 Based on amino acid sequence considerations, pannexins are homologous to innexins, the invertebrate gap junction family. However, mammalian pannexin1 does not form canonical gap junctions, instead forming hexameric oligomers in single plasma membranes and intracellularly. Pannexin1 acts as an ATP release channel, while less is known about the function of Pannexin2. We purified cellular membranes isolated from MDCK cells stably expressing rat Pannexin1 or rat Pannexin2 and identified pannexin channels (pannexons) in single membranes by negative stain and immuno-gold labeling. Protein gel and Western blot analysis confirmed Pannexin1 or Pannexin2 as the channel forming proteins. We expressed and purified Panx1 and Panx2 using a baculovirus-Sf9 expression system and obtained doughnut-like structures similar to those seen previously in purified connexin hemichannels (connexons) and mammalian membranes. Purified pannexons were comparable in size and overall appearance to Connexin46 and Connexin50 connexons. Pannexons and connexons were further analyzed by single particle averaging for oligomer and pore diameters. The oligomer diameter increased with increasing monomer molecular weight, and we found that measured oligomeric pore diameter for Panxs was larger than for Cx26. Furthermore, we showed that Panx1 and Panx2 form active homomeric channels in Xenopus oocytes and in vitro vesicle assays. Cross-linking and native gels of purified homomeric full-length and a C-terminal Panx2 truncation mutant showed a banding pattern more consistent with an octameric oligomerization number. We purified Panx1/Panx2 heteromeric channels and found they were unstable over time, possibly because Panx1 and Panx2 homomeric pannexons have different monomer sizes and oligomeric symmetry from each other.

Pannexins (Panxs), connexins (Cxs) and innexins (Inxs) belong to one superfamily (1). Panxs (Pan in ancient Greek means “whole”) were given this name because of their presumptive ubiquitous presence in multicellular animals (2). Although the term “pannexin” can encompass invertebrate forms, the mammalian branch commonly known as pannexins is formed by three members: Panx1, Panx2 and Panx3. In rat, Panx1 contains 426 amino acids (~48 kDa), Panx2 has 674 amino acids (~70 kDa) and Panx3 has 392 amino acids (~44.7kDa). There is very high conservation of amino acid sequence within individual Panx isoforms among mammalian species than between Panx1, Panx2 and Panx3. In rat, Panx1 contains 426 amino acids (~48 kDa), Panx2 has 674 amino acids (~70 kDa) and Panx3 has 392 amino acids (~44.7kDa). There is very high conservation of amino acid sequence within individual Panx isoforms among mammalian species than between Panx1, Panx2 and Panx3. Panx1 has also been implicated in ischemic, excitotoxic and ATP-dependent cell death (7) and is part of the immunological and neuronal inflammasome (8-10). The function of Panx2 in the CNS remains unknown. The role of Panx3 in tissues remains to
be established since it has not been shown to be functional by itself or in combination with Panx1 or Panx2 in electrophysiological assays (11), but has been shown to express at the plasma membrane in tissue culture cells (3).

Based on membrane topology folding and secondary structure prediction, Panxs were first proposed to form gap junction-like structures (12). More recent results established that while Cxs and Panxs share a number of topological similarities, there are also important differences. One is their sequence composition is very different from connexins. Four conserved cysteines are present in the pannexin extracellular loops rather than six as for connexins. These cysteines may influence the formation and properties of GJ channels (13). Our group and others established that Pannexins have glycosylation sites on their extracellular loops and are found in glycosylated forms in various cell types (3,14,15). Studies showed that while Cxs channels become coupled very soon after incorporation into the membrane (16), the active function for Panx1 pannexons is in non-junctional membranes (3,15,17-19). Both Panx1 homomeric pannexons and Panx1/Panx2 heteromeric pannexons are expressed at the plasma membrane (20). The Panx1/Panx2 heteromeric combination as well as Panx1 homomeric pannexons have been reported to make functional pannexons and gap junction-like channels in single and paired Xenopus oocytes respectively (11,21). However, while functional Panx1 channels have been found in tissues, Panx1 and Panx2 have not be seen to co-localize and form heteromeric Panx1/Panx2 channels at the tissue level. Thus, pannexins represent a novel class of connexin-like channel proteins. Since others and our group have found that Panx channels do not typically form gap junctions, in order to avoid confusion we distinguish between pannexons (single membrane oligomers of Panxs), connexons (single membrane hexamers of Cxs, also referred to as “hemichannels”) and gap junction channels (double membrane paired hexamers of Cxs).

In this study, we analyzed purified Panx1 and Panx2 channels (pannexons) by coordinated biochemical analysis and electron microscopy of mammalian membranes and as purified pannexons from baculovirus infected insect cells and compared them with Cxs connexons. Also, included are functional assays for Panx1 and Panx2 showing that Panx2 does form active channels as homomeric channels. We studied the oligomeric state of Panx2 and found that it most likely forms octamers as opposed to Panx1 that we previously established forms hexamers (15). Furthermore, we purified Panx1 and Panx2 heteromeric channels, but found that these are unstable, probably due to the different symmetries we see in homomeric channels and the consequent mis-match between the two isoforms.

**Experimental procedures**

**Antibodies** - Panx1 and Panx2 antibodies were generated against peptides using sequences in the N-terminus (Panx1 monoclonal), 1st extracellular loop (Panx2 polyclonal) and the C-terminus (Panx2 polyclonal) and custom produced and purified by Abgent, Inc. (San Diego). Full design and characterization of these antibodies are described in Boassa et al., in preparation. We also used two antibodies from Invitrogen, Inc. (Carlsbad, CA): mouse monoclonal anti-His6 tag antibody (Catalog # R930-25 or R931-25) and a mouse monoclonal for Cx26 that is directed against a cytoplasmic loop epitope (Catalog #13-8100).

**Generation of constructs for mammalian and baculovirus expression** - All constructs in this paper use amino acid sequences found in rat pannexins. Rat cDNAs encoding wild type rat Panx1 and Panx2 were originally and kindly provided by Dr. Roberto Bruzzone. Madin-Darby canine kidney (MDCK) cells were maintained at 37°C, and 10% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Another longer rat Panx2 construct was also generated according to the sequence found in (22). Development and characterization of Panx1-myc and Panx2-HA mammalian cell lines are described in (15) and in Boassa et al., in preparation. Experiments were conducted on stably expressing cell lines in selection with the antibiotic hygromycin.

Connexin (rCx26, rCx43, rCx46 and rCx46) and pannexin (rPanx1 and rPanx2) gene sequences were cloned into the baculovirus vector pBlueBac4.5 using XbaI and HindIII as restriction sites with a V5 epitope/hexa-histidine (V5-His6) added to the C-terminus. Isolation and purification of baculovirus expressed Sf9 proteins are described in (23). The truncation mutant for Panx2
was designed by first running secondary structure predictions on the Panx2 C-terminus from 3-4 different Internet available algorithms from the ExPASy proteomics server (http://expasy.org/tools/) to look for commonalities of α helices, β sheets and random coil in the Panx2 C-terminus among the five different algorithms: NNPredict (24); GOR (25); SCRATCH (26); PSIPRED (27) and JPRED (28). Truncations are done in a random coil sequence sections with a distance intermediate to any predicted consensus, consecutively ordered secondary structure (α-helices or β-strands).

**Gap junction and connexon/pannexon purifications** - Gap junction preparations were purified from HeLa cells stably expressing rCx26 wild type (rCx26WT) (29) and we used the same protocol to obtain Panx1 and Panx2 membranes from MDCK stably expressing rPanx1 or rPanx2. Pannexons or connexons were isolated from baculovirus-infected Sf9 cells according to our published methods using a Hi66 tag affinity purification protocol (23,30,31). Purified proteins were negatively stained with 2% uranyl acetate for EM visualization and analysis. Western blots were performed using denaturing 4-12% SDS gel PAGE as we previously published (23). Gels were stained using either a silver staining kit or a SYPRO Ruby gel stain (Invitrogen, Inc.). Buffer exchange was performed using Amicon Microcon centrifugal filter devices YM-100 (Millipore, Temecula, CA).

**Cross-linking of pannexons** - After purification in insect cells, proteins were cross-linked using 300 µg/ml DSP for 30 minutes at RT. If the sample was meant for electrophoresis the reaction was stopped by the addition of glycine following the protocol used in (15).

**Immunogold-labeling membranes on grids** - A 5 µl drop of isolated membranes in solution was placed on carbon coated grids and allowed to adsorb for approximately one minute at room temperature. The grids were then dipped into BSA-Tris buffer drops (20mM Tris HCl pH8, 1mg/ml BSA) plus 5% goat serum for 30 minutes. After that they were dipped in Primary Solution (BSA-Tris buffer +1% goat serum + primary antibody) for 1h. The Panxs and Cx26 primary antibodies were diluted 1:50 or 1:100. The grids were washed plunging them in three drops of BSA-Tris buffer and then incubated in Secondary Solution (BSA- Tris buffer +1% goat serum + 10 nm gold-conjugated secondary) for about 30 minutes. Secondary dilutions were 1:50. Finally the grids were washed in three drops of double distilled water and negatively stained with 2% uranyl acetate.

**Immunogold-labeling membranes in cross-section by thin section EM** - Membranes were spun down for 10 minutes at 18,300 rcf at RT. Then, the pellet was resuspended in the BSA-Tris buffer described in the previous section and again collected by centrifugation. The pellet was resuspended in Primary Solution (primary diluted 1:1 or 1:2 with BSA-Tris buffer) and incubated for 1h at RT. Membranes were then collected by centrifugation and resuspended in the BSA-Tris buffer. This wash procedure was performed 2 more times and then the membrane was resuspended in the Secondary Solution containing secondary 10 nm gold conjugated antibody (diluted 1:1 or 1:2 with BSA-Tris buffer) and incubated for 1h at RT. Three washes were then performed using BSA-Tris buffer. For both labeled and unlabeled membrane preparations analyzed by thin section EM, membranes were collected by centrifugation and the membrane pellet was fixed for 30 minutes in fixing solution (2% glutaraldehyde, 1% tannic acid pH 7-8, 0.1M cacodylate buffer) at RT. The pellet was washed three times with cacodylate buffer diluted 1:3 and stained in ice for 1h with 2% osmium tetroxide (diluted with cacodylate buffer) and then stained with 2% uranyl acetate in the same conditions. Following standard protocols, the dark pellet was dehydrated on ice with an increasing ratios of ethanol:water, followed by two changes of 100% ethanol. The pellet was then embedded in Durcupan resin for sectioning and EM analysis.

**Electron microscopy** - High dose, conventional transmission electron microscope images were recorded on a JEOL 1200 electron microscope operated at 80 kV. Thin sections were 80 nm thick and counterstained with lead. Specimens were negatively stained with 1 or 2% uranyl acetate. A 200 kV FEI Sphera microscope was used to image the negative stained grids in low dose (15-20 e⁻ /Å²) at 40,000 magnification. Images were recorded on film, scanned with Nikon LS-9000 digital film scanner and processed using EMAN1/EMAN2 using standard procedures for image alignment and averaging (32).
Cytochrome c loaded vesicle permeability assay - This protocol follows that used in several studies (33-36). Pannexons were reconstituted into vesicles using approx. 1 nmol of purified pannexin. Vesicles were prepared in 1 ml aqueous solution containing 50 mM KCl, 20 mM Tris, pH 7.4, 22 mg n-octyl-β-glucopyranoside, 6 mg cytochrome c and 10 mg 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). The protein-lipid-detergent solution was mixed and incubated for 30 min at 4 °C followed by detergent removal using Biobeads SM overnight at 4 °C. The formed proteoliposomes with an average diameter of 200 nm were applied to a Sephadex G-75 column to separate free cytochrome c from the cytochrome c loaded proteoliposomes. Fractions were collected and analyzed at 417 and 630 nm using a UV/Vis-spectrophotometer. The cytochrome c loaded proteoliposomes were collected in the void volume. Control vesicles without pannexons but with enclosed cytochrome c were prepared in parallel. Sodium ascorbate was used to reduce the intra-liposomal cytochrome c, which was monitored at 417 nm using a UV/Vis-spectrophotometer. A 200 µl vesicle aliquot was added to 790 µl of buffer (50 mM KCl, 20 mM Tris, pH 7.4), the carbenoxolone (CBX) concentration was adjusted and the vesicles were incubated for minimum 1 h at RT. After monitoring a stable baseline for about 30 seconds 10 µl of 30 mM sodium ascorbate in buffer was added and the absorbance was followed for 6 min. The total amount of entrapped cytochrome c in both vesicle suspensions (Panx proteoliposomes and control vesicles without pannexons) was determined by rupturing the vesicles with Triton X-100 and set to 100%. The control curves were subtracted from those obtained with the Panx proteoliposomes to extract the pannexon-mediated reduction of cytochrome c. To plot the concentration dependent blocking of the pannexon channels, the initial slopes were determined, normalized and plotted versus the CBX concentration.

Electrophysiology of Xenopus oocytes - Preparation of Panx1 mRNA, oocytes and electrophysiological recordings were performed as previously described (15,37). Pannexin2, in pRK5, was linearized with Hpa I. In vitro transcription was performed with the polymerases SP6, using the Message Machine kit (Ambion, Austin, TX). mRNAs were quantified by absorbance (260 nm), and the proportion of full-length transcripts was checked by agarose gel electrophoresis. In vitro transcribed mRNAs (~20 nl) were injected into Xenopus oocytes. Membrane conductance was determined using voltage pulses for oocytes expressing pannexins were held at −60 mV, and pulses to +60 mV were applied to transiently open the channels. A 70 sec -100 mV to +100 mV ramp was also applied to Panx2 expressing oocytes. Inhibitor concentrations used were 10 µM and 100 µM CBX and 1 mM Probenecid. Cytoplasmic acidification experiments using CO2 were performed as described in (38).

RESULTS
Membrane topology studies, hydrophobicity plots and secondary structure predictions revealed that these proteins have several similarities to connexins (four transmembrane segments, the C and N termini facing the cytoplasm and two extracellular loops containing conserved disulfide bonds) (3,4,12,15,17). Based on this, pannexins should form connexon-like or gap junction channel-like structures and early functional studies demonstrated that pannexons might perform a similar function as connexons (11,21).

Pannexin1 and Pannexin2 show connexin-like channels in isolated membrane preparation - We wanted to study the features of Panx1 and Panx2 channels in native mammalian membranes. In order to see if there is any structural homology between pannexin and connexin channels inside of mammalian cell membranes, MDCK cells stably expressing Panx1 or Panx2 were made. Following procedures used in our laboratory for connexin membrane purification by detergent solubilization and sucrose gradient cellular fractionation methods, Panx membranes were isolated, negatively stained with uranyl acetate and imaged by EM.

Under conventional imaging methods, our EM analysis showed an overall similarity between the channels formed by Panx1 or Panx2 in MDCK membranes (Fig. 1A and B respectively) and the ones showed by Cx26 in HeLa cells (Fig. 1C). As a negative control, membranes isolated from parental HeLa cells, deficient in connexin or pannexin expression, did not reveal any channel-like structures. These membrane preparations were of good purity as confirmed by stained protein gels.
and Western blots, confirming that the channels visible by EM in the membranes, are actually formed by Panx1 or Panx2 proteins (Fig. 1E and F).

While it is possible that channels visible in the membranes isolated from Panx2 stables, may be due to the induction of Cx43 found endogenously expressed in some MDCK lines (e.g. (39)), the Western blot filter containing Panx2 was stripped and re-hybridized with an antibody against Cx43. As expected only a very low signal was detected after overnight exposure of the filter indicative of either no or very minimal expression of Cx43. Thus, the channels we saw in EM images are formed by Panx2 oligomers and this was confirmed by immuno-gold labeling the membranes on the grid. The membranes showing Panx1 or Panx2 channels were adsorbed on carbon coated grids for 1 minute. The membrane-mounted grids were then dipped in a 50 µl drop of blocking solution (BSA-Tris buffer and 5% goat serum) for 30 minutes. After blocking, the grids were washed dipping them for few seconds in drops of BSA-Tris buffer (see experimental procedures). Incubation with Panx1 or Panx2 primary antibodies in BSA-Tris buffer with 1% of goat serum was made for 1h at RT. The grids were washed again as above and incubated with gold-conjugated secondary antibodies for 1h at RT. After the immunogold process, the grids were washed with double distilled water and negatively stained with uranyl acetate. The EM analysis showed Panx1 or Panx2 membranes covered by black spheres of gold beads, surrounded by gray areas with no or very few gold beads, confirming that the antibodies specifically recognized the channels present on the membranes. Membranes isolated from Cx26 expressing and parental HeLa cells were used for positive and negative controls, respectively for EM (Fig. 1C and D) and Western blot (Fig. 1G). In these Western blots, a clear band for Cx26 is visible in the positive control while no bands appeared on the negative control after hybridization with anti-Cx26, anti-Panx1 or anti-Panx2 antibodies.

Membrane cross-sections highlight different features between Panx1 or Panx2 membranes and Cx26 gap junctions - Fig. 2 contains thin sections through Panx1 and Panx2 membrane pellets that confirmed that we isolated channels in single membranes. These images were in contrast to Cx26 gap junction cross-sections having the classical double plasma membrane and channel striations typical for gap junctions. This data confirmed previous studies, including the one from our group, where Panx1 in mammalian cells was found to form only single membrane assemblies (3,15,17,40). Thin sections performed on immuno-gold labeled membranes often appeared circular (Fig. 2 B) with gold spheres only on the one external side presumed to be the cytoplasmic surface since the primary antibodies were against cytoplasmic domain peptides. Immuno-gold secondary antibodies against primary antibodies targeted to a cytoplasmic epitope of Cx26 specifically attached to both sides. Higher magnification of these membranes showing additional detail are shown in Fig. 2C.

Purification of Pannexin1 and Pannexin2 oligomers (pannexons) in insect cells provides an EM comparison with connexons - We purified Panx1, Panx2 and four connexins (Cx26, Cx43, Cx46 and Cx50) from Sf9 insect cells infected with baculoviruses expressing each protein. Cx26 was chosen because is the second smallest connexin and the structure has been solved to better than 10 Å resolution by EM (31) and 3.5 Å by X-ray crystallographic methods (41). We also compared Cx43, the best studied connexin. Cx46 and Cx50 were chosen because they have similar molecular masses. Fig. 3A contains images of purified oligomers of Cx26 (above left) to Panx2 (bottom right). The oligomers appear larger in a correspondence with the increasing monomer molecular weight. Cx26 oligomers tend to have a uniform appearance indicative of few orientations on the grid and display small doughnut-like structures with a clear pore. Oligomers of larger connexins or pannexins have a more heterogeneous appearance, perhaps indicating different orientations on the carbon coated grids. For Cx50 and Panx2 oligomers, it is clear that we have at least two different morphologies that we would attribute to two different orientations of the channels in combination with differential staining (upper and lower sides). In order to confirm the nature of our protein preparations and to check their purity, we performed gel staining and Western blots after electrophoresis in denaturing conditions (Fig. 3B). As expected, the molecular weight of these proteins increases from Cx26 (left) to Panx2 (right). Comparing stained protein gels.
(left lanes labeled with G for gel) and the Western blot (right lanes labeled with W for Western blot) for the same sample of each protein analyzed, we see a good correspondence of bands, however, dimeric bands are more intense in the Western blots because of the inherent nature of this enzymatic reaction enhancing the signal intensity. Thus, these samples contain homogeneous populations of connexons or pannexons.

Size analysis of pannexons and connexons after 2D image averaging - We next compared connexons and pannexons for their oligomeric size and pore diameters. Because these assemblies are small compared to most single particle reconstruction specimens, following a published method (42), we used negative staining in combination with cross-linking of the oligomers prior to imaging to maintain a stable structure. Particles were imaged and boxed for 2D reconstruction. At this resolution (low dose and 40,000 magnification), averaged images of views with a clear pore, showed similarities between connexons (30 particles averaged) and pannexons (50 particles averaged) (Supplement Fig. 1). We applied circular symmetry to these averaged images in order to get more accurate measurement of sizes and diameters of the particles. For Panx1 and Panx2 in these conditions we saw different morphologies of pannexons and the larger connexons (e.g. Cx50) within these images indicative of different orientations of the particle on the grid. While the pore diameters of the different isoforms of pannexons were similar, the oligomer diameters increased in correspondence with the protein molecular weight. Supplement Fig. 1 (middle column) contains two image averages of pannexons with different diameters (oligomer diameter ~120 and 160 Å for Panx1 and oligomer diameter ~183 and 190 Å for Panx2). On the other hand, Cx26, the smallest of the connexins we examined, tended to have a small number of unique orientations on the grid (oligomer diameter ~81 Å). In projection, the measured pore diameter for Cx26 is substantially smaller (~12.5 Å) than for Panx1 (~21 Å and 17 Å) or Panx2 (~29.5 Å and 30.5 Å).

Purified Panx1 and Panx2 pannexons form open channels - In order to test whether these pannexons were functional, Panx1 and Panx2 baculovirus Sf9 pannexon preparations were used in a cytochrome c liposome based assay used in several previous studies (33-36) (Fig. 4). Briefly, cytochrome c was trapped insight a 200 nm diameter proteoliposome. After the addition of ascorbate, and in the presence of open channels through which ascorbate can pass but not cytochrome c, cytochrome c was reduced (Fig. 4A). Using a Sephadex-G75 column free cytochrome c can effectively be separated from Panx proteoliposomes with entrapped cytochrome c (Fig. 4B). The reduction of cytochrome c was measured at 417 nm using an UV/Vis-spectrophotometer. For these vesicle assays, we did the negative control measurements of liposomes without protein to quantify the background signal. We measured the vesicles with protein and added CBX. Then, we measured the control vesicles without protein and added CXB. The control curves were subtracted from the curves gained with protein. As a positive control for this assay, we used a different protein/oligomer. Cx26 connexons inserted into liposomes containing entrapped cytochrome c were inhibited by CBX, α-cyclodextrin and calcium in good agreement with the literature data. For both, Panx1 and Panx2 proteoliposomes, we saw a time dependent significant reduction of intra-liposomal cytochrome c (Fig. 4C, D upper line). CBX was added at different concentrations to analyze whether it was able to block the reconstituted pannexons. While the reduction of cytochrome c itself was not influenced by CBX, the treatment of reconstituted Panx1 pannexons with CBX resulted in a concentration dependent decrease in channel activity with an IC₅₀ = (14 ± 4) µM being similar to published results (21) (Fig. 4C, E). Similarly, Panx2 channels were incorporated into proteoliposomes and their activity was inhibited by CBX with an IC₅₀ = (15 ± 4) µM (Fig. 4D, F). Thus, this is the first report of functional Panx2 homomeric pannexons, because the assay does not depend on the channels being expressed on the plasma membrane.

Panx1 and Panx2 homomeric channels have a different physiological profile from each other when expressed in single Xenopus oocytes - Panx2 has previously been reported to be non-functional in Xenopus oocytes except when in combination with Panx1 (11,21). However, it should be noted that the rat Panx2 construct we used in this study was 10 amino acids longer at the beginning of the
Supplement Figure 2). The large currents in CBX (100 µM, current was unaffected by high concentrations of CBX (100 µM, Fig. 5A, Table I) and these currents were unaffected by high concentrations of CBX (100 µM, Fig. 5B) or Probenecid (1 mM, Supplement Figure 2). The large currents in Panx2 expressing oocytes were detected in 21 out of 21 cells from 4 ovaries with little variation in amplitude (Table II). Uninjected oocytes exhibited significantly smaller currents. We also found that when Panx1 and Panx2 were co-expressed in Xenopus oocytes in a 1:1 ratio, there was a reduction in current from 1.96 ± 0.31 µA (n=6) to 0.86 ± 0.31 µA (n=6) (Table I) indicating a mutual inhibition between the two pannexins. This result is in agreement with measurements of dye uptake in Panx1 and Panx2 exogenously co-expressing HEK293T cells made by Penuela et al. (20).

In order to test whether these currents were due solely to the opening of Panx2 channels, we constructed four cysteine mutants. Panx2 has four conserved cysteines, two in each of the extracellular loops, as do all members of the pannexin/innexin superfamily. These four cysteines are C81, C99, C259 and C280. They may serve a similar function in disulfide bonding as the six cysteines in connexins. As we found previously for connexins and Panx1, mutation of any of these extracellular cysteines in Panx2 resulted in loss of function (Figure 5C, Table I), indicating that the currents observed with WT Panx2 channels are attributable to this protein and do not represent endogenous currents activated by the Panx2 expression. These Panx2 specific currents are also supported by the observation that Panx2 WT expressing oocytes were sensitive to cytoplasmic acidification as are all gap junction proteins (Supplement Figure 3, Table III). Perfusion of uninjected oocytes triggered the appearance of large membrane currents at positive potentials, which were not significantly enhanced in Panx2 expressing oocytes, indicating closure of Panx2 channels by cytoplasmic acidification (Table III).

Panx2 does not form hexamers - While we have previously established that Panx1 forms hexamers, we investigated whether a similar symmetry exists in Panx2 oligomers. Panx2-V5-His was expressed and purified using the baculovirus–Sf9 expression system. After purification we analyzed Panx2 oligomeric state by cross-linking with DSP 300 µg/ml following a protocol similar to the one applied to study Panx1 oligomers (15). Because of the larger size of Panx2 (~70 kDa) versus Panx1 (~48 kDa), we found that our gel analysis system was not accurate enough to definitely distinguish between hexamers, heptamers, or octamers. In Fig. 6B we show the Western blot of Panx2 X-linked on 4% Tris glycine gel PAGE. The cross-linked Panx2 band mapped well above the position of the high molecular weight marker at 500 kDa. Thus, Panx2 does not form a hexamer that would be expected below the 500 kDa marker band (~70 kDa x 6 = 420 kDa).

In order to better resolve the Panx2 oligomeric state, we constructed a truncated version of Panx2 sequence. Secondary structure prediction algorithms run on the C-terminus of Panx2 contained some predicted α-helices and β strands separated by extended areas of random coil. A truncation site between S340 and Q341 was chosen because it was close to the membrane (Figs. 6A and C) just after the first predicted helical segment and intermediate to next secondary structure element (Fig. 6D). This truncation mutant contained the first 340 amino acids plus a 30 amino acid V5-His6 tag (Panx2Trun340) for a predicted molecular weight of ~40.7 kDa. This molecular mass of Panx2Trun340 was somewhat smaller than Panx1 (~48 kDa). When Panx2Trun340-myc was expressed in HEK293 tissue culture cells, we found it had similar expression and trafficking to the full-length Panx2-myc.

A baculovirus containing Panx2Trun340-V5-His6 was generated and expressed in Sf9 cells.
Channel structures were purified and analyzed with two different gel systems as we had previously done in our initial Panx1 studies (15). In Fig. 6, we show two diagrams comparing the sequences of full-length and Panx2Trun340 (Fig. 6A and 6C) and the molecular weight of the monomer on denaturing gel (Fig. 6E). Cytochrome C vesicle assays as performed in Fig. 4 showed that truncated Panx2 pannexons inserted into liposomes were less permeable to ascorbate and full-length Panx1 and Panx2 (Fig. 6F). Since we observed that Panx2 truncated is still able to oligomerize and form channels by EM (Fig. 6G), we performed X-linking as described above for Panx2 full length. On Tris-glycine gel 4-20%, the highest band mapped above the 150 kDa of the marker, confirming that the oligomer cannot be a hexamer, which in this case was supposed to map around 246 kDa (41 kDa x 6 = 246 kDa). The X-linked sample boiled in presence of 5% BME (condition that breaks the X-linked bonds) showed the monomer as expected, mapping around 41 kDa (Fig. 6H). To confirm our result and better resolve our bands, we used a Tris-Acetate 3-8% gel (Fig. 6I) with HMW marker, which clearly showed the monomer, corresponding to 41 kDa, the dimer around 82 kDa and the upper band between above the 268 kDa, but lower than the 460 kDa marker band. This evidence confirms that the oligomeric state of Panx2 is not a hexamer but rather a heptamer or octamer. When purified cross-linked Panx1-V5-His6 oligomers were run in side-by-side experiments with Panx2Trun340-V5-His6 oligomers (Fig. 6J), the mobility of the hexameric band of Panx1 was higher than the truncated Panx2. Estimation of molecular weights indicates that the Panx1-V5-His6 band matches the expected calculated hexameric mass (~288 kDa) while the center of the Panx2Trun340-V5-His6 band (~326 kDa) can only be explained by an octameric oligomerization number (8 x 41 = 328). The calculated 287 kDa molecular mass for a heptameric Panx2Trun340-V5-His6 channel would be about the same molecular mass as Panx1-V5-His6 hexamers (288 kDa), however, the data in Fig. 6J shows a clear separation. Thus, this data clearly indicates Panx2 is an octamer. In our EM analysis, we found that Panx2Trun340-V5-His6 channels (Fig. 6G) were more labile than X-linked Panx2Trun340 channels (Fig. 6K) suggesting that the negative staining process can affect Panx2Trun340-V5-His6 stability. However, in each micrograph, many discrete channel structures are identifiable. Thus, these results indicate that the Panx2 channel does not have hexameric symmetry, but rather, it is most probably an octamer since this oligomer number matched better with the experimental data.

Panx1 and Panx2 form unstable heteromeric channels - Given the symmetry mis-match between Panx1 and Panx2 homomeric pannexons, we wondered how Panx1 and Panx2 could mix together in forming heteromeric channels described before by other authors (11,20). In order to isolate Panx1/Panx2 heteromeric channels in insect cells, we co-expressed Panx1-His6 tag and full-length Panx2-no tag baculoviruses in ratios 1:1, 1:2 or 1:4. Using this strategy as we have previously published (23), only homomeric Panx1 or heteromeric Panx1/Panx2 would be isolated, while we didn’t expect any homomeric channels for Panx2, giving the lack of the His tag for this construct. The only way Panx2-no tag could be isolated after co-infection in insect cells with Panx1-V5-His6, would be if it formed heteromers with Panx1-V-His6. After purification, the Western blot on these samples showed both the presence of Panx1 and Panx2 (Fig. 7C), suggesting that they co-purified. EM images of the same samples taken one or few hours after the purification showed a more heterogeneous looking preparation than Panx1 or Panx2 purified singularly, but contained “doughnut” like appearances (Fig. 7A), suggesting that we have Panx2 integrated in heteromeric channels together with Panx1, probably mixed to Panx1 homomeric channels as well. Twenty-four hours after the purification, the sample was highly heterogeneous without the appearance of channel structures (Fig. 7B), revealing high instability for these channels. In order to understand if the high instability would be caused by disaggregation of Panx1-V5-His6 and Panx2, we used a second resin purification step for the sample after 24 hours. Using a buffer exchange column, we replaced the elution buffer containing high concentration of l-histidine with a binding buffer (lacking l-histidine). We incubated our sample a second time with new Ni-NTA Nickel resin over-night. After elution with l-histidine, Western blots showed Panx1 bands around 50 kDa (unglycosylated and glycosylated), while the filters hybridized with anti-Panx2 antibody contained no Panx2 bands.
These data demonstrate that Panx1 and Panx2 can form heteromeric channels, but they are very unstable and disaggregate within few hours. Thus, this helps to explain why Panx1 and Panx2 can form heteromers in some expression systems such as Xenopus oocytes (11) and HEK293 cells (20), but our group and others (43) see a clear separation of homomeric Panx1 and Panx2 channels in stably expressed mammalian cells and brain tissue (Boassa et al., in preparation).

DISCUSSION

Pannexin channels represent a novel channel assembly with functions distinct from connexin channels or connexons, but sharing some structural features. Published studies have concentrated on their trafficking, expression and function in mammalian cells and tissues, while this work is focused on their structural characterization at the molecular level.

**Pannexin structure isolated material from mammalian cells and insect cells.** We showed for the first time that when exogenously expressed in mammalian cells, Panx1 and Panx2 oligomers in isolated membranes look very similar to connexin gap junction channels. Despite this structural homology between connexons and pannexons, our group and others have shown that Panxs do not form gap junctions (3,15,17). For Panx1 and Panx3, a carbohydrate tree at a unique Asn in the extracellular loops would sterically hinder gap junction formation (3,15,44). Panx2 also contains a unique glycosylation site (20) that may prevent docking, however, it is unclear if this isoform is fully glycosylated in native systems (43). In this study, we re-confirmed again using Panx1 and Panx2 membrane isolations that cross-sections through pelleted membrane preparations contain a single membrane layer with immunogold labels attached to only one side of the membrane in contrast to Cx26 preparations where we find double layers (gap junctions) and immuno-gold labels attached to both sides.

Pannexons purified from Panx-baculovirus infected insect cell membranes confirmed an overall structural homology between pannexons and connexons, both showing doughnut-like structures across a size range of connexins. Single particle 2D averages of selected Cx26 connexon and Panx1 or Panx2 pannexon images were compared for their size and pore diameter. Measurements revealed an increasing oligomer diameter commensurate with an increase in the monomer molecular mass. As the particle size increased, the images appeared more heterogeneous because of different orientations on the grid. Pore diameters for Panx1 and Panx2 were larger than for Cx26 and Panx1 pore diameters were smaller than Panx2. However, it should be emphasized that these measured from 2D averaged projection images and the expectation is that the pore has different diameters along its axis perpendicular to the membrane plane. The monomer molecular weight of Panx2 is about 45% larger than Panx1 with the major addition of mass predicted to be in the C-terminus (~128 amino acids for Panx1 C-terminus versus ~366 amino acids for Panx2 C-terminus). In addition, the N-terminus domain of Panx2 is also larger containing 52 amino acids versus 36 amino acids for Panx1 and 21 residues for Cx43. Surprisingly, cross-linking studies to determine stoichiometry using full-length and a Panx2 truncation mutant revealed the oligomeric state of this protein not being a hexamer as Panx1 (15), but fits the data best as an octamer. Given the conserved hexameric state of all connexons, of Panx1 pannexons and the conservation of folding of connexins and pannexins, this was unexpected. However, it is possible that in order to make a closed structure to accommodate the larger monomer size of Panx2 (and bulky cytoplasmic domains), the oligomer number needs to increase to eight.

**Panx2 channels are a unique functional entity, distinct from Panx1.** First reports by Bruzzone and co-workers indicated that Panx2 was only expressed at the plasma membrane in combination with Panx1 (11), as was found for co-expression of Drosophila Inx2 and Inx3 (45). Data from Lai et al., (43) and our lab show a clear separation between Panx1 at the plasma membrane and Panx2 intracellular pools when co-expressed in several cell lines or in high resolution light microscopic localization in tissues (Boassa et al, in preparation). Recently, Penuela et al. (20) showed that in HEK293T and NRK cells, Panx2 may form heteromers with Panx1, however, this based on co-immuno-purification of Panx1/Panx2 of whole cell lysates and light level immuno co-localizations of Panx1 and Panx2 at the plasma membrane. Within the resolution of the light microscope, it is unclear if in overlapping populations in the plasma
membranes, Panx1 and Panx2 make heteromeric channels or form domains of mixed populations of homomeric channels since the plasma membrane contains areas of only Panx1 or Panx2 labeling as well. It is worth noting that we can isolate Panx2 channel bearing membranes from intracellular compartments, because the isolation protocol we use is not an affinity based approach, but rather selects based on detergent resistance and density gradient fractionation methods. Our functional data from Xenopus oocyte studies confirms that Panx2 has an inhibitory effect on Panx1 although it is not known whether this is due to Panx1/Panx2 heteromeric channels being non-functional or Panx1 mis-trafficking when co-expressed with Panx2.

Here, we show for the first time that Panx2 homomeric channels are functional using a cytochrome c based proteoliposome assay. We also found that Panx2 channels in Xenopus oocytes could be opened, but only at higher, non-physiological voltages. These currents were eliminated by mutagenesis of any of the four cysteines in the extracellular loops, indicating that currents in oocytes expressing Panx2 WT could only be attributable to Panx2. In addition, cytoplasmic acidification closed Panx2 channels. The functional studies with the vesicle assay also demonstrated an inhibitor-induced closure of Panx1 and Panx2 channels. The effect on Panx1 channels is in accordance with previously published studies using single Xenopus oocytes (46) (this study). However, in contrast to the vesicle assay Panx2 channel opening events were not inhibited by CBX or Probenecid in Xenopus oocytes. While this result was surprising, Silverman et al. (10) showed that the potassium channel subunit, K,β3 eliminates inhibitor sensitivity in Panx1 when Panx1 and K,β3 are co-expressed. Here the reverse situation is presented, where it is possible that cellular factors not present in the in vitro proteoliposome assay can modulate Panx2 to eliminate CBX and Probenecid inhibition. Thus, we propose that Panx2 makes channels that are functionally and structurally different from Panx1 channels. It should also be noted that the elimination of most of the Panx2 C-terminus resulted in channels that showed reduced permeability to ascorbate in our vesicle assay system, but still made recognizable channel structures.

Finally, we find that when Panx1 and Panx2 are isolated from an over-expression system using an affinity tag for purification that the heteromers can form, but are unstable, reflecting the symmetry mis-match between the two isoforms. Taken together with other studies, we speculate that Panx1 and Panx2 do not oligomerize in vivo or if they do, their heteromeric associations are unstable or non-functional. While the in vivo function of Panx2 channels is still not established, it is clear that Panx2 has unique functional properties and molecular organization that set it apart from Panx1 channels. This structural and functional analysis on purified Panx1 and Panx2 channels lay the foundation for future structure-function studies to understand their unique role in cells and tissues.

REFERENCES


FOOTNOTES

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The abbreviations used are EM = electron microscopy; Panxs = pannexins; Panx1 = pannexin1, Panx2 = pannexin2; connexin = Cx; WT = wild type; CBX = carboloxylone

FIGURE LEGENDS

Fig. 1. Panx1 and Panx2 show a channel topology in mammalian cell membrane, very similar to gap junction proteins. Membranes isolated from Panx1(A) and Panx2 (B) exogenously over-expressed in MDCK cells contain channel-like structures similar in appearance to Cx26 exogenously expressed in HeLa cells (C). As a negative control, a membrane isolated from parental HeLa cells shows no channel-like structures (D). Gel staining (E) and Western blot (F) on denaturing PAGE gels show good purity and high specificity of our membrane purifications. (G) A positive control shows a Cx26 band, while after stripping and reprobing the same filter with several other antibodies shows no cross-reactivity. Antibodies used in this figure are mouse Cx26, our monoclonal Panx1 and polyclonal Panx2 antibodies described in the Experimental procedures section.

Fig. 2. Membrane cross-section comparison between Cx26 and Panx1 or Panx2, shows relevant differences. Membrane profiles of Cx26, Panx1 and Panx2 show: (A) double layers for Cx26 and single layer for Panx1 and Panx2. Immunolabeling with specific primary antibodies and with secondary gold conjugated antibodies shows that the gold labels both sides of the membrane profile for Cx26 and only one side for Panx1 and Panx2 (B). An enlarged view of an immuno-labeled membrane is shown in (C) for easier visualization.

Fig. 3. Isolated pannexin oligomers (pannexons) confirm similar features to connexons. Pannexons and connexons expressed and purified from baculovirus infected Sf9 cells. (A) EM analysis going from a smaller connexin (Cx26 first row left) to Panx2 (second row right). Larger proteins (Panx2 and Cx50) have more heterogeneous morphology of structures perhaps due to differential staining of different orientations. Images are displayed at the same magnification to show increasing channel size with increasing monomer size. (B) Stained protein gels (labeled with G for gel on the bottom) and Westerns blots (labeled with W for Western blot) by the side of each protein demonstrate good purity of these preparations. Note that Westerns can tend to over-emphasize dimeric bands.
Fig. 4. *Panx2* homomeric channels are functional. (A) Schematic of the cytochrome c based vesicle assay. If *Panx2* channels are functionally reconstituted, ascorbate crosses the lipid bilayer. Intra-liposomal cytochrome c gets reduced as monitored at 417 nm. (B) Fractions of a characteristic size exclusion chromatography to separate free cytochrome c from *Panx*-POPC-proteoliposomes with entrapped cytochrome c. Turbidity of the vesicle suspension is followed at 630 nm, cytochrome c containing fractions at 417 nm. (C) Time resolved reduction of intra-liposomal cytochrome c mediated by the transport of ascorbate via a transmembrane spanning active *Panx1* channel and inhibition with CBX. (D) As in (C) for *Panx2*. (E) Semi-logarithmic plot illustrating the concentration dependent blocking of the *Panx1* pannexons by CBX. Each data point represents the normalized initial slope $m_i$ of the *Panx1* mediated intra-liposomal cytochrome c reduction. The solid line is a fit to the data points using the Hill equation of the form: $m_i = m_{i,\text{MAX}} (c_{\text{CBX}}^{-n} / ([\text{IC}_{50}]^{-n} + c_{\text{CBX}}^{-n}))$ where $m_{i,\text{MAX}}$ is the initial slope recorded in buffer without CBX (set as unity), $c_{\text{CBX}}$ is the drug concentration, $\text{IC}_{50}$ the concentration giving half of the maximal inhibition and $n$ is the Hill coefficient. (F) As in (E) for *Panx2*.

Fig. 5. *Panx2* channels expressed in Xenopus oocytes opened at high positive membrane potentials and were insensitive to inhibitors. A voltage ramp from -100 mV to +100 mV over a span of 70 seconds applied to single, uninjected oocytes induced a transient inward current carried by endogenous channels (probably voltage activated sodium channels) (red traces in A). These currents were somewhat variable between oocytes and even within the same oocyte in response to repetitive activation as indicated by the variability of the amplitude and position of peak activity. Expression of *Panx2* resulted in large outward currents most prominent at potentials exceeding +75 mV (blue traces). Shown here are five representative traces from each condition. Membrane currents in *Panx2* expressing oocytes before (black traces), during (yellow) and after washout (red) of carbenoxolone (CBX) are shown in (B). CBX slightly attenuated the endogenous inward currents and led to larger peak currents as if stimulating rather than inhibiting *Panx2* currents, an effect that was not immediately reversed upon washout. This figure contains three traces each for *Panx2* WT injected oocytes and subsequent CBX treated oocytes and two traces for the CBX wash out oocytes. (C) Mutagenesis of cysteines in *Panx2* eliminated currents in injected oocytes (see also Table II). Shown here are five representative traces from the *Panx2*C81S mutant, *Panx2* WT and uninjected oocytes.

Fig. 6. Cross-linking of a *Panx2* truncation mutant for stoichiometric analysis reveal non-hexameric assemblies. (A) Folding diagrams for full length *Panx2*. (B) Cross-linked *Panx2* run on a 4% Tris-glycine gel reveals a band located well above a hexamer. This band corresponds to either a heptamer or octamer. To distinguish between an octamer and heptamer stoichiometry, a truncation mutant of *Panx2* was constructed so that *Panx2* is truncated after S340 plus a 30 amino acid tag (370 amino acids total). (C) Topology diagram for *Panx2*Trun340-V5-His6. (D) Predicted secondary structure in the *Panx2* C-terminus (amino acids 317-674) according to three different prediction algorithms NNPredict (*top*), GOR4 (*middle*) and PSIPRED (*bottom*). Heavy black lines mark stretches of putative $\alpha$-helices and heavy gray lines indicate $\beta$ structure propensity. An asterisk denotes predicted secondary structure elements longer than 3 amino acids common among these three predictions. The dotted arrow indicates the truncation position between S340 and Q341. (E) Purified *Panx2*Trun340-V5-His6 preparations were analyzed by gel stainings and Western blots that this protein maps at ~41 kDa as expected. *Panx2* truncated was X-linked with DSP 300 $\mu$g/ml after purification and analyzed on different gels. (F) Cytochrome c vesicle permeability measurements comparing *Panx1*-V5-His6, *Panx2* WT V5-His6 and *Panx2*Trun340 V5-His6 (as in Fig. 4). Comparison of the initial slopes $m_i$ of the fitted line to data points recorded during the first 25 seconds after the addition of ascorbate for the three pannexons indicated that *Panx2* truncation pannexons had a reduced permeability to ascorbate. Each category has an n=3. (G) Electron micrograph shows channels formed by *Panx2*Trun340. In (H) left lane *Panx2* shows the upper band mapping above 250 kDa, suggesting that it is not a hexamer (41 kDa x 6 = 246 kDa). In the right lane *Panx2* cross-linked was boiled in presence of 5% BME and shows the monomeric band mapping as expected around 41 kDa. (I) Cross-linked *Panx2*Trun340-V5-His6 is run on a higher resolving Tris Acetate gel system and is separated.
into the monomer (41 kDa), the dimer (82 kDa) and the upper band mapping between 268 and 460 kDa. (J) The cross-linked Panx2Trun340-V5-His$_6$ upper band maps higher than Panx1 hexameric band (~300 kDa). The measured position of the Panx2Trun340-V5-His$_6$ band is at a position that confirms that the Panx2 oligomer is mostly likely an octamer. (K) Electron micrograph shows Panx2Trun340 channel appearance after X-linking.

**Fig. 7.** Panx1/Panx2 heteromers are highly unstable. (A) EM image containing negatively stained Panx1/Panx2 oligomers examined 1 hour after purification. (B) After 24 hours, the channels are barely recognizable. (C) The 1 hour sample analyzed by Western blot contained both Panx1 and Panx2 bands. (D) After buffer exchange and a second Ni-NTA affinity column purification, Panx1 bands are evident, but not Panx2.

### TABLES AND TABLE LEGENDS

**Table I. Co-expression of Panx1 and Panx2 in Xenopus oocytes**

<table>
<thead>
<tr>
<th></th>
<th>Panx1</th>
<th>Panx2</th>
<th>Panx1 + Panx2</th>
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<tr>
<td>Mean current (µA)</td>
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<td>2.14</td>
<td>0.86</td>
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<td>SE</td>
<td>0.31</td>
<td>0.13</td>
<td>0.17</td>
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<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>4</td>
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The mRNAs for Panx1 and Panx2 were mixed with each other or with water at equal volumes to have identical concentrations for each experimental condition. The currents at +100 mV at the end of a voltage ramp from -100 to +100 mV were analyzed.

**Table II. Mutagenesis of extracellular cysteines cause elimination of currents in Xenopus oocytes**

<table>
<thead>
<tr>
<th></th>
<th>Uninjected</th>
<th>Panx2</th>
<th>C81S</th>
<th>C99S</th>
<th>C259S</th>
<th>C280S</th>
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<tr>
<td>Mean (µA)</td>
<td>0.54</td>
<td>1.78</td>
<td>0.71</td>
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<td>n</td>
<td>20</td>
<td>21</td>
<td>5</td>
<td>5</td>
<td>5</td>
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The mRNAs for Panx2 and four cysteine to serine substitution mutants were injected into oocytes and voltage ramps applied as previously described. Maximal current amplitudes at the end of voltage ramps (+100 mV) were analyzed and plotted.

**Table III. Effect of CO$_2$ on Panx2 channel currents**

<table>
<thead>
<tr>
<th></th>
<th>Uninjected</th>
<th>Uninjected$_{CO2}$</th>
<th>Panx2</th>
<th>Panx2$_{CO2}$</th>
<th>Panx2 - Uninjected</th>
<th>Panx2$<em>{CO2}$ - Uninjected$</em>{CO2}$</th>
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<tbody>
<tr>
<td>Mean (µA)</td>
<td>0.46</td>
<td>1.56</td>
<td>1.14</td>
<td>1.66</td>
<td>0.68</td>
<td>0.1</td>
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<tr>
<td>SEM</td>
<td>0.02</td>
<td>0.12</td>
<td>0.05</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Oocytes were treated with CO$_2$ that induces cytoplasmic acidification. The subscript $_{CO2}$ refers to oocytes treated with CO$_2$. Panx2 channel specific currents were assessed by subtracting currents measured in uninjected oocytes from currents from Panx2 channels that were subtracted with (second to right hand column) and without CO$_2$ perfusion (right hand column).
Figure 1
<table>
<thead>
<tr>
<th></th>
<th>Cx26</th>
<th>Panx1</th>
<th>Panx2</th>
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</table>

Figure 2
Figure 3
Figure 4

Figure 5
Figure 6
Figure 7
Pannexin1 and pannexin2 channels show quaternary similarities to connexons and different oligomerization numbers from each other
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