

# The actions of a novel potent islet beta-cell specific $K_{ATP}$ channel opener can be modulated by syntaxin-1A acting on sulfonylurea receptor 1

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**Abbreviation Footnote:**  $K_{ATP}$ , ATP-sensitive  $K^+$  channel;  $K_{ATP}$  channel opener, KCO; SUR, sulfonylurea receptor; Syn-1A, syntaxin-1A; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; NBF, nucleotide-binding fold; GST, glutathione S-transferase, TMD, transmembrane domain

## Abstract

**Objective:** Islet  $\beta$ -cell-specific  $K_{ATP}$  channel openers (KCOs) thiadiazine dioxides induce islet rest to improve insulin secretion, but their molecular basis of action remains unclear. We reported that Syntaxin-1A (Syn-1A) binds nucleotide binding folds (NBFs) of SUR1 in  $\beta$ -cells to inhibit  $K_{ATP}$  channels. As a strategy to elucidate the molecular mechanism of action of these KCOs, we explored the possibility that NNC 55-0462 might influence Syn-1A-SUR1 interactions or vice versa.

**Research Design and Methods:** Whole-cell and inside-out patch-clamp electrophysiology was utilized to examine the effects of GST-Syn-1A dialysis or GFP/Syn-1A co-transfection on NNC 55-0462 actions. *In vitro* pull-down binding studies were used to examine NNC 55-0462 influence on Syn-1A-SUR1 interactions.

**Results:** Dialysis of GST-Syn-1A into the cell cytoplasm reduced both potency and efficacy of extracellularly perfused NNC55-0462 in a HEK cell-line stably expressing Kir6.2/SUR1 (BA8 cells) and in rat islet  $\beta$ -cells. Moreover, inside-out membrane patches excised from BA8 cells showed that both GST-Syn-1A and its H3 domain inhibited  $K_{ATP}$  channels previously activated by NNC55-0462. This action on  $K_{ATP}$  channels is isoform-specific to Syn-1A, as Syn-2 was without effect. Furthermore, parent compound diazoxide showed similar sensitivity to GST-Syn-1A inhibition. NNC 55-0462 however did not influence Syn-1A-SUR1 binding interaction.

**Conclusion:** Our results demonstrated that Syn-1A interactions with SUR1 at its cytoplasmic domains can modulate the actions of KCOs-NNC 55-0462 and diazoxide on  $K_{ATP}$  channels. The reduced levels of islet Syn-1A in diabetes would thus be expected to exert a positive influence on the therapeutic effects of this class of KCOs.

Chronic exposure to hyperglycemia in diabetes can impair insulin secretory function (1) and accelerate apoptosis (2) in islet  $\beta$ -cells. Expression levels of soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins, the critical machinery for insulin granule exocytosis (3) particularly in first phase secretion (4), are severely reduced in islets of rodent models and human patients with type 2 diabetes (5-8). Hyperglycemia further reduced SNARE protein levels in Goto-Kakizaki (GK) rats, but could be partially restored by normoglycemia (6). Restoration of SNARE protein levels partially normalized insulin secretion in these animal models (6,7). In addition to direct glucotoxic effects, chronic hyperglycemia can lead to  $\beta$ -cell overstimulation (9). Prolonged exposure to high glucose and other secretagogues induces desensitization of islet  $\beta$ -cells to subsequent stimuli, with  $\text{Ca}^{2+}$ -influx-induced toxicity (10) and insulin stores depletion (11) suggested as possible mechanisms. In diabetes, chronic hyperglycemia and insulin resistance increase insulin secretory demand, and failure of  $\beta$ -cells to sustain this compensatory increase in workload may contribute to disease progression (12).

A novel therapeutic approach, therefore, is to pharmacologically induce “ $\beta$ -cell rest” by  $\text{K}_{\text{ATP}}$  channel openers (KCOs) to preserve residual secretory function and insulin stores (13). Pancreatic  $\text{K}_{\text{ATP}}$  channels are octamers of four sulfonylurea receptor-1 (SUR1) subunits in complex with four pore-forming Kir6.2 subunits, which act to modulate cell membrane potential, linking glucose-mediated changes in ATP/ADP ratio to insulin secretion (14,15). The opening of  $\text{K}_{\text{ATP}}$  channels leads to membrane hyperpolarization sufficient to induce  $\beta$ -cell rest. Nonselective KCO diazoxide can protect  $\beta$ -cells of human and rodents against hyperglycemia-induced desensitization *in*

*vitro* (16,17), restoring first phase and pulsatile insulin release (19), and protect against streptozotocin-induced injury (18,19). Remarkably, diazoxide treatment also preserves residual insulin secretion in patients with Type 1 (20,21) and Type 2 (22) diabetes. However, clinical use of diazoxide is limited because of its nonselective opening of extrapancreatic  $\text{K}_{\text{ATP}}$  channels causing cardiovascular side effects (23). Recently, a class of islet  $\beta$ -cell-specific KCOs (thiadiazine dioxides) being developed (24-27) are capable of inducing  $\beta$ -cell rest in rodent (10,28,29) and human islets (30,31), improving insulin stores and secretory responses, and protecting against autoimmune injury in an animal model of type 1 diabetes (32). These  $\beta$ -cell-specific KCOs interact with cytoplasmic and transmembrane domains (TMDs) of the SUR1 subunit, and displace  $^3\text{H}$ -glibenclamide binding to HEK cells stably expressing human Kir6.2/SUR1 (BA8 cells) (24,27). Their precise molecular mechanism of action, however, remains unknown (13).

We have recently reported that plasma membrane-bound SNARE protein Syntaxin-1A (Syn-1A) directly binds the cytoplasmic nucleotide binding folds (NBFs) of SUR proteins of  $\beta$ -cells (SUR1) (33,34) and cardiac myocytes (SUR2A) (35) to modulate  $\text{K}_{\text{ATP}}$  channels. We further demonstrated that the C-terminal H3 domain of Syn-1A is the putative domain mediating  $\text{K}_{\text{ATP}}$  channels inhibition (33). In the present study, we explored for the molecular basis by which a novel islet  $\beta$ -cell (SUR1)-specific KCO, NNC55-0462 (6-chloro-3-(1-methylcyclobutyl)amino-4*H*-thieno[3,2-*e*]-1,2,4-thiadiazine 1,1-dioxide) (FIG.1), modulates  $\text{K}_{\text{ATP}}$  channel opening. NNC55-0462 is very potent in inhibiting insulin release from rat islets, being >100 times more potent than its parent compound diazoxide (27). Using rat islet  $\beta$ -cells and BA8 cells,

we now examined the possibility that NNC55-0462 actions on pancreatic  $K_{ATP}$  channels could modulate or be modulated by Syn-1A-SUR1 interactions.

## Research Design and Methods

**Constructs and recombinant GST-fusion proteins.** The constructs pcDNA3-Syn-2 and pcDNA3-Syn-1A were from Richard Scheller (Genentech, San Francisco, CA), pGEX-4T-1-Syn-1A from W. Trimble (Hospital for Sick Children, Toronto, Canada), and pcDNA3-Syn-1B from G. Zamponi (U. of Calgary, Calgary, Canada). The coding sequences corresponding to Syn-1A (aa 1–266, without TMD), Syn-1A-Habc domain (aa 1-160) and Syn-1A-H3 domain (aa 191-256) were amplified by PCR and cloned into pGEX-4T-1 expression vector for generation of GST fusion proteins. GST fusion protein expression, purification and thrombin (Sigma) cleavage were performed following the manufacturer's instructions (Amersham Biosciences, NJ).

**Cell culture and transfection.** HEK-293 cells stably expressing human Kir6.2/SUR1 (BA8 cells) has been previously characterized (24,27), and cell culture and transfection were performed as previously described (33-35). Refer to Supplementary Appendix Online for detailed description.

**Preparation of rat islet  $\beta$ -cells.** Pancreatic islets were isolated from male Sprague-Dawley rats by collagenase digestion and dispersed into single cells by treatment with 0.05% trypsin in  $Ca^{2+}$ - and  $Mg^{2+}$ -free PBS as previously described (33,34). Islet cells were plated on glass cover-slips in 35mm tissue culture dishes and cultured overnight in RPMI-1640 medium (Invitrogen), containing 2.8mmol/l glucose and supplemented with 7.5% FBS, before electrophysiological recordings.

**Electrophysiology.** Current recordings from single islet  $\beta$ -cells and BA8 cells were performed using standard patch-clamp techniques in the whole-cell and inside-out configurations as previously described (33-35). Refer to Supplementary Appendix online for detailed description.

**In vitro binding assay and Western blotting analysis.** BA8 cells were washed with 1xPBS (pH 7.4) and harvested in binding buffer (25mmol/l HEPES- pH 7.4, 100mmol/l KCl, 1.5% Triton X-100, 2 $\mu$ mol/l pepstatin A, 1 $\mu$ g/ml leupeptin, 10 $\mu$ g/ml aprotinin). The cells were lysed by sonication and insoluble materials removed by centrifugation (55,000xg, 4 $^{\circ}$ C, 30 minutes). The detergent-extract (0.3ml, 1.2 $\mu$ g/ $\mu$ l protein) of BA8 cells was incubated for 2 hours at 4 $^{\circ}$ C with GST (as negative control) or GST-Syn-1A (500pmol protein bound to glutathione agarose beads) in absence or presence of indicated concentrations of NNC55-0462 or untagged Syn-1A and/or ATP. The beads were washed three times with binding buffer and the samples were separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and the proteins were identified with mouse anti-SUR1 antibody (a gift from J. Ferrer, University of Barcelona, Spain) (1:750). For Western blotting analysis of Syn-1A, Syn-1B and Syn-2 expression, whole cell lysate were prepared from rat brain (as positive control), rat islet, Syn-1A or Syn-1B expressing HEK293 cells (as negative or positive control) and rat pancreatic acinar cells (as positive control) by sonication or with homogenizer. Samples were separated by 12% SDS-PAGE and proteins of interest were identified with specific antibodies [Syn-1A and Syn-1B from Synaptic Systems (Goettingen, Germany) and Syn-2 from V. Olkkonen (Helsinki, Finland)].

**Data analysis.** Data analysis and curve fitting were performed using Origin 6.0

(Microcal Software Inc., Northampton, MA). Data are presented as means±S.E.M. Differences in means were compared using either one-way ANOVA followed by Dunnett's post-hoc test, or paired/unpaired Student's t-test, with  $p < 0.05$  considered as statistically significant. Concentration-response curves were fitted to the equation:  $y = \{(A_1 - A_2) / [1 + (X/X_0)^p]\} + A_2$  where  $y$  is the  $K_{ATP}$  current at different [NNC55-0462],  $X$  is the [NNC55-0462] applied externally to the cells,  $X_0$  is the [NNC55-0462] that produced half maximal  $K_{ATP}$  channel activation,  $A_1$  is the  $K_{ATP}$  current before NNC55-0462 was applied,  $A_2$  is the maximal  $K_{ATP}$  current level activated by NNC55-0462, and  $p$  is the slope of the curve.

## Results

**Syn-1A decreases the  $K_{ATP}$  opening action of NNC55-0462 in BA8 cells.** Previously, we had reported that Syn-1A inhibited Kir6.2/SUR1 currents by its actions on NBF-1 and NBF-2 of SUR1 (33,34). Here, we examined the possibility that NNC55-0462, belonging to a class of thiadiazine dioxide KCOs (FIG.1), acting on distinct domains in SUR1 (23,27), might influence the actions of Syn-1A on the NBFs, or vice versa. To examine the direct functional effects of Syn-1A on  $K_{ATP}$  channels, we again first utilized BA8 cells, a HEK cell-line stably expressing human Kir6.2/SUR1 (24,27) that does not contain endogenous Syn-1A (33); and dialyzed into the cytoplasm of these cells soluble GST-Syn-1A lacking its TMD. Here, external perfusion of NNC55-0462 dose-dependently activated Kir6.2/SUR1  $K_{ATP}$  channels, effective starting at  $0.1 \mu\text{mol/l}$  and achieving maximum effect at  $1 \mu\text{mol/l}$  (FIG.2A). Higher concentration of NNC55-0462 ( $3 \mu\text{mol/l}$ ) did not elicit a further increase in channel amplitude, but instead induced channel run-down. GST-Syn-1A ( $100 \text{ nmol/l}$ , FIG.2B), when compared to GST control

(Fig. 2A), reduced the ability of NNC55-0462 to open  $K_{ATP}$  channels. We analyzed the data in several ways to show that Syn-1A ( $10$  and  $100 \text{ nmol/l}$ ) reduced both drug potency (FIG.2C) and efficacy (FIG.2D).

FIG.2C examines drug potency, where dose-response curves were normalized to each group's maximal channel activation at  $1 \mu\text{mol/l}$  NNC55-0462, which demonstrated a rightward shift in potency caused by GST-Syn-1A dialysis. This is further illustrated in FIG.2E (*open bars*, left axis) showing  $EC_{50}$  of NNC55-0462 in GST control group was  $0.11 \pm 0.02 \mu\text{mol/l}$  ( $n=7$ ), while GST-Syn-1A dialysis at  $10 \text{ nmol/l}$  and  $100 \text{ nmol/l}$  increased it to  $0.15 \pm 0.05 \mu\text{mol/l}$  ( $n=5$ , not significant) and  $0.21 \pm 0.04 \mu\text{mol/l}$  ( $n=7$ ,  $p < 0.05$ ), respectively. FIG.2D examines drug efficacy, where dose-response curves were normalized to maximum channel activation observed with GST-dialyzed control group, which demonstrated a downward shift in efficacy caused by GST-Syn-1A dialysis. This is further illustrated in FIG.2E (*filled bars*, right axis), where maximum current density activated by NNC55-0462 was  $283 \pm 14 \text{ pA/pF}$  ( $n=7$ ) with GST dialysis, while Syn-1A dialysis at  $10 \text{ nmol/l}$  and  $100 \text{ nmol/l}$  decreased it to  $256 \pm 28 \text{ pA/pF}$  ( $n=5$ , not significant) and  $194 \pm 12 \text{ pA/pF}$  ( $n=7$ ,  $p < 0.01$ ), respectively, with the latter being a significant reduction of  $31 \pm 4\%$ .

To reiterate, these modulatory effects of Syn-1A on effectiveness of NNC55-0462 on BA8  $K_{ATP}$  channels are due to direct interactions with the SUR1 protein, since BA8 cells are devoid of endogenous Syn-1A and Syn-1A interacting proteins (33). Note that the concentrations of GST-Syn-1A required likely represent an overestimation due to suboptimal targeting of soluble GST-Syn-1A to the sites of  $K_{ATP}$  channels on the plasma membrane.

**Syn-1A decreases the  $K_{ATP}$  opening action of NNC55-0462 in rat islet  $\beta$ -cells.** To

demonstrate the physiological implication of the BA8 study, we performed similar studies on rat islet  $\beta$ -cells. NNC55-0462 dose-dependently activated  $K_{ATP}$  channel activity in rat islet  $\beta$ -cells, effective starting at  $0.03\mu\text{mol/l}$ , with maximal effects reached at  $1\mu\text{mol/l}$ . Like BA8 cells,  $\beta$ -cell  $K_{ATP}$  channels displayed run-down upon further perfusion with supramaximal concentration of NNC55-0462 at  $3\mu\text{mol/l}$ . The  $\beta$ -cell currents were sensitive to tolbutamide inhibition applied in the presence of  $3\mu\text{mol/l}$  NNC55-0462, confirming the identity of activated currents to be  $I_{KATP}$ . KCOs have negative allosteric effects on glibenclamide binding to SUR (36,37). In fact, such SUR1-specific KCOs could displace  $^3\text{H}$ -glibenclamide binding (24), which taken along with the high affinity of NNC55-0462 for SUR1, led us to use a higher concentration of tolbutamide to inhibit the channels.

Consistent with the BA8 results (FIG.2), both potency (FIG.3C) and efficacy (FIG.3D) of NNC55-0462 in opening islet  $\beta$ -cell  $K_{ATP}$  channels were decreased by dialysis of GST-Syn-1A into the  $\beta$ -cell cytoplasm. Here, GST-Syn-1A ( $1\mu\text{mol/l}$ ) increased  $EC_{50}$  of NNC55-0462 from  $0.050\pm 0.009\mu\text{mol/l}$  ( $n=10$ ) to  $0.11\pm 0.03\mu\text{mol/l}$  ( $n=7$ ,  $p<0.05$ ) (FIG.3E, *open bars*, left axis). With respect to efficacy, the maximum current density activated by NNC55-0462 was suppressed by GST-Syn-1A dialysis from  $157\pm 8\text{pA/pF}$  ( $n=10$ ) to  $110\pm 4\text{pA/pF}$  ( $n=7$ ,  $p<0.01$ ), which is a  $30\pm 3\%$  reduction (FIG.3E, *filled bars*, right axis). It is possible that soluble GST-Syn-1A lacking the TMD could bind a number of Syn-1A binding proteins in  $\beta$ -cells that might have independent effects on  $K_{ATP}$  channels, or alternatively sequester Syn-1A (i.e. endogenous v-SNAREs on insulin granules) to reduce the amount of GST-Syn-1A available for  $K_{ATP}$  channel interaction. Although we could not rule out these possibilities, their contributions are not likely to be significant in our study, as the results of

dialyzed Syn-1A on  $\beta$ -cell KCO potency and efficacy were remarkably similar to those of BA8 cells, albeit the  $K_{ATP}$  channel density is much higher in  $K_{ATP}$ -overexpressing BA8 cell line.

**Syn-1A, but not Syn-2, reduces the efficacy of NNC55-0462 in BA8 cells.** It is possible that other Syntaxins in the  $\beta$ -cell (38) might have similar actions as Syn-1A on  $K_{ATP}$  channels. Syn-1B and Syn-2 exhibit the highest homology to Syn-1A of  $>90\%$  and  $>70\%$ , respectively (39). Using Syntaxin-isoform specific antibodies and overexpressed proteins as controls, FIG.4A shows that Syn-2 is abundant in rat islets, but not Syn-1B, the latter confirming a previous report (40). We therefore proceeded to explore the specificity of action of Syn-1A on NNC55-0462-induced  $\beta$ -cell  $K_{ATP}$  channel opening by comparing the effects of Syn-1A versus Syn-2.

BA8 cells were transiently transfected with GFP alone as control, or co-transfected with full-length (including TMD) Syn-1A or Syn-2 to localize the expressed Syntaxin to the plasma membrane where  $K_{ATP}$  channels are located. FIG.4B shows representative current-voltage relationships demonstrating that maximum current density activated by  $1\mu\text{mol/l}$  NNC55-0462 was specifically reduced in Syn-1A- but not Syn-2-transfected group. Activated currents were confirmed to be of  $I_{KATP}$  by their sensitivity to tolbutamide inhibition (FIG.4C), applied in the presence of NNC55-0462 after maximal channel activation has occurred. FIG.4D summarizes these results showing that maximum current density activated by NNC55-0462 (at  $-120\text{mV}$ ) was reduced by GFP/Syn-1A transfection to  $206\pm 14\text{pA/pF}$  ( $n=10$ ,  $p<0.01$ ) from  $323\pm 14\text{pA/pF}$  in the GFP control group ( $n=9$ ), which is a  $\sim 36\%$  reduction. The lack of effect of GFP/Syn-2 transfection on  $K_{ATP}$  channel opening ( $349\pm 17\text{pA/pF}$ ,  $n=11$ , not significant) despite its high homology to Syn-1A indicate that Syn-1A effects on  $K_{ATP}$

channels are isoform-specific within the syntaxin family in  $\beta$ -cells.

**Syn-1A reverses the  $K_{ATP}$  opening action of NNC55-0462 in inside-out membrane patches.** We employed the inside-out patch configuration on BA8 cells (FIG.5) for patch-clamp recording to examine: 1) if NNC55-0462 can still open  $K_{ATP}$  channels when applied intracellularly, 2) if Syn-1A could influence these NNC55-0462 actions, and 3) which domain within Syn-1A (H3 vs Habc) effects these actions. Membrane patches were first exposed to 0 and 3mmol/l MgATP to confirm ATP-sensitivity of currents recorded. Patches were then held at 0.3mmol/l MgATP to produce partial channel inhibition and to prevent rapid channel run-down. NNC55-0462 (0.3 $\mu$ mol/l) was applied in 0.3mmol/l MgATP environment to induce channel opening, after which GST, GST-Syn-1A, GST-Syn-1A-Habc or GST-Syn-1A-H3 was applied to the solution. NNC55-0462 was able to induce  $K_{ATP}$  channel opening when applied on the intracellular side of the membrane, achieving a remarkable >80% of maximum channel opening at 0.3 $\mu$ mol/l (FIG.5A-5D). Application of GST (FIG.5A) or GST-Syn-1A-Habc (300nmol/l, FIG.5C) had no effect on NNC55-0462-mediated channel opening, whereas GST-Syn-1A (FIG.5B) and GST-Syn-1A-H3 (300nmol/l, FIG.5D) significantly suppressed the current activity activated by NNC55-0462. FIG.5E summarizes the data showing that percentage of maximal current activated by NNC55-0462 in presence of GST (92 $\pm$ 13%, n=9) was not significantly different from NNC55-0462 alone (84 $\pm$ 11%), whereas NNC55-0462 plus GST-Syn-1A showed a current of 39 $\pm$ 6% (n=9), which was a significant reduction of ~52% (p<0.01) from NNC55-0462 alone (91 $\pm$ 10%). While the percentage of current activation by NNC55-0462 plus GST-Syn-1A-Habc (88 $\pm$ 10%, n=6) was not significantly different from NNC55-0462

alone (93 $\pm$ 10%), NNC55-0462 plus GST-Syn-1A-H3 caused a similar (to GST-Syn-1A) ~54% reduction of current (38 $\pm$ 7%, n=6, p<0.01) compared to NNC55-0462 alone (92 $\pm$ 6%). These results indicate 1) NNC55-0462 can access its site of action on  $K_{ATP}$  channels when applied intracellularly, and 2) H3 domain of Syn-1A is the putative Syn-1A domain inhibiting  $K_{ATP}$  channels previously activated by this KCO.

**Syn-1A decreases the  $K_{ATP}$  opening action of diazoxide in rat islet  $\beta$ -cells.** We next examined whether the effect of Syn-1A is specific to this particular KCO compound. We chose to study the parent compound, diazoxide, which shares the backbone structure as NNC55-0462 (FIG.1) but interacts with SUR1 at slightly different domains. We repeated the experiment performed with NNC55-0462 on rat islet  $\beta$ -cells, but perfusing increasing concentrations of diazoxide (3–300 $\mu$ mol/l) instead. Similar to the NNC55-0462 study (FIG.3), GST-Syn-1A (FIG.6B) dialyzed into the cytoplasm of rat islet  $\beta$ -cells significantly reduced the potency and efficacy of diazoxide, when compared to GST dialysis (FIG.6A). Analysis of drug potency (FIG.6C and FIG.6E, *open bars*, left axis) shows EC<sub>50</sub> of diazoxide was increased from 21 $\pm$ 5 $\mu$ mol/l (n=10) to 42 $\pm$ 7 $\mu$ mol/l (n=7, p<0.05) by GST-Syn-1A dialysis. Analysis of drug efficacy (FIG.6D, and FIG.6E, *filled bars*, right axis) shows maximum current density activated by diazoxide was reduced by GST-Syn-1A from 136 $\pm$ 4pA/pF (n=10) to 92 $\pm$ 5pA/pF (n=7, p<0.01), which is a ~32% inhibition. These results together suggest that the effect of Syn-1A on KCOs opening of  $K_{ATP}$  channels is not specific to NNC55-0462 alone, but rather shared amongst different dioxide compounds.

**NNC55-0462 does not affect Syn-1A-SUR1 binding interactions.** Since we showed that NNC55-0462 was effective when applied not

only extracellularly but also intracellularly, the latter suggests the possibility that this KCO might share common binding domains with Syn-1A on SUR1 NBFs to influence  $K_{ATP}$  channel opening. We therefore examined whether this KCO could affect Syn-1A-SUR1 binding interactions. GST-Syn-1A bound to glutathione agarose beads was able to pull down the same amount of SUR1 protein from BA8 cell lysate extract with increasing NNC55-0462 concentrations (up to 10 $\mu$ mol/l) both in absence (FIG.7A) or presence of ATP (0.3mmol/l- FIG.7B, 5mmol/l- FIG.7C). ATP at 5mmol/l appeared to reduce the amount of SUR1 pulled down by GST-Syn-1A at all concentrations of NNC55-0462 (FIG.7C). A positive control with increasing concentrations of thrombin-cleaved Syn-1A (instead of NNC55-0462) showed that the assay employed is indeed capable of detecting competitive binding inhibition (FIG.7D). A negative control experiment showed that GST bound to beads could not pull down SUR1, representing nonspecific binding (FIG.7E); however, glutathione agarose beads alone did bind a small amount of SUR1 (~20% of that bound by GST-Syn-1A). This inability of NNC55-0462 to modulate Syn-1A binding to SUR1 suggests that this KCO does not likely bind the same domains that Syn-1A binds to in SUR1 at its NBFs (33,34). The ability of Syn-1A to block this KCO action on  $K_{ATP}$  channel suggests that Syn-1A could modulate NNC55-0462 binding to the SUR1 protein. However, due to the lack of a radiolabeled NNC55-0462, we were unable to investigate this possibility.

## Discussion

The current study is the first to demonstrate an effect of SNARE protein Syn-1A on the actions of NNC55-0462, a member of the novel class of thiadiazine dioxide KCOs, and its parent compound diazoxide. Our results showed that Syn-1A reduces the

potency, and more significantly the efficacy, of NNC55-0462 and diazoxide. In inside-out membrane patches, Syn-1A can also inhibit channels previously activated by NNC55-0462. Within the Syntaxin family present in  $\beta$ -cells, these actions were specific to Syn-1A, as Syn-2 which shares >70% homology with Syn-1A (39), had no effect on  $K_{ATP}$  channels. We further determined that H3 domain and not Habc domain to be the putative Syn-1A domain that modulated the KCO's actions on the  $K_{ATP}$  channel. Since Syn-1A also influenced the  $K_{ATP}$  channel opening actions of diazoxide, a non-specific KCO, this would suggest that Syn-1A might also influence the actions of other KCOs acting on SUR2 proteins, which will require further study.

The isoform specificity of Syn-1A's inhibition on NNC55-0462-mediated  $K_{ATP}$  channel opening is in line with previous studies examining other Syn-1A-ion channel interactions. Studies with Lc- and N-type calcium channels showed similar results, with the basis of such specificity mapped out to two critical cysteine residues found in Syn-1A but not in Syn-2 (41). However, these cysteine residues on the TMD of Syn-1A are unlikely candidates for the basis of its actions towards  $K_{ATP}$  channel modulation for the following reasons: 1) Soluble GST-Syn-1A fusion protein lacking the TMD inhibited NNC55-0462-mediated  $K_{ATP}$  channel opening as efficaciously as expression of the full length Syn-1A, and 2) Soluble GST-Syn-1A-H3 domain induced a similar inhibition as Syn-1A, consistent with our previous study which mapped out the NBF-interacting domain to be the H3 domain (33). Nonetheless, that report (41) does point out that differences as small as one to two amino acid residues are enough to produce isoform-specific action. Moreover, a study of Syn-1A versus Syn-1B, which shares 93% homology, shows that only Syn-1A is capable of mediating tonic voltage-dependent inhibition

on N-type calcium channels (42). With regards to other  $K^+$  channels, we previously reported that Syn-1A, but not Syn-2, reduced Kv2.1 current density by direct actions on specific Kv2.1 cytoplasmic domains and by inhibiting channel trafficking (43).

Although the mechanisms of action of Syn-1A on calcium channel regulation have been well characterized, those involved in  $K_{ATP}$  channel modulation are less clear. We postulated three possibilities: 1) Syn-1A may influence these KCOs' interaction with SUR1, or 2) conversely, these KCOs may influence Syn-1A interactions with SUR1, or 3) Syn-1A may influence the mechanism of action of KCOs by interfering with adenosine nucleotide binding and/or hydrolysis.

For the first possibility, Syn-1A interacting with the NBFs may induce conformational changes to SUR1 that affect the affinity of NNC55-0462 to its binding sites. Unfortunately, NNC55-0462 could not be labeled to directly test this possibility. Nonetheless, direct competitive inhibition is unlikely due to the inability of increasing concentrations of NNC55-0462 to overcome the reduced efficacy caused by Syn-1A. The SUR1 domains critical to the actions of a closely-related compound, NNC55-9216, were mapped out to TMDs 8-11 and both NBFs (24). Since Syn-1A can bind both NBFs of SUR1 (33,34), such interactions may induce negative allosteric modulation onto the KCO binding sites of SUR1, thereby decreasing the KCO binding affinity. In addition, previous studies have determined the sites of action of diazoxide to be TMDs 6-11 and NBF-1 (44). Therefore, it is not surprising that Syn-1A is capable of influencing the effects of both KCOs, due to potential overlapping binding or signal transduction sites. Further mutational studies will be required to elucidate these sites.

For the second possibility, NNC55-0462 may act to relieve Syn-1A inhibition on  $K_{ATP}$  channels, and thus, increased levels of

Syn-1A would reduce the effectiveness of this KCO. Our *in vitro* binding studies showed that increasing concentrations of NNC55-0462 did not affect Syn-1A binding to SUR1 protein, thus eliminating this possibility.

For the third possibility, the binding and activity of a wide range of KCOs are dependent on the presence of  $Mg^{2+}$  and hydrolysable ATP (45-47), and a postulated mechanism of action of these compounds is the acceleration of nucleotide hydrolysis by NBFs (48). Syn-1A may affect KCOs by interfering with this mechanism, either via slowing the rate of nucleotide hydrolysis, or by affecting the binding of MgATP or MgADP to their respective NBFs. In support, NNC55-9216 required both NBFs (24) whereas diazoxide required NBF-1 to exert their  $K_{ATP}$  channel opening action (44). Our *in vitro* binding study revealed reduced Syn-1A binding to SUR1 when MgATP concentration was increased to 5 mmol/l, which suggests competitive binding displacement at the NBFs. Should Syn-1A influence the efficacy of these KCOs via this postulated mechanism, further modulating effects may be observed with combinations of adenosine nucleotides and  $Mg^{2+}$ . These complex interactions are not within the scope of the present study and will require much further study.

Our previous report on a Syn-1A-overexpressing mice showed that a moderate increase of ~30% of islet Syn-1A levels was sufficient to cause hyperglycemia from reduction in  $\beta$ -cell insulin exocytosis and inhibition of L-type  $Ca^{2+}$  current density (49). However, there was no effect on  $K_{ATP}$  or Kv2.1 channels, suggesting that these distinct components of the insulin secretory process exhibit differing sensitivity to Syn-1A modulation. This also suggests that moderate increases in Syn-1A levels beyond normal islet levels (which do not occur physiologically) are not likely to influence KCO actions.

The converse however may not be true – that is when islet Syn-1A levels become reduced. Alemzadeh *et al.* reported that 6-weeks treatment with NN414, a SUR1-specific KCO of the same family as NNC-55-0462, led to improvements in fasting and post-IPGTT glucose levels in Zucker obese diabetic rats but not lean control rats, which was postulated to be due to lower insulin levels in lean animals during basal conditions and post-NN414 treatment (50). We propose an alternative explanation. Islet Syn-1A levels are severely reduced in type 2 diabetic rodent models and human patients (% of normal controls: obese Zucker rats: ~35%; GK rats: ~40%; human: ~20%) (5-8). This may at least partly explain the increased effectiveness of KCO therapy in the Zucker rats (50) by enhancing KCO potency and efficacy on  $\beta$ -cells, whereas normal islet levels of Syn-1A would dampen the KCO actions. Since brain levels of Syn-1A are high and do not fluctuate with diabetes (6), the KCO would not affect neuronal SUR1  $K_{ATP}$  channels, thereby increasing drug's specificity. Normoglycemic control partially restored islet Syn-1A levels in GK rats (6), which could dampen the KCO effects that

may be misconstrued as loss of drug effectiveness. These dynamics of islet Syn-1A expression occurring during the course of therapy may profoundly modulate KCO drug potency and efficacy on  $\beta$ -cells, rendering the drug more effective at stages of severe diabetes while dampening its actions as normoglycemic control is gradually attained. These exciting possibilities will require further experimental verification in diabetic rodent models.

### **Acknowledgements**

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## Figure Legends

**FIG. 1. Structure of NNC 55-0462 (6-chloro-3-(1-methylcyclobutyl) amino-4H-thieno[3,2-e]-1,2,4-thiadiazine 1,1-dioxide) compared with diazoxide.**

**FIG. 2. Syn-1A reduces potency and efficacy of NNC 55-0462 (NNC) on BA8  $K_{ATP}$  channels.** Shown in *A* and *B* are representative recordings of whole-cell  $K^+$  currents from BA8 cells subjected to extracellular perfusion of increasing NNC 55-0462 concentrations with 100 nmol/l GST (*A*) or 100 nmol/l GST-Syn-1A (*B*) dialysis. Currents were evoked by 500 ms hyperpolarizations to -120 mV in 10 seconds intervals from a holding potential of -70 mV. Intracellular solution contains 1 mmol/l MgATP. *C*: Summary of results with each curve (100 nmol/l GST-control, 10 nmol/l and 100 nmol/l GST-Syn-1A) normalized to its own maximum current level ( $I_{max}$ ) to illustrate shifts in potency. *D*: Summary of results with each curve normalized to the maximum current level of GST-control group ( $I_{max-GST}$ ) to illustrate changes in efficacy. *E*: Bar diagram summary showing GST-Syn-1A dialysis increased  $EC_{50}$  (*open bars*, left axis) and reduced efficacy (*filled bars*, right axis) of NNC 55-0462 on BA8  $K_{ATP}$  channel currents. Results are presented as means  $\pm$  S.E.M. of 5 – 7 cells in each group. One-way ANOVA followed by Dunnett's post-hoc test was used for statistical analysis (\* $p < 0.05$  compared to GST control group).

**FIG. 3. Syn-1A reduces potency and efficacy of NNC 55-0462 on rat islet  $\beta$ -cell  $K_{ATP}$  channels.** Rat islet  $\beta$ -cells were subjected to the same experimental protocol as BA8 cells in FIG. 2. Tolbutamide (Tolb.) was applied at the end of the experimental protocol, in the presence of the final dose of NNC 55-0462. *A*: Representative trace showing the effect of GST (1  $\mu$ mol/l) dialysis into the cell cytoplasm. *B*: Representative trace showing the effect of GST-Syn-1A (1  $\mu$ mol/l) dialysis into the cell cytoplasm. *C*: Summary of results with each curve (GST and GST-Syn-1A dialysis) normalized to its own maximum current level ( $I_{max}$ ) to illustrate a shift in potency. *D*: Summary of results with each curve normalized to the maximum current level of GST-control group ( $I_{max-GST}$ ) to illustrate changes in efficacy. *E*: Bar diagram summary showing GST-Syn-1A dialysis increased  $EC_{50}$  (*open bars*, left axis) and reduced efficacy (*filled bars*, right axis) of NNC 55-0462 on  $\beta$ -cell  $K_{ATP}$  channel currents. Results are presented as means  $\pm$  S.E.M. from 7 – 10 cells. Unpaired student's t-test was used for statistical analysis (\* $p < 0.05$ ).

**FIG. 4. Syn-1A, but not Syn-2, reduces efficacy of NNC 55-0462 in BA8 cells.** *A*. Syn-1A (*left upper panel*) and Syn -2 (*right panel*), but not Syn-1B (*left lower panel*), are expressed in rat pancreatic islets (30  $\mu$ g protein). Lysates were separated by 12% SDS-PAGE and probed with Syntaxin-isoform specific antibodies as indicated. Positive controls used include rat brain (2  $\mu$ g), HEK293 cells (15  $\mu$ g) overexpressing Syn-1A or Syn-1B, and pancreatic acinar cells (15 $\mu$ g, for Syn-2). Shown are representative blots of three similar experiments. Since Syn-1A and Syn-2 are present in rat islets which could modulate  $\beta$ -cell  $K_{ATP}$  channels, we next performed whole-cell recordings in BA8 cells transiently transfected with either GFP alone or co-transfected with Syn-1A or Syn-2 (*B-D*). *B*: Representative current-voltage (I-V) relationships, stepped from -140 mV to -20 mV at 20 mV intervals every 2 seconds from a holding potential of -70 mV, were recorded when maximum channel activation has occurred at 1  $\mu$ mol/l perfusion of NNC 55-0462. *C*: Representative I-V traces showing that currents activated were sensitive to tolbutamide

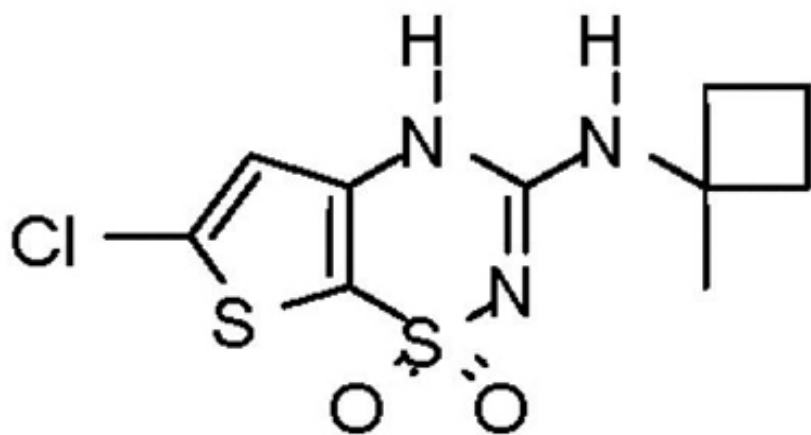
inhibition (3 mmol/l), applied in the presence of 1  $\mu\text{mol/l}$  NNC 55-0462 after maximum channel activation in the same experiment. **D:** Summary of results showing that Syn-1A transfection, but not Syn-2, reduced the maximum current density activated by NNC 55-0462 recorded at -120 mV ( $I_{-120 \text{ mV}}$ ). Results are presented as means  $\pm$  S.E.M. from 9 – 11 cells. One-way ANOVA followed by Dunnett's post-hoc test was used for statistical analysis (\* $p < 0.05$  compared to GST control group).

**FIG. 5. Syn-1A inhibits NNC 55-0462-activated  $K_{\text{ATP}}$  channels in inside-out membrane patches of BA8 cells.** Macroscopic currents were recorded from inside-out membrane patches of BA8 cells held at -100 mV in symmetrical 140 mmol/l  $K^+$  conditions. **A-D:** Representative traces showing the effect of GST (**A**), GST-Syn-1A (**B**), GST-Syn-1A-Habc (**C**) and GST-Syn-1A-H3 (**D**), 300 nmol/l each, on 0.3  $\mu\text{mol/l}$  NNC 55-0462 (NNC)-activated  $K_{\text{ATP}}$  currents. **E:** Summary of results presented as the means  $\pm$  S.E.M. with  $n = 6 - 9$  in each group. Paired student's t-test was used for statistical analyses (\* $p < 0.05$ ).

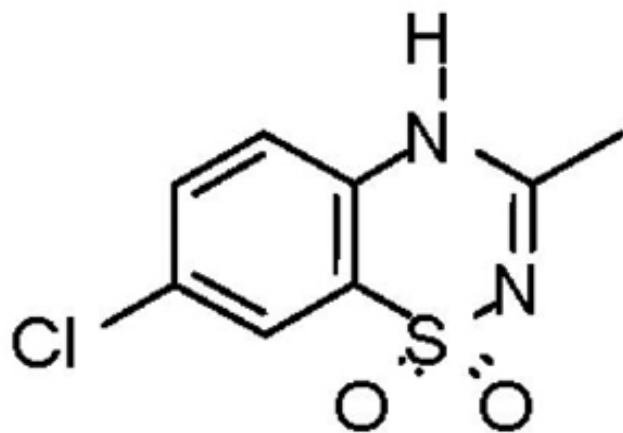
**FIG. 6. Syn-1A reduces potency and efficacy of diazoxide (DZX) on rat islet  $\beta$ -cell  $K_{\text{ATP}}$  channels.** Rat islet  $\beta$ -cells were subjected to the same experimental protocol as BA8 cells in FIG. 2. Tolbutamide (Tolb.) was applied at the end of the experimental protocol, in the presence of the final dose of DZX. **A:** Representative trace showing the effect of GST (1  $\mu\text{mol/l}$ ) dialysis into the cell cytoplasm. **B:** Representative trace showing the effect of GST-Syn-1A (1  $\mu\text{mol/l}$ ) dialysis into the cell cytoplasm. **C:** Summary of results with each curve (GST and GST-Syn-1A dialysis) normalized to its own maximum current level ( $I_{\text{max}}$ ) to illustrate a shift in potency. **D:** Summary of results with each curve normalized to the maximum current level of GST-control group ( $I_{\text{max-GST}}$ ) to illustrate changes in efficacy. **E:** Bar diagram summary showing GST-Syn-1A dialysis increased  $EC_{50}$  (*open bars*, left axis) and reduced efficacy (*filled bars*, right axis) of diazoxide on  $\beta$ -cell  $K_{\text{ATP}}$  channel currents. Results are presented as means  $\pm$  S.E.M. from 7 – 10 cells. Unpaired student's t-test was used for statistical analysis (\* $p < 0.05$ ).

**FIG. 7. NNC 55-0462 (NNC) does not influence Syn-1A binding to SUR1 protein.** GST-Syn-1A (2  $\mu\text{mol/l}$ ), bound to glutathione agarose beads, was incubated with BA8 cell lysate extract in the presence of increasing concentration of NNC 55-0462 at 0 (**A**), 0.3 (**B**) or 5 mmol/l ATP (**C**). (**D**) and (**E**) were performed as positive and negative control experiments, respectively. **D:** Increasing concentrations of thrombin-cleaved Syn-1A prevent Syn-1A bound to beads from pulling down SUR1. **E:** GST bound to beads does not pull down SUR1 compared to the strong binding of SUR1 to Syn-1A bound to beads. In these experiments, the proteins bound to beads were eluted, separated on SDS-PAGE, and probed with anti-SUR1 antibody. *Top panels* show representative blots, and *lower panels* show summaries of 3-4 experiments (mean  $\pm$  S.E.M.) expressed as a percentage of the control binding value determined at 0 ATP, in the absence of NNC 55-0462 (**A-C**) or thrombin-cleaved Syn-1A (**D**); and in (**E**), as a percentage of GST-Syn-1A bound to glutathione agarose beads. Molecular mass markers (kD) are indicated on the left. C indicates control lane.

Figure 1



NNC 55-0462



Diazoxide

**Figure 2**

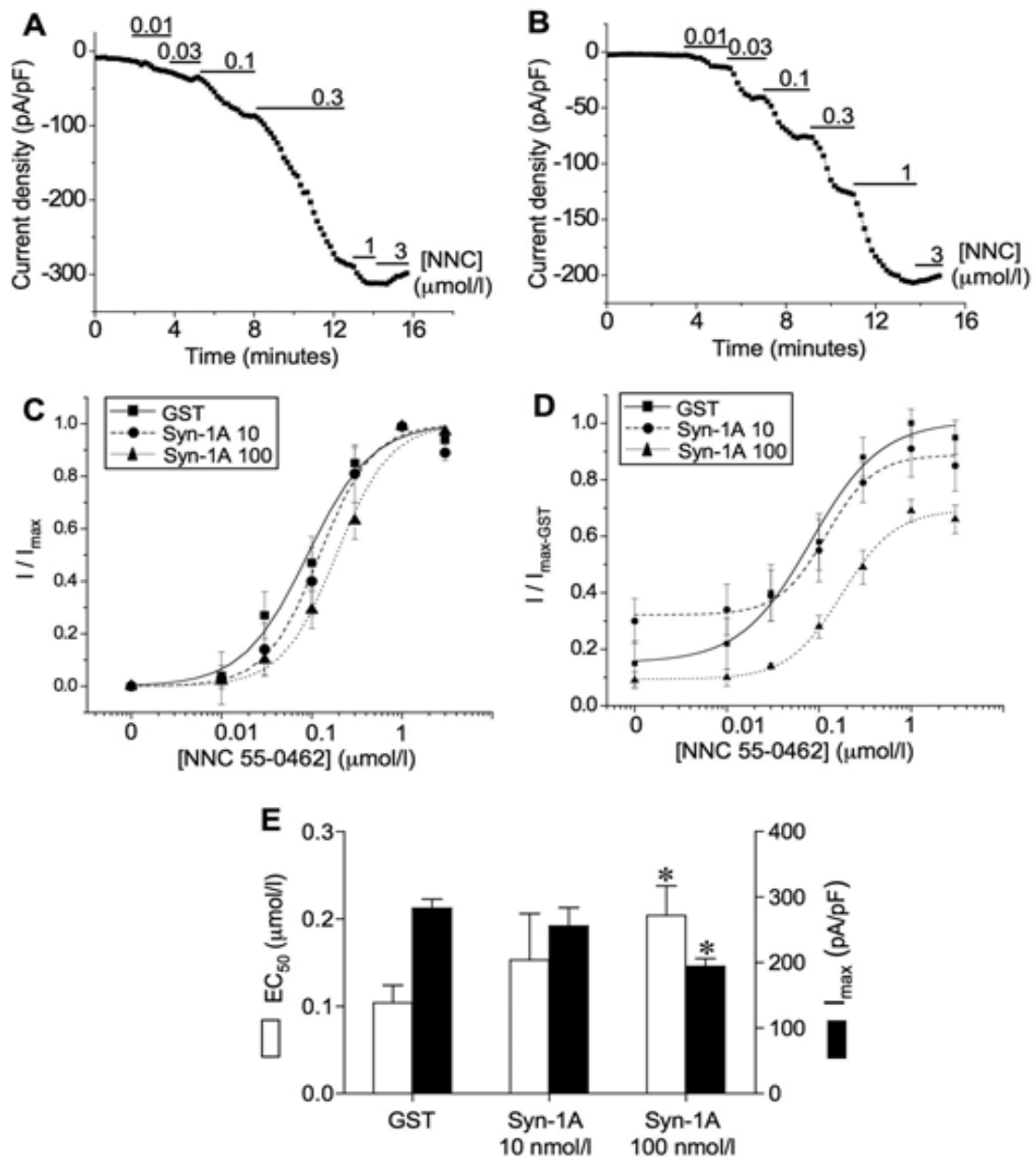


Figure 3

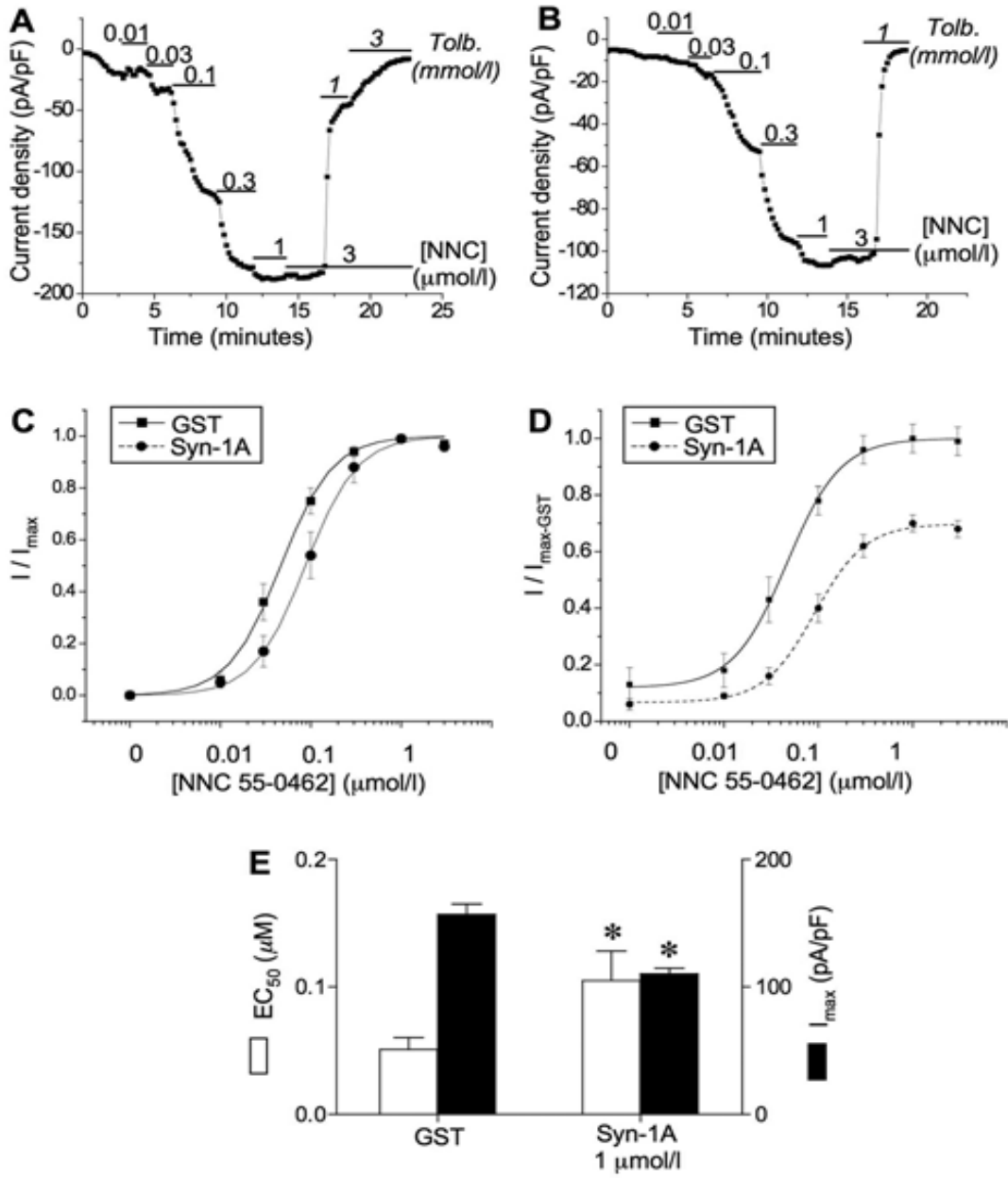
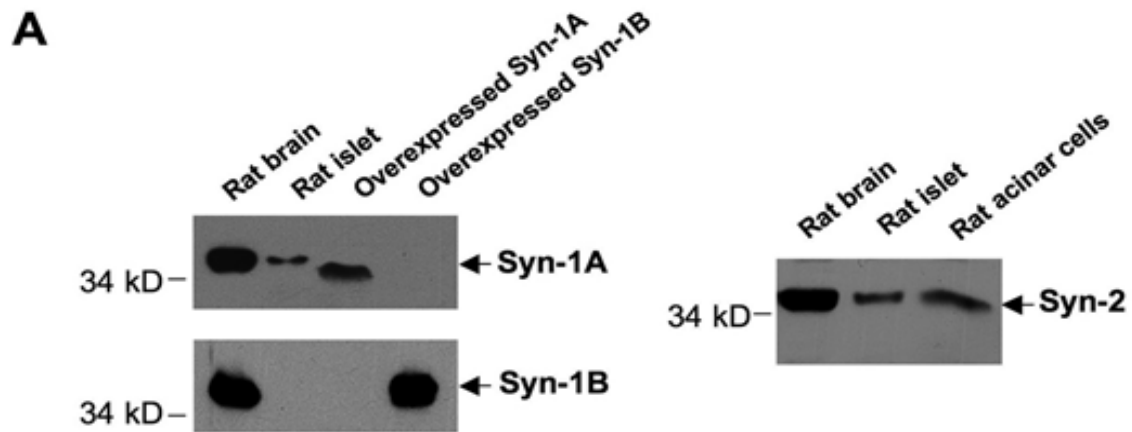
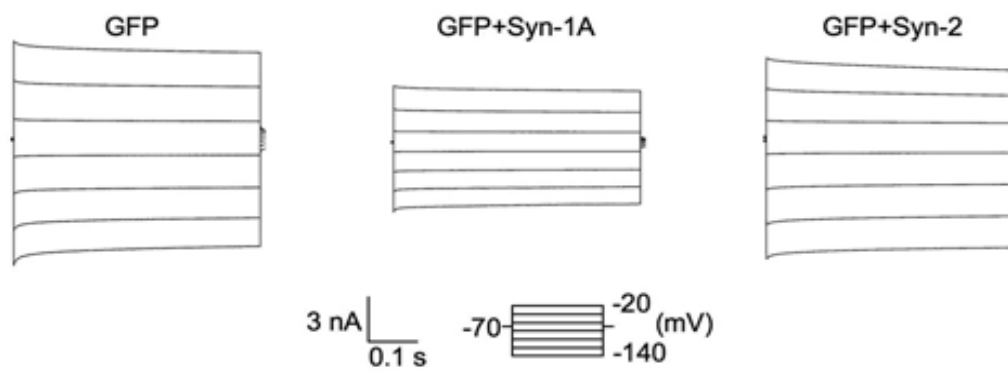


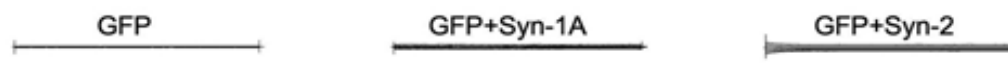
Figure 4



**B** NNC 55-0462 1  $\mu\text{mol/l}$



**C** NNC 55-0462 1  $\mu\text{mol/l}$  + Tolbutamide 3 mmol/l



**D**

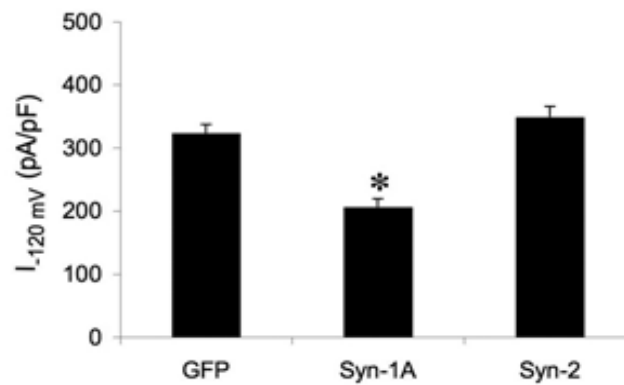


Figure 5

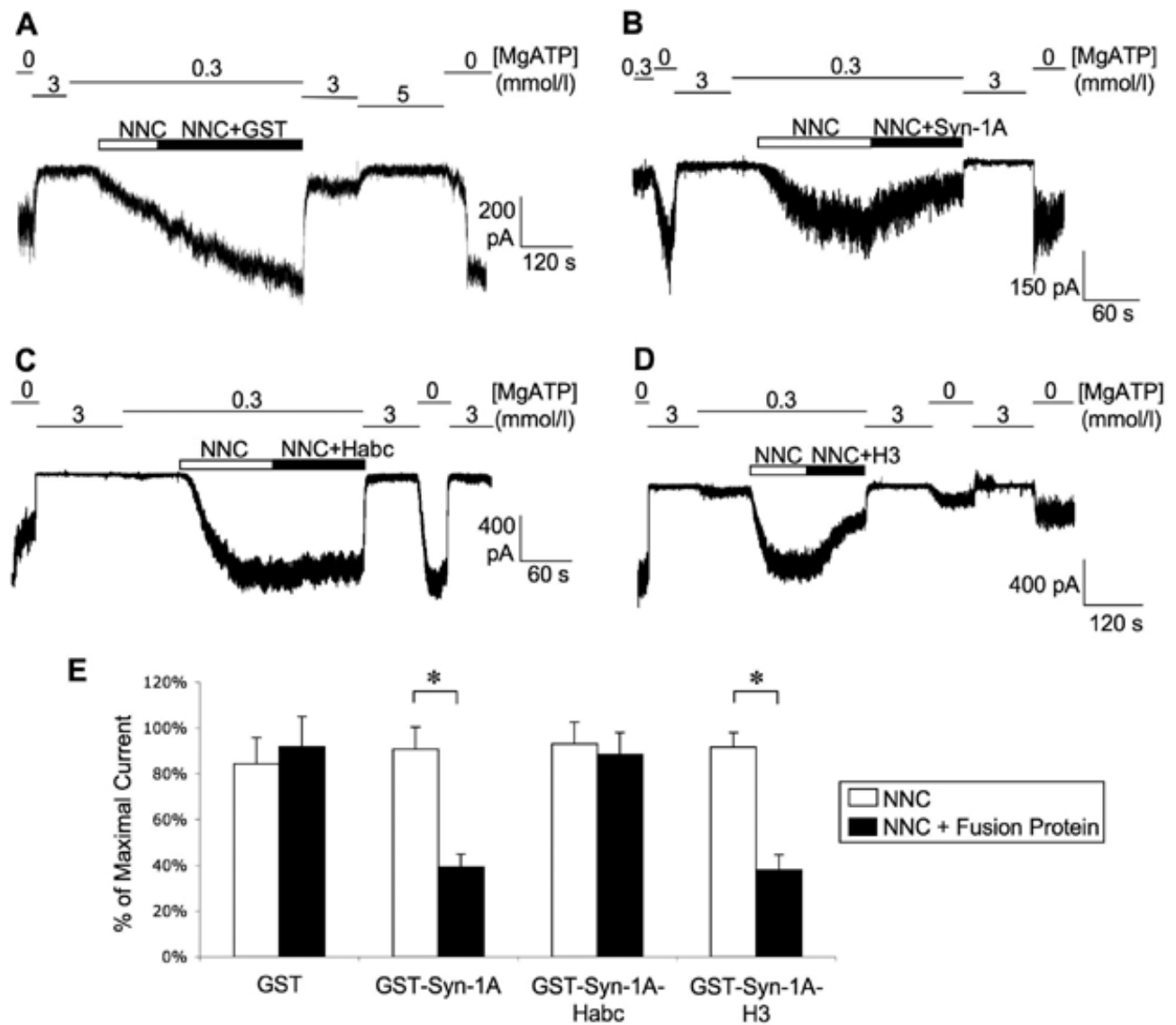


Figure 6

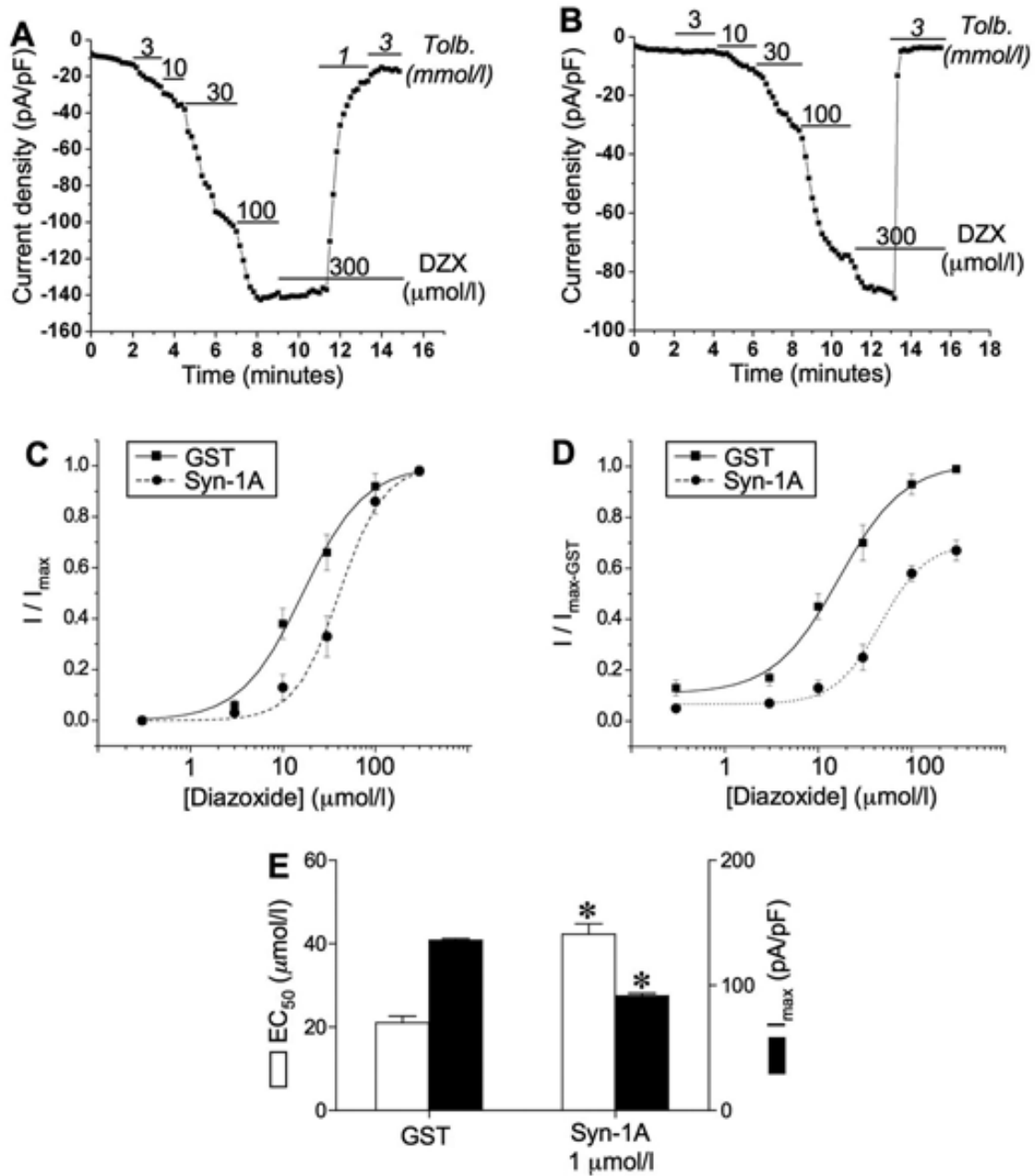


Figure 7

