Adrenal Chromaffin Cells Exhibit Impaired Granule Trafficking In NCAM Knockout Mice.

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ABSTRACT
Neural cell adhesion molecule (NCAM) plays several critical roles in neuron path-finding and intercellular communication during development. In the clinical setting serum NCAM levels are altered in both schizophrenic and autistic patients. NCAM knock out mice have been shown to exhibit deficits in neuronal functions including impaired hippocampal long term potentiation and motor coordination. Recent studies in NCAM null mice have indicated that synaptic vesicle trafficking and active zone targeting are impaired, resulting in periodic synaptic transmission failure under repetitive physiological stimulation. In this study we tested whether NCAM plays a role in vesicle trafficking that is limited to the neuromuscular junction, or whether it may also play a more general role in transmitter release from other cell systems. We tested catecholamine release from neuroendocrine chromaffin cells in the mouse adrenal tissue slice preparation. We utilize electrophysiological and electrochemical measures to assay granule recruitment and targeting in wild type and NCAM -/- mice. Our data show that NCAM -/- mice exhibit deficits in normal granule trafficking between the readily-releasable pool and the highly release-competent immediately releasable pool. This defect results in a decreased rate of granule fusion and thus catecholamine release under physiological stimulation. Our data indicate that NCAM plays a basic role in the transmitter release mechanism in neuroendocrine cells through mediation of granule recruitment and is not limited to the neuromuscular junction and central synapse active zones.

KEYWORDS
Electrophysiology, exocytosis, neuroendocrine, trafficking, NCAM
INTRODUCTION

Neural Cell Adhesion Molecule (NCAM) was originally identified as a neural integral membrane protein that facilitated cell-cell adhesion and axonal fasciculation (Rutishauser et al. 1978; Rutishauser et al. 1983). NCAM is found in 3 major isoforms that are derived through alternative splicing of a single gene (Cunningham et al. 1987). NCAM 120, 140 and 180 all exhibit an IgG domain-containing extracellular portion that plays a role in cell-cell interactions. The 140 and 180 KD isoforms also contain intracellular domains whose functions are less well understood but include nFκB-binding, cytoskeletal binding and other signaling motifs (Crossin and Krushel 2000; Suter and Forscher 1998). Despite the role NCAM plays in developmental processes, mice in which the NCAM gene has been knocked out are viable and grow to maturity (Cremer et al. 1994).

NCAM is widely distributed throughout neuronal tissues, including neurons and glia of both the central and peripheral nervous systems (Seki and Arai 1993). It is also expressed in neuroendocrine cells of pancreatic islets and neurohypophysis (Langley et al. 1989) as well as chromaffin cells of the adrenal medulla (Grant et al. 1992). NCAM knock-out mice show deficits in normal formation of the islets of Langerhans (Esni et al. 1999) where it was suggested that lack of NCAM may affect cadherin-mediated adhesion, leading to abnormal cell sorting and aggregation during development. Cancerous neuroendocrine cells express an altered isoform-specific NCAM expression profile compared to non-cancerous cells, indicating that post-translational modification may affect tumor-genesis in this cell class (Lahr and Mayerhofer 1995). However, the functional role and thus effect of the NCAM knock-out in individual neuroendocrine cells has not been addressed.

Recent studies have demonstrated a functional role for NCAM in regulation and maintenance of transmitter release in neuronal tissues. NCAM knockout mice exhibit neuromuscular synaptic transmission deficits under physiological stimulation that lead to locomotor impairment (Polo-Parada...
NCAM in catecholamine release. Transmission failure in these mice may be the result of an improper development of the synaptic vesicle trafficking process. This was demonstrated by the co-existence of two separate modes of synaptic vesicle cycling in the neuromuscular junction (NMJ) of adult NCAM -/- mice (Polo-Parada et al. 2001). One route is characterized by vesicle release that is not limited to the active zones but occurs throughout the nerve terminal. This release mechanism is mainly evoked by calcium influx through L-type Ca\(^{2+}\) channels. Exocytosis is then followed by an endocytic path that is sensitive to brefeldin-A (BFA), an agent that inhibits vesicular budding from the endoplasmic reticulum. This mechanism is classified as an immature trafficking process and is normally only observed during development in wild-type mice. The second trafficking mechanism is characterized by exocytosis that is preferentially evoked by calcium influx through P/Q-type channels and leads to endosomal trafficking that is insensitive to BFA. This P/Q-channel-dependent, BFA-insensitive vesicle exo-endocytosis cycle has been classified as a mature phenotype in that it is normally observed as the sole trafficking route in NMJs of the adult mouse (Polo-Parada et al. 2001). In addition, vesicle fusion in the normal adult NMJ is restricted to active zones at the nerve-muscle contact site (Polo-Parada et al. 2001). It is not yet understood how loss of NCAM affects synaptic maturation, vesicle cycling and targeting on the molecular level. However, some insight is offered by mice lacking only the NCAM 180 isoform, where synaptic transmission relies on the mature P/Q-type Ca\(^{2+}\) influx path, is not BFA sensitive, but does lack the proper vesicle targeting to the active zones at the nerve-muscular contact site. Like the total NCAM -/- mice, the NCAM 180 -/- mice also exhibit locomotor deficits (Polo-Parada et al. 2004). Taken together these data point to improper targeting of vesicles to the active zone, and not the persistence of BFA-sensitive cycling or P/Q type-dependent Ca\(^{2+}\) influx, as the cause of the synaptic deficit in NCAM knockout mice.

Neuroendocrine cells lack the specialized synaptic structures found at the NMJ. Given this difference we wondered whether NCAM -/- mice would express any exocytic abnormalities. Neuroendocrine
chromaffin cells release catecholamine through exocytosis from large dense-core granules that employ molecular fusion machinery very similar to that utilized in the neuromuscular junction with the exception that the large dense core secretory granules dock and fuse with the cell surface in the absence of a specialized active zone structure. We tested several parameters of catecholamine release from chromaffin cells in tissue slices prepared from NCAM +/+ as well as NCAM -/- mice. Electrochemical and electrophysiological techniques were employed to determine granule recruitment to the releasable state, fusion competence and sustained levels of release under repetitive physiological stimulation. We report here that chromaffin cells from NCAM -/- mice exhibit deficiencies in transmitter release that are analogous to those found in the neuromuscular junction. We expand on this finding and provide evidence that show the deficit is due to an impaired recruitment of granules from the Readily Releasable Pool (RRP) to the final highly-fusogenic Immediately Releasable Pool (IRP). These data indicate an elemental role for NCAM in the granule/vesicle trafficking mechanism in diverse secretory cell types and thus is not limited to the specialized neuronal active zone.

METHODS

Adrenal slice preparation: Adult NCAM -/- and wild-type C57/BL6 mice (4 to 8 weeks old, Jackson Laboratories, Bar Harbor ME) were used in this study. NCAM -/- mice were originally generated by Cremer et al. (1994) in a C57/BL6 background. NCAM -/- homozygotes were confirmed by PCR analysis. All anesthesia and euthanasia protocols were reviewed and approved by the institutional animal care and use committee (IACUC) of Case Western Reserve University, an accredited oversight body (Federal animal welfare assurance # A3145-01). Animals were deeply anesthetized by halothane (Sigma, St. Louis MO) inhalation and sacrificed by decapitation. Adrenal glands were immediately removed and immersed in ice-cold, low calcium bicarbonate buffered saline (BBS) containing (in mM) 140 NaCl, 2 KCl, 0.1 CaCl₂, 5 MgCl₂, 26 NaHCO₃, 10 Glucose that was gassed with 95% O₂/5% CO₂ (all chemical were acquired from Fisher Scientific, Cleveland OH, unless
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otherwise noted). Osmolarity of the BBS was approximately 310 mOsm. Glands were trimmed of excess fat and embedded in 3% low gelling point agarose (Sigma) that was prepared before hand by melting agar in low calcium BBS at 110°C followed by equilibration to 35°C. The agarose was gelled on ice following tissue embedding. The agarose block was trimmed to approximately 3 mm cubes that contained a single gland each and glued to a tissue stand of a vibrotome (WPI, Sarasota FL). The tissue stand was then placed in a slicing chamber filled with ice-cold, bubbled, low Ca\(^{2+}\) BBS. Adrenal glands were sectioned into 200 μm thick slices. The slices were collected and kept in the low calcium BBS first at 35°C for 30 minutes, then at 25°C until recording.

**Immunohistochemical analysis:** Animals were deeply anesthetized and fixed by transcardiac perfusion with 60 ml of 3.7% formaldehyde (Sigma) in phosphate buffered saline (PBS). Adrenal glands were removed post-fix, stored in 30% sucrose over night at 4°C, embedded in OCT, cryosectioned at 14 μm and mounted on superfrost slides (Fisher Scientific). Slides were then washed twice in PBS and permeabilized with PBS + 0.1% triton for 30 minutes. Sections were blocked with a solution of 4% BSA in PBS for 1 hour and incubated with anti-NCAM antibody (R025b; a gift of Dr. Urs Rutishauser, Memorial Sloan-Kettering Cancer Center, New York, NY) overnight at 4°C. Sections were washed 5 times each for 15 minutes with PBS, and incubated with a FITC-conjugated secondary antibody (Zymed, San Francisco, CA) for 2 hours at room temperature. Sections were then washed with PBS 5 times each for 15 minutes and mounted with anti-fade medium (Prolong, Molecular Probes, Eugene, OR). Fluorescence signals were visualized on an Olympus IX70 microscope at either 40x or 100x magnification. Image stacks were captured at pixel-width z-resolution (square voxels) and deconvolved with a constrained-iterative algorithm built into SlideBook image processing software (v 4.0; Intelligent Imaging Innovations Inc, Denver, CO).
Western blot analysis: Mice were anesthetized, sacrificed and the adrenal glands were quickly removed. The adrenal medulla was dissected out and homogenized in HEPES extraction buffer (in mM: 25 HEPES, 150 NaCl, 1 EDTA) containing 1% NP-40 and protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). The solubilized protein concentrations were determined (BCA method; Pierce, Rockford, IL) and adjusted to 2 μg/ml. To remove sialic acid, the extract was incubated for 1 hr at 37°C with 0.08 U/ml Neuraminidase Type X (Calbiochem, Sand Diego, CA). SDS sample buffer containing dithiotheritol was added to each sample and the proteins separated by SDS-PAGE according to the Lamellae method on a 5 or 15% gel. The proteins were transferred onto polyvinylidene fluoride membranes pre-absorbed with a solution of PBS and 4% milk. The membranes were then probed with primary antibodies in PBS and 4% milk overnight at 4 °C. All antibodies were obtained from Transduction Labs (BD, San Diego, CA) except for actin (Sigma). The membranes were then washed in PBS, 0.1% Triton X-100 and HRP-conjugated secondary antibody for 2 hours. The membranes were again washed 5 times, 15 minutes each in PBS. An enhanced chemi-luminescence kit (Promega, Madison WI) was used to expose the blots onto film.

Electrophysiological recording: All experiments were performed 2 to 8 hours after slice preparation. Tissue slices were constantly superfused with normal Ca²⁺ BBS during recording (adapted from (Barbara et al. 1998)) containing (in mM): 140 NaCl, 2 KCl, 3 CaCl₂, 2 MgCl₂, 26 NaHCO₃, 10 Glucose and gassed with 95% O₂/5% CO₂. The osmolarity was approximately 310 mOsm. The recording chamber volume was about 1.5 ml and the buffer flow was set at 1 ml per minute. Tissue slices were held in place by silver wires placed over the agar margin and visualized using an upright microscope (Olympus, Melville, NY) equipped with a 40X water-immersion objective.

Patch pipettes were pulled from borosilicate glass (4-5 MΩ). They were partially coated with molten dental wax and lightly polished by a microforge (Narashige, Tokyo, Japan). All recordings in this study
were performed in the perforated patch configuration. The perforated patch pipette solution contained (in mM); 135 CsGlutamate, 10 HEPES-H, 9.5 NaCl, 0.5 TEA-Cl and 0.53 amphotericin B. pH was adjusted to 7.3 and osmolarity was 305 mOsm. Amphotericin B was prepared as a 100x stock solution in DMSO (tissue culture-tested, Sigma) twice daily and diluted into internal pipette solution every 2 hours. The internal solution was back-filled into patch pipettes without tip-dipping. No correction for liquid junction potential was made in this study. Cells were chosen for patching based on their appearance and physical orientation within the slice. Preference was given to cells that exhibited homogeneous cytosol (un-mottled) and an accessible clean surface. An orientation was considered preferable if it left access for the carbon fiber on the capillary pole. Cells were perforated to a series resistance of no more than 30 MΩ (mean = 19.9 ± 0.56 MΩ, n= 112) and held at -80 mV. Leak currents were not corrected. Voltage clamp records were acquired through an EPC-9 voltage-clamp amplifier (HEKA Elektronik, Lambrecht Germany) under control of ‘Pulse’ software (v 8.53; HEKA Elektronik). Cell capacitance (Cm) was estimated using a software lock-in module based on the Lindau-Neher technique (Gillis 1995) implemented as the “Sine + D.C.” mode. A 323 Hz, 25 mV peak amplitude sine wave was applied to the holding potential, and the reversal potential of the lock-in module was set to 0 mV. Membrane current was sampled at 20 kHz, and Cm was calculated at 323 Hz from the average value of 62 points per sine cycle. Non-secretory capacitance signals were eliminated from dual-pulse recordings as described in the literature (Moser and Neher 1997; Voets et al. 1999). Only cells with less than -60 pA leak current were analyzed for this study (leak current was -28.4 ± 6.58 pA; pre-stimulus cell capacitance = 8.86 ± 1.01 pF, n = 112 cells).

Peptide transfection protocol: The PP-19 peptide was synthesized by Sigma Genosys (The Woodlands, TX) after a previously published sequence (Gad et al. 2000). Cells were transfected by using the Chariot protein transfection method (Active Motif, Carlsbad, CA). The transfection reaction was altered from the manufacture’s suggested protocol to increase efficiency in the adrenal slice.
NCAM in catecholamine release. The reactions were prepared as follows: 2 ng Chariot reagent were dissolved in 50 µl distilled water, the peptide was prepared as a solution of 500 ng protein in 50 µl normal BBS. These solutions were mixed, sonicated and kept at room temperature for 30 minutes to allow the Chariot-peptide complex to form. The Chariot/peptide complex mixture was then diluted to a final volume of 5 ml by low Ca\(^{2+}\) BBS, and bubbled with 95% O\(_2\)/5% CO\(_2\). Maximum transfection efficiency was determined as the PP-19 concentration at which no further increase in PP-19 resulted in a greater difference in recorded behavior from control cells. Incomplete or sub-optimal transfection of cells with PP-19 would lead to an increased variance in measured granule exocytosis. However, this potential error would equally effect both NCAM +/+ and -/- cells and thus would decrease the likelihood for detection of any knock out-dependent phenotype. Slices were incubated in this mixture for at least 1 hour prior to recording.

*Electrochemical catecholamine detection:* Commercially available carbon fiber electrodes (ALA Scientific, Longneck, NY) of 5 µm tip diameter were used for amperometric catecholamine detection (Wightman et al. 1991). Carbon fibers were cut prior to each recording. A +650 mV potential was placed on the carbon fiber once it was in the bath, and the background current was allowed to relax to a steady value. If the resting current was larger than 15 pA or was unstable, the fiber was re-cut or replaced. Amperometric currents were recorded through a dedicated amplifier (VA-10, ALA Scientific). The head stages for the VA-10 and EPC-9 amplifiers shared a common Ag-AgCl bath ground. To minimize cross-talk between them a 10 Ω resistor was placed into the ground wire of the VA-10 to separate the ground planes. During recordings the fiber was placed as close to the capillary pole of the patched cell as possible without physically distorting the cell. Oxidative amperometric currents, indicating catecholamine release, were passed through an analogue 1 kHz Bessel filter and sampled at 20 kHz simultaneous to the voltage clamp current records in the Pulse software.
Voltage-clamped chromaffin cells were stimulated by trains of simulated action potential waveforms or by square pulse depolarization. Simulated action potentials were composed of a 3-step ramp as follows (start potential [mV], end potential [mV], duration [ms]): -80, 50, 2.5; 50, -90, 2.5; -90, -80, 2.5. This waveform evoked Na$^+$ and Ca$^{2+}$ currents statistically identical to native action potentials (Chan and Smith 2001). All experiments were carried out at 24 – 27 °C, and data analysis was performed with IGOR Pro software (WaveMetrics Inc., Lake Oswego, OR). All data are presented as mean ± S.E.M.. Statistical analysis of data sets was determined by the student’s paired T test. Asterisks in figures indicate a statistical difference (p < 0.02) from control values.

RESULTS

Adrenal NCAM expression is limited to the medulla. We assayed expression of NCAM within the adrenal medulla by immuno-histochemistry. Tissue slices were cut from NCAM wild type (+/+) and knock out (-/-) mice and stained with a pan-anti-NCAM antibody raised against the extracellular domain (R025b). Following staining, slides were imaged under bright field illumination to identify the medulla (Fig. 1A). The adrenal medulla is made up nearly entirely by adrenal chromaffin cells with a few vascular endothelial cells and terminals from the innervating splanchnic nerve (Carmichael 1986). The NCAM +/+ slice showed strong immuno-fluorescence signal in the medulla but little in the cortex. The right image pair was taken of slices prepared from NCAM -/- mice and as expected, exhibit almost no signal. A higher magnification image of the NCAM +/+ slice indicates that the anti-NCAM stains the periphery of the chromaffin cells and is not due to signal from only the vascular endothelium or nerve terminals (Fig. 1B). Western blot analysis of isolated adrenal medulla was performed as described in Methods. Equal amounts of protein from adrenal medullae harvested from NCAM +/+ and -/- mice were loaded into adjacent lanes, separated on a 15% poly-acrylamide gel, transferred to membrane and probed with the same pan anti-NCAM antibody (R025B). As shown in
Fig. 1C all 3 isoforms of NCAM are present in the wild type medulla, with the 140 KD form expressed at the highest level. As expected, no NCAM immuno-fluorescence signals were detectable in the NCAM -/- medulla. Taken together these data indicate that NCAM is expressed in the adult adrenal medulla of wild type mice and that the 140 KD isoforms is the most prevalent.

Physiological stimulation of NCAM -/- chromaffin cells elicits less catecholamine release. We measured granule fusion with the capacitance technique and catecholamine release by electrochemical amperometry. Normally capacitance measurements would reflect the sum activity of exocytic membrane addition as well as endocytic membrane retrieval from the cell surface. In order to better quantify exocytosis only, we inhibited endocytosis by acute cell transfection with the clathrin-blocking peptide PP-19. PP-19 disrupts the association of synaptojanin and endophilin, a necessary step in clathrin-mediated endocytosis (Gad et al. 2000) and has successfully been applied in this preparation. We have previously shown that mouse chromaffin cells stimulated at 15 Hz in situ recycle granule membrane exclusively by clathrin-mediated endocytosis and that PP-19 blocks this process (Chan and Smith 2003). We confirmed this to also be true in NCAM -/- cells (Figure 2 Ai); the evoked capacitance response was significantly larger in the PP-19 transfected cells, while catecholamine release measured by amperometry was equivalent (data not shown).

NCAM -/- mice exhibit periodic neuromuscular transmission failures under physiological stimulation (Polo-Parada et al. 2001). In order to determine if NCAM -/- chromaffin cells share this characteristic, we stimulated NCAM +/- and -/- cells in situ at 15 Hz with action potential wave forms to mimic native sympathetic stimulation (Chan and Smith 2001). We measured evoked capacitance increases in PP-19 transfected cells to estimate granule fusion and carbon fiber amperometry to simultaneously measure catecholamine output. Ca$^{2+}$ influx was measured as previously reported (Chan et al. 2003) as the peak Ca$^{2+}$ current during the action potential depolarization. Records from NCAM +/- and
NCAM -/- cells (n = 9 and 8, respectively) were averaged and are plotted in figure 2. The averaged capacitance traces (Fig. 2Aii) reveal that the NCAM -/- mice exhibit a deficit in evoked granule fusion. The difference is not due to genotype-specific differences in evoked Ca\(^{2+}\) influx, which was equivalent in both cell types (inset). Representative amperometric records from single cells are provided in panel Bi. The average number of spikes per cell for each condition confirms that stimulation of NCAM -/- cells resulted in fewer exocytic events than in NCAM +/+ cells (2Bii). We next estimated total catecholamine output by integrating capacitance signals (Fig. 2C). Total catecholamine release was significantly lower in NCAM -/- cells compared to +/+ cells (p < 0.02). Thus, as measured by membrane insertion, numbers of amperometric spikes and total catecholamine release, NCAM -/- cells show a secretory deficit under normal physiological stimulation. Thus we set out to elucidate the underlying mechanism.

**The secretory deficit is due to a decreased number of granules in the IRP.** NCAM -/- mice exhibit a deficit in stimulus-evoked granule fusion and catecholamine release. We wondered what caused this secretory deficit. In figure 2, we showed that evoked Ca\(^{2+}\) influx in both NCAM +/+ and -/- mice is equivalent and therefore not the cause (Fig. 2Aii inset). Two other potential sources for the phenotype were considered: 1) The NCAM -/- cells exhibit a deficit in the ‘readily releasable’ granule population that defines and limits stimulus-dependent secretion (Heinemann et al. 1993) - or - 2) The NCAM -/- mice express an exocytic machinery with impaired Ca\(^{2+}\) sensing, thus causing a less steep stimulus-secretion function. We tested both possibilities.

The recruitment and maturation of chromaffin secretory granules to the fusion competent state has been well studied in bovine and mouse chromaffin and clonal PC-12 cells (Hay et al. 1995; Heinemann et al. 1993; Parsons et al. 1995; Steyer et al. 1997). A prevailing mechanism describes a series of sequential recruitment steps from a large reserve granule pool to a ‘readily releasable pool’
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(RRP). From the RRP granules can either revert to the reserve pool, undergo exocytosis or enter a more fusogenic ‘immediately releasable pool’ (IRP) through which granules fuse approximately 5-fold faster than through the RRP (Voets et al. 1999). Action potential stimulation provides a very modest and brief Ca$^{2+}$ signal, thus granules from the IRP can be expected to dominate the release process under physiological stimulation. We utilized a paired-pulse depression protocol to quantify the size of RRP and IRP in both wild-type and NCAM -/- mice. The RRP paired-pulse depression protocol was based on a previous study in isolated chromaffin cells (Gillis et al. 1996) and has been used extensively to quantify the number of granules resident at defined steps along the secretion path (Smith 1999; Smith et al. 1998; Voets et al. 1999; Yang et al. 2002). The dual-pulse protocol separates Ca$^{2+}$ influx from the secretion process in that Ca$^{2+}$ influx is sufficiently large that it is not the limiting factor for secretion, rather the number of granules defines the magnitude of the response. Briefly, two 100 ms square pulse stimuli were delivered at a 100 ms interval and their amplitudes were adjusted to evoke equivalent amounts of Ca$^{2+}$ entry (Fig. 3Ai). The first Ca$^{2+}$ influx causes secretion and depletion of the RRP, leaving fewer granules for release during to the second Ca$^{2+}$ influx. This results in a depletion-dependent secretory depression in response to the second pulse. The evoked capacitance response for each pulse was measured and their magnitudes used to calculate the total number of release-ready granules (see Fig. 3 legend and Gillis et al. (1996) for a full description of the technique). The results from this analysis are provided in figure 3Aii and show that the RRP is significantly larger in the NCAM -/- mice than in the wild-types. We then quantified the IRP using a protocol similar to that used to measure the RRP. This approach is described in a study performed in the same cell preparation (Voets et al. 1999). The stimulus protocol consists of two 10 ms pulses separated by a 10 ms interval. The brief depolarizations allow only a modest amount of Ca$^{2+}$ to enter the cell, accessing only the highly fusogenic IRP but not the RRP. IRP size is calculated in the same manner as the RRP (Voets et al. 1999). NCAM -/- mice exhibited a statistically significant decrease in the size of the IRP (Fig. 3Bii).
We considered two possible scenarios to explain the larger RRP and smaller IRP found in NCAM -/- cells. Both scenarios are based on a sequential model for granule trafficking modified from Heinemann et al. (1993) except that we add a transition from the RRP into the IRP:

\[
A \xrightarrow[k_1]{k_{1\to RRP}} RRP \xrightarrow[k_2]{k_{2\to IRP}} IRP
\]

where A is the reserve granule pool, \(k_1\) and \(k_{1\to RRP}\) are the rate constants for transfer from A to the RRP and \(k_2\) and \(k_{2\to IRP}\) are the rate constants for transfer between the RRP and the IRP. The rates of exocytosis from the RRP and IRP were determined by Voets et al. (1999) in the same cell preparation. Where the refill kinetic for the RRP is described by:

\[
\tau_{RRP} = \frac{1}{k_{1} + k_{2}}
\]

and the refill kinetic for the IRP is described by:

\[
\tau_{IRP} = \frac{1}{k_{2}}
\]

Scenario 1 dictates that the NCAM -/- mice express a larger RRP and smaller IRP than +/+ due to a deficit in retention of granules in the IRP (\(k_2\) is normal, but \(k_{2\to IRP}\) is larger). Thus scenario 1 would predict a similar \(\tau_{RRP}\) for both NCAM +/+ and -/- mice but a smaller \(\tau_{IRP}\) for the -/- cells. On the other hand, scenario 2 is based on a less efficient transfer of granules from the RRP to the IRP, but a normal back reaction (\(k_2\) is smaller in the -/- cells, but \(k_{2\to IRP}\) remains unchanged). Thus scenario 2 would predict a faster \(\tau_{RRP}\) in -/- cells but no change in \(\tau_{IRP}\). We set out to test these scenarios. We again utilized the dual-pulse stimulus to determine the rates of refilling for the RRP and IRP in both NCAM +/+ and -/- cell types. This approach has been used previously in bovine chromaffin cells (Smith et al. 1998) as well as in mouse chromaffin cells in situ (Voets et al. 1999). Briefly, two sets of paired-pulse
depolarizations are delivered at variable intervals such that the first pair depletes the granule pool and the second pair probes for recovery after a delay. Results from this protocol are presented in Figure 2C. Next, we determined \( \tau_{\text{IRP}} \) by fitting the IRP refill curves with a single exponential (\( \tau_{\text{IRP}^+/-} = 0.61 \text{s} \) and \( \tau_{\text{IRP}^-/-} = 0.68 \text{s} \); Fig. 3Ci). Since the depletion protocol used to quantify the RRP will also deplete the IRP as a subset, we fit the RRP refill curve with a double-exponential function, holding the fit parameters determined for the IRP as one of the terms and fitting the second RRP-specific term (\( \tau_{\text{RRP}^+/-} = 5.66 \text{s} \) and \( \tau_{\text{RRP}^-/-} = 3.59 \text{s} \); Fig. 3Cii). The shorter \( \tau_{\text{RRP}} \) measured in the -/- cells along with an equivalent \( \tau_{\text{IRP}} \) in both genotypes indicate that scenario 2 is responsible for the secretory deficit in NCAM -/- cells. Granules traffic into the IRP at a slower rate in NCAM -/- cells compared to the +/- cells.

We next examined the possibility that the secretory discrepancy between wild-type and NCAM -/- mice may be due to a lowered affinity of the fusion mechanism for Ca\(^{2+}\). In order to test this possibility we stimulated cells with square pulse depolarizations ranging from 5 to 100 ms to generate a stimulus-secretion function. The order of the stimuli was randomized to guard against a bias due to granule depletion. Evoked Ca\(^{2+}\) influx was integrated to provide a quantification of stimulus strength. The measured capacitance jumps are reported as a function of integrated Ca\(^{2+}\) influx in figure 4A. The stimulus-secretion relationship is supra-linear and is similar to previous reports in isolated bovine chromaffin cells (Engisch et al. 1997; Engisch and Nowycky 1996; Smith 1999). A closer examination of the data presented in Fig. 4A show that at low Ca\(^{2+}\) influx, wild type mice exhibited larger capacitance jumps than did NCAM -/- mice (Fig 4B). However as Ca\(^{2+}\) influx became greater, the NCAM -/- mice exhibited a steeper stimulus-secretion function than wild-type (\( P<0.02 \)) and thus capacitance increases were larger in the knock outs than in control as stimulus amplitude (Ca\(^{2+}\) influx) became larger. The secretory behavior under low Ca\(^{2+}\) influx is consistent with the secretory deficit reported in figure 2, where action potential stimulation results in only modest Ca\(^{2+}\) influx (Fig.
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4B, grey box and inset) and is likely to be due to release through the IRP. The augmentation in secretion from NCAM -/- cells as compared to +/+ cells under elevated Ca\(^{2+}\) influx is addressed below.

Our data indicate that the secretory deficit measured in NCAM -/- cells under action potential stimulation is the consequence of fewer granules in the IRP for rapid release upon physiological Ca\(^{2+}\) influx. We also find a larger RRP in the knockout mice, perhaps due to a backlog of granules awaiting transfer into the IRP (fig. 3C). But this shift in granules from the IRP to the RRP in NCAM -/- mice is not large enough to quantitatively explain the secretory differences seen between +/+ and -/- mice under very strong stimulation where the RRP supplies the majority of granule for exocytosis (Fig 4A, > 30 pC Ca\(^{2+}\) influx). Therefore there may be a second contributing factor. We turned to Western blot analysis to determine the relative expression levels of key SNARE-complex proteins known to be involved in neuroendocrine granule docking prior to exocytosis (Burgoyne and Morgan 1998; Burgoyne et al. 1996; Graham et al. 2002), a step that defines the functional RRP (Xu et al. 1998). We measured the levels of SNAP-25, Syntaxin and Munc-13 in NCAM +/+ and -/- mice (Fig. 8) and found a significant increase in expression in each case. This may indicate that the NCAM -/- cells are over-expressing molecular components of the fusion machinery in order to compensate for the deficit in exocytosis and catecholamine release under physiological stimulation.

**PMA and ML-9 invert NCAM -/- and +/+ phenotypes.** We previously showed that treatment of NCAM -/- NMJs with the phorbol ester phorbol-12-myristate-13-acetate (PMA) rescues transmission failures normally observed under repetitive stimulation (Polo-Parada et al. 2001; Polo-Parada et al. 2004). We also demonstrated that granule recruitment and secretion is regulated by a PMA-sensitive process in isolated bovine chromaffin cells through activation of protein kinase C (PKC) (Smith 1999; Smith et al. 1998). Lastly, we found that block of myosin light chain kinase with the agent ML-9 induces synaptic
failures in NMJs of NCAM +/+ mice, mimicking the phenotype of NCAM -/- mice. Work by others has shown a link between MLCK and granule availability in chromaffin cells (Gasman et al. 1998; Li et al. 2003). We asked if PMA and ML-9 would produce effects in mouse chromaffin cells under physiological stimulation similar to those observed in the NMJ. We blocked clathrin-mediated endocytosis as previously described (Chan and Smith 2003) to isolate exocytosis in the capacitance recording (see also Fig. 2). We then pre-treated NCAM -/- and NCAM +/+ tissue slices for 5 minutes with 100 nM PMA or 15 µM ML-9 respectively and stimulated the cells at 15 Hz. The results from these experiments are presented in Fig. 6 and show that, as in the NMJ, phorbol ester treatment of the NCAM -/- cells completely rescues the secretory deficit seen in untreated cells. Furthermore, the static IRP measured from PMA-treated NCAM -/- cells was identical to that measured in untreated NCAM +/+ cells (66.3 ± 7.96 fF and 68.96 ± 7.05 fF respectively). Conversely, pre-treatment of NCAM +/+ cells with ML-9 decreased secretory output to a level similar to that observed in untreated NCAM -/- cells (Fig. 6). Lastly, as a control we treated NCAM -/- cells with ML-9, which would be predicted to have no effect. Indeed this was the case, no change in secretion was measured between untreated and ML-9-treated NCAM -/- cells.

DISCUSSION

A rich literature describes a critical role for neural cell adhesion molecule (NCAM) in axon guidance and stabilization of central synapses during development. However, more recently NCAM has been shown to retain an important role following development, it has been shown to be important in aspects of synaptic stabilization and function in the adult animal (Cremer et al. 1998; Muller et al. 1996; Polo-Parada et al. 2001; Polo-Parada et al. 2004; Rafuse et al. 2000). Data from these studies combine to demonstrate that NCAM -/- mice exhibit deficits in both central hippocampal and cerebellar synaptic plasticity as well as peripheral neuromuscular synaptic transmission. The synaptic systems in all these studies signal through the fusion of vesicles at specialized presynaptic active
zones. This shared characteristic lead to the suggestion that NCAM may play a structural role in active zone stabilization, somewhat similar to the intercellular adhesion function served during development (Moscoso et al. 1998; Rutishauser and Landmesser 1996, 1991). However, the data collected from the neuroendocrine chromaffin cells point to a more basic function for NCAM in that this cell-type does not release granules at a structurally identifiable active zone. This opens the possibility that NCAM may be an important molecule in the regulation and proper function of secretory cells in general and is not restricted to specialized active zones found in neurons. In the current study we present data demonstrating that evoked catecholamine release is decreased in NCAM knock out mice. In addition, we demonstrate that the secretory deficit in NCAM -/- mice is due to a slower transfer of granules from the readily-releasable pool (RRP) into the immediately releasable pool (IRP). Granule fusion and transmitter release can occur either from the RRP or the IRP. However, the rate constant for granule fusion from the IRP is at least 5-fold faster than that from the RRP (Voets et al. 1999) and is expected to represent the vast majority of catecholamine release under mild stimulation. Thus, the smaller IRP found in the NCAM -/- mice would directly decreases the rate of granule exocytosis under physiological stimulation in which Ca$$^{2+}$$ influx is modest.

Such functional abnormalities would be predicted to result in detrimental misregulation of many organ and homeostatic systems that fall under the control of the sympathetic nervous system and catecholamine release. We hypothesized that lowered catecholamine levels would lead to a lower vascular tone and thus decreased mean arterial pressure in the knock out mice. Indeed, we did measure lower blood pressure in the NCAM -/- versus +/- mice, but it failed to reach significance. This could be partially attributed to alteration of other secretory systems (i.e. increased renin secretion from the kidney). The lack of patho-physiologies that are expected from disruption of neuroendocrine output points to a compensation for the secretory deficit. This may reside at the cellular level. We found that the NCAM -/- mice over-express proteins vital to the secretory process
NCAM in catecholamine release. (SNAP-25, Syntaxin and Munc-13). Such an over-expression is consistent with a compensation mechanism designed to facilitate more complete fusion of the granules within the remaining IRP or to enhance the rate of fusion from the RRP. This would be achieved by increasing the copy number of fusion machines per granule. However, this over-expression is insufficient to rescue the NCAM-dependent trafficking deficit responsible for impaired catecholamine release. Additional potential compensatory sites could include the up-regulation of end-organ adrenergic receptor expression and/or down regulation of the corresponding parasympathetic nervous system activity. Possible compensation for NCAM-dependent neuroendocrine abnormalities have been described in other secretory systems. Organization and polarity of insulin-secreting pancreatic β-cells is altered in NCAM knock out mice and is similar to that found in humans suffering from type-II diabetes mellitus (Esni et al. 1999). However, Esni and colleagues found glucose levels in these mice to be normal.

Potential systems-level compensatory mechanisms notwithstanding, on the cellular level NCAM -/- chromaffin cells exhibit a secretory phenotype that is due to a decreased retention of granules in the IRP. This, combined with the quantitative experimental and analytic techniques available in chromaffin cells, will allow further definition of the regulatory role played by NCAM in the exocytic process. Previous studies in the neuromuscular junction have shown that the NCAM-dependent secretory deficit can be overcome by treating the cells with phorbol esters such as PMA (Polo-Parada et al. 2001). Here we confirm this finding to be true in chromaffin cells (Fig. 6); indeed PMA treatment restores the IRP in NCAM -/- mice to control values. PMA increases stimulus-dependent exocytosis in chromaffin cells (Gillis et al. 1996) as well as in other neuronal preparations (Stevens and Sullivan 1998) by facilitating the recruitment of granules to the readily releasable pool through activation of protein kinase C. Indeed, prolonged stimulation of chromaffin cells with depolarizing trains results in a persistent secretory facilitation through Ca$^{2+}$-dependent activation of PKC (Smith 1999). Further
experiments will determine the exact nature and orientation of NCAM and PKC in the secretory granule recruitment process.

Other trafficking mechanisms may involve an NCAM-associated vesicle mobilization step. For example, it is known that in chromaffin cells (Matsumura et al. 1999; Neco et al. 2002), hippocampal cultured neurons (Ryan 1999), and superior cervical ganglion cells (Mochida 1995), vesicle mobilization is dependent on the normal function of myosin light-chain kinase (MLCK). Similar results were obtained in the neuromuscular junction where block of MLCK mimics the synaptic transmission failures observed in NCAM -/- mice (Polo-Parada et al. 2001; Polo-Parada et al. 2004). This finding was also repeated in the current study where ML-9 treatment of NCAM +/+ cells depresses granule fusion to a level observed in untreated NCAM -/- cells. The function of MLCK in catecholamine exocytosis would be part of a more complex signaling path; it has been shown recently that specific NCAM isoforms interact with components of the cytoskeleton including α- and β-tubulin, α- and β-actin, tropomyosin, microtubulin-associated protein (MAP 1A) and rho kinase (Buttner et al. 2003). Rho-kinases play a role in the modulation of MLCK activity and have recently been shown to regulate activity-dependent mobilization and fusion of chromaffin granules (Gasman et al. 1998; Li et al. 2003). Together these results point to a potential association of NCAM and MLCK-dependent processes in the recruitment of secretory organelles in general. These elements may provide a molecular complex allowing proper granule trafficking in normal cells and may explain the deficit in the IRP in the NCAM -/- mice. Ongoing and future experiments in chromaffin cells will clarify the NCAM-dependent mechanism in the evoked release of signaling molecules.
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LITERATURE CITED


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FIGURE LEGENDS

Figure 1. Adrenal NCAM expression is limited to the chromaffin cells of the medulla.
A. Bright field (top row) and NCAM immuno-fluorescence (bottom row) are shown for NCAM +/+ (left) and -/- (right) tissues. NCAM was labeled with the pan-isoform antibody RO25b. Dotted lines delineate the adrenal medulla from the cortex. In NCAM +/+ mice, fluorescence signal is present in chromaffin cells (medulla; M) while no NCAM signal is detected in the adrenal cortex (C). Scale = 40 µm. B. Higher magnification reveals cell surface expression of NCAM in the wild-type mice. Scale bar = 10 µm. C. Western blot analysis to determine NCAM isoform expression in the adrenal medulla. The blot demonstrates that all three isoforms of NCAM are present in NCAM +/+ mice, but none are expressed in the NCAM -/- mice.

Figure 2. NCAM -/- cells exhibit decreased exocytosis under physiological stimulation.
Ai. PP-19 transfection blocks endocytosis in NCAM -/- chromaffin cells. Tissue slices cut from NCAM -/- mice were transfected with the PP-19 peptide (see Methods for details) and stimulated with AP wave forms at 15 Hz. Data from this experiment are plotted together with data collected from untransfected cells (n = 6 and 8, respectively). The larger capacitance response in the PP-19-treated cells is due to a lack of endocytic membrane retrieval. Error bars at the end of each record represent the S.E.M.. Aii. Wild type and NCAM -/- chromaffin cells were stimulated at 15 Hz. Cells were transfected with the PP-19 peptide, thus capacitance reflects exocytosis alone. Cell capacitance records for each group were averaged (n= 9 +/-; n = 8 -/-) and plotted. Mean calcium currents evoked during each train were measured and averaged for all cells in both the NCAM +/+ and -/- cell-types (inset). Bi. Catecholamine release was measured under each condition by carbon fiber amperometry. Representative recordings from NCAM +/+ and -/- cells are provided in panel i. Bii. This protocol was repeated on wild type and NCAM knock out mice (n = 9 and n = 8, respectively). Total numbers of
spikes were counted for each condition and are plotted. C. Total catecholamine release for each group was determined by integrating the entire amperometric records from panel B. The resulting average integrals are plotted. Error bars at the end-point represent the S.E.M. of each data set.

**Figure 3. The releasable granule pools are altered in NCAM -/- mice.**

Readily and immediately releasable granule pools (RRP and IRP, respectively) were quantified in both NCAM +/+ and -/- chromaffin cells. Ai. The RRP was quantified as described (Gillis et al. 1996). Briefly cells were voltage clamped at -80 mV and stimulated with a pair of 100 ms depolarizations first to 0 mV and then to +5 mV to balance Ca\(^{2+}\) influx. The top trace shows the evoked current influx. In the example recording provided, the first pulse resulted in a greater Cm increase than the second pulse (Cm\(_1\) and Cm\(_2\) respectively). This is broadly interpreted as a consumption of release-ready granules by the first response leaving few for the second pulse to access. Formally, the RRP can thus be quantified as 
\[
RRP = S / (1 - R^2)
\]
where S = the sum of Cm\(_1\) and Cm\(_2\) and R is the ratio of Cm\(_2\) to Cm\(_1\). ii. This dual-pulse RRP protocol was repeated on NCAM +/+ and -/- cells. Only cells that exhibit strong depression (Cm\(_2\) is small compared to Cm\(_1\)) provide an accurate estimate of the releasable pool size by the dual pulse method. For this reason only cells that exhibited a depression ration (Cm\(_2\)/Cm\(_1\)) of 0.7 or less were included for further analysis. The quantified RRP sizes from NCAM +/+ and NCAM -/- records (n = 36 and 32 respectively) are summarized and show that the RRP was significantly larger in the NCAM -/- cells. Bi. The immediately releasable granule pool (IRP) was measured as previously described (Voets et al. 1999) in NCAM +/+ and -/- mice. This pool was measured as the paired-pulse depression in response to 10 ms stimuli to +5 mV. As in panel A, data from a representative experiment are pictured. ii. This protocol was repeated on NCAM +/+ and -/- cells with those that exhibited a depression ratio of at least 0.7 chosen for further analysis (n = 22 and 23, respectively). The IRP was found to be significantly smaller in NCAM -/- cells. C. Kinetic analysis of IRP (i) and RRP (ii) refilling. For each cell type, the IRP (+/+ n = 25, -/- n = 23) or RRP (+/+ n = 21,
was depleted by delivering a dual-pulse protocol as in panels A and B. A second pulse-pair was then delivered at various times following initial depletion to probe the degree of refilling in a time-dependent manner. Since the IRP is considered a subset of the RRP, we first fit the IRP refilling curves with a mono-exponential function (solid lines represent the best fit with dotted lines showing 1/e confidence bands, Ci) and then used the measured refilling rate constant as a fixed term in a double exponential fit for the RRP refilling curves (solid lines represent the best fit with dotted lines showing 1/e confidence bands, Cii). The second slower exponential thus represents the refilling of the RRP alone.

**Figure 4. A granule recruitment deficit is responsible for the lessened exocytosis under physiological stimulation.**

**A.** Cells were stimulated in order to quantify secretion as a function of stimulus strength. In this protocol cells were stimulated with randomized, variable duration square pulse depolarizations to +5 mV. Cell capacitance values are plotted against integrated Ca²⁺ influx, utilized as an index of stimulus strength. At lower stimulus amplitudes the NCAM -/- cells exhibited a weaker stimulus-secretion coupling, but stronger coupling with increased stimulation. **B.** The lower stimulus range of the plot in A is shown to better examine the secretory behavior under Ca²⁺ influx levels expected during physiological stimulation (grey box). The inset shows mean Ca²⁺ influx evoked per action potential during 15 Hz stimulation in NCAM -/- and +/+ mice. Despite the over-all steeper stimulus-secretion function found in the NCAM -/- mice, the expected release at levels matching physiological stimulation is smaller in the knock-outs.

**Figure 5. SNARE complex proteins are over-expressed in NCAM -/- mice.** A. A potential compensation mechanism for the granule trafficking deficit may explain the steeper stimulus-secretion function found in NCAM -/- mice. Western blot analysis was performed on equal amounts of
protein collected from NCAM -/- and +/- adrenal medullary tissues. Proteins were separated by PAGE electrophoresis and probed with antibodies to SNAP-25, Syntaxin and Munc-13, all key SNARE complex proteins. Actin levels serve as a loading control. B. Densitometry was performed on the blots to quantify expression of each protein in the NCAM -/- adrenal medullae.

Figure 6. PKC rescues the NCAM -/- phenotype and ML-9 mimics the knockout phenotype. The protocol presented in Fig. 2 was repeated on NCAM -/- tissue slices pre-treated with either 100 nM PMA or NCAM +/- slices pre-treated with 15 µM ML-9 for 5 minutes (n = 11 and 22 respectively). PMA treatment of NCAM -/- cells resulted in secretion levels nearly identical to wild-type while ML-9 treatment of NCAM +/- cells decreased evoked capacitance signals to below those observed in the NCAM -/- cells. We also treated NCAM -/- cells with ML-9 and found no effect (n = 6). Data for untreated cells are re-plotted from Fig. 2 for comparison. Error bars are shown for the final point in each record and represent the S.E.M..
Figure 1

A

B

C

(+/+)  (-/-)

180kD  140kD  120kD
Figure 2

Ai

ΔCm (pF)

NCAM -/- with PP-19

NCAM -/- without PP-19

Bi

+/+

-/-

Σ Amperometry (µC)

NCAM +/+  
NCAM -/-

Σ Amperometry (µC)

C

NCAM +/+  
NCAM -/-

Σ Amperometry (µC)

s
Figure 3

Ai

\[ I_{\text{Mon.}} \text{ (nA)} \]

\[ C_m \text{ (pF)} \]

\[ \begin{array}{c}
0.0 \\
0.4 \\
0.8 \\
\end{array} \]

\[ s \]

ii

\[ \begin{array}{c}
0 \\
5 \\
10 \\
15 \\
20 \\
\end{array} \]

\[ s \]

Bi

\[ I_{\text{Mon.}} \text{ (nA)} \]

\[ C_m \text{ (pF)} \]

\[ \begin{array}{c}
0.0 \\
0.1 \\
0.2 \\
\end{array} \]

\[ s \]

ii

Ci

\[ I_{\text{Mon.}} \text{ (nA)} \]

\[ C_m \text{ (pF)} \]

\[ \begin{array}{c}
0 \\
1 \\
2 \\
3 \\
4 \\
\end{array} \]

\[ s \]

ii
Figure 4

A

Calcium influx (pC)

ΔCm (fF)

NCAM +/+  
NCAM -/-

B

Calcium influx (pC)

ΔCm (fF)

NCAM +/+  
NCAM -/-

ICa

ICa^2+ (pC)
Figure 5

A

NCAM (+/+)  NCAM (-/-)

SNAP-25
Syntaxin
Munc-13
Actin

B

![Bar graph showing the percentage control of SNAP-25, Syntaxin, Munc-13, and Actin for NCAM (+/+) and NCAM (-/-).]
Figure 6

\[ \Delta C_m \text{ (pF)} \]

- NCAM \(-/-\), PMA
- NCAM \(+/+\)
- NCAM \(-/-\)
- NCAM \(-/-, ML-9\)
- NCAM \(+/+\), ML-9

\[ s \]

0 20 40 60