/-} mice and a small though statistically insignificant reduction in *Casr*^{+/-} mice compared with the *Casr*^{+/+} mice (Fig. 6d). To exclude the possibility that decreased tyrosine hydroxylase immunofluorescence in the iris of *Casr*^{-/-} mice was not simply due to down regulation of tyrosine hydroxylase expression in the innervating neurons, we quantified TH mRNA levels and tyrosine hydroxylase immunofluorescence in the SCG of these mice and wild type littermates. These studies revealed no significant differences in the levels of either tyrosine hydroxylase mRNA (Fig. 6e) or tyrosine hydroxylase immunofluorescence intensity (Supplementary Fig 7a). Reduced sympathetic innervation density could not only result from defective axonal growth and branching but from a decrease in the size of the innervating population of neurons. To investigate if excessive loss of SCG neurons in *Casr*^{-/-} mice contributes to the innervation defect, we counted the number of neurons in the SCG of *Casr*^{+/+} and *Casr*^{-/-} mice at P1 and found no significant differences (Fig. 6f). There were also no significant differences in the nuclear diameter of SCG neurons (Supplementary Fig 7b) or in the volume of the SCG (Supplementary Fig 7c) between genotypes. Taken together, these results demonstrate that

the CaSR plays a crucial role in establishing appropriate sympathetic target innervation density but is not required for the survival of developing sympathetic neurons *in vivo*.

CaSR regulates dendritic growth in the hippocampus

Our discovery that CaSR plays a crucial role in regulating axonal growth from sympathetic neurons at a stage in development when they express high levels of CaSR raised the possibility that CaSR may play a similar role in regulating the growth of neural processes from other neurons that express this receptor. To test this possibility we studied the consequences of disrupting CaSR function in hippocampal pyramidal neurons as these neurons express increasingly levels of the CaSR in rats several days after birth 11, 19. Dendrites start growing from hippocampal pyramidal neurons during late fetal development and become larger and increasingly elaborate during subsequent postnatal development 20. We first confirmed expression of the CaSR in mouse hippocampal neurons at P4, a stage at which organotypic brain slice cultures can be efficiently established and the pyramidal neurons ballistically transfected 21. RT/PCR demonstrated that the hippocampus contains transcripts encoding full-length CaSR at this stage (Supplementary Fig. 8a), and immunocytochemistry revealed that the CaSR is expressed by both neurons and glial cells in dissociated cultures established from P4 mouse hippocampus (Supplementary Fig. 8b). We transfected pyramidal neurons with the dominant-negative CaSR plasmid or an empty control plasmid by firing gold microcarriers coated with these plasmids into organotypic slice cultures 21. Dominant negative CaSR-expressing and control-transfected neurons were recognised by coating the microcarriers with plasmids that express different fluorescent proteins (red fluorescent protein, RFP or yellow fluorescent protein, YFP, respectively). We scanned pyramidal neurons in each labeled group in the CA2 and CA3 regions of the hippocampus 48 hours after transfection and analyzed the resulting Z stack images of the dendritic trees 21. The dendritic arbors of dominant-negative CaSR expressing neurons were significantly shorter and less branched than those of control-transfected neurons (Fig. 7a). Overexpressing wild type CaSR in these neurons did not increase the size and complexity of the dendritic arbors (Fig. 7b), indicating that the endogenous level of CaSR in these neurons, like that in E18 SCG neurons, is not limiting. The dendritic arbors of representative dominant-negative CaSR-transfected and control-transfected neurons are illustrated in Figure 7c. These findings suggest that CaSR plays a significant role in regulating the growth and complexity of the dendritic arbors of hippocampal pyramidal neurons in post-natal mice.

DISCUSSION

Our study has demonstrated a novel and unexpected function for the CaSR in regulating the growth of neural processes in the peripheral and central nervous system. Activating the CaSR in late fetal sympathetic neurons with either elevated $[Ca^{2+}]_o$ or a calcimimetic in the presence of lower $[Ca^{2+}]_o$ enhances axonal growth in culture, whereas a calcilytic, dominant-negative CaSR or deletion of the CaSR eliminate the effects of elevated $[Ca^{2+}]_o$ on axonal growth. The influence of the CaSR on sympathetic axon growth is restricted to the immediate perinatal period of development when the expression of CaSR in sympathetic neurons is high and when sympathetic axons are growing and branching extensively within their target fields 8. Our finding that the sympathetic innervation density of the iris is significantly lower in CaSR-deficient newborn mice compared with wild type littermates demonstrates that the effect of the CaSR on axonal growth from sympathetic neurons is physiologically relevant for the establishment of normal sympathetic innervation density *in vivo*. While the CaSR enhances sympathetic axonal growth and target field innervation, it is not required for sympathetic neuron survival either *in vitro* or *in vivo*.

The influence of the CaSR on sympathetic axon growth declines rapidly following birth with the decrease in CaSR expression in sympathetic neurons. It is known that the fetus is

relatively hypercalcemic compared to the adult, with a $[Ca^{2+}]_o$ of around 1.7 mM at late stages of gestation 22. Because the neurite growth-promoting effect of $[Ca^{2+}]_o$ reaches saturation around 1.4 mM (Fig. 2), our findings suggest that the ambient $[Ca^{2+}]_o$ to which sympathetic neurons are exposed *in utero* prior to birth constitutively stimulates axonal growth via the CaSR. This implies that the effect of the CaSR on axonal growth *in utero* is controlled primarily by its level of expression rather than by fluctuations in $[Ca^{2+}]_o$. Our finding that overexpressing the CaSR in E18 SCG neurons does not enhance neurite growth in medium containing a maximally activating level of $[Ca^{2+}]_o$ but does confer responsiveness to elevated $[Ca^{2+}]_o$ in P1 SCG neurons implies that CaSR expression falls from a maximally effective level for promoting neurite growth at E18 to a functionally ineffective level by the end of the first postnatal day. This developmental decrease in CaSR expression together with the abrupt decrease in $[Ca^{2+}]_o$ that follows parturition 22 effectively curtail the influence of the CaSR on sympathetic axon growth *in vivo*. Taken together, our findings demonstrate that in sympathetic neurons of the peripheral nervous system, the constitutive activation of CaSR *in utero* enhances axon growth late in gestation, and this is crucial for the establishment of appropriate target innervation.

Our demonstration that the dominant-negative CaSR markedly reduces the size and complexity of hippocampal pyramidal dendritic arbors in the intact neuropil of postnatal organotypic slice cultures shows that the CaSR also enhances dendritic growth in the developing central nervous system. In the developing rat, CSF and brain interstitial fluid $[Ca^{2+}]_o$ falls from 1.6 mM in the foetus to between 1.1 and 1.2 mM by postnatal day 20 23. Thus, during postnatal development, brain $[Ca^{2+}]_o$ lies within the range capable of partially activating the CaSR expressed by hippocampal pyramidal neurons. Because the CaSR can detect local changes in $[Ca^{2+}]_o$ caused by extrusion of Ca^{2+} from neighbouring cells 24 and because synaptic activity in the pyramidal layers of the hippocampus causes marked changes in $[Ca^{2+}]_o$ 25, the CaSR may play a key role in modifying dendritic architecture in response to changes in synaptic activity. As such, the CaSR may participate in processes underlying learning and memory. This potential neural function of CaSR may help explain the developmental delay and neurological deficits of individuals with inactivating mutations of the CaSR 26,27.

METHODS

Real-time PCR quantification of mRNA levels

Extracted total RNA (RNeasy Mini extraction kit, Qiagen, Hilden, Germany) was reverse transcribed for 1 hr at 37°C with StrataScript reverse transcriptase (Stratagene) in a 40 µl reaction with 5 mM dNTPs (Stratagene) and 10 µM random hexamers (Amersham). For quantification of CaSR and tyrosine hydroxylase mRNA levels, 3 µl of each reaction product was amplified in a 25 µl reaction using the Brilliant QPCR kit (Stratagene). CaSR primers: 5'-ACCTGCTTACCCGGAAGAGGGCTTT-3' and 5'-AATTCAGGTGCCGTAGGTGTTTCAG-3'. Tyrosine hydroxylase primers: 5'-AAGGAAAGTGTCTAGAGTTG-3' and 5'-ACCCTGCTTGTATTGGAA-3'. Data were normalized to GAPDH mRNA. GAPDH primers: 5'-TCCCACTCTCCACCTTC-3' and 5'-CTGTAGCCGTATTCATTGTC-3'. PCR was performed with the Mx3000P (Stratagene) with 40 cycles of 95°C for 30sec, 58°C (CaSR) or 55°C (tyrosine hydroxylase) or 51°C (GAPDH) for 1min and 72°C for 30sec. These cycles were followed by 95°C for 1min, 65°C for 30sec and 95°C for 30sec. A melting curve confirmed that the SYBR green signal corresponded to a unique and specific amplicon. Standard curves were generated for every real-time PCR run by using serial three-fold dilutions of reverse transcribed RNA extracted from E13 embryos.

RT-PCR detection of full-length and splice variant CaSR was carried out with forward primer 5'-ACCTGCTTACCCGGAAGAGGGCTTT-3' and reverse primer 5'-GCACAAAGGCGGTCAGGAAAATGCC-3'. β -actin cDNA was also amplified as control using forward primer 5'-TCCTAGCACCATGAAGATC-3' and reverse primer 5'-AAACGCAGCTCAGTAACAG-3'. PCR was carried out for 35 cycles of: 94 °C for 30 sec, 56 °C (CaSR) or 50 °C (β -actin) for 30 sec and 72 °C for 1 min. The PCR reaction was terminated with a final extension at 72 °C for 8 min.

Immunoblotting

20-25 ganglia per age were lysed in RIPA buffer and insoluble debris removed by centrifugation. Protein concentration was determined by BSATM Protein Assay Kit (Pierce) and 18 μ g protein per lane was resolved on 10 % denaturing polyacrylamide gels. Samples were transferred to PVDF membranes using the Bio-Rad TransBlot (Bio-Rad, CA-USA). Membranes were blocked with 5% dried milk in PBS with 0.1% Tween-20. CaSR immunoreactive bands were detected using an anti-CaSR N-terminus polyclonal antibody 28 (1:5,000; Imgenex) and an HRP-conjugated anti-rabbit secondary antibody (1:2000; Promega, UK) and ECL-plus (Amersham)

Neuron cultures

Dissociated cultures of SCG neurons were set up from CD-1 mice or from E18 *CaSR*^{+/+}, *CaSR*^{+/-} and *CaSR*^{-/-} C57/Bl6 mice 17 and were grown on poly-ornithine/laminin coated 35 mm tissue culture dishes in defined medium 28 supplemented with 0.4 ng/ml NGF and 100 μ M Boc-D-FMK. Organotypic slice cultures of P4 CD1 mouse hippocampi 21 were grown in neurobasal medium (GIBCO) supplemented with 2 % B-27 and 2 % heat inactivated horse serum. $[Ca^{2+}]_o$ in the tissue culture medium was measured using a radiometer 125 and adjusted to the required level using EGTA or $CaCl_2$.

Ballistic transfection

Ballistic transfection of dissociated neuron cultures and organotypic brain slice cultures was carried out as previously described 21. Gold particles were coated with either pYFP or pRFP (Clontech, CA, USA) together with human parathyroid CaSR-pcDNA3.1, dominant negative CaSR (DN CaSR-pcDNA3.1) or pcDNA3.1 control plasmid. DN CaSR-pcDNA3.1 expressed the R185Q-CaSR that has been described and characterized previously 15. For organotypic brain slice culture transfections, a Bcl-X_L expression plasmid was adsorbed to all gold particle to ensure similar neuronal survival in both experimental and control transfected neurons. For dissociated cultures, the neurons were transfected 3 hours after plating.

Quantification of neuronal survival

The survival of transfected neurons in dissociated cultures was quantified by counting the numbers of transfected YFP-labelled neurons 12 hours after transfection and again at 48 hours, and expressing the number surviving at 48 hours as a percentage of the initial number of YFP-labelled neurons. The area counted was defined by the area in which the gold particles could be seen embedded in the bottom of the culture dish. Survival in non-transfected cultures was quantified by counting the number of neurons in a 12 $\times$ 12 mm grid in the centre of the dish 3 hr after plating and again at 24 hr, and expressing the number of neurons surviving at 24 h as a percentage of the initial number of neurons counted. Duplicate cultures were set up for all conditions and the data were compiled from three or more separate experiments.

Analysis of neuritic arbors

The neurite arbors of transfected neurons in dissociated cultures or organotypic cultures were visualized by expression of either YFP or RFP. In non-transfection experiments, neurite arbors were labelled with the fluorescent dye calcein-AM (Invitrogen) at the end of the experiment. For every condition in each experiment, images of at least 100 neurons were digitally acquired using an Axioplan Zeiss laser scanning confocal microscope, and neuritic arbors were traced using the LSM510 software. These traces were used to ascertain total neurite length and number of branch points. Fast-Sholl analysis was also carried out on these traces as described 29. Pair-wise comparisons were made using the student T-test (two-tailed). For multiple comparisons ANOVA was performed followed by Tukey's post-hoc test.

Immunocytochemistry

Cultured SCG or hippocampal pyramidal neurons were fixed in ice-cold methanol for 10 mins and were washed twice in phosphate-buffered-saline (PBS) before blocking non-specific binding and permeablizing the cells with 5% BSA and 0.02% Triton-X100 in PBS for 1 h at room temperature. The cells were incubated with primary antibody in 1% BSA at 4°C for approximately 18 hours. The primary antibodies used were: an anti- β -III tubulin monoclonal antibody (Promega, 1:1000), an anti-CaSR polyclonal antibody raised to the CaSR N-terminus 30 (Imgenex, 1:200) and an anti-CaSR polyclonal raised to a carboxy-terminal sequence of rat CaSR 31 (gift from Dr D. Shoback, 1:100). After 3 washes, the cells were incubated with the appropriate secondary antibody: either Alexa 594-conjugated anti-mouse IgG (Invitrogen, UK) or Alexa 488-conjugated anti-rabbit IgG (Invitrogen, UK) diluted 1:500 in PBS containing 1% BSA and 0.02% Triton-X100 for 90 minutes at room temperature. (Alexa-Fluor, Invitrogen, 1:500). Negative controls (no primary antibody) were set up in all cases.

Immunohistochemistry

The eyes of *Casr^{+/+}*, *Casr^{+/-}* and *Casr^{-/-}* littermates were fixed in formalin (BDH, UK) overnight at 4°C and were cryoprotected in 25% sucrose in PBS overnight at 4°C before being frozen. 15 μ m serial sections were cut at right angles to the visual axis. The sections were mounted onto poly-lysine-coated slides (BDH) and were blocked with 10% normal goat serum containing 0.1% tritonX-100 in PBS for 1 h at room temperature. After incubation for 18 hr at 4°C with rabbit anti-tyrosine hydroxylase polyclonal antibody (Chemicon) diluted 1:200 in PBS with 2% normal goat serum, the sections were washed three times in PBS before being incubated with an Alexa 594-conjugated goat anti-rabbit secondary antibody (Alexa-Fluor, Invitrogen, 1:500). All sections were counterstained with DAPI (Chemicon). Four images were digitally acquired from sections passing through the iris, each one from a different quadrant of the iris. The outline of the iris in these images was traced using Adobe Photoshop 7. The total iris area and the area containing tyrosine hydroxylase-positive fibres were estimated by automated pixel counts, and the ratio tyrosine hydroxylase-positive area to total iris area was calculated as a percentage. Six eyes for each genotype were sectioned and analyzed before the genotypes were ascertained so as to avoid any sampling bias. For tyrosine hydroxylase immunohistochemistry of SCG, P1 *Casr^{+/+}* and *Casr^{-/-}* heads were fixed in formalin (BDH, UK) for 3 days at 4°C before dehydration and wax embedding. 8 μ m microtome sections were stained for tyrosine hydroxylase using methods described above, and digital images were acquired using identical settings for both genotypes.

Quantification of neuronal number in the SCG.

Estimates of the numbers of neurons in the SCG of P1 *Casr*^{+/+} and *Casr*^{-/-} pups were carried out using stereology on serial sections as previously described 32.

CaSR mutant mice

The *Casr* mutant mice used have a deletion of exon 5 17. Because these mice express a truncated receptor that might have some activity 33, we carried out RT-PCR using primers that amplify both the full-length and splice variant transcripts to ascertain whether this splice variant is expressed in SCG neurons in wild type and/or knockout mice. As expected, both transcripts were amplified from the positive control tissue (kidney of *Casr*^{+/+} mice ³⁴), and the full-length transcript was amplified from the SCG of P1 *Casr*^{+/+} and *Casr*^{+/+} mice. However, transcripts for the splice variant were not detectable in the SCG of P1 *Casr*^{+/+}, *Casr*^{+/+} or *Casr*^{-/-} mice (Supplementary Fig. 9). This indicates that the truncated, in-frame CaSR receptor could not be impacting on our results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CaSR expression in the developing SCG

a, Level of CaSR mRNA relative to GAPDH mRNA (arbitrary units) in developing SCG (mean $\pm$ sem, $n = 3$ per age). **b**, CaSR staining in E18 SCG cultures with an N-terminus anti-CaSR polyclonal antibody (neurons were double labelled with anti- β III tubulin). Scale bar = 50 μ M.

Figure 2. $[\text{Ca}^{2+}]_o$ influences neurite growth from SCG neurons at the peak of CaSR expression
 Total neurite length (**a**) and Sholl profiles (**b**) of E18 SCG neurons cultured with different $[\text{Ca}^{2+}]_o$ over the physiological range of activation of the CaSR. Mean $\pm$ sem of data from 179 to 279 neurons per condition from 3 separate experiments of each type, *** $P < 0.001$, statistical comparison with 0.7 mM $[\text{Ca}^{2+}]_o$, two-tailed, unpaired T-test. **c**, Representative examples of E18 SCG neurons cultured with 0.7 mM and 2.3 mM $[\text{Ca}^{2+}]_o$. Scale bar = 50 μM .

Figure 3. Developmental time-course of effects of $[\text{Ca}^{2+}]_o$ on neurite growth from SCG neurons
 Total neurite length (**a**) and Sholl profiles (**b**) of E16, E18, P0 and P1 SCG neurons cultured with either 0.7 mM or 2.3 mM $[\text{Ca}^{2+}]_o$. Mean $\pm$ sem of data from 160 to 405 neurons per condition from 3 to 6 separate experiments of each type, *** $P < 0.001$, statistical comparison with 0.7 mM Ca^{2+}_o , two-tailed, unpaired T-test.

Figure 4. CaSR calcimimetic and calcilytic compounds respectively enhance and inhibit the effects of $[Ca^{2+}]_o$ on neurite growth from E18 SCG neurons

Total neurite length and Sholl profiles of E18 SCG neurons grown with 0.7 mM $[Ca^{2+}]_o$ with and without 10 nM of the calcimimetic NPS-R467 (**a**) and with 2.3 mM $[Ca^{2+}]_o$ with and without 10 nM of the calcilytic NPS-89636 (**b**). Mean $\pm$ sem of data from 3-4 separate experiments of each type ($n = 363$ neurons for **a** and 254 neurons for **b**)., *** $P < 0.001$, statistical comparison with control (CTR), two-tailed, unpaired T-test.

Figure 5. DNCaSR and wild type CaSR have opposing effects on neurite growth from cultured SCG neurons

Total neurite length and Sholl profiles of E18 SCG (**a** and **d**) and P1 (**b** and **c**) SCG neurons transfected with plasmids expressing either dominant-negative CaSR (DNCaSR) (**a** and **b**) or wild-type CaSR (WTCaSR) (**c** and **d**). These neurons and control transfected neurons (CTR) were also transfected with pYFP to identify transfected neurons and label their processes. Neurons were transfected 3 hours after plating and were incubated for a further 24 hours in medium containing 2.3 mM $[Ca^{2+}]_o$ before fluorescent images were acquired and analysed. Mean $\pm$ sem of data from 114 to 204 neurons per condition from 3 separate experiments of each type, *** $P < 0.001$, statistical comparison with control transfected neurons, two-tailed, unpaired T-test.

Figure 6. Deletion of *Casr* reduces sympathetic axon growth *in vitro* and *in vivo*

Total neurite length (**a**) and Sholl profiles (**b**) of cultured E18 SCG neurons isolated from *Casr*^{+/+} (WT), *Casr*^{+/-} (HET) and *Casr*^{-/-} (KO) mice. Neurons were cultured in medium containing 2.3 mM [Ca²⁺]_o. Mean ± sem of data from 94 to 254 neurons per genotype from 4 independent experiments, set up from 3 to 4 embryos per genotype. **c**, Representative fields of irises from P1 *Casr*^{+/+}, *Casr*^{+/-} and *Casr*^{-/-} mice stained for tyrosine hydroxylase-positive sympathetic axons (Scale bar = 50 μm). **d**, Quantification of density of tyrosine hydroxylase -positive sympathetic axons in the irides of P1 *Casr*^{+/+}, *Casr*^{+/-} and *Casr*^{-/-} littermates. Mean ± sem of percentage tyrosine hydroxylase -positive staining in 157 to 167 iris fields per genotype from 3 mice of each genotype. ****P* < 0.001 One way ANOVA with Tukey's post hoc test, statistical comparison with WT. **e**, Estimates of neuronal number in the SCG of P1 *Casr*^{+/+} and *Casr*^{-/-} littermates. Mean ± sem of data from 3 mice of each genotype. **f**, Level of tyrosine hydroxylase mRNA relative to GAPDH mRNA (arbitrary units) in P1 SCG dissected from *Casr*^{+/+}, *Casr*^{+/-} and *Casr*^{-/-} mice. Mean ± sem of data from 5 mice of each genotype are shown.

Figure 7. DNCaSR impairs the growth of postnatal hippocampal pyramidal dendrites
a, Total dendrite length and Sholl profiles of CA2 and CA3 hippocampal pyramidal neurons in P4 organotypic slice cultures triple transfected with either of two gold microcarriers: pYFP, Bcl-X_L and pcDNA3.1 (control transfections, CTR) or pRFP, Bcl-X_L and dominant-negative CaSR-pcDNA3.1 (DNCaSR). Mean $\pm$ sem of data from 71 control and 77 DNCaSR transfected neurons, 10 separate cultures. **b**, Total dendrite length and Sholl profiles of CA2 and CA3 hippocampal pyramidal neurons in P4 organotypic slice cultures triple transfected with: either pYFP, Bcl-X_L and pcDNA3.1 (control transfections, CTR) or pRFP, Bcl-X_L and wild type CaSR-pcDNA3.1 (WTCaSR). Mean $\pm$ sem of data from 36 control and 37 wild type neurons, 5 separate cultures. **c**, Typical examples of control transfected neurons (green) and DNCaSR-transfected neurons (yellow / red) in the same field are also illustrated. *** $P < 0.001$, statistical comparison with control, two-tailed, unpaired T-test. Scale bars = 50 μ M.