

**Figure 5 | Cardiac conduction defects after TRPV2 elimination.** (a) Change in connexin 43 localization in TRPV2<sup>flox/flox</sup>; Cre<sup>+/-</sup> hearts after tamoxifen treatment. Scale bar, 100  $\mu$ m. (b) Intraventricular conduction delay after TRPV2 depletion. Electrocardiographs (comparable with lead II) obtained by telemetry.

with reduced amplitude and a slow decay speed (Fig. 6d,e). In addition, the Ca<sup>2+</sup> content of the myocyte sarcoplasmic reticulum was reduced (Fig. 6f), although intracellular free Ca<sup>2+</sup> concentrations under basal conditions were in line with the controls.

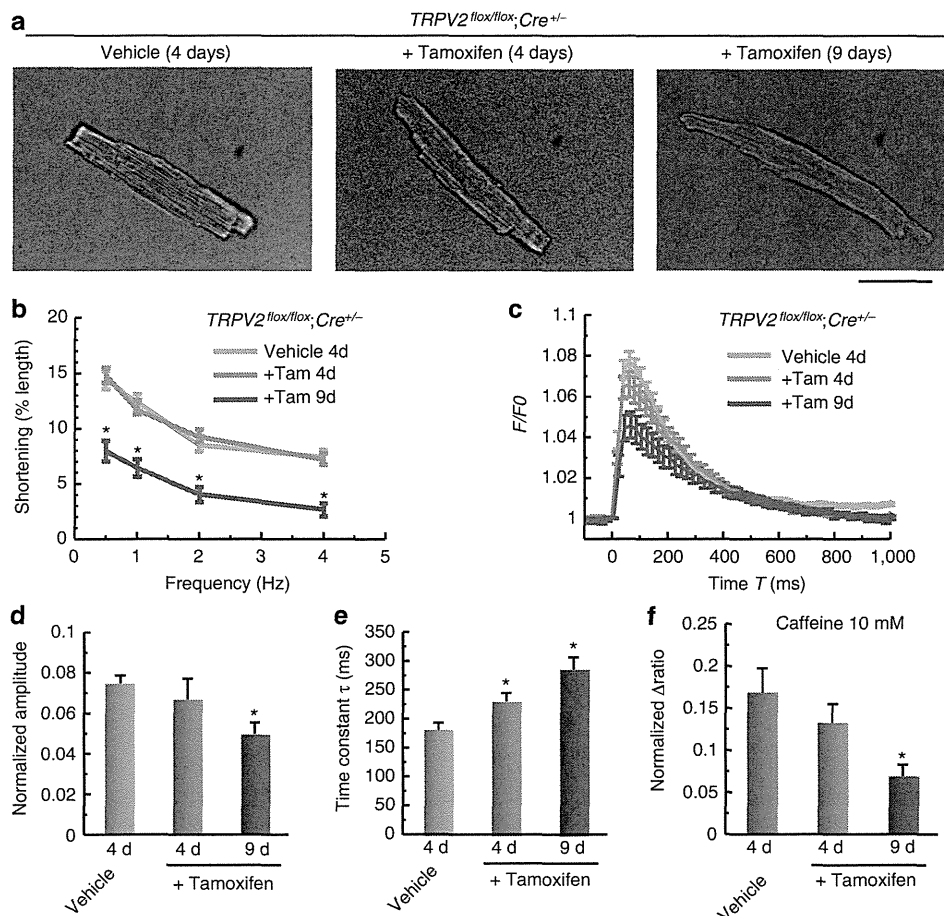
The subcellular structure of cardiomyocytes and the localization of Ca<sup>2+</sup> regulatory proteins are well suited to their cellular functions<sup>21</sup>. In cardiomyocytes, Ca<sup>2+</sup> release for contraction occurs at distinct structures (dyads) along T-tubules, which are critical for efficient E-C coupling<sup>22</sup>. In the dyad space in control vehicle-treated hearts, L-type Ca<sup>2+</sup> channels and Na<sup>+</sup>/Ca<sup>2+</sup> exchangers were localized in T-tubules, and ryanodine receptors were located on sarcoplasmic reticulum membranes, so that these immunofluorescence signals appeared as well-ordered patterns (Fig. 7a). In TRPV2<sup>flox/flox</sup>; MerCreMer<sup>+/-</sup> mice treated with tamoxifen for 4 days, the key Ca<sup>2+</sup> regulatory proteins in cardiomyocyte E-C coupling (that is, L-type Ca<sup>2+</sup> channels, ryanodine receptors, and Na<sup>+</sup>/Ca<sup>2+</sup> exchangers) were normally expressed, and localized in T-tubules and sarcoplasmic reticulum membranes, as in vehicle controls, suggesting normal Ca<sup>2+</sup> handling in the cardiomyocytes (Fig. 7a,b). The myofilament structures were normal in these tamoxifen-treated mice for 4 days (Fig. 7c). Thus, the main cause of the rapid and severe decline in cardiac pump function in TRPV2-deficient hearts was not abnormal Ca<sup>2+</sup> handling affecting myocyte E-C coupling or disorganization of contractile filaments.

**Abnormal Ca<sup>2+</sup> handling in TRPV2-deficient myocytes.** In TRPV2<sup>flox/flox</sup>; MerCreMer<sup>+/-</sup> hearts, 9 days after the onset of tamoxifen treatment the subcellular localization of L-type Ca<sup>2+</sup> channels, ryanodine receptors and Na<sup>+</sup>/Ca<sup>2+</sup> exchangers was abnormal, representing a defect in intracellular Ca<sup>2+</sup> handling (Fig. 7a), although the levels of expression of these Ca<sup>2+</sup> regulatory proteins were similar to controls (Fig. 7b). It was possible that T-tubules and dyad structures were disordered in TRPV2-deficient hearts by day 9. In isolated cardiomyocytes at

this stage, RyR activity was not only significantly lower (Fig. 6c,d), but the reaction area of Ca<sup>2+</sup> sparks elicited by electrical stimulation was also reduced and showed subcellular heterogeneity (Supplementary Fig. 4). TRPV2-deficient myocytes on day 9 also showed extensive disorganization of the myofilaments needed to generate force (Fig. 7c). At this later stage, TRPV2-deficient myocytes showed impaired Ca<sup>2+</sup> handling (Fig. 6b-f and Supplementary Fig. 4) and disorganization of the contractile cytoskeletal apparatus (Fig. 7c). Thus, eliminating TRPV2 not only led to a rapid and severe decline in normal cardiac pump function, but also resulted in a subsequent cellular dysfunction in individual myocytes.

#### Characterization of TRPV2-deficient neonatal cardiomyocytes.

Intra-, inter- and extracellular mechanical forces play vital roles in the differentiation and maturation of cardiomyocytes, both in *in vivo* and *in vitro*<sup>1-3</sup>. In particular, the formation of mature intercalated discs is important during development for directing myofibrogenesis, appropriate Ca<sup>2+</sup> handling for E-C coupling and differentiation into synchronously beating myocytes<sup>6</sup>. In cultured newborn cardiomyocytes, we were able to follow the formation of intercalated discs and the reorganization of myofibrils with time in myocytes isolated by enzymatic dissociation from neonatal heart tissue<sup>23</sup>. When we examined the formation of intercalated discs in cultured neonatal cardiomyocytes, by staining for the gap junction protein, connexin 43, and N-cadherin (Fig. 8a, upper and middle, first three panels), we observed that the intercalated disc structures at cell-cell interfaces showed many zigzags, sharp turns and a high degree of convolution. In control cardiomyocytes isolated from TRPV2<sup>flox/flox</sup>; MerCreMer<sup>-/-</sup> with or without tamoxifen treatment or TRPV2<sup>flox/flox</sup>; MerCreMer<sup>+/-</sup> mice cultured without tamoxifen (Fig. 8a, all panels except for right column), we observed well-developed sarcomeres, typical localization of connexin 43 and N-cadherin to intercalated discs at cell-cell interfaces and synchronously beating myocytes. In these



**Figure 6 | Cardiomyocyte contractility and  $\text{Ca}^{2+}$  handling by isolated cells from TRPV2-deficient hearts.** (a) Morphology of isolated cardiomyocytes. Scale bar, 50  $\mu\text{m}$ . (b) Frequency-dependent shortening of myocytes ( $n=100, 175,$  and  $58$  cells measured from  $8, 6$  and  $3$  hearts). (c) Indo-1 fluorescence in single cardiomyocytes stimulated at  $1\text{ Hz}$  ( $n=28, 18$  and  $12$  cells from  $7, 4$  and  $3$  hearts). (d) Peak amplitude of  $\text{Ca}^{2+}$  transients. (e) Decay time constant obtained by fitting the decline phase. (f) Estimation of sarcoplasmic reticulum  $\text{Ca}^{2+}$  content. Data are means  $\pm$  s.e.m.  $*P < 0.05$  versus vehicle control.

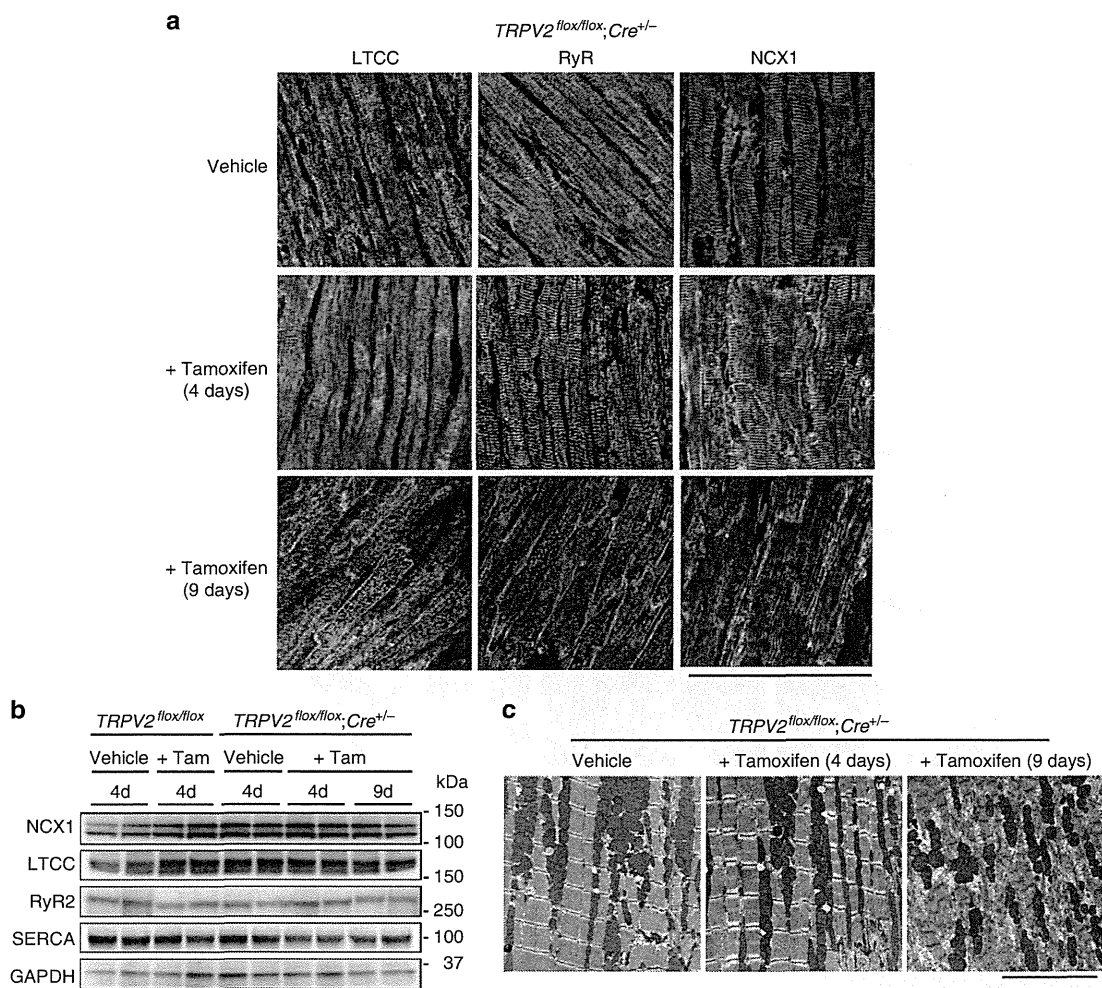
cardiomyocytes, NCX1 was highly expressed at the cell surface and at intercalated areas, indicating their maturation and differentiation into synchronously beating myocytes (Fig. 8a, first three bottom panels). By contrast, TRPV2-deficient myocytes cultured with  $0.2\ \mu\text{g ml}^{-1}$  tamoxifen for 48 h formed no intercalated discs between neighbouring cells, and showed aberrant intracytoplasmic accumulation of connexin 43, internally diffuse N-cadherin, and reduced expression of NCX1 (Fig. 8a, right panels). Although we observed spontaneous weak beating in TRPV2-deficient myocytes, it was not synchronous.

Previously, we reported that recombinant TRPV2 in Chinese hamster ovary cells could be activated by stretch-induced mechanical stimulation<sup>17,18</sup>. We investigated whether neonatal cardiomyocytes showed an intracellular  $\text{Ca}^{2+}$  increase after stretch stimulation dependent upon TRPV2. In  $\text{TRPV2}^{\text{fllox/fllox}}$ ;  $\text{MerCreMer}^{+/-}$  myocytes untreated with tamoxifen, an extracellular  $\text{Ca}^{2+}$ -dependent intracellular  $\text{Ca}^{2+}$  increase was detected after stretch stimulation, which was blocked by inhibitors of stretch-activated channels,  $\text{Gd}^{3+}$  or ruthenium red (RR) (Fig. 8b–d). Although RR is known to block RyR, its effect is not permanent, so using a bath application should limit its effects to plasma membrane channels<sup>24</sup>. By contrast, no stretch-evoked  $\text{Ca}^{2+}$  increase was observed in tamoxifen-treated myocytes. In addition, we confirmed the stretch-evoked  $\text{Ca}^{2+}$

increase in HEK293 cells expressing TRPV2 and the blockade of this response by  $\text{Gd}^{3+}$  or RR (Fig. 8e). These results indicate that TRPV2 is a key molecule in the stretch-induced  $\text{Ca}^{2+}$  response of cardiomyocytes. Interestingly, the formation of intercalated discs and the maturation into cardiomyocytes showing the synchronized beating and alignment of myofilaments seen in vehicle-treated  $\text{TRPV2}^{\text{fllox/fllox}}$ ;  $\text{MerCreMer}^{+/-}$  myocytes were disturbed in the presence of RR at the same level as in TRPV2-deficient myocytes (Fig. 8f). Taken together, these findings suggest that TRPV2 activation is required for the formation of intercalated discs connecting neighbouring myocytes and their maturation into synchronously beating myocytes.

#### Stretch-dependent TRPV2 activation controls IGF-1 secretion.

Substantial evidence from genetic mouse models has demonstrated the critical role of the IGF-1 receptor/PI3K/Akt pathway in maintaining physiological cardiac morphology and function<sup>25</sup>. Surprisingly, the connection with neighbouring myocytes and sarcomere orientation was maintained in TRPV2-deficient myocytes, when IGF-1 was added to cultures (Fig. 9a). Therefore, we measured IGF-1 secretion from cardiomyocytes in culture in response to cyclic stretch stimulation at  $1\text{ Hz}$  for 30 min. IGF-1 secretion was markedly elevated in control



**Figure 7 | Expression of Ca<sup>2+</sup> handling protein by isolated cardiomyocytes and electron micrographs of myofibrils in TRPV2-deficient hearts.**

(a) Time-dependent changes in the localization of the Ca<sup>2+</sup> regulatory proteins LTCC, RyR and NCX1 in tamoxifen-treated TRPV2<sup>lox/lox</sup>, Cre<sup>+/-</sup> mice. Double staining with anti-LTCC antibody (green) and DAPI (blue) in cardiac muscle (left panels). Double staining with anti-RyR antibody (green) and DAPI (blue) in cardiac muscle (middle panels). Double staining with anti-NCX1 antibody (green) and DAPI (blue) in cardiomyocytes (right panels). Scale bar, 100  $\mu$ m. (b) Expression of NCX1, LTCC, RyR and SERCA proteins in TRPV2-deficient heart extracts (10  $\mu$ g per lane), using GAPDH as the internal control in cardiac muscle, (see full blots in Supplementary Fig. 6). (c) Electron micrographs of myofibrils in cardiomyocytes from TRPV2<sup>lox/lox</sup>, Cre<sup>+/-</sup> mice, untreated or treated with tamoxifen. Scale bar, 5  $\mu$ m.

myocytes and was blocked by the inhibitors of stretch-activated channels, Gd<sup>3+</sup> or RR, whereas it was significantly reduced in TRPV2-deficient myocytes (Fig. 9b).

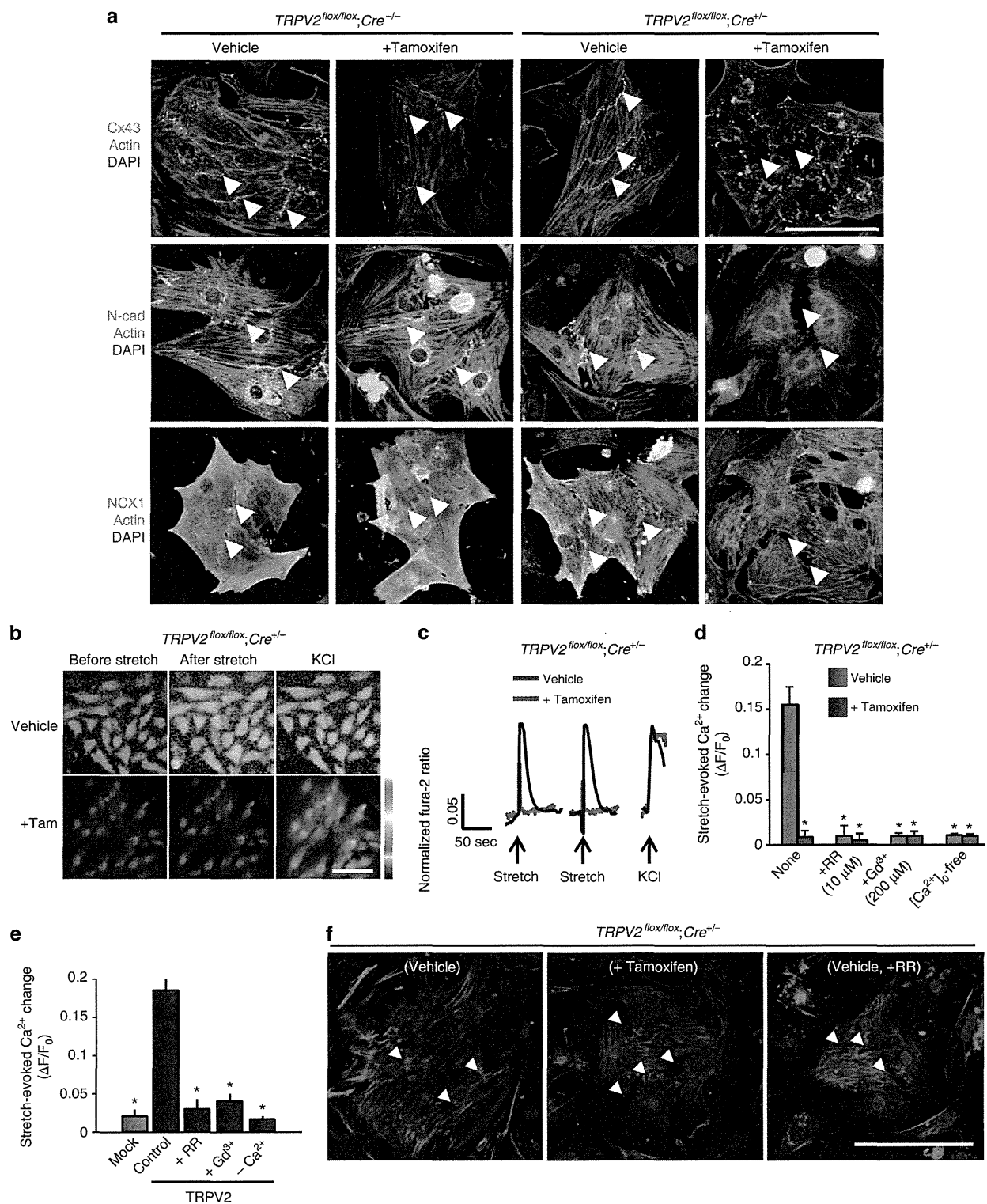
IGF-1 secreted from cardiomyocytes and fibroblast promotes the cardioprotective response to severe pressure overload by activating PI3K and its downstream effector Akt<sup>26,27</sup>. Patients with heart failure have been reported to have significantly lower IGF-1 levels compared with controls<sup>28</sup>. In TRPV2<sup>lox/lox</sup>, MerCreMer<sup>+/-</sup> mice treated with tamoxifen for 3 or 4 days, IGF-1, IGF-1 receptor, PI3K $\alpha$  and Akt1 expression was significantly reduced (Fig. 9c–f). These observations suggest that the IGF-1 receptor/PI3K/Akt pathway is downregulated in TRPV2-deficient hearts.

**Effects of IGF-1 administration in TRPV2-deficient hearts.** To investigate whether the reduced IGF-1 signalling seen in TRPV2-deficient mice represented a significant molecular basis for their observed heart failure phenotype, we treated TRPV2-deficient mice with recombinant IGF-1. In normal mice, IGF-1

administration for 1 week resulted in an increase in cardiac mass and heart-to-body weight ratio (Fig. 9g,h). Remarkably, in TRPV2-deficient mice, administering IGF-1 prevented both the enlargement of the left ventricular diastolic dimensions and cardiac dysfunction (Fig. 9i,j). In addition, expression of IGF-1 receptor, PI3K $\alpha$  and Akt1 were all significantly increased by the administration of IGF-1 in TRPV2-deficient hearts (Supplementary Fig. 5). These results suggest that reduced IGF-1 signalling is a major factor in the heart failure observed in TRPV2-deficient mice. However, IGF-1 administration did not result in complete recovery, suggesting that other signalling pathways may also be impaired in TRPV2-deficient hearts.

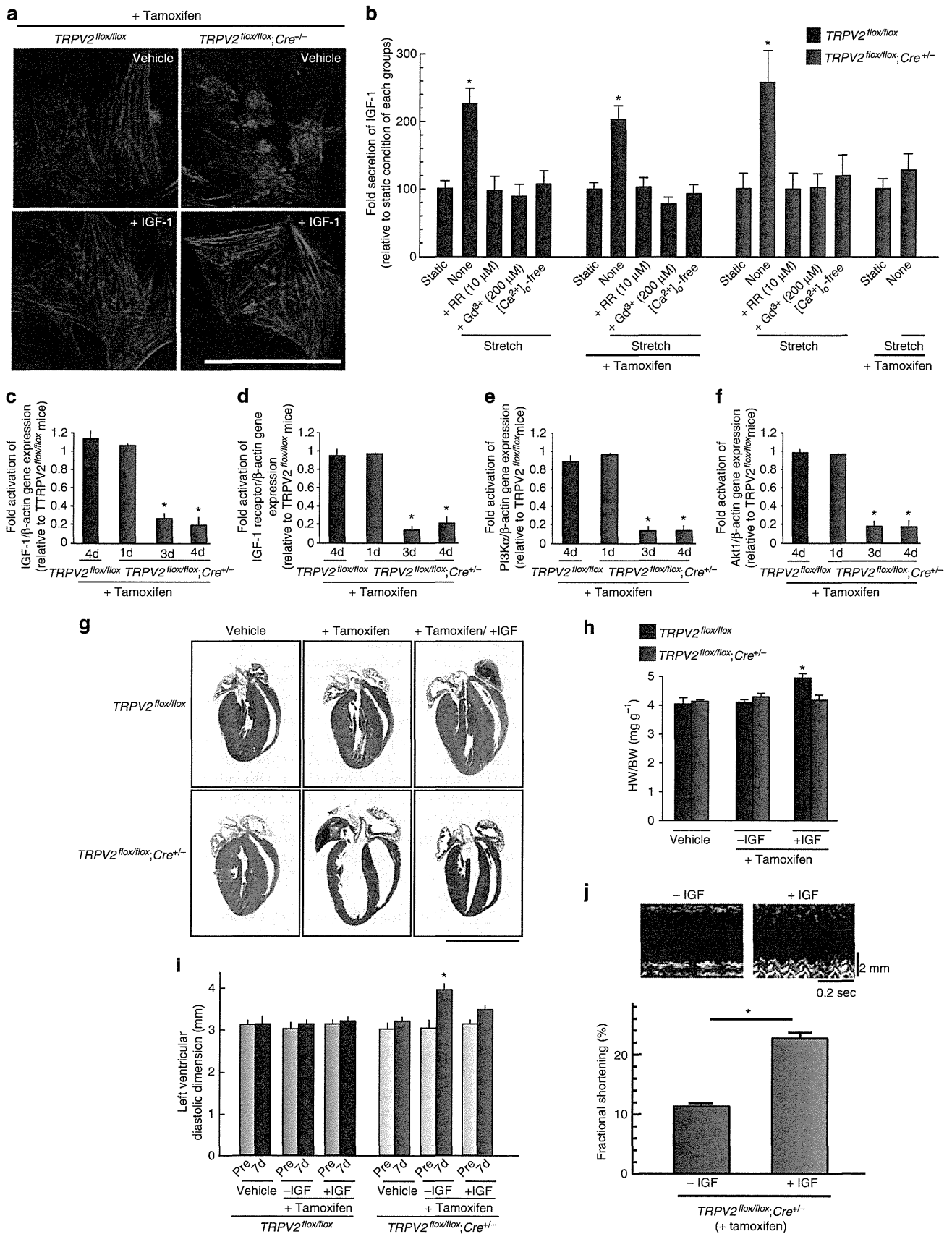
## Discussion

This study has shown a critical role for TRPV2 in the normal heart. TRPV2 is localized at intercalated discs, which are critical for detecting the mechanical forces generated by myocyte contraction. The elimination of TRPV2 from the mouse heart resulted in severe cardiac dysfunction within a few days (Fig. 3),



**Figure 8 | Impairment of maturation into functional syncytia with synchronously beating cardiomyocytes in TRPV2-deficient newborn mice.**

(a) Isolated cardiomyocytes from *TRPV2<sup>flox/flox</sup>; Cre<sup>-/-</sup>* or *TRPV2<sup>flox/flox</sup>; Cre<sup>+/-</sup>* newborn mice. Triple staining with anti-connexin 43 antibody (green), phalloidin (red) and DAPI (blue) in cardiomyocytes (upper panels). Triple staining with anti-N-cadherin antibody (green), phalloidin (red) and DAPI (blue) in cardiomyocytes (middle panels). Triple staining with anti-NCX1 antibody (green), phalloidin (red) and DAPI (blue) in cardiomyocytes (lower panels). Scale bar, 100  $\mu$ m. (b) Fura-2-imaging of stretch-induced intracellular  $Ca^{2+}$  dynamics in neonatal cardiomyocytes. Scale bar, 100  $\mu$ m. (c) Representative trace of stretch-induced intracellular  $Ca^{2+}$  increase. (d) Effect of mechanosensitive channel inhibitors. Data are means  $\pm$  s.e.m. \* $P$  < 0.01 versus vehicle control ( $n$  = 19–44). (e) Stretch-induced  $Ca^{2+}$  changes in ectopically expressed TRPV2 in HEK293 cells. Data are means  $\pm$  s.e.m. \* $P$  < 0.01 versus control. ( $n$  = 23–37) (f) Isolated cardiomyocytes from newborn mice cultured with 10  $\mu$ M rubidium red. Scale bar, 100  $\mu$ m. Arrowheads, cell-cell junctions.



despite no impairment of myocyte contraction or E–C coupling efficiency (Fig. 6). These observations suggest that the functional integration with neighbouring cardiomyocytes was weakened in TRPV2-deficient hearts. It seems that cardiomyocytes sense inadequate functional output at a cellular level and respond by increasing the number of myofibrillar attachment sites in intercalated area<sup>7</sup>. In a failing heart, the elevated expression of adherens junction proteins and the increase in membrane convolution and interdigitation between neighbouring cardiomyocytes seems to lead to a decrease in the flexibility of the contractile tissue and increased stiffness<sup>7</sup>. In TRPV2-deficient hearts, the myofilaments were disassembled at their attachment sites to intercalated discs (Fig. 4a). The TRPV2-deficient myocytes did not seem to sense the inadequate functional output or the severe cardiac dysfunction and therefore might have difficulty maintaining the integrity of their intercalated discs. On the other hand, cultured neonatal cardiomyocytes from TRPV2-deficient mice showed impaired formation of intercalated discs and translocation of connexin 43 and N-cadherin into cell–cell adhesion sites, and no spontaneous, synchronized beating (Fig. 8a). The TRPV2 inhibitor, RR, also affected intercalated disc formation in neonatal cardiomyocytes (Fig. 8f), implicating TRPV2 activity in the maintenance of intercalated disc integrity as well as their formation. Further experiments are needed to elucidate the molecular mechanism involved in maintaining intercalated disc structure and exactly how this function depends upon TRPV2.

It is possible that TRPV2 has a pivotal role as an anchoring protein for myofibrils at intercalated discs, coordinating the transmission of mechanical forces regardless of its channel activity. The transmission of the force generated by contracting myofibrils is mediated by so-called adherens junctions, which are composed of transmembrane proteins of the cadherin family and which, in the cytoplasm, are coupled to the contracting sarcomeres by members of the catenin family<sup>7</sup>. Reportedly, TRPV4 associates with adherens junctions via  $\alpha$ -catenin in the human bladder urothelium<sup>29</sup>. In common with several members of other TRP subfamilies<sup>30</sup>, the cytoplasmic region of TRPV2 is likely to couple to a rigid intracellular structural network and form ‘mechanosensitive  $\text{Ca}^{2+}$ -signalosomes’ at intercalated discs. If so, eliminating TRPV2 is therefore likely to interrupt mechanical coupling with neighbouring myocytes at intercalated discs, causing severe cardiac dysfunction.

In addition, we showed a significant reduction of stretch-dependent IGF-1 secretion in TRPV2-deficient myocytes (Fig. 9b), and a downregulation of IGF-1 receptor/PI3K/Akt signalling in TRPV2-deficient hearts (Fig. 9c–f). IGF-1 is continuously secreted in the normal state and in response to mechanical stress dependent on the intracellular  $\text{Ca}^{2+}$  concentration<sup>25,26,31</sup>; this in turn modulates myocyte PI3K signalling, which is involved in myocardial hypertrophy and heart failure<sup>25</sup>. It appears that the TRPV2 activation-dependent IGF-1 secretion of cardiomyocytes controls the equilibrium balance of intracellular IGF-1 receptor/PI3K/Akt signalling in response to haemodynamic stress. This forms a molecular basis for the myocyte mechanical feedback mechanism in the normal state and

in response to haemodynamic stress, which is required to maintain cardiac structure and function. Therefore, loss of TRPV2 not only leads to a rapid and severe decline in normal cardiac pump function, but also triggers subsequent cellular dysfunction in individual myocytes.

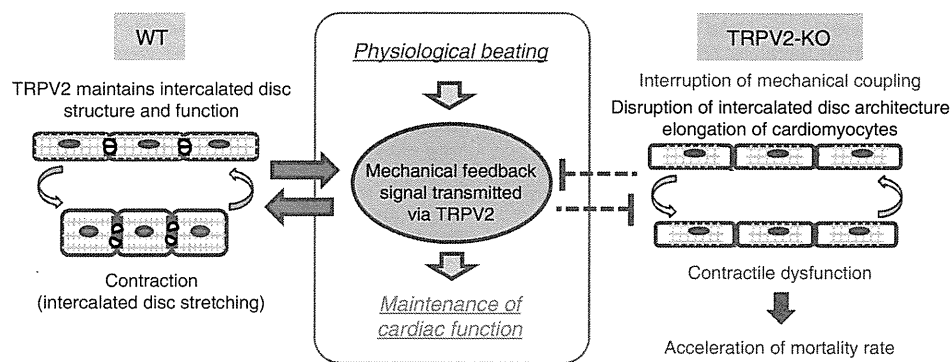
TRP channels are unique cellular sensors responding to a wide variety of extracellular and intracellular signals, including mechanical and osmotic stress<sup>32</sup>. The present study shows that TRPV2 is a candidate molecule for stretch-activated channels in myocyte intercalated discs, and is crucial for the mechanical stimulation-dependent  $\text{Ca}^{2+}$  signalling of cardiomyocytes. However, it is unclear whether TRPV2 channels directly transduce mechanical stimuli or are part of a downstream signalling pathway, as discussed below. Several regulatory interactions between TRPV2 cytoplasmic domains and various signalling molecules have been investigated. Mercado *et al.*<sup>33</sup> proposed that  $\text{PIP}_2$  is constitutively associated with the C-terminal domain of TRPV2, promoting its inhibition  $\text{Ca}^{2+}$ -dependently. It is not yet certain that TRPV2 directly senses membrane lipid stretching through  $\text{PIP}_2$  breakdown and diacylglycerol production, as has been shown for other mechanosensitive channels<sup>34,35</sup>. Therefore, to clarify the regulatory mechanism of TRPV2 activation, further electrophysiological investigations will be needed in the future.

TRPV2 has been reported to translocate from an intracellular compartment to the plasma membrane in response to IGF-1 and intracellular  $\text{Ca}^{2+}$  concentration<sup>17,36</sup>. Therefore, the appropriate control of these mediators might be needed for the localization of TRPV2 to the membrane in intercalated discs in working hearts. It is possible that the transmission and generation of mechanical force during contraction in response to haemodynamic stress provides the control for IGF-1 production and intracellular  $\text{Ca}^{2+}$  concentrations, in which TRPV2 plays a pivotal role by continuously monitoring mechanical stress. TRPV2 activity is also directly controlled by PI3K activity, and this is independent of channel translocation to the plasma membrane<sup>37</sup>. Activation of PI3K by growth factors results in  $\text{PIP}_3$  synthesis, by the phosphorylation of  $\text{PIP}_2$  (ref. 25). Thus, TRPV2 activity is also likely to be regulated by the  $\text{PIP}_2$  content, dependent on PI3K activity, and interactions with other lipids in intercalated discs. However, little is known about the lipid composition of intercalated discs. Taken together, these findings suggest that the lipid composition and potentiation of the IGF-1 receptor/PI3K/Akt signalling cascade in response to haemodynamic stress are key factors for TRPV2 activation at intercalated discs in the heart.

This study has identified a molecular basis for the maintenance of cardiac structure and function, through myocyte mechanical feedback systems, in which TRPV2 plays a pivotal role (Fig. 10). The integrity of intercalated discs has a large impact on force transmission and generation during muscle contraction, and is reflected in the effect of mechanical feedback on the quality of myocytes in the heart. Eliminating TRPV2 had no effect on  $\text{Ca}^{2+}$  handling in myocyte E–C coupling (Fig. 6b–f), which is generated at dyads formed between a T-tubule and the junctional part of the sarcoplasmic reticulum. It appears that the  $\text{Ca}^{2+}$  signalling

**Figure 9 | Impairment of stretch-dependent IGF-1 secretion and IGF-1 receptor/PI3K/Akt pathway in TRPV2-deficient myocytes.** (a) Isolated cardiomyocytes from newborn mice cultured with 10  $\mu\text{M}$  IGF-1. Scale bar, 100  $\mu\text{m}$ . (b) Stretch-dependent IGF-1 secretion by cardiomyocytes. \* $P < 0.05$  versus the other groups. Data are means  $\pm$  s.e.m. (c–f) IGF-1, IGF-1 receptor, PI3K $\alpha$  and Akt expression level in TRPV2-deficient hearts ( $n = 3$  mice per group). \* $P < 0.05$  versus non-treated TRPV2<sup>flx/flx</sup> mice. Data are means  $\pm$  s.e.m. (g) Histological analysis 7 days after onset of IGF-1 administration and tamoxifen treatment in TRPV2-deficient mice. Scale bar, 5 mm. (h) HW/BW ratio. Data are means  $\pm$  s.e.m. ( $n = 5$ –12 per group) \* $P < 0.05$  for tamoxifen-treated TRPV2<sup>flx/flx</sup>; Cre<sup>+/-</sup> mice versus IGF-1-treated TRPV2<sup>flx/flx</sup>; Cre<sup>+/-</sup> mice after 7 days. (i, j) Echocardiographic parameters ( $n = 3$  per group). (i) Data are means  $\pm$  s.e.m. \* $P < 0.05$  versus all the other groups. by two-way analysis of variance with Bonferroni's *post hoc* test.





**Figure 10 | Role of TRPV2 within the working heart.** In wild-type mice, TRPV2 protein (yellow) is located in the intercalated discs (red), which is cyclically stretched and accompanied by cardiac contractions within the working heart. The mechanical feedback signal transmitted via TRPV2 is crucial for the maintenance of cardiomyocytes. TRPV2 ablation leads to the disruption of the intercalated disc architecture (red), and disorganization of sarcomere myofibril proteins at the intercalated discs (orange). These TRPV2-KO myocytes show contractile dysfunction. Therefore, the mortality rate of TRPV2-KO mice is accelerated. Thus, the mechanical feedback signal within the working heart integrates myocyte structure and function, with TRPV2 playing a pivotal role.

involved in myocyte E–C coupling and the mechanical signal mediated by TRPV2 are spatially and temporally controlled by different signals in cardiomyocytes. Further studies might clarify the molecular mechanism of mechanotransduction mediated by TRPV2 in cardiomyocytes, and so improve our understanding of cardiac development and maturation, hypertrophic remodelling in the heart and the pathophysiology of heart disease.

Recently, Rubinstein *et al.*<sup>38</sup> reported the cardiac function of TRPV2-KO mice expressing *Cre* under the control of the cytomegalovirus promoter generated by Park *et al.*<sup>39</sup>. They observed that cardiac function declined in TRPV2-deficient mice compared with controls, despite no histological abnormality<sup>38</sup>. Although there are differences in the severity of cardiac dysfunction and the depression of myocyte contractility, these results are consistent with our observations. However, the construction of their TRPV2-KO mice differed from our model in the site at which the partial elimination occurred, which corresponded to the channel pore and carboxy-terminal region of TRPV2, using a ubiquitous promoter, and the genetic background of the mice (B6129SF2/J)<sup>39</sup>. Park *et al.*<sup>39</sup> have reported that their model was susceptible to perinatal lethality but displayed normal thermal and mechanical nociception. They therefore suggested the possibility that compensatory mechanisms prevented the obvious phenotype of TRPV2-deficient cells being reflected in their KO mice<sup>39</sup>. On the other hand, the cardiac-specific elimination of TRPV2 in this study affected neither the embryonic development nor the growth after birth, in the absence of tamoxifen administration. Taken together, we believe that the acute elimination of TRPV2 function in our model might circumvent any hypothetical compensatory process.

## Methods

**Animals.** Mice were housed under a 12-h light–dark cycle in a temperature-controlled environment. All the experiments were performed in male mice aged 10 weeks old (weighing 22–24 g). Littermates were used in this study to randomize genetic variation. All animal experiments were approved by the Animal Research Committee of Okayama University (Okayama, Japan), and were performed in accordance with institutional guidelines.

**Generation of TRPV2 conditional KO mice.** All experiments requiring gene recombination in this study were carried out in accordance with the institutional guideline of Okayama University (Okayama, Japan). Using cloned TRPV2 cDNA (accession code. NM011706) as a probe, we screened a genomic library constructed from C57/BL6j mouse DNA. The genomic clone was used to generate the targeting

vector shown in Supplementary Fig. 1, which was linearized for electroporation into C57/BL6j ES cells. After selection, G418-resistant ES clones were screened for the presence of the targeted locus by southern blot analysis. Targeted ES cells were microinjected into Balb/c mouse blastocysts, and germline transmission of the TRPV2 conditional null alleles was confirmed by southern blotting and PCR genotyping using genomic DNA extracted from mouse tail veins. We crossed mice carrying a *TRPV2<sup>fllox</sup>* allele with transgenic mice (*MerCreMer*) expressing tamoxifen-inducible cardiomyocyte-specific Cre recombinase to produce *TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>+/-</sup>* mice. Germline transmission of TRPV2 conditional null alleles was confirmed by southern blotting and PCR genotyping using the primer pair 5'-TTAAATGACTTGTGAGGGAGATAGC-3' and 5'-CAAGTAACA CAATCTACCAAGGTC-3', yielding 322 (wild-type) and 369 (null allele) bp products. To induce *Cre*-mediated recombination, we injected 10-week-old male *TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>+/-</sup>*, *TRPV2<sup>fllox/+</sup>;MerCreMer<sup>+/-</sup>* and *TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>-/-</sup>* mice intraperitoneally with 8 mg kg<sup>-1</sup> tamoxifen (Sigma) once daily for 4 consecutive days. Tamoxifen injection and subsequent analyses were performed in a blinded fashion.

**Electrocardiography.** Transthoracic electrocardiography (ECG) was used to evaluate cardiac function with the Aplio 300 (Toshiba Medical System) and a 14-MHz transducer. The 10-week-old male mice were anesthetized initially with 2% isoflurane, and then at 1% during the examination. Left ventricular short-axis dimensions at the tip of the papillary muscles were measured on M-mode. Fractional shortening was calculated as (LVDd–LVDs)/LVDd × 100 (%).

**Blood pressure measurement.** Chronic measurements of blood pressure and ECG were performed on unrestrained, conscious mice (10-week-old, male) using a commercially available telemetry and computer-based data acquisition system (Data Sciences International) according to the manufacturer's instructions. Briefly, a pressure-sensing catheter was implanted in the thoracic aorta via the left carotid artery, and two electrodes were placed subcutaneously on the right shoulder and left inguinal region to record lead II ECG under anaesthesia with 2% isoflurane inhalation. Mice were returned to their home cage (placed on top of telemetry receivers), and blood pressure and ECG were continuously monitored and recorded.

**Administration of IGF-1.** Recombinant human IGF-1 was purchased from Cell Science, and diluted with 0.9% NaCl at a concentration of 10 mg ml<sup>-1</sup> and administered to mice (60 µg per day) by continuous infusion (0.25 µl h<sup>-1</sup>) using a mini osmotic pump (Alzert 1002). IGF-1 administration and tamoxifen treatment were started at the same time. Control mice received vehicle alone.

**Neonatal cardiomyocyte culture.** Primary cardiomyocyte cultures were prepared from ventricles of 1-day-old mice by very gentle trypsinization at room temperature, by a modification of preparation methods from rat neonatal hearts<sup>40</sup>. Hearts were rapidly removed from neonatal *TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>+/-</sup>* or *TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>-/-</sup>* mice anesthetized with an overdose of diethyl ether. The ventricles were excised, cut into several pieces and washed three times with 10 ml ice-cold phosphate-buffered saline for 1 min by gentle shaking. The tissue pieces were digested three times with 0.06% trypsin in DMEM (8 ml) for 8 min at 37 °C by gentle agitation. The cell suspension was resuspended in DMEM with 10% fetal calf

serum (FCS) to stop trypsinization, and was centrifuged at 14g for 3 min. The cell pellets were resuspended in fresh DMEM containing 10% FCS, and plated on collagen-coated 24-well dishes at a density of  $4 \times 10^4$  cells per well and maintained in DMEM containing 10% FCS. The formation of myocytes clusters and the spontaneous synchronized beating were confirmed by inverted microscope (CKX41, Olympus).

**Stretch stimulation of cardiomyocytes.** For stretch stimulation of cardiomyocytes, the cell suspension were plated on 1 cm<sup>2</sup> collagen-coated polydimethylsiloxane stretch chambers at  $2 \times 10^5$  cells per well, and cultured. After 24 h, primary cardiomyocytes were divided into two groups and maintained for up to 2 days in DMEM containing 10% FCS, with or without tamoxifen ( $0.2 \mu\text{M ml}^{-1}$ ). Membranes were uniformly stretched by 20% for 3 s, using a computer-controlled stepping motor machine (STB-150, STREX), by a slight modification of cell-stretch culture methods<sup>41</sup>. One end of the chamber was firmly attached to a fixed frame, while the other was attached to a movable frame connected to a motor-driven shaft. The amplitude and frequency of stretch were controlled by a programmable microcomputer. The silicon membrane was uniformly stretched over the whole membrane area, and the lateral thinning did not exceed 1% at 20% stretch. TRPV2 deficiency did not have a discernible impact on the cardiomyocytes' ability to adhere to the membrane, although the cell-cell interfaces with neighbouring myocytes were expanded.

**Stretch-induced Ca<sup>2+</sup>-transients in newborn cardiomyocytes.** Stretch-induced Ca<sup>2+</sup> transients were examined in cardiomyocytes loaded with 2  $\mu\text{M}$  fura-2 acetoxymethyl ester (fura-2) for 30 min at 37 °C and maintained in standard Tyrode's solution under continuous flow using a microperfusion system. Fura-2-loaded cells were alternately excited at 340 and 380 nm using a Lambda DG-4 Ultra High Speed Wavelength Switcher (Sutter Instruments) coupled to an inverted IX71 microscope with a UApo 20  $\times$  /0.75 objective lens (Olympus). Fura-2 fluorescent signals were recorded (ORCA-Flash 2.8; Hamamatsu Photonics) and analysed by a ratiometric fluorescence method using MetaFluor software (version 7.7.5.0; Molecular Devices).

**Isolation of adult mouse ventricular myocytes.** Ventricular myocytes were obtained from 10-week-old male TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>+/-</sup> and TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>-/-</sup> mice by a slight modification of Shioya's methods<sup>42</sup>. Hearts were rapidly removed from adult TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>+/-</sup> or TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>-/-</sup> mice anesthetized with an overdose of pentobarbital ( $300 \text{ mg kg}^{-1}$ , intraperitoneally), and Langendorff perfused at a constant hydrostatic pressure of 70 cm H<sub>2</sub>O at 37 °C using cell isolation buffer (CIB) supplemented with 0.4 mM EGTA (EGTA-CIB), which chelates calcium within the heart. CIB contained 130 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl<sub>2</sub>, 0.33 mM NaH<sub>2</sub>PO<sub>4</sub>, 22 mM glucose, 50 nM ml<sup>-1</sup> bovine insulin (Sigma) and 25 HEPES-NaOH (pH = 7.4). Insulin was used from 1 U ml<sup>-1</sup> stock solution in 0.1 mM HCl (pH = 4.0). EGTA was from 400 mM stock in 1 M NaOH (pH = 7.8). The perfusate was then switched to the enzyme solution (15 ml), which was CIB supplemented with 0.3 mM CaCl<sub>2</sub>, 1 mg ml<sup>-1</sup> collagenase (Worthington Biochemical), 0.06 mg ml<sup>-1</sup> trypsin (Sigma) and 0.06 mg ml<sup>-1</sup> protease (Sigma). Once the tissue had undergone complete digestion, the ventricles were excised, cut into several pieces and further digested in fresh enzyme solution (15 ml) for 15–20 min at 37 °C until they were mostly dissociated. In this enzyme solution, the CaCl<sub>2</sub> level was increased to 0.7 mM, and 2 mg ml<sup>-1</sup> BSA (Sigma) was supplemented. The cell suspension was centrifuged at 14g for 3 min. The cell pellet (~0.1 ml) was resuspended in CIB supplemented with 1.2 mM CaCl<sub>2</sub> and 2 mg ml<sup>-1</sup> BSA, and then incubated at 37 °C for 10 min, centrifuged (14g, 3 min) and resuspended in 10 ml Tyrode solution supplemented with 2 mg ml<sup>-1</sup> BSA. Tyrode's solution contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.33 mM NaH<sub>2</sub>PO<sub>4</sub>, 11 mM glucose and 5 mM HEPES-NaOH (pH = 7.4)<sup>40,42</sup>.

**Cell shortening and Ca<sup>2+</sup> transients in adult cardiomyocytes.** Isolated cardiomyocytes were loaded with 10  $\mu\text{mol l}^{-1}$  Indo-1 AM (Invitrogen) and electrically stimulated at 1 Hz using a two-platinum electrode insert connected to a bipolar stimulator (Nihon Kohden, SEN-3301) on the stage of an inverted microscope (IX71, Olympus) with a  $\times 20$  water immersion objective lens (UApo N340, Olympus). Calcium transients were measured as the ratio of fluorescence emitted at 405/480 nm after excitation at 340 nm using a high-performance Evolve EMCCD camera (Photometrics). Cardiomyocytes were maintained under continuous flow in standard Tyrode's solution, exchanged using a microperfusion system. For measuring caffeine-induced calcium transients, cells were paced at 1 Hz prior to induction of caffeine contractures. Electrical stimulation was stopped 15 s before rapid perfusion with a 10 mmol l<sup>-1</sup> caffeine solution. The experiments were recorded and analysed using MetaMorph software (version 7.7.1.0; Molecular Devices). Results were the means of the fluorescent signals from 10–20 cardiomyocytes from a single heart.

**Measure of IGF-1 concentration.** IGF-1 concentrations were measured in conditioned media from stretched and unstretched myocytes in a sandwich ELISA using mouse standards, according to the manufacturer's guidelines (R&D Systems),

which quotes the sensitivity of this assay as 30 ng ml<sup>-1</sup>. Standard curves and positive controls were included in each assay, and IGF-1 concentrations were obtained by interpolation.

**Real-time PCR.** The Mouse PI3K-AKT Signalling Pathway RT<sup>2</sup> Profiler PCR Array was purchased from Qiagen. Total RNA extracted from TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>+/-</sup> or TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>-/-</sup> hearts with or without tamoxifen were reverse transcribed into cDNA with oligo (dT) primers using Superscript III. Real-time PCR was performed with Step-One plus<sup>TM</sup> (Applied Biosystem). Statistical analysis of the results was performed with the  $\Delta\text{Ct}$  value ( $\text{Ct}_{\text{gene of interest}} - \text{Ct}_{\beta\text{-actin}}$ ). Relative gene expression was obtained using the  $\Delta\Delta\text{Ct}$  method ( $\text{Ct}_{\text{sample}} - \text{Ct}_{\text{calibrator}}$ ) using the TRPV2<sup>fllox/fllox</sup>;MerCreMer<sup>-/-</sup> hearts without tamoxifen as a calibrator.

**Histology.** Hearts were excised and immediately fixed in buffered 4% paraformaldehyde, embedded in paraffin and sectioned to a thickness of 4  $\mu\text{m}$ . We stained serial sections of samples with Masson's trichrome to evaluate gross morphology and fibrosis. The preparations were examined under a light microscope (SZX7 or BX43, Olympus).

**Electron microscopy.** For electron microscopy, excised hearts were fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer, postfixed with 2% OsO<sub>4</sub> in 0.1 M phosphate buffer and stained with uranyl acetate and lead citrate. The microtome sections were examined under a JEM-1200 electron microscope (Nihondenshi Co., Japan).

**Antibodies.** The following antibodies were used for immunostaining or immunoblot analysis: anti-TRPV2 (1:200 dilution, AB5398, Millipore); anti-vinculin (1:100 dilution, V9131, Sigma); anti-connexin 43 (1:100 dilution, C6219, Sigma); anti-N-cadherin (1:100 dilution, 3B9, life technologies); anti-Cav3 (1:1,000 dilution, 610420, BD Pharmingen.); anti-LTCC (1:1,000 dilution, ACC033, Alomone); anti-SERCA (1:1,000 dilution, MA3919, Thermo); and anti-RyR (1:1,000 dilution, MA3916, Thermo). The anti-NCX antibody was generated in our laboratory (1:1,000 dilution).

**Immunocytochemistry.** For immunocytochemistry, 5  $\mu\text{m}$  frozen heart sections embedded in OCT compound (Tissue-Tek) were permeabilized with 0.1% Triton X-100 and incubated with primary antibodies. For immunostaining of rat cardiomyocytes, cells immobilized on collagen-coated glass slides were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 and then stained with primary antibodies. These samples were then treated with Alexa Flour 488-conjugated anti-rabbit IgG (A11008, Life Technologies) or Alexa Flour 488-conjugated anti-mouse IgG (A11001, Life Technologies). Cells or sections were examined using a confocal microscope (Fluoview FV1000, Olympus) mounted on an Olympus IX81 epifluorescence microscope with a UPlanSApo  $\times 60/1.35$  oil immersion objective lens (Olympus).

**Immunoblotting.** Mice hearts and kidneys were homogenized in a Hiscotron homogenizer (NITI-ON) in lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% SDS, 2  $\mu\text{g ml}^{-1}$  leupeptin, 1  $\mu\text{g ml}^{-1}$  aprotinin, 200  $\mu\text{M}$  phenylmethylsulfonyl fluoride, and 200  $\mu\text{M}$  benzamide hydrochloride. The lysates were centrifuged at 100,000 g for 20 min and the supernatants were used for immunoblot analysis. Immunoreactive bands were visualized using a chemiluminescence detection system (Perkin Elmer) and an LAS3000 Luminescent Image Analyzer (Fuji Film).

**Data analysis.** Data were analysed by individuals who were blinded to the genotype, drug treatment or operation. Data presented here were reproducible in at least three independent experiments. Results are shown as the mean  $\pm$  s.e.m. Paired data were evaluated using a Student's *t*-test. Two-way analysis of variance with Bonferroni's *post hoc* test was used for multiple comparisons wherever appropriate. The Kaplan–Meier method with a log-rank test was used for survival analysis.  $P < 0.05$  was considered statistically significant.

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## Author contributions

Y.K. planned and designed the study, performed the experiments, and wrote the paper. K.I., Y.U., S.T., K.N., M.K., A.S., T.T., K.K., and S.M. carried out the experiments and analysed data. K.N. gave conceptual advice.

## Additional information

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# The Role of Pak-Interacting Exchange Factor- $\beta$ Phosphorylation at Serines 340 and 583 by PKC $\gamma$ in Dopamine Release

Toshihiko Shirafuji,<sup>1</sup> Takehiko Ueyama,<sup>1</sup> Ken-ichi Yoshino,<sup>1</sup> Hideyuki Takahashi,<sup>1</sup> Naoko Adachi,<sup>1</sup> Yukio Ago,<sup>2</sup> Ken Koda,<sup>2</sup> Tetsuaki Nashida,<sup>2</sup> Naoki Hiramatsu,<sup>2</sup> Toshio Matsuda,<sup>2</sup> Tatsushi Toda,<sup>3</sup> Norio Sakai,<sup>4</sup> and Naoaki Saito<sup>1</sup>

<sup>1</sup>Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, Kobe 657-8501, Japan, <sup>2</sup>Laboratory of Medicinal Pharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan, <sup>3</sup>Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan, and <sup>4</sup>Department of Molecular and Pharmacological Neuroscience, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8551, Japan

Protein kinase C (PKC) has been implicated in the control of neurotransmitter release. The AS/AGU rat, which has a nonsense mutation in PKC $\gamma$ , shows symptoms of parkinsonian syndrome, including dopamine release impairments in the striatum. Here, we found that the AS/AGU rat is PKC $\gamma$ -knock-out (KO) and that PKC $\gamma$ -KO mice showed parkinsonian syndrome. However, the PKC $\gamma$  substrates responsible for the regulated exocytosis of dopamine *in vivo* have not yet been elucidated. To identify the PKC $\gamma$  substrates involved in dopamine release, we used PKC $\gamma$ -KO mice and a phosphoproteome analysis. We found 10 candidate phosphoproteins that had decreased phosphorylation levels in the striatum of PKC $\gamma$ -KO mice. We focused on Pak-interacting exchange factor- $\beta$  ( $\beta$ PIX), a Cdc42/Rac1 guanine nucleotide exchange factor, and found that PKC $\gamma$  directly phosphorylates  $\beta$ PIX at Ser583 and indirectly at Ser340 in cells. Furthermore, we found that PKC phosphorylated  $\beta$ PIX *in vivo*. Classical PKC inhibitors and  $\beta$ PIX knock-down (KD) significantly suppressed Ca<sup>2+</sup>-evoked dopamine release in PC12 cells. Wild-type  $\beta$ PIX, and not the  $\beta$ PIX mutants Ser340 Ala or Ser583 Ala, fully rescued the decreased dopamine release by  $\beta$ PIX KD. Double KD of Cdc42 and Rac1 decreased dopamine release from PC12 cells. These findings indicate that the phosphorylation of  $\beta$ PIX at Ser340 and Ser583 has pivotal roles in Ca<sup>2+</sup>-evoked dopamine release in the striatum. Therefore, we propose that PKC $\gamma$  positively modulates dopamine release through  $\beta$ PIX phosphorylation. The PKC $\gamma$ - $\beta$ PIX-Cdc42/Rac1 phosphorylation axis may provide a new therapeutic target for the treatment of parkinsonian syndrome.

**Key words:**  $\beta$ PIX; Cdc42; dopamine; Parkinson's disease; phosphoproteome; PKC

## Introduction

Protein kinase C (PKC) is an important kinase in the enhancement of Ca<sup>2+</sup>-triggered exocytosis (Iwasaki et al., 2000; Barclay et al., 2003). The PKC family consists of at least 10 subtypes and is divided into the following three subfamilies: conventional PKC (cPKC), novel PKC, and atypical PKC (Nishizuka, 1988, 1992). Among PKCs, only cPKCs (including PKC $\gamma$ , which is a neuron-specific PKC isoform; Saito and Shirai, 2002) are activated by Ca<sup>2+</sup> because they contain a C2 domain that specifically binds to Ca<sup>2+</sup> and phosphatidylserine (PS; Murray and Honig, 2002).

The AS/AGU rat, a spontaneously occurring mutated animal that exhibits locomotor abnormalities, progressive dopaminergic (DAergic) neuronal degeneration in the substantia nigra (SN), and lower extracellular levels of dopamine (DA) in the striatum, has been used as a valuable model for parkinsonian syndrome (Payne et al., 2000). It is noteworthy that a mutation in PKC $\gamma$  that leads to the early termination at the C2 domain without possessing the catalytic domain causes parkinsonian syndrome in AS/AGU rats (Craig et al., 2001). The mutation in AS/AGU rats should result in the kinase-dead form of PKC $\gamma$ , but it is still unclear how the mutation causes parkinsonian symptoms.

PKC has been shown to modify exocytosis in at least three steps: (1) increased vesicle recruitment into readily releasable pools (Gillis et al., 1996; Stevens and Sullivan, 1998), (2) acceleration of fusion pore expansion (Scepek et al., 1998), and (3) changes in the kinetics of exocytosis (Graham et al., 2002). However, only the functional consequences of the phosphorylation of SNAP25 (Iwasaki et al., 2000), synaptotagmin I (Hilfiker et al., 1999), and Munc18 (Barclay et al., 2003) by PKC have been established on exocytosis *in vivo*. Furthermore, no attempts have been made to achieve a comprehensive understanding of DA exocytosis through an identification of

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Correspondence should be addressed to Naoaki Saito, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501 Japan. E-mail: naosaito@kobe-u.ac.jp.

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PKC $\gamma$  substrates. Therefore, we attempted to identify the PKC substrates involved in exocytosis to reveal the mechanisms of regulated exocytosis.

Pak-interacting exchange factor- $\beta$  ( $\beta$ PIX) is a Rho guanine nucleotide exchange factor (GEF) that specifically activates Rac1 and Cdc42 (Shin et al., 2002; Shin et al., 2004; Chahdi et al., 2005; Feng et al., 2006; Shin et al., 2006; ten Klooster et al., 2006; Chahdi and Sorokin, 2008).  $\beta$ PIX has been reported to be an essential element of the exocytotic machinery in neuroendocrine cells (Audebert et al., 2004; Mombisse et al., 2009). To date, there have been several studies on  $\beta$ PIX phosphorylation (Shin et al., 2002; Chahdi et al., 2005; Shin et al., 2006; Mayhew et al., 2007), but there have been no reports of the involvement of  $\beta$ PIX phosphorylation in DA release.

In the present study, we found that the AS/AGU rat is indeed a PKC $\gamma$ -knock-out (KO) animal, and our phosphoproteome analysis using PKC $\gamma$  KO mice found 10 candidates in the striatum that are phosphorylated by PKC $\gamma$ . Among the 10 candidates, we demonstrated that PKC $\gamma$  activated DA release through the phosphorylation of  $\beta$ PIX.

## Materials and Methods

**Antibodies.** The anti-GFP antibody (Ab) and the anti-vesicular monoamine transporter 2 (VMAT2) Ab recognizing the C-terminal of mouse VMAT2 were prepared in house. The anti-PKC $\gamma$  (C2-domain) monoclonal Abs specifically recognizing C2-domain of PKC $\gamma$  have been described previously (Kose et al., 1990). The following Abs were purchased: anti-FLAG from Sigma-Aldrich (catalog #P2983 RRID:AB\_439685); anti- $\beta$ PIX (SH3 domain) from Millipore; anti- $\beta$ -actin (catalog #ab66338 RRID:AB\_2289239) and anti-PKC $\gamma$  (N-terminal) specifically recognizing N-terminal of PKC $\gamma$  from Abcam; anti-glutathione S-transferase (GST) (catalog #sc-33613 RRID:AB\_647588), anti-PKC $\alpha$  (catalog #sc-208 RRID:AB\_2168668), anti-PKC $\beta$ 1 (catalog #sc-209 RRID:AB\_2168968), anti-PKC $\beta$ 2 (catalog #sc-210 RRID:AB\_2252825), and anti-PKC $\gamma$  (catalog #sc-211 RRID:AB\_632234) from Santa Cruz Biotechnology; and anti-serPKC motif (catalog #2261S RRID:AB\_330310), anti- $\beta$ PIX (catalog #4515S RRID:AB\_2274365) for immunoprecipitation (IP), and anti-postsynaptic density-95 (PSD95; catalog #2507S RRID:AB\_10695259) from Cell Signaling Technology.

**Production of anti-phosphoThr76, anti-phosphoSer340, anti-phosphoSer583, and anti- $\beta$ 2PIX Abs.** The production of anti-phospho Abs was performed as described previously (Matsubara et al., 2012). For the preparation of anti-phospho-Thr76 (pT76), anti-phospho-Ser340 (pS340), and anti-phospho-Ser583 (pS583)  $\beta$ PIX Abs, oligopeptides corresponding to the amino acids of human  $\beta$ PIX containing pT76 [VSPKSG(pT)LKSP], pS340 [SASPRM(pS)GFIYQ], and pS583 [SLGRRS(pS)LSRLE] were used as antigens, respectively. After the fifth boost, serum was collected and purified with an affinity column and the non-phospho-antigen peptide. The anti- $\beta$ 2PIX Ab was obtained by eluting the IgG from those that were bound to the nonphospho-Ser583 peptide column.

**Animals.** The AS/AGU rats were provided by R.W. Davies (Payne et al., 2000). The PKC $\gamma$ -Cre knockin (KI) mouse was provided by Z.F. Chen (Ding et al., 2005). After the sixth backcross, homozygous littermates obtained by crossing the heterozygous PKC $\gamma$ -Cre KI mouse were used as the PKC $\gamma$  KO and wild-type (WT) mice in the studies. All animal studies were approved by the Institutional Animal Care and Use Committee and conducted according to the Kobe University Animal Experimentation Regulations.

**Sample preparation and Western blot analysis.** The brains from AS/AGU rats and mice were homogenized and the concentrations of the proteins were measured with a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). SDS-PAGE and immunoblot analyses were performed as described previously (Adachi et al., 2005).

**Preparation of P2 synaptosomal fraction.** Adult male mouse brains were collected and homogenized in ice-cold 0.32 M sucrose solution containing 1 mM phenylmethylsulfonyl fluoride, 20  $\mu$ g/ml leupeptin, and a phosphatase-inhibitor cocktail (Nacalai Tesque). The total homogenate

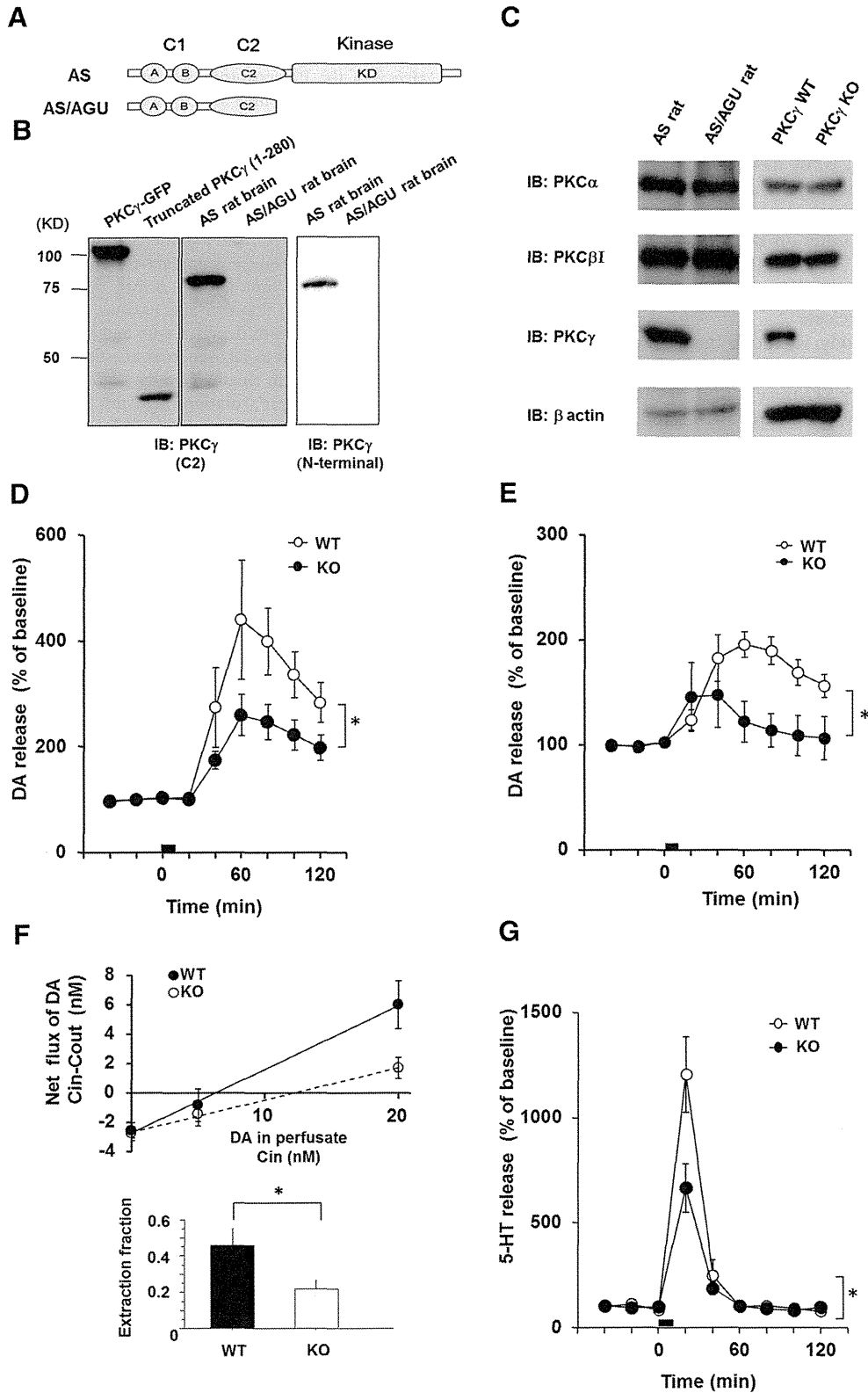
was subjected to centrifugation at  $800 \times g$  for 12 min at 4°C to remove the nuclei and the supernatant, which we defined as the total fraction, was further centrifuged at high speed at  $22,000 \times g$  for 20 min at 4°C. The pellet was used as the P2 synaptosomal fraction. To determine the efficiency of the P2 synaptosomal extraction process, we compared the amount of VMAT2 and PSD95 proteins between the total fraction and P2 synaptosomal fraction in the same amount of protein (50  $\mu$ g), which was calculated using the BCA protein assay kit.

**In vivo microdialysis.** *In vivo* microdialysis was performed with male mice essentially as described previously (Koda et al., 2010; Ago et al., 2013). In brief, mice were anesthetized by injection of sodium pentobarbital (40 mg/kg, i.p.), and a guide cannula (one site per animal) for a dialysis probe (Eicom) was implanted stereotaxically in the dorsal striatum (anterior 0.1 mm, lateral 1.8 mm, ventral 2.2 mm relative to the bregma and skull; Franklin and Paxinos, 1997). The cannula was cemented in place with dental acrylic and the animals were maintained warm and allowed to recover from anesthesia. The active probe membrane was 1 mm in length. Two days after the surgery, the probe was perfused with Ringer's solution (147.2 mM NaCl, 4.0 mM KCl, and 2.2 mM CaCl<sub>2</sub>, pH 6.0; Fuso Pharmaceutical Industries) at a constant flow rate of 1  $\mu$ l/min. To prepare the Ringer's solution containing 100 mM K<sup>+</sup>, an identical amount of sodium was replaced for maintaining iso-osmolality. Experiments were initiated after a stabilization period of 3 h. Microdialysis samples (20  $\mu$ l) were collected every 20 min and were assayed for DA by high-performance liquid chromatography (HPLC) with electrochemical detection. No-net-flux microdialysis experiments were conducted in a PKC $\gamma$  KO and WT mice as described previously (Justice, 1993; Chefer et al., 2005; Hewett et al., 2010). Three different concentrations of DA in Ringer's solution ( $C_{in}$  of 0, 5 and 20 nM DA) were perfused through the probe and DA in the perfusates ( $C_{out}$ ) was measured in the fifth fraction following 4 fractions (equilibration period) at each applied DA concentration. A slope was calculated for the linear regression for DA applied ( $C_{in}$ ) and the difference between dopamine applied and DA measured ( $C_{in} - C_{out}$ ). The slope (extraction fraction) is an indirect measure of dopamine transporter (DAT) dynamics *in vivo* to remove extracellular DA.

**Measurement of DA and DA metabolite levels in striatum.** The concentrations of DA were quantified by HPLC with an electrochemical detector (ECD-100; Eicom; Kawasaki et al., 2006; Kawasaki et al., 2007). Tissue samples were homogenized in 0.2 M perchloric acid containing 100  $\mu$ M EDTA and isoproterenol as an internal standard. The homogenate was centrifuged at  $15,000 \times g$  for 15 min at 0°C. The supernatant was filtered through a 0.22  $\mu$ m membrane filter (Millipore), and then a 10  $\mu$ l aliquot of the sample was injected onto the HPLC column every 30 min for the DA assay. An Eicompak SC-50DS column (3.0 mm i.d.  $\times$  150 mm; Eicom) was used, and the potential of the graphite electrode (Eicom) was set to +750 mV against an Ag/AgCl reference electrode. The mobile phase contained 0.1 M sodium acetate/0.1 M citrate buffer, pH 3.5, 190 mg/L octanesulfonic acid, 5 mg/L EDTA, and 17% (v/v) methanol. Data were calculated by analyzing the peak area of the external standard of dopamine hydrochloride (Sigma-Aldrich).

**Cell culture.** COS7 and HEK293 cells were cultured in DMEM and Eagle's minimum essential medium (Nacalai Tesque), respectively, which were supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100  $\mu$ g/ml). Nonessential amino acids (100  $\mu$ M) were added for HEK293 cells. PC12 cells were cultured in DMEM containing 10% fetal bovine serum and 5% horse serum. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Construction of plasmids.** WT PKC $\gamma$  was cloned into pcDNA3.1 (Life Technologies) and the subdomains of PKC $\gamma$  were cloned into pcDNA3.1 with GFP, as described previously (Seki et al., 2005). Human  $\beta$ PIX was provided by the RIKEN BioResource Center through the National BioResource Project of MEXT in Ibaraki, Japan (Ota et al., 2004). For the construction of plasmids encoding full-length  $\beta$ 2PIX that was fused with 3xFLAG at the N terminal,  $\beta$ 2PIX with a NotI/BamHI site that was produced by PCR was cloned into a 3xpFLAG-CMV10 vector (Sigma-Aldrich). Because the target sequence for rat  $\beta$ PIX knock-down (KD; sh369) was located in the coding region of  $\beta$ PIX, sh369-resistant  $\beta$ 2PIX in the 3xpFLAG-CMV10 vector was made by placing 6-base silent



**Figure 1.** The PKC $\gamma$  KO model exhibits symptoms of parkinsonian syndrome. *A*, Schematic illustrations of the PKC $\gamma$  protein and AS/AGU mutations. The truncated PKC $\gamma$  polypeptide terminates within the C2 domain. *B*, Recombinant truncated PKC $\gamma$  (1–280 aa), which was transfected in COS-7 cells, the AS rat brain lysate, and the AS/AGU rat brain lysate, was detected by immunoblot analysis with an anti-PKC $\gamma$  (C2-domain) monoclonal antibody and anti-PKC $\gamma$  (N terminal) antibody, respectively. *C*, The PKC $\gamma$  KO was confirmed by immunoblot analysis of the whole brain of the AS/AGU rats and PKC $\gamma$  KO mice. Arrowheads are recombinant PKC $\gamma$ -GFP and PKC $\gamma$  (1–280aa)-GFP, which were used as positive controls. *D*, *In vivo* microdialysis in the striatum of the PKC $\gamma$  KO mice. A high level of K<sup>+</sup> was perfused into the striatum through a dialysis probe for the time indicated by the square at 3–4 months. The results are expressed as mean  $\pm$  SEM ( $n = 4–5$ , interaction of the genotype and time for DA release that was stimulated by high K<sup>+</sup> levels; \* $p < 0.05$ ,  $F_{(8,48)} = 2.31$ , repeated two-way ANOVA). *E*, *In vivo* microdialysis for DA in the striatum of the PKC $\gamma$  KO (KO) mice that were stimulated with METH. METH (1 mg/kg) was perfused into the striatum through a dialysis probe for the time indicated by the square at 3 months (*Figure legend continues*).

changes within the targeting sequence (5'-CAACGAGtGAAaAATTa-3') with a QuikChange Multisite-Directed Mutagenesis Kit (Agilent Technologies). For construction of plasmids encoding full-length  $\beta$ 2PIX or fragments that were fused to GST, full-length  $\beta$ 2PIX and the SH3 (1–92 aa), DH (93–274 aa), and PH (275–400 aa) C-terminal (401–625 aa) regions were amplified by PCR with a NotI/BamHI site and cloned into the pGEX-6P1 vector. Substitutions of Ser or Thr to Ala or Glu at the identified phosphorylation sites (Thr76Ala, Ser215Ala, Ser340Ala or Glu, and Ser583Ala or Glu) were introduced with a QuikChange kit.

**Protein expression.** Protein expression was performed as described previously (Kawasaki et al., 2010). In brief, BL21 pLys *Escherichia coli* and Sf9 cells were transformed with expression plasmids. *E. coli* and Sf9 cells were harvested and lysed. For the purification of recombinant proteins, GST-fusion proteins were purified with glutathione-Sepharose 4B resin (GE Healthcare Biosciences).

**RNAi: short hairpin RNA and small interfering RNA.** Double-stranded oligonucleotides were cloned into the short hairpin RNA (shRNA) expression vector, pSuper (puro; Oligoengine). The target sequence for the shRNA rat  $\beta$ PIX KD was 5'-GCAGACCAGCGAGAAGTTGAG-3' (sh369; coding nucleotides 369–389). Because the  $\beta$ PIX shRNA sequence that we used was common to both the  $\beta$ 1 and  $\beta$ 2 isoforms, KDs of both  $\beta$ 1 and  $\beta$ 2 in PC12 cells were examined with  $\beta$ PIX SH3- and  $\beta$ 2-specific antibodies. The synthesized small interfering RNA (siRNA) for rat  $\beta$ PIX was composed of a mixture of 4 oligonucleotides (si878: 5'-GGGAUGACAUAAGACGUU-3', si806: 5'-AGUGUCAAGAAGUACGAAA-3', si1115: 5'-GGAGCAUGAUCGAGCGCAU-3', and si1153: 5'-CAACAGGACUUGCACGAAU-3') was purchased from Thermo Fisher Scientific (SmartPool). Verified shRNA plasmids for KD of Cdc42 (sh197; 5'-GATTACGACCGCTGAGTTA-3'; Ueyama et al., 2014) and Rac1 (sh618; 5'-CCTTTGTACGCTTTGCTCA-3'; Ueyama et al., 2006) were described previously.

**In vitro PKC phosphorylation assay.** An *in vitro* PKC phosphorylation assay was performed as described previously (Kawasaki et al., 2010). In brief, precipitated FLAG-tagged  $\beta$ 2PIX proteins or purified GST-tagged  $\beta$ 2PIX were incubated with 200 ng of GST-tagged PKC $\gamma$  or GST and the following buffers: 20 mM Tris, pH 7.4, 0.5 mM CaCl<sub>2</sub>, 10  $\mu$ M ATP, 0.5 mCi [ $\gamma$ -<sup>32</sup>P] ATP, 8  $\mu$ g/ml PS, and 0.8  $\mu$ g/ml ( $\pm$ )-1,2-didecanoylglycerol (DO) in a 50  $\mu$ l final volume. The samples were incubated with or without PKC inhibitors, including GF109203X (GFX), which was used as a pan PKC inhibitor, and G86976, which was used as a cPKC inhibitor, at 30°C for 15 min. For the calculation of the relative phosphorylation levels, the densitometries of the autoradiography were normalized with the total protein levels. The average relative phosphorylation levels of PKC $\gamma$  stimulation were defined as 1.00.

**PKC phosphorylation assay in cells.** A PKC phosphorylation assay in cells was performed as described previously (Kawasaki et al., 2010) but with slight modifications. In brief, HEK293 cells were transfected with WT  $\beta$ 2PIX in 3xpFLAG-CMV10 with a NEPA21 electroporator (Nepa Gene). After 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulation with or without PKC inhibitors for 30 min in HEPES buffer at 37°C, the cells were collected and resuspended in homogenization buffer containing 150 mM NaCl, 10 mM ethylene glycol tetraacetic acid, 2 mM ethylenediamine tetracetic acid, 10 mM HEPES, pH 7.4, 1 mM phenylmethylsulfonyl

fluoride, 20  $\mu$ g/ml leupeptin, and a phosphatase-inhibitor cocktail. The precipitated proteins were separated by SDS-PAGE. The phosphorylated proteins were visualized with phospho-Abs. For the calculation of the relative phosphorylation levels, the densitometries of the immunoblots of the phospho-Abs were normalized to the total protein levels in each experiment and the averages of the relative levels of phosphorylation in more than three independent experiments are presented. The phosphorylation levels of the prestimulations were defined as 1.00.

**PKC phosphorylation assay in vivo.** The mice P2 synaptosomal fraction was resuspended in HEPES buffer containing 1 mM phenylmethylsulfonyl fluoride, 20  $\mu$ g/ml leupeptin, and a phosphatase-inhibitor mixture and used for the *in vivo* PKC phosphorylation assay (Wu et al., 1982). After 2  $\mu$ M TPA stimulation with or without 2  $\mu$ M GFX for 30 min in HEPES buffer at 37°C, the P2 fraction was collected and resuspended in homogenization buffer containing 150 mM NaCl, 10 mM ethylene glycol tetraacetic acid, 2 mM ethylenediamine tetracetic acid, 10 mM HEPES, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 20  $\mu$ g/ml leupeptin, and the phosphatase-inhibitor mixture. The precipitated proteins were separated by SDS-PAGE. The phosphorylated proteins were visualized with ser-PKC motif Abs.

**DA release assay.** DA release assays in  $\beta$ PIX KD cells were performed 96 h after transfection by NEPA21. PC12 cells were washed thrice with 500  $\mu$ l of incubation solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 2 mM glucose, 20  $\mu$ M pargyline, and 10 mM HEPES, pH 7.5) and then incubated for 60 min in 500  $\mu$ l of incubation solution with <sup>3</sup>H-DA (PerkinElmer), followed by three washes with 500  $\mu$ l of incubation solution. The cells were allowed to rest or were stimulated with 500  $\mu$ l of a high-K<sup>+</sup> solution containing 100 mM KCl and 35 mM NaCl for 10 min. The supernatant was collected and the cells were harvested in 500  $\mu$ l of incubation solution with 2% Triton X-100. The amount of DA that was secreted into the medium and retained in the cells was measured in 500  $\mu$ l of samples with a scintillation counter LS-6500 (Beckman Coulter). DA secretion was expressed by the following formula: %DA = (<sup>3</sup>H in supernatant) / (<sup>3</sup>H in supernatant + <sup>3</sup>H in cell lysate). A collagen-IV-coated six-well plate (BD Biosciences) was used for the DA release assay.

**Mass spectrometry for  $\beta$ 2PIX-phosphorylation site identification.** Mass spectrometry for  $\beta$ 2PIX-phosphorylation site identification was performed as described previously (Sakuma et al., 2012). After the *in vitro* PKC phosphorylation assay and electrophoresis, silver-stained bands that corresponded to GST-tagged  $\beta$ 2PIX proteins were excised and destained. After reduction-alkylation reactions, the proteins in the gels were digested with porcine trypsin (sequencing grade; Promega) in 50 mM ammonium bicarbonate for 15 h at 37°C. The peptide fragments extracted from the gels were subjected to liquid chromatography/tandem mass spectrometry (LC/MS/MS) with a high-performance liquid chromatography system (Paradigm MS4; Michrom Bioresources) coupled to a linear ion trap mass spectrometer (Finnigan LTQ Orbitrap XL; Thermo Fisher Scientific). The LC/MS/MS data were interpreted with a MASCOT MS/MS ions search (Matrix Science).

**Phosphoproteome analysis.** Phosphoproteome analysis was performed as described previously with some modifications (Saito et al., 2006). Mice striata were dissolved in 50 mM Tris HCl, pH 9.0, 8 M urea, 10 mM ethylenediamine tetracetic acid, 1 mM phenylmethylsulfonyl fluoride, 20  $\mu$ g/ml leupeptin, and a phosphatase inhibitor mixture. After homogenization with a Dounce homogenizer (10 strokes), the resultant solution was centrifuged at 2000  $\times$  g for 5 min and the supernatant was collected. The protein amounts were measured with a BCA protein assay kit. The proteins from the striatum were dried and resuspended in 50 mM Tris HCl buffer, pH 9.0, containing 8 M urea at a concentration of 10  $\mu$ g/ $\mu$ l. These mixtures were subsequently reduced with dithiothreitol, alkylated with acrylamide, and digested with Lys-C endopeptidase at 37°C overnight, followed by trypsin digestion at 37°C overnight. The digested solutions were desalted and concentrated with Empore high-performance extraction disk cartridges (3M). Phosphopeptide enrichment was performed with hydroxy acid-modified metal oxide chromatography (HAMMO; Titansphere Phos-TiO Kit; GL Sciences; Kyono et al., 2008). For elution of the phosphopeptides, 50  $\mu$ l of 5% NH<sub>3</sub> and 5% pyrrolidine were used. The fractions were immediately acidified and desalted with SPE-C tips (GL Sciences). A Tomy CC-105 vacuum evap-

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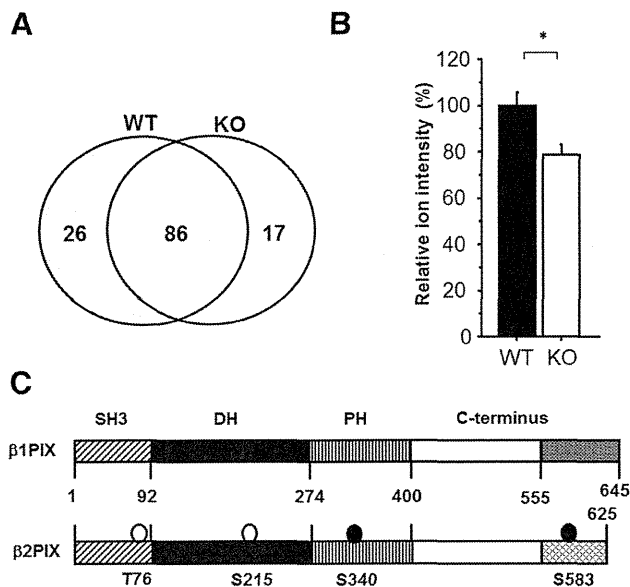
(Figure legend continued.) (n = 4, interaction of the genotype and time for DA release stimulated by METH,  $F_{(8,48)} = 3.37$ ; \* $p < 0.01$ , repeated two-way ANOVA). **F**, No-net flux microdialysis to quantitate basal DAT activity in PKC $\gamma$ -KO mice. Three different concentrations of DA in CSF (0, 5, and 20 nM DA) were perfused through the probes to determine the extracellular DA concentration and extraction fraction. Linear regression for the DA perfused and DA measured provided extraction fraction (slope) as an indirect measure of DAT activity *in vivo* to remove extracellular DA. Extraction fraction for WT and KO mice are shown. Data represent mean  $\pm$  SEM (n = 4 and 5 for WT and KO mice, respectively, \* $p < 0.05$ ). **G**, *In vivo* microdialysis of serotonin in the striatum of the PKC $\gamma$ -KO mice that were stimulated by high K<sup>+</sup> levels. K<sup>+</sup> (100 mM) was perfused into the striatum through a dialysis probe for the time indicated by the square at 3–4 months. The results are expressed as mean  $\pm$  SEM (n = 4, interaction of the genotype and time for serotonin release that was stimulated by high K<sup>+</sup> levels,  $F_{(8,48)} = 5.399$ ; \* $p < 0.001$ , repeated two-way ANOVA).

**Table 1. DA and DA metabolite levels in the striatum of PKC $\gamma$  WT and KO mice**

Age (mo)	Genotype	DA (ng/g weight)	DOPAC (ng/g weight)	HVA (ng/g weight)
3	KO	19629.5 $\pm$ 625.4	1838.6 $\pm$ 68.9	1490.6 $\pm$ 89.0
	WT	19516.4 $\pm$ 361.1	1999.1 $\pm$ 103.4	1652.1 $\pm$ 18.4
6	KO	20667.2 $\pm$ 803.5	1671.2 $\pm$ 113.0	1620.0 $\pm$ 93.9
	WT	17416.8 $\pm$ 781.3	1559.2 $\pm$ 77.2	1334.6 $\pm$ 63.7
12	KO	28911.0 $\pm$ 1801.4	2576.0 $\pm$ 277.9	2378.1 $\pm$ 161.3
	WT	29564.9 $\pm$ 2581.4	2253.1 $\pm$ 202.2	2392.2 $\pm$ 128.4

Results are expressed as mean  $\pm$  SEM of 4–5 mice.

HVA, Homovanillic acid.



**Figure 2.** Phosphoproteome analysis revealed 10 phosphopeptides with decreased average ion intensity in PKC $\gamma$  KO mice striatum. *A*, Overview of the phosphoproteome (WT,  $n = 5$ ; KO,  $n = 4$ ). *B*, Average ion intensity of the Ser340  $\beta$ PIX phosphopeptide is calculated in both PKC $\gamma$  KO and WT mice ( $n = 4$ – $5$ ;  $*p < 0.05$ , unpaired  $t$  test). The relative average ion intensity of WT mice was defined as 100%. The results are expressed as mean  $\pm$  SEM. *C*, Schematic illustrations of the  $\beta 1$  and  $\beta 2$  PIX protein. The phosphorylation sites that were determined by the *in vitro* PKC phosphorylation assay and the phosphoproteome analysis are circled. The open circles are the phosphorylation sites that were determined only *in vitro*. The closed circles are the phosphorylation sites that were determined *in vitro* and *in vivo*.

**Table 2. List of phosphopeptides with a PKC phosphorylation motif**

Protein name	Accession no.	Sequence	Site	No. of phosphopeptides		Lowest peptide score		Highest peptide score		Average ion intensity		
				WT	KO	WT	KO	WT	KO	WT	KO	WT/KO
Gap junction alpha-1 protein	NP 034418	KVAAGHELQPLAIVDQRPS $\underline{S}$ RA	S365	7	0	11	—	28	—	3	0.4	7.3
Disks large-associated protein 1	NP 808307	RSLDSLDPAGLLT $\underline{S}$ PKF	S437	5	0	48	—	77	—	3.4	0.8	4.4
MAP kinase-activating death domain protein	NP 001171190	RA $\underline{I}$ LS $\underline{D}$ SEI $\underline{T}$ NSATS $\underline{A}$ IFGKA	T1235	8	4	40	26	90	58	3.7	1	3.9
DnaJ homolog subfamily C member 5	NP 001258513	RSL $\underline{S}$ TSGESLYHVLGLDKN	S10	54	55	3	4	88	85	969	299.5	3.2
Calnexin	NP 001103969	KAEDEILNRS $\underline{P}$ RN	S582	6	2	25	22	31	34	3.1	1.8	1.8
Stathmin	NP 062615	KRASGQAFELIL $\underline{S}$ PRS	S16	26	23	8	6	93	84	12.1	7.5	1.6
Stathmin	NP 062615	RASGQAFELIL $\underline{S}$ PRS	S25	25	17	5	19	81	83	7.4	5.9	1.3
Rho guanine nucleotide exchange factor 7	NP 001106989	RM $\underline{S}$ GFIYQGLK	S340	3	0	24	—	59	—	1.8	1.4	1.4
Reticulon-4	NP 077188	RRGSGSVDEL $\underline{F}$ ALPAASEPVPSSAEKI	S165	15	8	34	41	70	59	2.3	1.7	1.3
$\alpha$ -adducin	NP 001019629	KFRTP $\underline{S}$ FLKK	S724	3	2	7	9	29	25	6.1	4.8	1.3
$\beta$ -adducin	NP 001258786	KDIATEK $\underline{P}$ GSPVKS	S594	10	8	10	17	50	56	0.4	0.3	1.2

Phosphopeptides with a WT/KO ratio  $> 1.0$  are listed. The numbers of phosphorylation sites are based on the results of mice in PhosphoSitePlus ([www.phosphosite.org](http://www.phosphosite.org)).  $\underline{S}$  and  $\underline{I}$  are the identified phosphorylation sites. Number of phosphopeptides means the total numbers of the phosphopeptides in each group ( $n = 4$ ). Highest or lowest peptide score means the highest or lowest peptide score in each group ( $n = 4$ ). Peptides that were not detected are shown as —.

orator was used to concentrate the sample and the phosphopeptides were analyzed by LC/MS/MS.

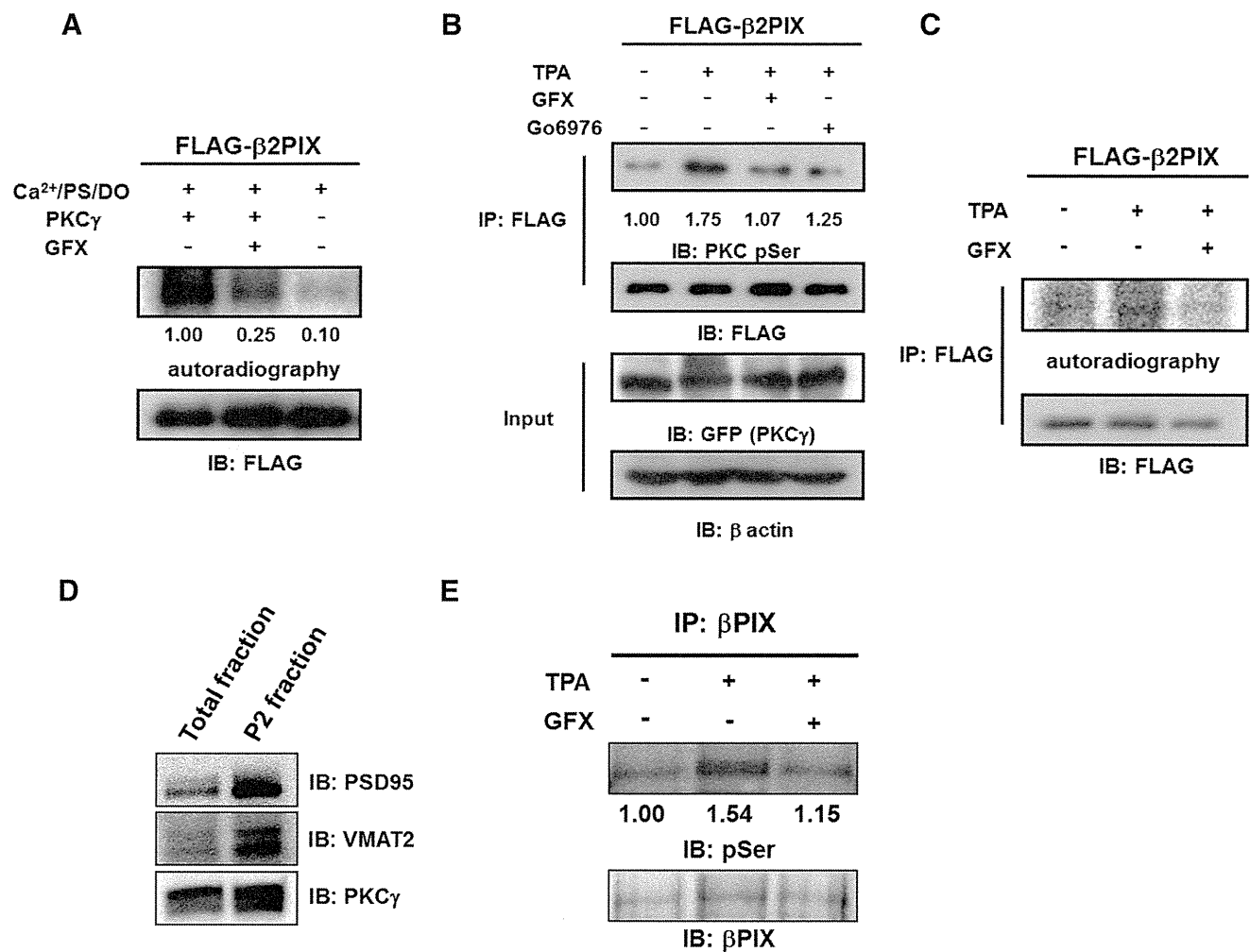
**Statistical analysis.** The data are presented as mean  $\pm$  SEM and were analyzed with unpaired  $t$  tests, one-way ANOVA with a *post hoc* Dunnett's test, or a repeated two-way ANOVA. The statistical analyses were performed with the Statview 5.0J software package (SAS Institute).  $p$ -values of 5% or less were considered statistically significant.

## Results

### PKC $\gamma$ KO animals exhibited parkinsonian symptoms, including DA release impairment in the striatum

AS/AGU rats show altered behaviors (Craig et al., 2001), DA release impairment, and the pathology of the nigrostriatal system resembles the pathology observed in human patients with parkinsonian syndrome (Campbell et al., 1996; Payne et al., 2000). AS/AGU rats have a spontaneously occurring mutation that changes the CAG (Glu281) codon to a TAG (stop codon) in PKC $\gamma$ , and the putatively truncated PKC $\gamma$ , if produced, will terminate at the fifth residue from the C terminus of the last strand of the  $\beta$ -sheet structure of the C2 domain (Fig. 1A). The truncated protein may result in a severe or complete loss of the kinase function of PKC $\gamma$ , although it has not yet been clarified whether the truncated protein is expressed in AS/AGU rats. Although both anti-PKC $\gamma$  (N terminal) Ab and anti-PKC $\gamma$  (C2-domain) Ab detected an 80 kDa single band in AS rats, no band was detected in the AS/AGU rats (Fig. 1B). These findings indicated that PKC $\gamma$  was not expressed in AS/AGU rats, suggesting the hypothesis that PKC $\gamma$  KO mice show similar symptoms as those observed in AS/AGU rats. We first confirmed that our PKC $\gamma$  KO mice did not express PKC $\gamma$ , whereas the expression levels of other members of cPKCs were unaltered (Fig. 1C). To determine the effects of the PKC $\gamma$  KO on DA release in the striatum, we performed *in vivo* microdialysis in the striatum. The basal line extracellular levels of DA in the striatum did not differ significantly between the PKC $\gamma$  WT and KO mice. After treatment with high levels of K $^{+}$  (Fig. 1D) and methamphetamine (METH; Fig. 1E), the increase in DA release in the WT was significantly larger than that in the KO 3–4 months after birth. Although high K $^{+}$  levels induced DA release is mediated by exocytosis and not by DAT, PKC induces DAT endocytosis (Daniels and Amara, 1999), which may decrease the extracellular DA levels by increasing





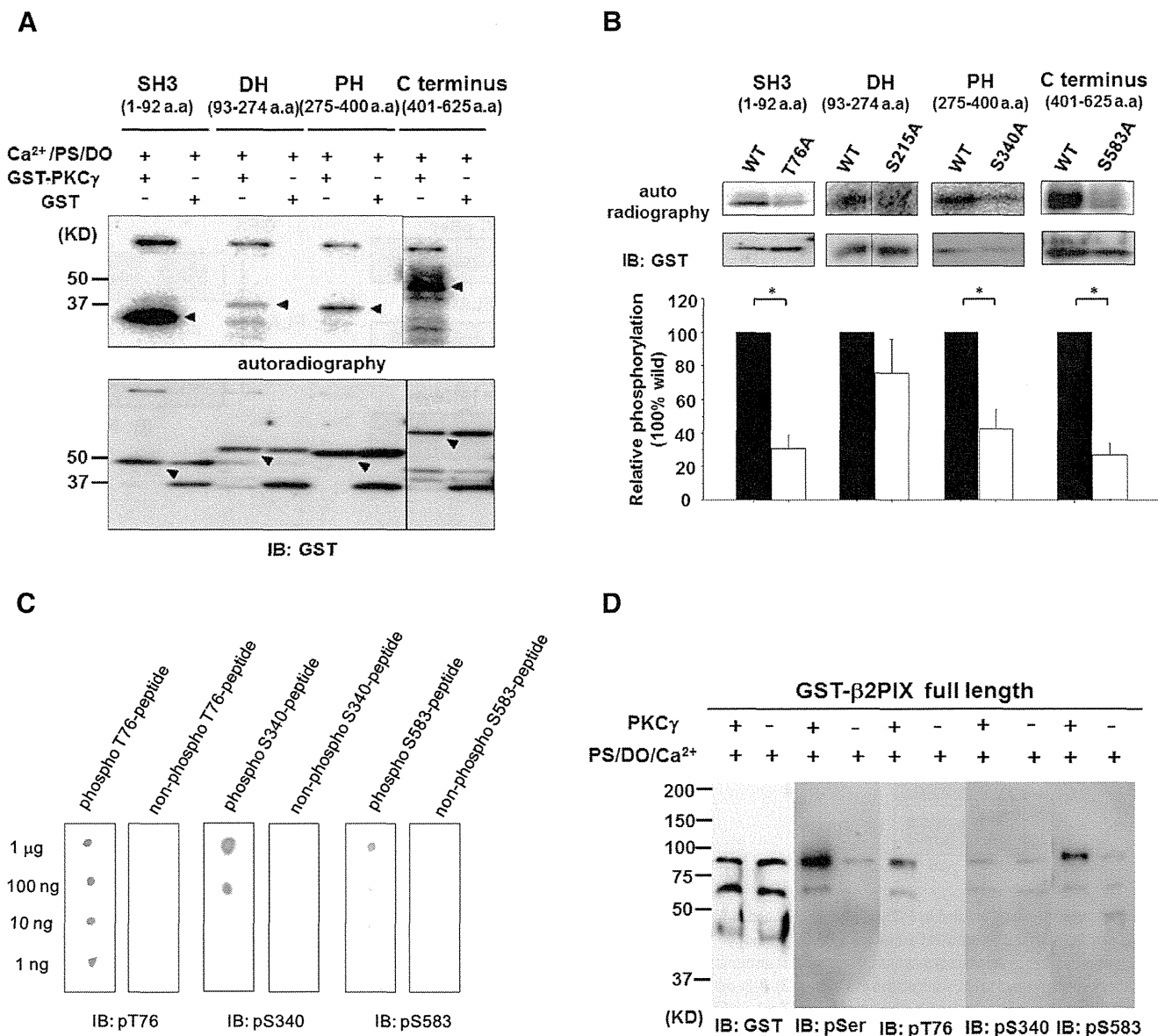
**Figure 3.**  $\beta$ PIX is phosphorylated by PKC $\gamma$  *in vitro*, in cells, and *in vivo*. **A**, *In vitro* phosphorylation of  $\beta$ 2PIX. FLAG-tagged  $\beta$ 2PIX proteins were purified and incubated with or without recombinant PKC $\gamma$  in the presence of PKC activator (PS/DO/Ca<sup>2+</sup>) and [ $\gamma$ -<sup>32</sup>P]ATP for 20 min. The *in vitro* phosphorylation of  $\beta$ 2PIX was also performed in the presence of GFX. The phosphorylated proteins were detected by autoradiography and the levels of protein expression were determined by Western blotting with an anti-FLAG antibody. The numbers show the relative phosphorylation levels that were normalized to PKC $\gamma$  stimulation as 1.00 ( $n = 3$ ). **B**, *In-cell* phosphorylation of  $\beta$ 2PIX. HEK293 cells expressing FLAG-tagged  $\beta$ 2PIX and GFP-tagged PKC $\gamma$  were stimulated with 1  $\mu$ M 12-O-TPA for 20 min in the presence or absence of 1  $\mu$ M GFX or 1  $\mu$ M G6976. FLAG-tagged  $\beta$ 2PIX proteins were purified with anti-FLAG agarose resin. Phosphorylated proteins were detected by an immunoblot analysis with an anti-pSer PKC motif antibody. Protein expression was determined by Western blotting with an anti-FLAG antibody. The average relative phosphorylation levels for each experimental condition were normalized to the prestimulation signal set as 1.00 ( $n = 5$ ). **C**, PC12 cells expressing FLAG-tagged  $\beta$ 2PIX were incubated with <sup>32</sup>P monosodium phosphate and stimulated with 1  $\mu$ M 12-O-TPA in the absence or presence of 1  $\mu$ M GFX. FLAG-tagged  $\beta$ 2PIX proteins were purified with anti-FLAG agarose resin. Phosphorylated proteins were detected by autoradiography, and protein expression was determined by immunoblots with an anti-FLAG antibody. **D**, The same amount of samples of total fraction and the P2 synaptosomal fraction were immunoblotted by anti-PSD95 antibody as a postsynaptic marker, anti-VMAT2 antibody as a presynaptic marker, and anti-PKC $\gamma$  antibody. **E**, the P2 synaptosomal fraction was stimulated with 2  $\mu$ M 12-O-TPA in the absence or presence of 2  $\mu$ M GFX.  $\beta$ PIX proteins were purified with anti- $\beta$ PIX antibody. Phosphorylated proteins were detected by phospho-Abs. The numbers show the relative phosphorylation levels that were normalized to pretreatment as 1.00 ( $n = 3$ ).

DAT activity in PKC $\gamma$  KO mice striatum. To evaluate the DAT activity in PKC $\gamma$  KO mice, we performed the no-net-flux microdialysis experiment. The slope (extraction fraction) is the measure of the activity of DAT *in vivo*. Fig. 1G shows decreased DAT activity in the striatum of PKC $\gamma$  KO mice, suggesting that PKC $\gamma$  KO mice tend to have increased rather than decreased extracellular DA levels compared with WT mice (Fig. 1F). Consistent with our results, the increase in the serotonin release stimulated by high K<sup>+</sup> levels in WT was also significantly larger than that of the KO (Fig. 1G), as described previously in AS/AGU rats (Al-Fayez et al., 2005). Because we had preliminary data that suggested slight loss of DAergic neurons in 12-month-old, but not 3- or 6-month-old, PKC $\gamma$  KO mice, there was a possibility that the decrease in DA release in PKC $\gamma$  KO mice was due to the decrease in DA in the striatum. We measured DA and its metabolites using

HPLC in the striatum. No difference in DA or DA metabolite levels in the striatum was observed between PKC $\gamma$  KO mice and WT mice at 3, 6, and 12 months (Table 1), suggesting that DA release disorder was from the exocytotic machinery disorder instead of the lack of the DAergic neurons in the nigrostriatum system. These results suggested that the PKC $\gamma$  KO mice would show similar parkinsonian symptoms as those observed in the AS/AGU rats. From the analysis of the PKC $\gamma$  KO mice and AS/AGU rats, we concluded that PKC $\gamma$  KO animals could be used as a practical model of parkinsonian syndrome.

#### Phosphoproteome analysis identified 10 phosphoproteins, including $\beta$ PIX, with a PKC-phosphorylation motif

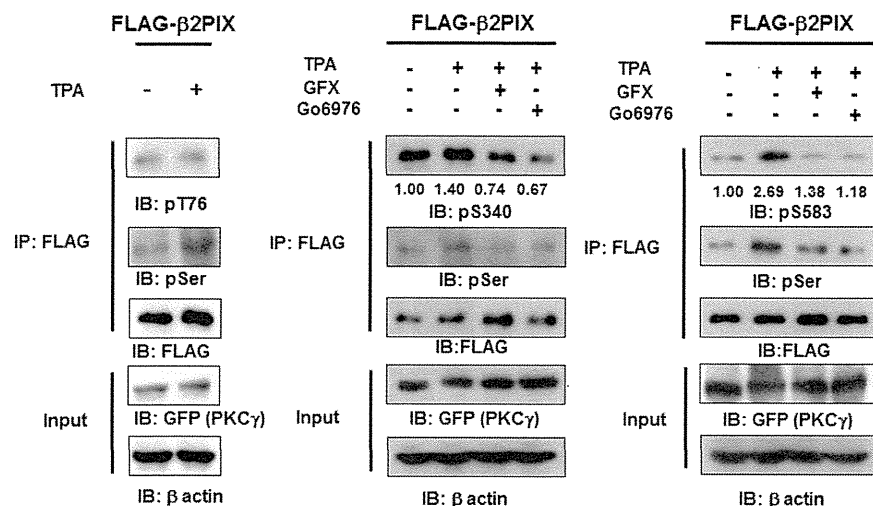
We hypothesized that a loss or decrease of PKC $\gamma$ -mediated phosphorylation in the nigrostriatal system results in DA release im-



**Figure 4.** Analysis of the *in vitro* PKC $\gamma$  phosphorylation sites in  $\beta$ 2PIX. **A**, *In vitro* phosphorylation of the GST-tagged  $\beta$ 2PIX SH3, DH, PH, and C terminus regions. Each protein region was expressed in *E. coli*, purified, and incubated with [ $\gamma$ -<sup>32</sup>P] ATP and recombinant PKC $\gamma$  in the presence of Ca<sup>2+</sup>, PS, and DO. The phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography (top). The protein levels of the recombinant  $\beta$ 2PIX protein regions were detected by Western blotting (bottom). The arrowheads on the top indicate the autoradiography and those on the bottom indicate the total protein for each domain. **B**, *In vitro* phosphorylation of the  $\beta$ 2PIX SH3, DH, PH, and  $\beta$ 2 C terminus regions containing the indicated mutations determined by an *in vitro* phosphorylation assay that was followed by mass spectrometry and phosphoproteome analysis. Phosphorylation levels were normalized to the WT phosphorylation signal for each domain, which were set to 100%: SH3 ( $n = 5$ ), DH ( $n = 4$ ), PH ( $n = 5$ ),  $\beta$ 2 C terminus ( $n = 3$ ); The results are expressed as mean  $\pm$  SEM ( $*p < 0.05$ , unpaired  $t$  test). **C**, Specificity of anti-phospho-Thr76, Ser340, and Ser583 antibodies. The indicated amount of the phospho-peptide (pT76 [VSPKSG(pT)LKSP], pS340 [SASPRM-(pS)GFIYQ], and pS583 [SLGRRS(pS)LSRLE]) or non-phosphopeptide was dotted on the PVDF membrane. Immunostaining was performed using purified anti-pT76, pS340, and pS583 antibodies. **D**, *In vitro* phosphorylation assay of full-length  $\beta$ 2PIX. GST-tagged full-length  $\beta$ 2PIX was expressed in *E. coli*, purified, and incubated with ATP and recombinant PKC $\gamma$  in the presence of Ca<sup>2+</sup>, PS, and DO. Phosphorylated proteins were separated by SDS-PAGE and detected by an anti-pSer PKC motif antibody, an anti-pT76 antibody, an anti-pS340 antibody, or an anti-pS583 antibody, respectively.

pairment. To identify the substrates for PKC $\gamma$ , we performed a phosphoproteome analysis using HAMMOC methods (Kyono et al., 2008; Fig. 2A). Among the phosphopeptides in the WT group, we chose the proteins that may have a relationship to exocytosis and then calculated the WT/KO ratio of the average ion intensity. The average ion intensity ratio of the phosphopeptides that included the PKC phosphorylation motif are shown in Table 2. Among these 10 candidates, we focused on  $\beta$ PIX (Fig. 2B), even when the degree of the phosphorylation decrease was small, because it is expressed in DAergic neurons in the

SNpc (<http://www.informatics.jax.org/assay/MGI:4944920>) and has been reported to play important roles in the machinery of exocytosis (Audebert et al., 2004; Mombosse et al., 2009). In the present phosphoproteome analysis, phosphorylation of Ser340 in  $\beta$ 2PIX was detected. The  $\beta$ PIX family consists of two splicing forms,  $\beta$ 1 and  $\beta$ 2; the difference between  $\beta$ 1 and  $\beta$ 2 PIX exists in the C terminus (Fig. 2C). Although both  $\beta$ 1 and  $\beta$ 2 contain Ser340,  $\beta$ 2PIX is the predominant form in the CNS (Koh et al., 2001). Therefore,  $\beta$ 2PIX was used in the present study.



**Figure 5.** PKC mediates the phosphorylation of full-length  $\beta$ PIX at Ser340 and Ser583 in cells. HEK293 cells transfected with FLAG-tagged WT  $\beta$ PIX and GFP-tagged PKC $\gamma$  were stimulated with 1  $\mu$ M TPA in the presence or absence of 1  $\mu$ M GFX, which is a pan-PKC inhibitor, or G6976, which is a cPKC inhibitor. FLAG-tagged  $\beta$ PIX was precipitated and separated by SDS-PAGE. The total amounts of protein were determined by immunoblot analyses with an anti-FLAG antibody. GFP-tagged PKC $\gamma$  or  $\beta$  actin was detected by anti-GFP or anti- $\beta$  actin antibodies. The phosphorylation levels of the FLAG-tagged  $\beta$ PIX proteins that were determined with an anti-pT76 antibody (**A**;  $n = 2$ ), an anti-pS340 (**B**;  $n = 4$ ), or an anti-pS583 (**C**;  $n = 4$ ) antibody were normalized to the phosphorylation levels of the WT. The numbers show the average relative phosphorylation levels that were normalized to prestimulation levels set as 1.00.

### $\beta$ PIX is phosphorylated by PKC $\gamma$ *in vitro*, in cells, and *in vivo*

To determine whether  $\beta$ PIX was phosphorylated by PKC $\gamma$ , we performed *in vitro*, in cells, and *in vivo* phosphorylation experiments.  $\beta$ PIX was phosphorylated by PKC $\gamma$  *in vitro*, whereas the inhibition of PKC activity with GFX abolished the phosphorylation (Fig. 3A). Next, we investigated whether  $\beta$ PIX was phosphorylated by PKC in cells. Enhanced phosphorylation of FLAG-tagged  $\beta$ PIX extracted from HEK293 cells transfected with FLAG-tagged  $\beta$ PIX and GFP-tagged PKC $\gamma$  after treatment with 1  $\mu$ M TPA was observed with an anti-Ser PKC motif Ab (Fig. 3B). Furthermore, the TPA-induced phosphorylation of  $\beta$ PIX in cells was reduced by the PKC inhibitors G6976 and GFX (Fig. 3B). These results were further confirmed in PC12 cells (Fig. 3C) and in the P2 synaptosomal fraction of the mouse brain (Fig. 3D). Collectively, these results showed that  $\beta$ PIX was phosphorylated by Ca<sup>2+</sup>-dependent PKC *in vitro*, in cells, and *in vivo*.

### PKC $\gamma$ phosphorylates Thr76 in SH3, Ser340 in PH, and Ser583 in the C terminus of $\beta$ PIX *in vitro*

$\beta$ PIX consists of the following four regions: SH3 (1–92 aa), DH (93–274 aa), PH (275–400 aa), and the C terminus (401–625 aa). Because each region of  $\beta$ PIX contains one or more predicted PKC phosphorylation sites, we performed an MS analysis combined with an *in vitro* phosphorylation assay using full-length GST-tagged  $\beta$ PIX for screening the phosphorylation sites by PKC $\gamma$ . The *in vitro* PKC assay, followed by the MS analysis, revealed the phosphorylation of Thr76, Ser215, and Ser583 in addition to that of Ser340, which was revealed by the phosphoproteome analysis of PKC $\gamma$  KO mice (Fig. 2C). Thr76, Ser215, and Ser340 are common sites in both  $\beta$ 1 and  $\beta$ 2PIX. However, Ser583 is found only in  $\beta$ 2PIX. To examine the PKC $\gamma$ -mediated phosphorylation of these sites, recombinant proteins were used in *in vitro* phosphorylation assays. Importantly, all domains of  $\beta$ 2PIX were phosphorylated by PKC $\gamma$  *in vitro*, but the SH3 and C-terminal domains were phosphorylated much more

strongly than the DH and PH domains (Fig. 4A). For the next step, we generated recombinant mutant proteins in which each Ser or Thr residue was replaced with Ala (Thr76Ala in SH3, Ser215Ala in DH, Ser340Ala in PH, and Ser583Ala in the C terminus) and examined which Ser/Thr residues of Thr76, Ser215, Ser340, or Ser583 could be phosphorylated by PKC $\gamma$  *in vitro*. We observed that the Thr76Ala and Ser583Ala mutant proteins exhibited impaired phosphorylation of the SH3 and C-terminal domains (Fig. 4B). The Ser340Ala in the PH domain showed a moderately reduced phosphorylation. The Ser215Ala showed no significant reduction of phosphorylation in the DH domain. These findings suggested that Thr76, Ser340, and Ser583, but not Ser215, are PKC $\gamma$  phosphorylation sites *in vitro*.

### cPKC phosphorylates $\beta$ PIX Ser340 and Ser583 in cells

To further confirm that  $\beta$ PIX is phosphorylated by cPKC in cells, we produced Abs that specifically recognized phosphorylated Thr76, Ser340, and Ser583 (Fig. 4C). The purified full-length  $\beta$ PIX was phosphorylated by recombinant PKC $\gamma$  *in vitro* and then applied to SDS-PAGE, which was followed by Western blotting with the anti-pT76, anti-pS340, and anti-pS583 Abs. The  $\beta$ PIX that was phosphorylated by PKC $\gamma$  was detected by anti-pT76 and anti-pS583, but not the anti-pS340 Ab (Fig. 4D), suggesting that Ser340 was not a direct PKC phosphorylation site. Next, we investigated whether  $\beta$ PIX was phosphorylated at Thr76, Ser340, and Ser583 in cells. Figure 5 shows that Ser340 and Ser583, but not Thr76, were phosphorylated in TPA-treated HEK293 cells. Moreover, the PKC-dependent phosphorylation of Ser340 and Ser583 was confirmed with the PKC inhibitors G6976 and GFX (Fig. 5). These findings suggested that Ser340 and Ser583 were phosphorylated by cPKC in cells. It is noteworthy that Ser583 was phosphorylated directly, whereas Ser340 was phosphorylated indirectly by PKC $\gamma$ .

### Involvement of cPKC in the regulation of DA release

A <sup>3</sup>H-DA release assay in PC12 cells, a cell line in a DAergic neuronal model, was used to study the functional role of PKC and  $\beta$ PIX in the regulation of DA release. PC12 cells expressed endogenous  $\beta$ PIXs and cPKCs, including PKC $\gamma$  (Fig. 6A). To determine the degree of DA release stimulated by K<sup>+</sup>, PC12 cells were stimulated with various K<sup>+</sup> concentrations and the DA release was increased in a K<sup>+</sup> concentration-dependent manner (Fig. 6B). The functional role of cPKC in the regulation of K<sup>+</sup>-induced DA release was monitored with G6976 and GFX. These PKC inhibitors significantly decreased the high K<sup>+</sup>-stimulated DA release (Fig. 6C). It is noted that there were no differences of <sup>3</sup>H-DA uptake into the PC12 cells between the control and PKC inhibitor groups (Fig. 6D,E). G6976 and GFX also significantly suppressed the TPA-stimulated DA release (Fig. 6F). The activation of PKC $\gamma$  after K<sup>+</sup>-induced depolarization was confirmed by monitoring the translocation of GFP-tagged PKC $\gamma$  from the cytosol to the plasma membrane in PC12 cells (Fig. 7A). These

results suggested that cPKC plays a crucial role in  $K^+$ -induced DA release machinery in PC12 cells.

### $\beta$ PIX KD suppressed DA release

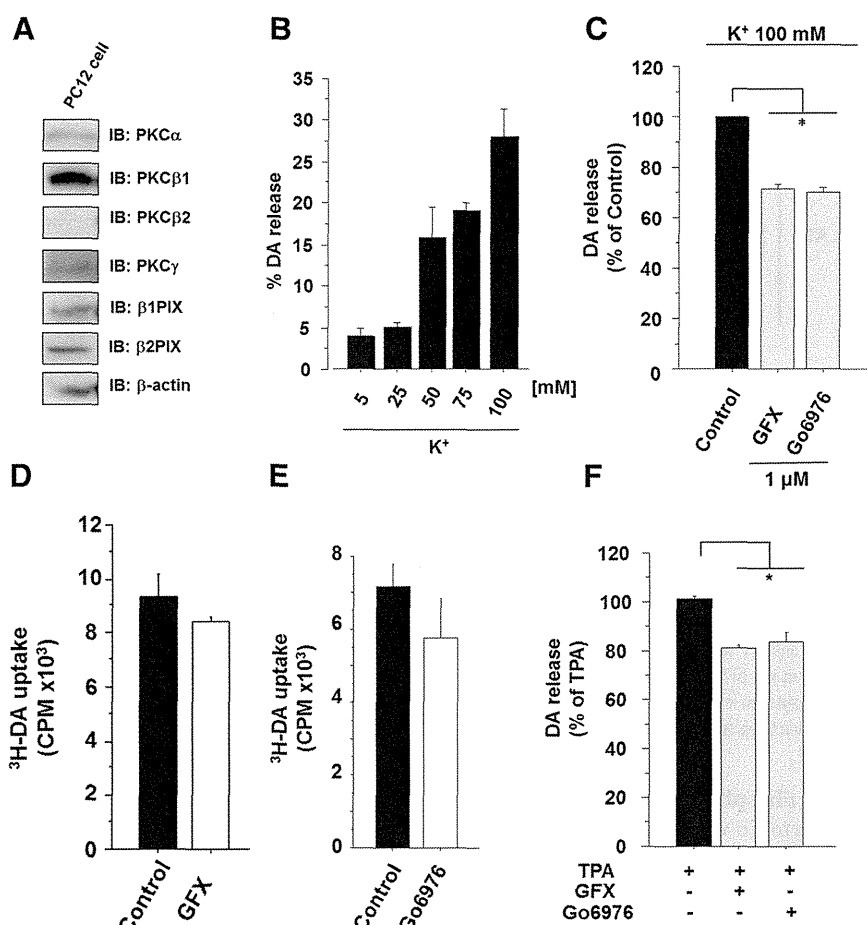
Because  $\beta$ PIX is able to stimulate growth hormone secretion from PC12 cells (Mombouisse et al., 2009; Mombouisse et al., 2010), we investigated the possible relationship between  $\beta$ PIX and DA release. KD of  $\beta$ PIX in PC12 cells by shRNA (sh369) resulted in a significant inhibition of  $K^+$ -evoked DA secretion from PC12 cells (Fig. 7B). A similar significant reduction in DA release was reproduced with synthetic  $\beta$ PIX siRNA (Fig. 7C). These results were consistent with the idea that  $\beta$ PIX is an important element of the DA exocytotic machinery.

### PKC-mediated phosphorylation of $\beta$ 2PIX at Ser340 and Ser583 promoted DA release

To determine the role of the PKC-mediated phosphorylation of  $\beta$ PIX in DA release, we exogenously and simultaneously introduced the shRNA-resistant forms of  $\beta$ 2PIX mutant, including  $\beta$ 2PIX Thr76Ala, Ser340Ala, or Ser583Ala mutants and  $\beta$ PIX sh369, into PC12 cells. DA release from PC12 cells transfected with sh369 was completely rescued by the reintroduction of WT FLAG-tagged  $\beta$ 2PIX (Fig. 8A). These results suggested that  $\beta$ 2PIX, which is a dominant isoform in the CNS, has important roles in DA release. Moreover, we examined the effects of the  $\beta$ 2PIX phosphorylation on DA release.  $\beta$ 2PIX Ser340Ala and Ser583Ala mutants failed to rescue the reduced DA release by  $\beta$ PIX sh369, whereas the  $\beta$ 2PIX Thr76Ala mutant acted similarly to WT  $\beta$ 2PIX (Fig. 8A). Together, our results suggested that the PKC-mediated phosphorylation of Ser340 and Ser583 on  $\beta$ 2PIX positively regulates DA release. Finally, we examined the effects of Cdc42 and Rac1 KD on DA release. When Cdc42 was knocked down, Rac1 was upregulated and vice versa. Therefore, we knocked down both Cdc42 and Rac1. The KD of both Cdc42 and Rac1 in PC12 cells by shRNAs resulted in a significant inhibition of  $K^+$ -evoked DA release from PC12 cells (Fig. 8B).

### Discussion

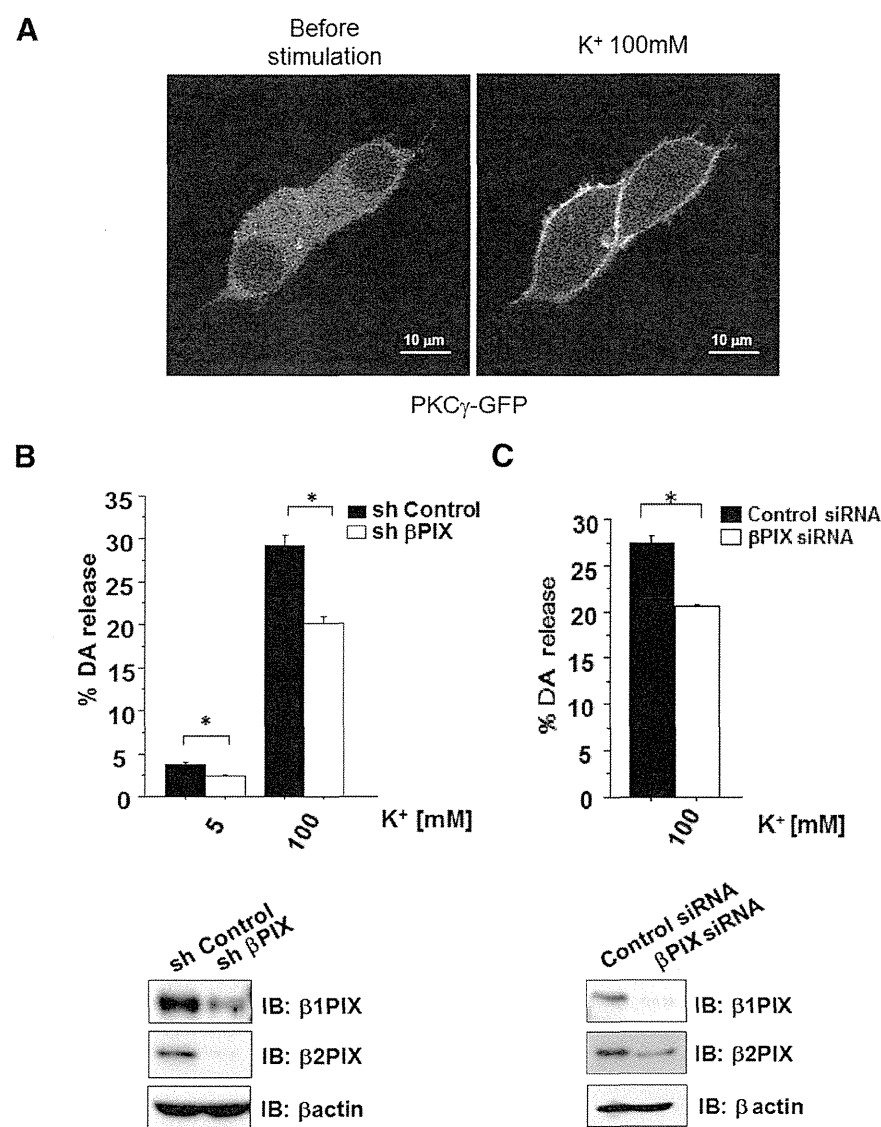
In this study, we revealed that AS/AGU rats did not express full-length PKC $\gamma$  or the truncated form of PKC $\gamma$ , indicating that a loss, and not a gain, of function is the cause of the parkinsonian symptoms exhibited by AS/AGU rats. In agreement with these findings, we demonstrated decreased DA release stimulated by high levels of  $K^+$  and METH in the striatum. It is noteworthy that altered DA release is the predominant characteristic of parkinsonian symptoms in PKC $\gamma$  KO mice rather than loss of DAergic neurons in the SNpc. We propose that PKC $\gamma$  KO animals are useful models for the study of parkinsonian syndrome because



**Figure 6.** cPKC positively regulates  $K^+$ -stimulated DA release. **A**, cPKCs and  $\beta$ PIX are expressed in PC12 cells. PC12 cell lysates were analyzed with immunoblotting with anti-PKC $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\gamma$ , and  $\beta$ PIX antibodies. **B**, DA release in PC12 cells was measured with various  $K^+$  concentrations. The results are expressed as mean  $\pm$  SEM ( $n = 3$ ). **C**, Amounts of DA release that were stimulated in response to 100 mM  $K^+$  with 1  $\mu$ M GFX or 1  $\mu$ M Gö6976 were measured in PC12 cells ( $n = 3$ ;  $*p < 0.05$ , unpaired  $t$  test). DA release levels were normalized to the levels released in response to 100 mM  $K^+$ , which were set to 100%. The results are expressed as mean  $\pm$  SEM ( $n = 3$ ). **D, E**, Uptake of  $^3H$ -DA into PC12 cells with or without GFX and Gö6976 were measured. The results are expressed as mean  $\pm$  SEM ( $n = 3$ ). **F**, TPA-stimulated DA release in PC12 cells. DA release in PC12 cells was measured with 1  $\mu$ M TPA in the absence or presence of 1  $\mu$ M GFX. DA release levels were normalized to that of 1  $\mu$ M TPA stimulation, which was set to 100%. The results are expressed as mean  $\pm$  SEM ( $n = 3$ ,  $*p < 0.01$ , unpaired  $t$  test).

PKC $\gamma$  KO mice and AS/AGU rats have damage not only in the DAergic system, but also in the serotonergic system (Al-Fayez et al., 2005), as has been observed in patients with sporadic Parkinson's disease. Furthermore, the use of the PKC $\gamma$  KO mouse model has an advantage because it is possible to perform gene manipulations in them, such as PKC $\gamma$  rescue, compared with the AS/AGU rats.

Although it is generally accepted that DA release in the striatum induced by high  $K^+$  levels is mediated by exocytosis and not by DAT, PKC was reported to induce DAT endocytosis (Daniels and Amara, 1999), which, by altering DA reuptake, can change the amplitude of  $K^+$ -induced DA release as measured by microdialysis. We have ruled out this possibility because DAT activity was instead decreased in PKC $\gamma$  KO mice (Fig. 1F). Moreover,  $^3H$ -DA uptake does not differ between the control and PKC inhibitors in PC12 cells (Fig. 6D,E). These results suggested that the reduced  $K^+$ -induced DA release was not through the change of the DAT endocytosis by the PKC $\gamma$  KO. There are also reports that activation of PKC induces DAT-mediated DA reverse transport (Kantor and Gnegy, 1998; Cowell et al., 2000). Although



**Figure 7.**  $\beta$ PIX positively regulates K<sup>+</sup>-stimulated DA release. **A**, PKC $\gamma$  was activated by high K<sup>+</sup> level stimulation in PC12 cells. Activation of PKC $\gamma$  by K<sup>+</sup> stimulation was examined by monitoring the translocation of GFP-tagged PKC $\gamma$  in PC12 cells. **B**, DA release was measured in PC12 cells that were transfected with shRNA for  $\beta$ PIX and sh Control, which was used as a negative control. The results are expressed as mean  $\pm$  SEM ( $n = 6$ ,  $*p < 0.01$ , unpaired  $t$  test). **C**, DA release was measured in PC12 cells transfected with siRNA for  $\beta$ PIX. The expression of siRNAs for  $\beta$ PIX in PC12 cells and lysate was analyzed by immunoblot analyses with anti- $\beta$ PIX antibodies. The results are expressed as mean  $\pm$  SEM ( $n = 5$ ,  $*p < 0.01$ , unpaired  $t$  test).

these studies focused more on PKC $\beta$  than on PKC $\gamma$ , our study showed that PKC $\gamma$  also play important roles for DAT-mediated DA reverse transport (Fig. 1*F*), suggesting that PKC $\gamma$  play roles for both K<sup>+</sup>-mediated DA release and DAT-mediated DA reverse transport. Therefore, PKC $\gamma$  KO resulted in decreased DAT activity, including uptaking DA and DA reverse transport through DAT. The decreased level of K<sup>+</sup>-induced DA release in PKC $\gamma$  KO mice is probably underestimated: DA release may be more significantly decreased when the disordered DAT activity is normalized.

We identified 10 phosphoproteins that were decreased in PKC $\gamma$  KO mice under the hypothesis that decreased levels of phosphorylated substrates of PKC $\gamma$  in the nigrostriatal system lead to impairments in DA release. Our results indicated that  $\beta$ PIX was one of the pivotal targets of PKC $\gamma$  or cPKC in enhancing Ca<sup>2+</sup>-evoked DA release based on the following several lines

of evidence: (1) PKC $\gamma$  phosphorylated  $\beta$ PIX *in vitro*, in cells, and *in vivo*; (2) Ser583 of  $\beta$ PIX was phosphorylated by PKC $\gamma$  both *in vitro* and in cells, whereas Ser340 was phosphorylated by cPKC only in cells, suggesting that cPKC indirectly phosphorylated Ser340; (3) cPKC inhibitors and the KD of endogenous  $\beta$ PIX also suppressed the DA release from PC12 cells, suggesting that both PKC $\gamma$  or cPKC and  $\beta$ PIX play pivotal roles in Ca<sup>2+</sup>-evoked DA release from DAergic neurons; and (4) WT  $\beta$ 2PIX, but not Ser340Ala or Ser583Ala, rescued the decreased DA release from PC12 cells by sh369 for  $\beta$ PIX, suggesting that both phosphorylation sites are necessary for regulating DA release. Why does PKC $\gamma$  KO cause the parkinsonian syndrome phenotype, even when other cPKCs are expressed in PKC $\gamma$  KO animals? Although the substrate specificity of each PKC subtype appears to be low *in vitro*, there have been several confirmatory reports of subtype-specific functions of cPKC under physiological conditions (Uchino et al., 2004; Ueyama et al., 2004; Al-Fayez et al., 2005; Kawasaki et al., 2010; Sakuma et al., 2012). Our data suggest that PKC $\gamma$  has important roles in DA release in mice.

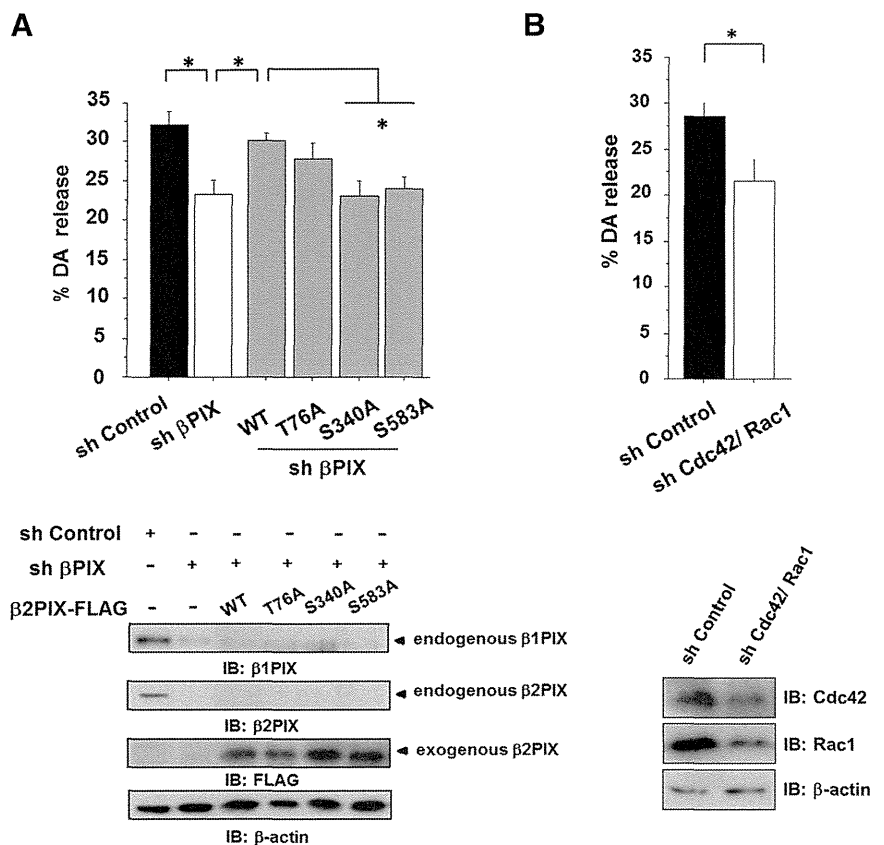
In neurons, Ca<sup>2+</sup> entry triggers exocytosis, suggesting that certain Ca<sup>2+</sup> sensors may be necessary for Ca<sup>2+</sup>-dependent exocytosis. Candidates for Ca<sup>2+</sup> sensors include molecules that possess EF hand motifs or C2 domains, which were first defined in cPKC, and annexin family proteins, as has been reported previously (Burgoyne and Morgan, 1998). PKC has been reported to modify exocytosis in the steps both before and after docking to the plasma membrane through the following mechanisms: (1) increased vesicle recruitment into the readily releasable pool (Gillis et al., 1996; Stevens and Sullivan, 1998), (2) acceleration of fusion pore expansion (Scepek et al., 1998), and (3) changes in the kinetics of exocytosis (Graham et al., 2002). However,  $\beta$ PIX is related to the exocytosis step after docking (Momboisse et al., 2009; Momboisse et al., 2010). The cPKC- $\beta$ PIX-Cdc42/Rac1 axis possibly has important roles in DA release in the step after docking through the  $\beta$ PIX phosphorylation by cPKC.

How can  $\beta$ PIX regulate DA release through the phosphorylation at Ser340 or Ser583? Previous reports have revealed that  $\beta$ PIX phosphorylation results in  $\beta$ PIX translocation to the membrane and the subsequent activation of Rac1 and/or Cdc42. In PC12 cells, basic fibroblast growth factor induces the phosphorylation of  $\beta$ PIX at Ser525 and Thr526, resulting in neurite outgrowth by the activation of Rac1 through the translocation of the  $\beta$ PIX complex at neuronal growth cones (Shin et al., 2002; Shin et al., 2006). In human mesangial cells, endothelin-1 and cAMP inducers cause the PKA-dependent phosphorylation of  $\beta$ PIX at Ser516 and Thr526, resulting in cytoskeletal rearrangement by

the activation of Cdc42 through  $\beta$ PIX translocation (Chahdi et al., 2005). Therefore,  $\beta$ PIX may be differently phosphorylated in response to various stimuli and this may result in its stimulus-dependent specific targeting and Cdc42/Rac1 activation at the targeted sites. We have demonstrated for the first time that Ser583 is a PKC $\gamma$  or cPKC phosphorylation site. Ser583 and the previously reported sites Ser516, Ser525, and Thr526 are all located in the C-terminal domain of  $\beta$ PIX and may have similar functions for the translocation and subsequent activation of Cdc42/Rac1. Phosphorylation at Ser340 is located in the PH domain, which interacts with phosphatidylinositol lipids within biological membranes (Wang and Shaw, 1995) and may also play important roles in the membrane targeting of  $\beta$ PIX. In fact, the PH domain has been reported to interact with PKC (Yao et al., 1994) and Cdc42 (Rossman et al., 2002) and it has been implicated in the regulation of the GEF activity of SOS (Ras-GEF; Karlovich et al., 1995) and p140Ras-GRF (Ras and Rac GEF; Buchsbaum et al., 1996). Therefore, through PH-domain-mediated membrane targeting and interaction, the cPKC- $\beta$ PIX-Cdc42/Rac1 axis may act at specific membranes (Chen et al., 1997; Falasca et al., 1998; Maffucci and Falasca, 2001). Downstream of the  $\beta$ PIX phosphorylation, we speculate that Cdc42/Rac1 are the targets of phosphorylated  $\beta$ PIX, as suggested in previous studies (Shin et al., 2002; Shin et al., 2004; Chahdi et al., 2005; Shin et al., 2006). Although we have not proved a direct relationship between phosphorylated  $\beta$ PIX and Cdc42/Rac1, we speculate that  $\beta$ PIX phosphorylated by PKC $\gamma$  regulates Cdc42/Rac1 functions based on our data that the double KD of Cdc42 and Rac1 resulted in decreased DA release (Fig. 8B).

Although Ser340 is common to both  $\beta$ 1PIX and  $\beta$ 2PIX, Ser583 only exists in the unique C terminus of  $\beta$ 2PIX, which is the neuron-dominant isoform (Koh et al., 2001) that emerged from and was conserved after *Xenopus laevis* (Fig. 9). Therefore, Ser583 phosphorylation by neuron-specific PKC $\gamma$  may have important roles in the modulation of  $\beta$ PIX function in the highly developed CNS and highly elaborated function, including DA release. It is also of note that the reported phosphorylation sites at Ser340, Ser516, Ser525, and Thr526 are conserved through evolution. Although we detected the TPA-induced phosphorylation of  $\beta$ 2PIX *in vivo* using the synaptosomal P2 fraction of PKC $\gamma$  WT mice by pSer PKC motif Ab (Fig. 3D), we could not show clear TPA-induced phosphorylation at Ser340 and Ser583 in PKC $\gamma$  WT using our site-specific phospho-Abs for pS340 and pS583, likely due to the sensitivity of anti-pS340 and pS583 Abs.

In the present study, we have demonstrated that PKC $\gamma$  KO mice can be a useful model of parkinsonian syndrome. In addition, we found for the first time that DA release was positively regulated by the PKC-mediated direct phosphorylation of  $\beta$ PIX at Ser583 and indirect phosphorylation at Ser340. The phospho-



**Figure 8.** Phosphorylation of  $\beta$ 2PIX at Ser340 and Ser583 promotes DA release. *A*, DA release was measured in PC12 cells that were transfected with sh $\beta$ PIX and  $\beta$ 2PIX (WT and Ser-Ala mutants) with shRNA-resistant sequences. The levels of endogenous  $\beta$ 1,  $\beta$ 2PIX, and exogenous  $\beta$ 2PIX were confirmed. Comparable levels of all ectopically expressed  $\beta$ 2PIX proteins were confirmed by Western blot analyses. The results are expressed as mean  $\pm$  SEM ( $n = 3-6$ ,  $*p < 0.05$ , one-way ANOVA with *post hoc* Dunnett's test). *B*, DA release was measured in PC12 cells that were transfected with shRNA for both Cdc42 and Rac1 and sh Control, which was used as a negative control. The results are expressed as mean  $\pm$  SEM ( $n = 3$ ,  $*p < 0.01$ , unpaired *t* test).

	340
H. sapiens	ASPRMSGFIYQ
M. musculus	ASPRMSGFIYQ
B. taurus	ASSRMSGFIYQ
G. gallus	ASPRMSGFIYQ
X. Laevis	ASPRMSGFIYQ
D. rerio	ASPRMSGFIYQ
D. melanogaster	VSQRMSAFIYE
	516                      525/526
H. sapiens	PERKPSDEEFASRKSTAALEE
M. musculus	PERKPSDEEF AVRKSTAALEE
B. taurus	PERKPSDEEFALRKSTAALEE
G. gallus	PERKPSDEEFALRKSTAALEE
X. Laevis	PERKPSDEEFALRKSTAALEE
D. rerio	PERKPSDEEF AVRKSTAALEE
	583
H. sapiens	LGRSSLSRLE
M. musculus	LGRSSLSRLE
B. taurus	LGRSSLSRVE
G. gallus	LGRSSLSRLE
X. Laevis	LGRSSLSRLE

**Figure 9.** Schematic comparisons of Ser340 and Ser583 through evolution. Ser340 and Ser583 are evolutionally conserved in many species. It is noted that  $\beta$ 2PIX emerged from *X. laevis*, although  $\beta$ 1PIX is expressed in *Drosophila melanogaster*. Ser516, Ser525, and Thr526, as reported previously, are also conserved in many species.