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## **$\alpha$ II-spectrin controls calcium-regulated exocytosis in neuroendocrine chromaffin cells through N-WASP interaction**

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**$\alpha$ II-spectrin controls calcium-regulated exocytosis in neuroendocrine chromaffin cells through N-WASP interaction**

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5  **$\alpha$ II-spectrin controls calcium-regulated exocytosis in neuroendocrine**  
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7 **chromaffin cells through N-WASP interaction**  
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12 Sébastien Houy<sup>1</sup>, Gaël Nicolas<sup>2,3</sup>, Fanny Momboisse<sup>1</sup>, Magali Malacombe<sup>1</sup>, Marie-France Bader<sup>1</sup>,  
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52 **Running title:** Spectrin/N-WASP interaction in exocytosis  
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56 **Key words:** Spectrin, exocytosis, PC12 and chromaffin cells, N-WASP  
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**Abstract**

Besides a fundamental structural role at the plasma membrane, spectrin- and actin-based skeletons have been proposed to participate to various processes including vesicular trafficking. Neuroendocrine cells release hormones and neuropeptides through calcium-regulated exocytosis, a process that is coordinated by a fine remodelling of the actin cytoskeleton. We describe here that calcium-regulated exocytosis is impaired in chromaffin and PC12 cells with reduced  $\alpha$ II-spectrin expression levels. Using yeast two-hybrid screening, we show that N-WASP is a partner of the  $\alpha$ II-spectrin SH3 domain and demonstrate that secretagogue-evoked N-WASP recruitment at cell periphery is blocked in absence of  $\alpha$ II-spectrin. Additionally, experiments performed with ectopically expressed  $\alpha$ II-spectrin mutant unable to bind N-WASP indicated that the interaction between SH3 domain and N-WASP is pivotal for neuroendocrine secretion. Our results extend the list of spectrin interactors and strengthen the idea that  $\alpha$ II-spectrin is an important scaffold protein that gathers crucial actin related players of the exocytic machinery.

**Abbreviations:**

N-WASP: Neuronal Wiskott–Aldrich Syndrome protein

Ena/VASP: Ena/Vasodilator-stimulated phosphoprotein

EVL: Ena/Vasodilator-stimulated phosphoprotein-like protein

## Introduction

Most secretory cells display a dense network of filamentous actin beneath their plasma membrane. In neuroendocrine cells, actin dynamics play an important and composite function in calcium-regulated exocytosis in order to achieve secretory vesicle recruitment, docking and fusion with the plasma membrane. At first, cell stimulation with a secretagogue leads to a loss of cortical actin filaments. Thus, for a long time actin filament network was uniquely considered to be a physical barrier preventing the reserve pool of secretory vesicles to gain access to the plasma membrane. However today this view has evolved towards a dynamic role of actin in which a complex process of actin filament remodelling is critically required for efficient exocytosis (1-4). Particularly, we have shown that N-WASP is recruited at exocytic sites where its ability to promote actin filament assembly is necessary for exocytosis (1).

Actin filaments are thought to be physically linked to spectrin family proteins, which represent another important element of the membrane skeleton network in neuronal cells (5). Spectrins are long stretchable proteins composed of intertwined  $\alpha$  and  $\beta$  subunits to form  $\alpha\beta$  heterodimers. Spectrin exists as elongated tetramers resulting from self-association of heterodimers. These tetramers form filaments that have been involved in various processes including membrane stabilisation, membrane protein-sorting, vesicular trafficking and neurite outgrowth. Two and five genes encode  $\alpha$  and  $\beta$  chains, respectively. The involvement of  $\alpha$ II-spectrin (previously named fodrin) in calcium-regulated exocytosis has been previously suggested both in neurons and neuroendocrine cells (6, 7). However, besides these studies, no further evidence has been provided and the underlying molecular mechanism by which  $\alpha$ II-spectrin may control exocytosis remains unknown.

The present study was undertaken to investigate the role of  $\alpha$ II-spectrin in calcium-regulated exocytosis. Using neuroendocrine PC12 and chromaffin cells as a secretory cell models, we describe here the interaction between  $\alpha$ II-spectrin and N-WASP and propose that this interaction is pivotal for the exocytotic process.

## Material and methods

### DNA constructs, cell culture and transfection

Plasmids encoding Flag- $\alpha$ 8-11, Flag- $\alpha$ 8-11<sup>P1017L</sup>, N-WASP-EGFP, and the vectors pGHsuper and pEGFP-RNAi were described previously (1, 8-10). DNA fragment encoding the unrelated siRNA sequence (ATTCTATCACTAGCGTGAC) or the siRNA sequence of rat/bovine  $\alpha$ II-spectrin (CCAAGCTTATTCAGAACAA /CCAAGCTGATTCAGAACAA) separated from its reverse complement by a short spacer were annealed and cloned in the BglII and HindIII sites in front of the H1 promoter of either the pGHsuper vector or the pEGFP-N2-RNAi (9, 11). Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with collagenase, purified on self-generating Percoll gradients and maintained in culture as previously described (12). PC12 cells culture and transfection conditions were as described (1, 11).

### Antibodies and immunofluorescence

The following antibodies were used: rabbit polyclonal anti- $\alpha$ II-spectrin antibodies (6); mouse monoclonal anti-flag antibodies (Sigma); rabbit polyclonal anti-N-WASP antibodies (Tebu Santa Cruz); mouse monoclonal anti-actin antibodies (Sigma) and rabbit polyclonal anti-GH antibodies (kindly provided by Dr. A. F. Parlow, NIDDK, NIH, Bethesda, MD). Alexa-labelled secondary antibodies were obtained from Molecular Probes. Blots were processed using the Super Signal detection system (Pierce). For immunocytochemistry, chromaffin cells grown on fibronectine- or PC12 cells grown on poly D-lysine-coated glass coverslips were fixed and immunostained as described previously (13). Stained cells were visualized using a confocal microscope LSM 510 (Carl Zeiss, Jena, Germany).

### Growth hormone (GH) release assay

PC12 cells were washed twice with Locke's solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 11 mM glucose and 15 mM Hepes, pH 7.2) and then incubated for 10 min with Locke's solution (basal release at resting) or stimulated with an elevated K<sup>+</sup> solution (Locke's containing 59 mM KCl and 85 mM NaCl). The supernatant was collected, and the cells were harvested by scraping in 10 mM phosphate-buffered saline. The amounts of GH secreted into the medium and retained in the cells were measured using an ELISA assay (Roche). GH secretion is expressed as a percentage of total GH present in the cells before stimulation.

### **Carbon fiber amperometry**

Carbon-fiber electrode of 5  $\mu\text{m}$  diameter (ALA Scientific) was held at a potential of +650 mV compared with the reference electrode (Ag/AgCl) and approached closely to chromaffin cells. Catecholamine's secretion was induced by 10 seconds pressure ejection of 100 mM  $\text{K}^+$  solution from a micropipette positioned at 10  $\mu\text{m}$  from the cell and recorded during 100 seconds. Amperometric recordings were performed with an AMU130 (Radiometer Analytical) amplifier, sampled at 5 kHz, and digitally low-passed filtered at 1 kHz. Analysis of amperometric recordings was done as previously described (14) with a custom-written macro (obtained from Dr. R. Borges laboratory) running under Igor software (Wavemetrics), allowing automatic spike detection and extraction of spike parameters. The number of amperometric spikes was counted as the total number of spikes with an amplitude  $>5$  pA within 100 seconds. The spike parameters analysis was restricted to spikes with amplitudes of 5 pA. Data of amperometric spikes were averaged by individual cell.

### **Yeast two-hybrid screening**

Yeast two-hybrid screening using the  $\alpha\text{II}$ -spectrin SH3 domain as a bait was performed as described in (15, 8). The sequence encoding from residues E970 to P1026 (accession number U83867) was cloned into the pLEX10 vector in frame with the C-terminus of the LEXA DNA-binding domain. The yeast strain L40 established with the  $\alpha\text{II}$ -Sp-bait was transformed with 100  $\mu\text{g}$  of plasmid cDNA originating from a rat kidney library made in the laboratory. As the baits did not induce background,  $\text{His}^+$  clones were selected on medium lacking tryptophan, leucine and histidine (DO-WLH) after 3 to 4 days of growth at 30°C. Beta-galactosidase activity was analysed on filter and liquid assays using X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) and ONPG (*o*-nitrophenyl- $\beta$ -D-galactopyranoside) as substrates, respectively. Recombinant pGAD plasmids were recovered from  $\text{His}^+\text{lacZ}^+$  phenotype yeasts and selected after transformation of an HB101 *E. coli* strain grown on a selective medium lacking leucine. Analyses of interaction specificity were performed by yeast mating of the L40 and AMR70 strains.

### **Expression and purification of recombinant peptides in *E. coli***

The sequences of human  $\alpha\text{II}$ -spectrin corresponding to the SH3 domain (E970 to P1026) and to the  $\alpha 9$  repeat containing the SH3 domain (residues E885 to K1089) were cloned into pGEX-

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3 KG vector (16). GST-tagged peptides were expressed in BL21 Gold *E. coli* strain after  
4 induction with 0.5 mM IPTG and purified according to the manufacturer's instructions.  
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### 8 ***In Vitro* protein-protein interactions and immunoprecipitation**

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10 *In vitro* interactions were performed overnight at 4°C with 20 µg of recombinant GST-tagged  
11 peptides immobilised on Sepharose 4B glutathione (Amersham Biosciences) and S<sup>35</sup> labelled-  
12 N-WASP obtained by transcription and translation *in vitro* (TNT® T7 Quick kit for PCR DNA,  
13 Promega) in 100 mM KCl, 0.2 mM EDTA, 20 mM Hepes buffer, pH 7.9, containing proteases  
14 inhibitors. After five washes in 0.01% NP 40, Hepes buffer, bound proteins were eluted by GSH  
15 (10 mM). Amounts of eluted, radiolabelled proteins were evaluated after SDS-PAGE using  
16 “Instant Imager” apparatus (Packard).  
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22 Anti-α2 spectrin or anti-flag antibodies were covalently immobilized on AminoLink coupling  
23 resin and co-immunoprecipitation experiments were performed using Pierce™ Direct IP Kit  
24 (ThermoFisher) according to the manufacture instructions.  
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### 29 **Data analysis**

30 In all the Figures, data are given as the mean values ± S.D. obtained at least in three independent  
31 experiments performed on different cell cultures. n represents the number of experiments.  
32 Statistical significance has been established using ANOVA test and data were considered  
33 significantly different when p value was lower than 0.05. Gaussian distribution and variance  
34 equality of the data were verified.  
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## Results

### Calcium-regulated exocytosis requires $\alpha$ II-spectrin

To directly assess the functional importance of endogenous spectrin in the exocytotic machinery, we employed a short interference RNA (siRNA) strategy in PC12 cells, using a plasmid (pGHsuper) that expresses full-length human growth hormone (GH) and a siRNA targeting the sequence of  $\alpha$ II-spectrin. GH specifically stored into secretory granules is used as a reporter for exocytosis in the subpopulation of cells that transiently express the siRNA (9). As revealed by immunocytochemistry analysis, cells expressing  $\alpha$ II-spectrin siRNA, identified by the expression of GH, presented significant loss of spectrin staining but no alteration of the expression level of GH or its distribution in secretory granules (Fig. 1A). Fluorescence quantification revealed that the level of  $\alpha$ II-spectrin in cells expressing siRNAs was reduced by  $\sim 90\%$  (Fig. 1B). The effect of spectrin silencing on PC12 cell secretory activity was then analyzed. Reduction of endogenous spectrin significantly inhibited the secretion of GH stimulated by a depolarizing concentration of  $K^+$  (59mM; Figure 1C), indicating that  $\alpha$ II-spectrin is a key component of the exocytic machinery allowing large dense core vesicle to release hormones and proteins from neuroendocrine cells.

To further analyze the role of  $\alpha$ II-spectrin in hormone secretion, we monitored real-time single granule catecholamine exocytosis by carbon fiber amperometry. Figure 2A illustrates representative amperometric traces obtained from chromaffin cells expressing either an unrelated siRNA or a  $\alpha$ II-spectrin siRNA. The number of amperometric events was found to be significantly reduced in  $\alpha$ II-spectrin knocked down cells (Figure 2B), indicating that  $\alpha$ II-spectrin plays a role in the number of granules undergoing exocytosis. Figure 2C summarizes the detailed analysis of each individual amperometric spikes. None of the spike parameters were significantly altered by the reduction of endogenous  $\alpha$ II-spectrin levels (Table 1), suggesting that  $\alpha$ II-spectrin is not directly implicated in the fusion pore expansion or in the size and catecholamine content of the secretory granules.

### Interaction between $\alpha$ II-spectrin and N-WASP

To further investigate the putative mechanisms by which  $\alpha$ II-spectrin could regulate exocytosis, we looked for potential partners using a yeast two-hybrid. The backbone of  $\alpha$ II-spectrin is composed of 20 repeat units ( $\alpha 1$ - $\alpha 20$ ) and the  $\alpha 9$  unit contains a SH3 domain (Figure 3A). This SH3 domain (residues E970 to P1026) fused to the DNA binding domain of LexA was used as a bait to screen a rat kidney cDNA library. Six clones containing incomplete sequences of

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3 Wiskott-Aldrich Syndrome protein N-WASP (accession number D88461/BAA21534) were  
4 retrieved. The largest sequence encodes a peptide starting from residue D67. Proline1017 of the  
5  $\alpha$ II-spectrin SH3 corresponds to a highly conserved proline residue involved in SH3 domain  
6 interactions with its binding partners (15). We probed interaction of  $\alpha$ II-spectrin SH3 peptides  
7 with N-WASP D67-A190 using the yeast two-hybrid system and found that P1017L mutation  
8 totally abolished the interaction as revealed by the absence of growth on selective medium  
9 (Figure 3B).

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15 The interaction between spectrin and N-WASP was then validated through an independent  
16 biochemical approach using  $^{35}$ S-labelled N-WASP and the  $\alpha$ II-spectrin SH3 domain fused to  
17 GST. As shown in figure 3C, the GST-SH3 domain bound N-WASP peptide whereas GST  
18 alone did not, thus confirming that the SH3 domain of  $\alpha$ II-spectrin is able to interact with N-  
19 WASP. Interestingly, a spectrin peptide containing the SH3 domain and the adjoining  $\alpha$ 9 repeat  
20 retained higher amount of N-WASP suggesting that the sequence surrounding the SH3 domain  
21 improved the spectrin-N-WASP interaction (Figure 3C). This interaction was further confirmed  
22 ex-vivo as endogenous N-WASP was co-immunoprecipitated with  $\alpha$ II-spectrin from PC12  
23 cells (Figure 3D).

### 33 **$\alpha$ II-spectrin/N-WASP interaction is required for secretagogue-induced hormone release**

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37 In PC12 cells, upon  $K^+$ -stimulation ectopically expressed GFP-N-WASP is efficiently recruited  
38 from the cytoplasm to the cell periphery to stimulate exocytotic activity by promoting actin  
39 filaments formation (1). To explore the functional importance of the N-WASP/spectrin  
40 interaction during exocytosis, we used two different approaches. First, the distribution of GFP-  
41 N-WASP was investigated in cells expressing  $\alpha$ II-spectrin siRNA. We found that the  
42 translocation of N-WASP to the plasma membrane in response to **59 mM**  $K^+$ -stimulation was  
43 strongly inhibited in cells depleted for  $\alpha$ II-spectrin compared to cells expressing an unrelated  
44 siRNAs (Figure 4A, B). These data strongly suggest that  $\alpha$ II-spectrin actively participates to  
45 the recruitment and/or stabilization of N-WASP at the plasma membrane upon cell stimulation  
46 for secretion. Second, we expressed in PC12 cells a flag tag-fused peptide of truncated  $\alpha$ II-  
47 spectrin corresponding to the region between  $\alpha$ 8 and  $\alpha$ 11 repeat units (Figure 3A) containing  
48 the N-WASP interacting domain. This peptide should sequester N-WASP and prevent its  
49 interaction with endogenous  $\alpha$ II-spectrin. **Overexpressed Flag-Spectrin  $\alpha$ 8-11 displayed a**  
50 **predominant cytosolic staining in resting PC12 cells and was efficiently translocated to the cell**  
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3 periphery upon 59 mM K<sup>+</sup>-stimulation of the cells (Figure 4C). On contrary, the mutant  
4 defective in N-WASP binding, Flag-Spectrin  $\alpha$ 8-11<sup>P1017L</sup>, remained cytosolic upon cell  
5 stimulation (Figure 4C). Moreover, endogenous N-WASP was found to co-immunoprecipitate  
6 with flag-spectrin  $\alpha$ 8-11 peptide, but not with the corresponding peptide carrying the P1017L  
7 mutation (Figure 4D). These results confirm that the peptide  $\alpha$ 8-11 could act as a dominant  
8 negative truncated mutant by sequestering N-WASP and thus by preventing the endogenous  
9 interaction  $\alpha$ II-spectrin-N-WASP.

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16 Expression of flag-spectrin  $\alpha$ 8-11 in PC12 cells significantly inhibited GH release, by nearly  
17 60% as efficiently as the knockdown of  $\alpha$ II-spectrin (Figure 4E). This inhibitory effect was  
18 dependent on the ability of flag-Spectrin  $\alpha$ 8-11 to bind N-WASP since it was not observed with  
19 the peptide carrying the P1017L mutation (Figure 4E). Altogether, these results demonstrate  
20 for the first time that in neuroendocrine cells  $\alpha$ II-spectrin is able to recruit N-WASP to the  
21 plasma membrane and that this interaction is required for large dense core granule exocytosis.  
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## Discussion

The potential involvement of  $\alpha$ II-spectrin in adrenal chromaffin cell exocytosis was first suggested in the late 80's in studies showing that injection of antibodies raised against  $\alpha$ II-spectrin inhibits calcium-mediated secretion (6). Using a loss of function approach, we have observed here that knocking down expression of  $\alpha$ II-spectrin inhibits calcium-regulated exocytosis in chromaffin and PC 12 cells, directly demonstrating that endogenous  $\alpha$ -spectrin is pivotal for neuroendocrine hormone release. A role for spectrin in neurotransmitter release from neurons has also been proposed in neuromuscular junctions of fruit flies *Drosophila* as presynaptic neurotransmitter release is impaired in  $\alpha$ -spectrin null *Drosophila* mutants or in mutants displaying a large decrease of pre-synaptic level of  $\alpha$ -spectrin (7, 17). Yet, until now the mechanism by which  $\alpha$ -spectrin regulates calcium-regulated exocytosis of synaptic and large dense core vesicles remains unexplored.

We show here by amperometric analysis of catecholamine secretion from chromaffin cells that silencing of  $\alpha$ II-spectrin expression decreases in the number of individual exocytic events/spikes without changing the shape of the remaining spikes. This observation rules out a direct role for  $\alpha$ II-spectrin in the membrane fusion process itself, but is consistent with a role in earlier stages of exocytosis such as the recruitment and/or docking of large dense core secretory granules to the exocytotic sites. Sikorski and collaborators have previously proposed in cultured hippocampal neurons that brain spectrin is involved in synaptic vesicle recruitment to the presynaptic active zone through its interaction with the vesicle-bound synapsin (18). Whether synapsin interacts with  $\alpha$ II-spectrin in chromaffin cells is not known and remains to be tested, although synapsin has been proposed to negatively regulate catecholamine release from chromaffin cells (19).  $\alpha$ II-spectrin has been also shown to interact with syntaxin1, a member of the soluble N-ethyl maleimide-sensitive factor attachment protein receptor (SNARE) machinery involved in granule docking and fusion (20), but the importance of this interaction during exocytosis has never been explored. On the other hand, spectrin was first described as a regulator of actin polymerization (21-23).  $\alpha$ II-spectrin is also known to interact with several actin-binding proteins including Tes and the Ena/VASP protein family members, EVL and VASP (8, 24, 25). The present findings describing the actin binding protein N-WASP as a functional partner of  $\alpha$ II-spectrin in the secretory pathway, replaces spectrin in the frame of the cortical actin organization that supervises the exocytotic machinery.

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3 We have previously described that N-WASP is recruited to the plasma membrane upon  
4 PC12 cell stimulation thanks to its interaction with the Rho-GTPase Cdc42 which is activated  
5 at the plasma membrane during hormone release (1). Once located at the plasma membrane, N-  
6 WASP may interact with the secretory granule-bound actin nucleating complex ARP2/3 to  
7 promote actin polymerization at the exocytotic site and then enhance hormone release (1). Here,  
8 we observed that knocking down  $\alpha$ II-spectrin prevents N-WASP recruitment to the cell  
9 periphery of stimulated PC12 cells suggesting that  $\alpha$ II-spectrin is also implicated in the  
10 recruitment of N-WASP to the exocytotic sites. Whether N-WASP interacts simultaneously  
11 with Cdc42 and  $\alpha$ II-spectrin is not known. However, N-WASP is known to be maintained in  
12 an autoinhibited conformation through intramolecular bounds that are relieved upon Cdc42  
13 binding (26). As activation of N-WASP by Cdc42 unmasks its proline-rich domain, it is  
14 tempting to imagine that Cdc42 binding occurs first hence allowing the subsequent binding  
15 with  $\alpha$ II-spectrin SH3 binding domain.  $\alpha$ II-spectrin is a long protein (200 nm for tetramers)  
16 with a SH3 domain that might constitute an active spot to recruit the exocytic machinery  
17 including N-WASP. In line, several presynaptic proteins from the active zone seem to be mis-  
18 localized in the absence of spectrin (7). It is worth to mention that annexin-A2 has also been  
19 identified among the spectrin interacting partners (27). Annexin-A2 is a cytosolic protein that  
20 triggers the formation of actin bundles essential for the formation of active exocytotic sites in  
21 stimulated chromaffin cells (3). Whether spectrin is a hub involved in the recruitment to the  
22 exocytotic sites of the actin-binding proteins (N-WASP, annexin-A2) required for building the  
23 actin architecture necessary for exocytosis remains to be determined.  
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**Figures legends:**

**Figure 1:** Reduction of endogenous  $\alpha$ II-spectrin by RNA interference inhibits GH secretion from PC12 cells. PC12 cells were transfected with pGHsuper (Control) or pGHsuper encoding unrelated shRNA or pGHsuper encoding  $\alpha$ II-spectrin shRNA for 72h. **(A)** Cells were fixed and stained with anti-GH antibodies revealed by Alexa 488-labeled anti-rabbit antibodies. Cells were then post-fixed, stained with anti- $\alpha$ II-spectrin antibodies visualized with Alexa-647 conjugated anti-rabbit antibodies and analysed by confocal microscopy. Transfected cells are indicated with an asterisk. **Note that neither cell shape nor cell survival is affected by the expression of the  $\alpha$ II-spectrin siRNA.** Bar, 5 $\mu$ m. **(B)** Histogram representing analysis of  $\alpha$ II-spectrin immunoreactivity detected in non-transfected cells (NT), in pGHsuper-transfected cells (Control) and in cells expressing unrelated siRNA (UnR) or  $\alpha$ II-spectrin siRNA. n= 25 cells **(C)** Assay for GH secretory activity in cells expressing  $\alpha$ II-spectrin siRNA. Transfected cells were incubated for 10 min in Locke's solution (Resting) or stimulated for 10 min with 59 mM K<sup>+</sup> (K<sup>+</sup>-stimulated). GH secreted into the medium and retained in the cells was then estimated by an ELISA assay. GH release is expressed as the percentage of total GH present in the cells before stimulation (n = 3). \*\*\*P<0.001, NS (not significant) compared to control cells (ANOVA)

**Figure 2:** Amperometric analysis of catecholamine secretion from bovine chromaffin cells knocked-down for  $\alpha$ II-spectrin. Chromaffin cells were transfected with pEGFP-N2-RNAi encoding unrelated shRNA or  $\alpha$ II-spectrin shRNA for 72h. **(A)** Representative amperometric recordings from transfected chromaffin cells. Cells were stimulated 10 s (horizontal bar) by a local application of 100 mM K<sup>+</sup>. **(B)** Cumulative number of spikes during 100s of recording of cells expressing unrelated shRNA (n=27 cells) and cells expressing  $\alpha$ II-spectrin shRNA (n= 22 cells) \*P<0.05, (t-test). **(C)** Scheme of an amperometric spike describing the following parameters: the surface area (quantal size, Q) being proportional to the amount of catecholamine released per event, the half-width (t half) reflecting the duration of the exocytotic event, the spike height value (I max) reflecting the maximal flux of catecholamine. I<sub>max</sub>, t half and Q in cells expressing unrelated shRNA (black bars) or  $\alpha$ II-spectrin shRNA (white bars) are presented as histograms.

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3 **Figure 3:** In vitro interaction between N-WASP and SH3 domain of  $\alpha$ II-spectrin. **(A)**  
4 Schematic representation of  $\alpha$ II-spectrin.  $\alpha$ II-spectrin is composed of 20 repeat units ( $\alpha$ 1- $\alpha$ 20)  
5 flanked by a small N-terminal region and C-terminus EF-hand motifs. Repeat  $\alpha$ 9 contains the  
6 SH3 domain. SH3- $\alpha$ 9 and  $\alpha$ 8-11 constructs are indicated. Numbers represent amino acid  
7 position. **(B)** L40 yeast cells transformed with pLEX vector empty or containing the indicated  
8 spectrin constructs were mated with AMR70 yeast cells transformed with pGAD vector empty  
9 or containing N-WASP (D67-E501). As revealed by yeast growth on triple-selective medium  
10 (DO-WLH),  $\alpha$ II-spectrin SH3 domain (SH3<sup>WT</sup>) interacts with N-WASP. Note that the mutation  
11 (P1017L) abolished the interaction with N-WASP. **(C)** GST, GST fused to  $\alpha$ II-SH3 or fused to  
12  $\alpha$ II-SH3- $\alpha$ 9 were immobilized on sepharose 4B beads and incubated with rat N-WASP (D67-  
13 E501) expressed as <sup>35</sup>S-methionine labelled peptide using in vitro transcription and translation.  
14 After extensive washings, retained N-WASP peptide were eluted with 10 mM glutathione then  
15 analyzed by SDS-PAGE. <sup>35</sup>S-methionine-labelled N-WASP peptide was analyzed by  
16 autoradiography. **(D)** Immunoprecipitation experiments from lysates of PC12 using  $\alpha$ II-  
17 spectrin antibodies. Endogenous N-WASP, revealed by anti-N-WASP antibodies, co-  
18 precipitated with  $\alpha$ II-spectrin. Control with non-immune (NI) serum is shown.

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33 **Figure 4:** **(A-B)** PC12 cells transfected with pGHsuper encoding unrelated shRNA (control) or  
34 with pGHsuper encoding  $\alpha$ II-spectrin shRNA were plated on four-well plates and transfected  
35 again, 48 hours later, with the N-WASP-EGFP plasmid. 24 hours after the second transfection,  
36 cells were stimulated for 10 minutes with 59 mM K<sup>+</sup> (stimulated) or incubated for 10 minutes  
37 in Locke's solution (resting). Cells expressing siRNA were identified by GH staining (not  
38 shown) whereas  $\alpha$ II-spectrin was observed with anti- $\alpha$ II-spectrin antibodies visualized with  
39 Alexa-647 conjugated anti-rabbit antibodies. Bar, 5  $\mu$ m. The effect of  $\alpha$ II-spectrin knocking  
40 down on intracellular distribution of N-WASP was then analyzed by confocal microscopy and  
41 quantified in B. **(C)** PC12 cells expressing the Flag-tagged spectrin  $\alpha$ 8-11 peptide or the P1017  
42 mutant unable to interact with N-WASP (Flag-tagged  $\alpha$ 8-11<sup>P1017L</sup>) were labelled with the  
43 monoclonal anti-flag antibodies revealed by Alexa-488 conjugated anti-mouse antibodies. Bar,  
44 5 $\mu$ m. **(D)** Immunoprecipitation (IP) experiments from lysates of PC12 cells expressing flag-  
45 tagged spectrin  $\alpha$ 8-11 peptides using anti-flag antibodies. Precipitated spectrin  $\alpha$ 8-11 peptides  
46 were revealed by western blot with anti-flag antibodies and N-WASP with anti-N-WASP  
47 antibodies. Control with non-immune (NI) serum is shown. **(E)** GH secretory activity was  
48 measured in PC12 cells co-expressing GH and either Flag- $\alpha$ 8-11 or Flag- $\alpha$ 8-11<sup>P1017L</sup>. GH

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3 release is expressed as the percentage of total GH (n = 3). \*\*P<0.01, compared to control cells  
4 (ANOVA).  
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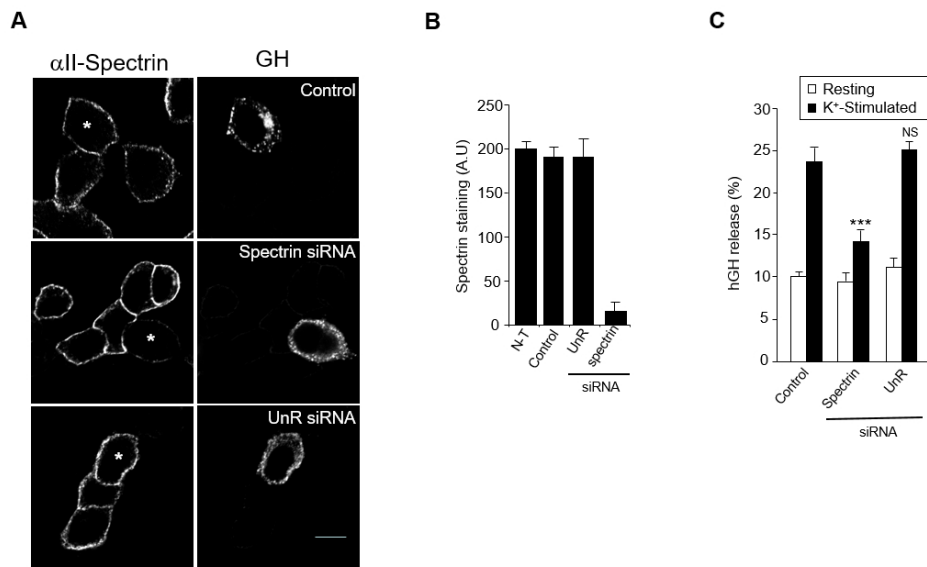


Figure 1, Houy et al.,

Figure1

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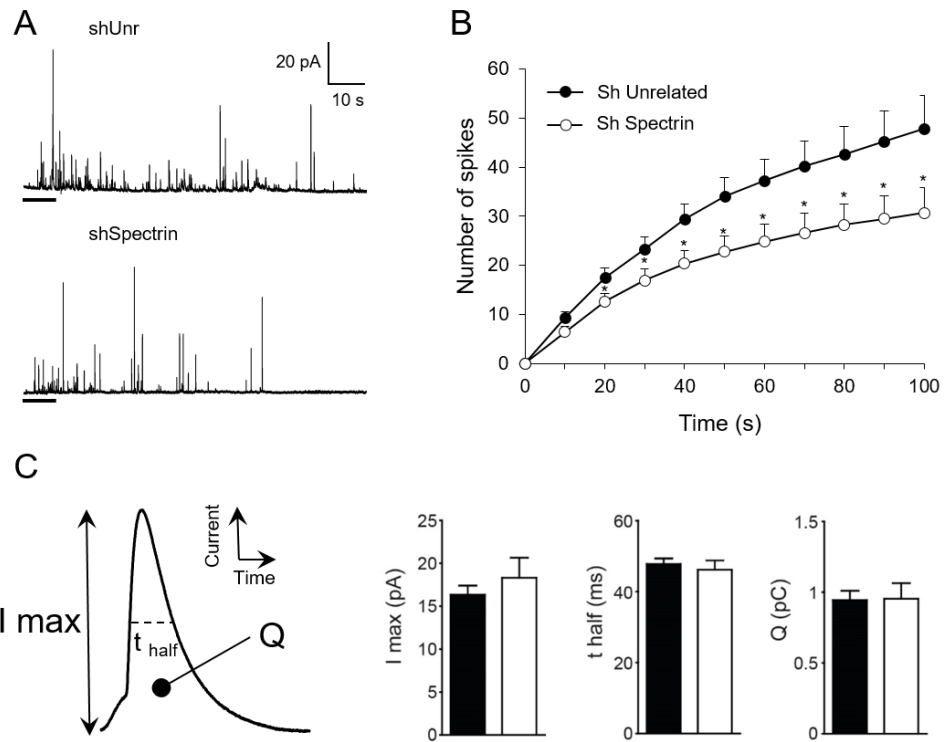
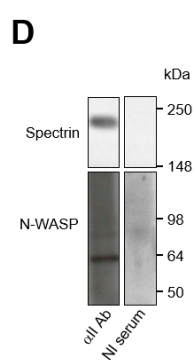
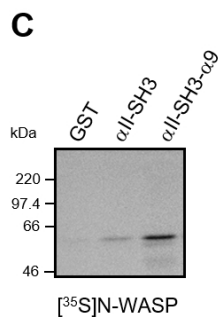
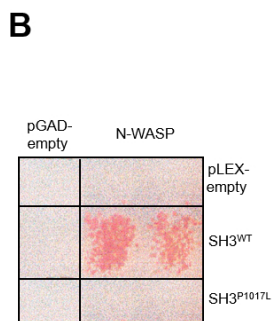
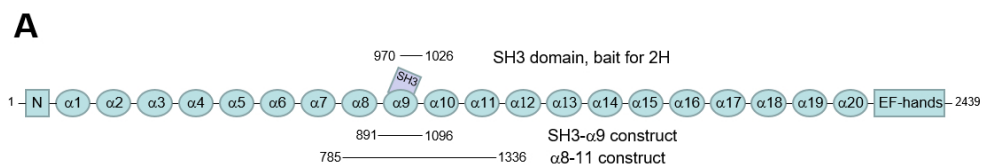


Figure 2, Houy et al.,

Figure2

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**Figure 3, Houy et al.,**

Figure3

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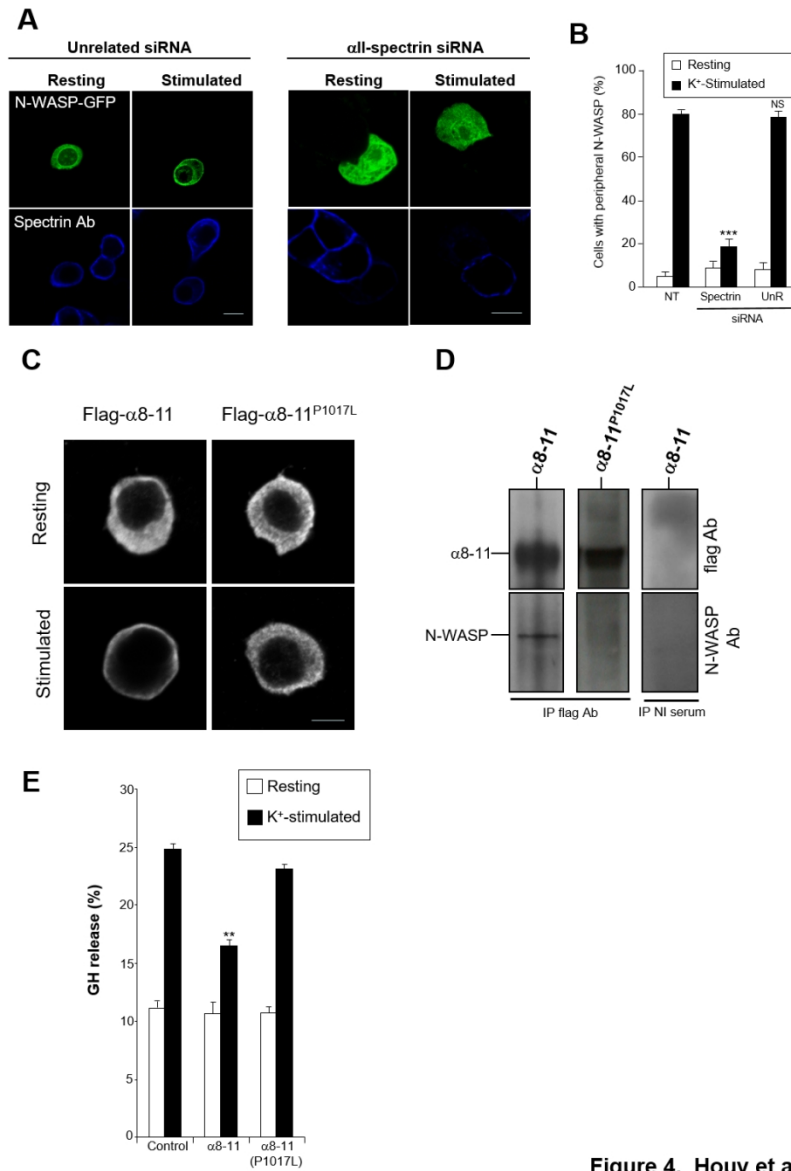


Figure 4, Houy et al.,

revised figure 4

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**Table 1: Table summarizing the amperometric parameters of the exocytic events recorded from chromaffin cells knocked-down for  $\alpha$ II-spectrin.**

Amperometric parameters	Sh Unr	Sh spectrin
Event per cell (n)	47.88 $\pm$ 9.9	30.68 $\pm$ 5.1
Spikes analysed	781	425
Q (pC)	0.94 $\pm$ 0.06	0.95 $\pm$ 0.11
T half (ms)	47.8 $\pm$ 1.5	46.2 $\pm$ 2.6
I max (pA)	16.3 $\pm$ 1.04	18.3 $\pm$ 2.3
Cells (n)	27	22

Value are given as the mean  $\pm$  SEM. Q, quantal size or charge; t half, half-width, I max, spike amplitude.