Published in final edited form as:

Eur J Neurosci. 2017 June ; 45(12): 1594–1605. doi:10.1111/ejn.13602.

Connexin36 localization to pinealocytes in the pineal gland of mouse and rat

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Abstract

Several cell types in the pineal gland are known to establish intercellular gap junctions, but the connexin constituents of those junctions have not been fully characterized. Specifically, the expression of connexin36 (Cx36) protein and mRNA has been examined in the pineal, but the identity of cells that produce Cx36 and that form Cx36-containing gap junctions has not been determined. We used immunofluorescence and freeze fracture replica immunogold labelling (FRIL) of Cx36 to investigate the cellular and subcellular localization of Cx36 in the pineal gland of adult mouse and rat. Immunofluorescence labelling of Cx36 was visualized exclusively as puncta or short immunopositive strands that were distributed throughout the pineal, and which were absent in pineal sections from Cx36 null mice. By double immunofluorescence labelling, Cx36 was localized to tryptophan hydroxylase-positive and 5-hydroxytryptamine-positive pinealocyte cell bodies and their large initial processes, including at intersections of those processes and at sites displaying a confluence of processes. Labelling for the cell junction marker zonula occludens-1 (ZO-1) either overlapped or was closely associated with labelling for Cx36. Pinealocytes thus form Cx36-containing gap junctions that also incorporate the scaffolding protein ZO-1. FRIL revealed labelling of Cx36 at ultrastructurally-defined gap junctions between pinealocytes, most of which was at gap junctions having reticular, ribbon or string configurations. The results suggest that the endocrine functions of pinealocytes and their secretion of melatonin is supported by their intercellular communication via Cx36-containing gap junctions, which may now be tested by the use of Cx36 null mice.

Graphical Abstract

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Author contributions

S.G.W., D.D.T. and J.I.N. conducted immunofluorescence studies, K.V. and J.E.R. conducted FRIL studies, T.Y. prepared FRIL replicas, all authors participated in data analysis, and J.I.N., K.G.V., and J.E.R wrote the final manuscript.

Competing financial interests

The authors declare no competing financial interests.

Data statement

The raw immunofluorescence and FRIL images will not be deposited in a public repository due to the large sizes of these image files and the complexity of annotating each of the many hundreds of separate images. However, those data can be obtained upon request to the corresponding author.

Several neuroendocrine cell types are linked by gap junctions consisting of connexin36, including pancreatic β -cells, adrenal medullary chromaffin cells and hypothalamic neuroendocrine cells. Here, we show the localization of connexin36-containing gap junctions to pinealocytes in the pineal of mice and rats. Our results suggest that, as in other neuroendocrine organs, intercellular communication mediated by these gap junctions contribute to the hormone secretory functions of pinealocytes.

Keywords

gap junctions; immunofluorescence; zonula occludens-1; melatonin

Introduction

Myriad cell types in vertebrates form gap junctions that mediate coupling between cells by allowing the exchange of ions and small molecules via intercellular channels that link plasma membranes of apposing cells. The channels are composed of one or a few of the twenty members of gap junction forming connexin proteins that are differentially expressed among different cell types. The functional importance of gap junctions in organizing the collective activity of cells has been demonstrated by reports of physiological deficits in organ function following gene deletion of particular connexins in transgenic mice and by the variety of human diseases caused by connexin mutations (Goodenough & Paul, 2009; Laird, 2009). Among tissues in which gap junction-mediated intercellular communication supports coordinated cellular activity are the endocrine (neuroendocrine) glands, including pancreatic islets, the anterior and posterior pituitary, adrenal cortex and medulla, thyroid and parathyroid, and neuroendocrine hypothalamus (Michon *et al.*, 2005; Hodson *et al.*, 2015; Bell & Murray, 2016). In some of these systems, it has been established that normal secretion of the endocrine gland's resident hormone is critically dependent on expression of particular connexins and functional cell-cell coupling via gap junctions (Bosco *et al.*, 2011; Hodson *et al.*, 2012; Desarmenien *et al.*, 2013). The pineal gland is also an endocrine organ that secretes melatonin in response to sympathetic noradrenergic innervation from the superior cervical ganglia to regulate circadian rhythms, and it potentially participates in a

variety of other physiological processes (Sapède & Cau, 2013; Liu *et al.*, 2015; Opie *et al.*, 2015). Little is known whether gap junctional-coupling within the pineal contributes to the normal function of this gland.

The pineal gland consists primarily of pinealocytes that produce melatonin (Pevet, 1977, 1981; Vollrath, 1981; Moller & Baeres, 2002; Sapède & Cau, 2013) and which can be identified by immunolabelling for their markers tryptophan hydroxylase (TrypH) (Rath *et al.*, 2016), 5-hydroxytryptamine (5-HT) (Tsao *et al.*, 2017), or S-antigen (aka, arrestin) (Schomerus *et al.*, 1994). Additional cells include a set of spatially-restricted astrocytes identified by their labelling for glial fibrillary acidic protein (GFAP) and located largely in anterior regions of the gland (Moller *et al.*, 1978; Schachner *et al.*, 1984; Yamamoto *et al.*, 1990), and widely-distributed perivascular interstitial cells that represent about 10–15% of the pineal cell population (Vollrath, 1981) and that can be identified, or at least a portion thereof, by their robust immunohistochemical labelling for calbindin-D28K (Yamamoto *et al.*, 1990; Tsao *et al.*, 2017). Early studies described ultrastructurally-identified gap junction in these cell types in the pineal of various species, including rat (Krstic, 1974; Taugner *et al.*, 1981; Cieciora & Krakowski, 1991), guinea-pig (Huang & Taugner, 1984), rabbit (Romijn, 1973), monkey (Ichimura *et al.*, 1986; Ichimura, 1992), and humans (Moller, 1976). Consistent with the presence of gap junctions, the expression of several connexins in the pineal has been reported, including connexin26 (Cx26) in rat pinealocytes (Saez *et al.*, 1991), connexin43 (Cx43) in rat pineal astrocytes (Saez *et al.*, 1991; Berthoud & Saez, 1993), Cx43 and Cx45 in various pineal cell types in chicken (Berthoud *et al.*, 2000), and connexin36 (Cx36) in unidentified cell types of rat pineal (Belluardo *et al.*, 2000; Condorelli *et al.*, 2000). To further determine which of the connexin family members are expressed in the pineal and to establish the cell types that express particular connexins, we used immunofluorescence labelling of various connexins in combination with labelling of pineal cell types, as well as freeze-fracture immunogold labelling (FRIL) of Cx36 and Cx43 to determine the presence of these connexins in gap junctions of pinealocytes *vs.* other cell types. Our present results focus specifically on the cellular localization of Cx36 to pinealocytes in the pineal gland of mouse and rat. In addition, because gap junctions between pinealocytes were reported to have intimate associations with tight junctions (Taugner *et al.*, 1981), and because Cx36 has been shown to interact with the cell junction protein zonula occludens-1 (ZO-1) (Li *et al.*, 2004), we used immunofluorescence to examine the localization of Cx36 in relation to ZO-1.

Materials and methods

Animals and antibodies

A total of fifteen adult male Sprague-Dawley rats, three adult male CD1 mice, eight adult male C57BL/6 wild-type mice, and three adult male C57BL/6 Cx36 null mice were used for immunofluorescence approaches in the present study. The C57BL/6 mouse strain was used because this represented the genetic background for mice with Cx36 gene ablation, allowing tests for specificity of immunofluorescence labelling of Cx36. However, C57BL/6 mice compared with other mouse strains exhibit impaired melatonin production (Vivien-Roels *et al.*, 1998; Kennaway *et al.*, 2002). Thus, we also examined another mouse strain (*i.e.*, CD1)

to assess possible differences in immunolabelling patterns of Cx36 in the pineal of C57BL/6 vs. CD1 mice. Tissues from three of the rats were taken at midnight, and tissues from all of the other animals were taken during the day (9:00 to 11:00 am). Animals were obtained from breeding facilities at the University of Manitoba, were housed under standard conditions, with free access to food and water, and were utilized according to protocols approved by the Central Animal Care Committee of University of Manitoba, with minimization of the numbers animals used. Acquisition of immunofluorescence data involved the use of multiple primary antibodies. These antibodies and the dilutions at which they were used included: mouse monoclonal Ab39-4200, rabbit polyclonal Ab51-6300 and rabbit polyclonal Ab51-6200 antibodies against Cx36, each used at a concentration of 2 µg/ml; sheep polyclonal PA1-4678 against TrypH, used at a dilution of 1:175; rabbit polyclonal Ab61-7300 against ZO-1, used at a concentration of 2 µg/ml; and goat polyclonal Ab20079 against 5-HT, used at a dilution of 1:100. The first five of these antibodies were obtained from ThermoFisher (Rockford, IL, USA), and the antibody against 5-HT was obtained from ImmunoStar (Hudson, WI, USA). Additional antibodies included anti-calbindin-D28K (Sigma, Oakville, ON, Canada) used at a dilution of 1:200, anti-GFAP (Neuromics, Edina, MN, USA) used at a dilution of 1:1000, and anti-S-antigen (aka, arrestin) (MyBioSource, San Diego, CA, USA) used at a dilution of 1:50. Antibodies against several other connexins examined in the pineal, including Cx30, Cx32, Cx43, Cx45, Cx47 and Cx57, were all obtained from ThermoFisher, and were used at a concentration of 2 µg/ml. Donkey Cy3-conjugated, Cy5-conjugated and AlexaFluor 488-conjugated secondary antibodies (ThermoFisher, Rockford, IL, USA; Jackson ImmunoResearch, West Grove, PA, USA) were used at a dilution of 1:600 to 1:1000, respectively. All primary and secondary antibodies were diluted in 50 mM Tris-HCl, pH 7.4, containing 1.5% sodium chloride (TBS), 0.3% Triton X-100 (TBST) and 10% normal goat or normal donkey serum.

Tissue preparation and immunofluorescence labelling

All animals were deeply anaesthetized with equithesin (3 ml/kg), and perfused transcardially with cold (4°C) pre-fixative containing 50 mM sodium phosphate buffer (pH 7.4), 0.1% sodium nitrite, 0.9% NaCl, and 1 unit/ml heparin, followed by perfusion with cold fixative consisting of 0.16 mM sodium phosphate buffer (pH 7.4), 0.2% picric acid, and either 1% or 2% formaldehyde that had been stored in sealed ampules (Electron Microscopy Sciences, Hatfield, PA, USA). Mice weighing 25–35 grams were perfused with 40–50 ml of fixative, and rats weighing 225 to 300 grams were perfused with 150–300 ml of fixative. Following fixation, animals were perfused with a cold solution containing 10% sucrose in 25 mM sodium phosphate buffer (pH 7.4). Brains with pineal gland attached were removed and stored at 4°C for 24–48 hr in cryoprotectant solution containing 25 mM sodium phosphate buffer (pH 7.4), 10% sucrose and 0.04% sodium azide. Tissues were frozen and sectioned transversely or sagittally at a thickness of 15 µm using a cryostat, and sections were collected on gelatinized glass slides. Slide mounted sections were incubated overnight at 4°C with solution containing two primary antibodies. Sections were then washed in TBST for 1 hr, incubated for 1.5 hr with appropriate combinations of secondary antibodies at room temperature, washed in TBST for 20 minutes and then washed in TBS for 30 minutes. Some sections were counterstained with deep red Nissl fluorescent NeuroTrace (ThermoFisher). Sections were coverslipped with Fluoromount G anti-fade medium (SouthernBiotech,

Birmingham, AL, USA). Immunofluorescence labelling was examined on a Zeiss Z2 Axiovision Imager microscope and a Zeiss 710 laser scanning confocal microscope. Data from wide-field and confocal microscopes were collected as either single scans or as through-focus z-stack images consisting of multiple scans taken at z-intervals of 0.43 μm , with stacks consisting of 8–12 scans. Images were processed using Zeiss ZEN software, and images with Cy5 immunolabelling were pseudocoloured red.

Quantitative analysis of the density of immunofluorescence labelling for Cx36 was conducted on pineal taken from rats during morning hours and on pineal taken from rats a midnight. A total of 8 fields in sections of each pineal from the six rats were photographed at x20 objective magnification, with equal illumination intensity settings. Zeiss Zen software was then used to obtain quantitative values for percentage of pixels representing labelling for Cx36 in each image, after appropriate setting of thresholds for imaging of immunofluorescent Cx36-puncta and excluding background fluorescence. Value for the 8 fields were averaged for each rat, and the means from the pineals of the three day rats vs. the means from those of the three night rats were taken for statistical analysis using Student's T test.

Freeze fracture replica immunogold labelling

Pineal tissue was used for FRIL studies from one postnatal day 13 Sprague-Dawley rat (29 g, male). The pineal in rat was examined at postnatal day 13 because pineal tissue was readily available from this age of a rat that had been appropriately prepared for studies of other tissues by FRIL, and because previous studies of developing pineal suggest that the pineal has reached maturity by day 13. The rat were anesthetized using ketamine/xylazine, and prepared for electron microscopy by transcardiac perfusion with 4% formaldehyde in Sorenson's phosphate buffer (SPB), the pineal gland was dissected and cut to 125 micron slices using a refrigerated Lancer Vibratome 3000 (Technical Products International, St. Louis, MO, USA) that maintained samples at 4 °C. These sections were infiltrated with 30% glycerol as a cryoprotectant to minimize ice crystal damage during freezing, mounted on aluminum "Slammer" supports (Heuser et al., 1981), and frozen by contact with a liquid nitrogen-cooled metal mirror (Ultra-Freeze MF7000, RMC Products, Tucson, AZ, USA).

Frozen samples were fractured and replicated in a JEOL JFD-2 freeze-fracture device (JEOL, Tokyo, Japan), and while still frozen, were bonded to gold "index" grids (Electron Microscopy Sciences, Fort Washington, PA, USA) using 1.5–2% Lexan (SABIC, Houston, TX, USA) dissolved in ethylene dichloride, according to our detailed procedures (Rash et al., 1995, 2004; Rash & Yasumura, 1999). The Lexan-stabilized samples were thawed and photomapped with a Zeiss LSM510 Meta laser scanning confocal microscope using tissue autofluorescence. Bulk tissue remnants were removed from the confocal-mapped replica by washing with constant agitation in SDS detergent in 0.16% Tris-HCl buffer (pH 8.9). Initially, the replica was washed in 1.7% SDS solution with 30 mM sucrose (pH 8.9) at 60°C for 2 hr, then 80 °C for 2 hr, and back to 60°C for a total of 7.5 hr washing. The samples were then digested for one hr in 4% collagenase D (Roche Applied Science, Indianapolis, IN, USA) in 0.15 M Sorenson's phosphate buffer (SPB; pH 7.4) at 40°C, followed by an additional 11 hr digestion in 3% SDS (pH 9.1) without sucrose at 80 °C.

SDS-washed replicas were rinsed in blocking buffer (Dinchuk et al., 1987) consisting of 10% heat-inactivated goat serum plus 1.5% fish gelatin in SPB, and antibodies diluted to 10 µg/ml in blocking buffer. We used Thermo-Fisher antibodies to monoclonal Cx36 (39-4200) and polyclonal Cx43 (71-0700). After primary labelling with Cx36 and Cx43 for 8.5 hr at 4 °C, one mid-sagittal sample of pineal was rinsed and counter-labelled for 13 hr at 4°C plus 1.5 hr at 27 °C using goat anti-mouse IgG (10-nm plus 30-nm for Cx36) and goat anti-rabbit IgG (5-nm and 20-nm for Cx43) (all gold labels were from BBI Solutions, Cardiff, UK). Two sizes of gold beads were used to label each connexin (“dual double-labeling”) because the smaller gold beads have higher labelling efficiency than the larger beads but are visible only at high magnifications, whereas the larger immunogold beads (20- and 30-nm) served as highly-visible “flags” that were readily detected at much lower magnifications than used when searching for gap junctions by conventional freeze fracture (*i.e.*, in the absence of immunogold labelling), and hence allowed rapid examination of greater tissue areas.

After immunogold labelling, each sample was air-dried and coated on the labelled side with 10 to 20 nm of evaporated carbon to anneal cracks in the replica, as well as to stabilize the immunogold beads. Before viewing by transmission electron microscopy, the Lexan support film was removed by immersing the grids in ethylene dichloride solvent for 2–3 hr, and the samples were again air-dried.

For TEM studies, samples were photographed as stereoscopic pairs with a 10° included angle using a JEOL JEM 1400 TEM equipped with a Gatan Orius SC1000 11 Megapixel digital camera. The contrast range for each image was optimized using ‘levels’. Slight image compressions created by photography of samples tilted for stereoscopy were corrected by using the ‘image distort’ functions and trimmed so that the ‘stereo windows’ appear rectangular and level.

Results

Cx36 distribution in pineal of mouse and rat

An overview of immunofluorescence labelling for Cx36 in the mouse pineal gland is presented in Figure 1. To visualize sections of the pineal, the main cellular constituent of the gland was immunolabelled for the pinealocyte marker TrypH, which is the first enzyme in the pathway for synthesis of melatonin. As shown in sections of the whole pineal in mouse, pinealocyte cell bodies were well labelled for TrypH (Fig. 1A1 and 1B1), but their initial processes were only weakly immunoreactive and their fine, more distal processes were largely unlabelled, as shown at higher magnifications in subsequent images. In wild-type mice, immunofluorescence for Cx36 was consistently robust, at least with the necessarily weak fixation conditions required to reveal labelling, and was distributed throughout the pineal, often as irregularly shaped patches of immunolabelling (Fig. 1A2). Specificity of Cx36 detection with the several antibodies used was confirmed in the pineal of Cx36 null mice where, in similar sections of the whole pineal labelled for TrypH (Fig. 1B1), immunofluorescence labelling for Cx36 was entirely absent (Fig. 1B2). In rat, the specificity of Cx36 detection was supported by immunolabelling with two different antibodies directed at different non-overlapping amino acid sequences within Cx36. Patterns of labelling produced by mouse monoclonal Ab39-4200 (Fig. 1C1) were qualitatively identical to those

obtained with rabbit polyclonal Ab51-6300 (Fig. 1C2), and double labelling with these antibodies showed total overlap of labelled structures in merged images (Fig. 1C3) over distances $>20\text{ }\mu\text{m}$ (arrowheads), potentially involving continuity over multiple cells, as illustrated at higher magnification below. Similar labelling for Cx36 was obtained with a third anti-Cx36 Ab51-6200 (not shown).

Immunolabelling in both rat and mouse consisted exclusively of either fine immunofluorescent puncta (Cx36-puncta) or short immunofluorescent strands (Cx36-strands). Both Cx36-puncta and Cx36-strands were prominent in the rat pineal (Fig. 1D), where the puncta were frequently organized in curvilinear arrangements (Fig. 1C). The mouse pineal contained Cx36-puncta that were organized in small and large clusters (Fig. 1E). The Cx36-puncta were fairly uniform in size measuring 0.3 to $0.8\text{ }\mu\text{m}$ in diameter, whereas the dimensions of strands were more variable ranging from 3 to $10\text{ }\mu\text{m}$ or more in length. Although C57BL/6 mice compared with other mouse strains exhibit impaired melatonin production (Vivien-Roels *et al.*, 1998; Kennaway *et al.*, 2002), no differences in patterns or density of Cx36 labelling were observed in the pineal of CD1 *vs.* C57 mice (not shown). As shown in images of tissue from rat (Fig. 1F), the pineal stalk also displayed similarly dense labelling for Cx36, consisting of fine puncta and patches of immunofluorescence around pineal stalk tissue surrounding a central lumen, as seen in transverse section (Fig. 1F1, asterisk). At higher magnification, the patches were resolved to consist also of fine puncta, as shown in sagittal section (Fig. 1F2). The course of the stalk connecting the pineal with subcortical structures and labelling for Cx36 along its length was also visualized in sagittal section at lower magnification (not shown). Visual inspection of sections indicated that patterns of labelling for Cx36 in rat pineal tissue taken during the day *vs.* at midnight (not shown) were qualitatively similar, and labelling density also appeared similar. Comparative quantitative analysis was nevertheless undertaken, yielding an average percentage pixel density for Cx36 labelling of 0.78 ± 0.12 and 0.62 ± 0.10 for fields of pineal examined in animals taken during the day *vs.* those taken at midnight, respectively, which was a non-significant difference.

Cellular localization of Cx36

The cellular localization of Cx36 in the pineal was examined by immunolabelling for Cx36 in combination with labelling either for TrypH or its downstream product, 5-hydroxytryptamine (5-HT). Labelling for TrypH is an adequate marker of pinealocytes, as tested in sections of adult rat pineal that were taken for fluorescence Nissl staining and simultaneous immunolabelling for TrypH (Tsao *et al.*, 2017). The vast majority of Nissl-stained cells were TrypH-positive (quantitative data??? not shown), indicating that TrypH is likely expressed by all pinealocytes and can be readily detected in these cells by immunofluorescence. Confocal overlay images of double immunofluorescence labelling for Cx36 and TrypH in the pineal gland of adult rat and mouse are shown in Figure 2. In mouse, clusters of Cx36-puncta were often distributed at sites where pinealocyte cell bodies were in close proximity and where their main processes appeared to converge (Fig. 2A). Detailed examination of Cx36-puncta associated with pinealocyte cell bodies and thick initial processes conducted by confocal 3D rotation of image z-stacks, such as that shown of mouse pineal in Figure 2B, indicated that Cx36-puncta were localized to the periphery of cells and

likely their plasma membranes. In rat, scattered Cx36-puncta, clusters of these puncta, and Cx36-strands were invariably localized to TrypH-positive pinealocyte cell bodies and their initial processes (Fig. 2C). Occasionally, intersecting initial processes from adjacent cells displayed labelling at points of intersection (Fig. 2D), suggesting gap junction formation at these points of apparent contact. Strands of Cx36 labelling tended to follow along the periphery of TrypH-positive cell bodies or along processes (Fig. 2E), which however were only poorly labelled for TrypH, as noted above. Intracellular labelling appeared to be absent or below the level of immunofluorescence detectability in both rat and mouse pinealocytes. In additional studies, we found that Cx36 was similarly localized to pinealocytes immunolabelled for their marker, S-antigen (not shown).

Immunofluorescence for Cx36 examined in combination with labelling of 5-HT produced visualization of pinealocyte cell bodies comparable to that obtained with labelling of TrypH, as shown by comparison with fluorescence Nissl counterstaining, but in addition better revealed immunopositive initial and more distal pinealocyte processes, as shown in rat pineal (Fig. 3A). 5-HT-immunopositive elements also included bundles of sympathetic fibers (Fig. 3A, arrowheads), which are known to accumulate 5-HT and to be highly concentrated in perivascular spaces (Vollrath, 1981; Nishimura *et al.*, 1999), thus clearly distinguishing these fibers from pinealocyte processes, both in extra-lobular location and staining properties. Images of double immunofluorescence labelling for 5-HT and Cx36 in the pineal of adult rat and mouse are presented in Figure 3B–F. In rat, Cx36-strands were seen to be associated with finer pinealocyte processes (Fig. 3B), which occasionally could be followed back to their cell bodies. In mouse, double labelling revealed not only clusters of Cx36-puncta localized to relatively large initial processes of pinealocytes (Fig. 3C), but also individual Cx36-puncta along their processes, and at locations where these processes were highly intermingled (Fig. 3D). Labelling for Cx36 and 5-HT clearly showed localization of singlets and clusters of Cx36-puncta to pinealocyte cell bodies, notably among clusters of these somata at their apparent close appositions (Fig. 3E). Most illustrative of the configurations in which Cx36-puncta occur in relation to pinealocytes is their occurrence at confluences of and close contacts between initial processes emanating from multiple pinealocytes (Fig. 3F). Confocal analysis indicated lack of Cx36 association with 5-HT-immunopositive sympathetic fibers (not shown).

In double-labelling studies with other cell markers in combination with Cx36, our analyses indicated that Cx36 lacked association with either GFAP-positive astrocytes or calbindin-D28K-positive interstitial cells (not shown). Further, our examination of several other connexins in mouse and rat pineal showed an absence of immunolabelling for Cx30, Cx32, Cx45, Cx47 and Cx57 in either species (not shown).

Cx36 association with ZO-1

The scaffolding protein ZO-1 was shown to be associated with gap junctions and tight junctions in a wide variety of tissues (Li *et al.*, 2004; Li *et al.*, 2008; Ciolofan *et al.*, 2006; Hervé *et al.*, 2012), and close structural associations have been described between these two types of junctions in the pineal gland (Taugner *et al.*, 1981). We therefore examined localization of Cx36 in relation to ZO-1 by double immunofluorescence labelling for these

two proteins in the rat pineal. Labelling of ZO-1 was localized to Cx36-puncta and to both short strands and to longer strands (up to 10 μm) (Fig. 4A2). In addition, much longer immunopositive filaments (up to 20 μm) that were devoid of labelling for Cx36 were observed (Fig. 4B,C). Cx36-puncta and Cx36-strands were invariably co-localized with labelling for ZO-1 (Fig. 4A), whereas the longer ZO-1-positive filaments were devoid of labelling for Cx36 and likely represented tight junctions of other cell types traversing these 15 μm -thick sections (Fig. 4C). Besides Cx36/ZO-1 co-localization along strands, additional ZO-1-strands lacking labelling for Cx36 were seen meandering among strands showing Cx36/ZO-1 overlap, including regions where they were either closely adjacent to or in linear continuity with such areas of overlap (Fig. 4B), demonstrating clear distinction between Cx36/ZO-1 colocalization vs. separate areas localized for ZO-1 without Cx36.

Cx36 localization by FRIL

Rat pinealocytes were identified based on established anatomical and ultrastructural criteria, including: 1) radial distribution around the lumen within each lobule (Figs. 5 and 6); 2) linear and reticular gap junctions at the narrow apices of these cells; and 3) abundant smooth endoplasmic reticulum (Huang and Taugner, 1984). Pinealocyte gap junctions that were immunogold labelled for Cx36 were localized to appositions between the thin apical processes that surround and delineate the lumen within each lobule (Figs. 5 and 6). (See explanatory diagram in Przybylska-Gornowicz *et al.*, 2012). Most of these gap junctions conformed to the reticular/string/ribbon types previously described by us in the OFF sublamina of the retina (Kamasawa *et al.*, 2006) and as originally shown in pineal (Taugner *et al.*, 1981). The longer of these strands are presumed to correspond to the linear strands seen by immunofluorescence (Figs. 1C, 1F2 and 2E). Finally, a few isolated “plaque” gap junctions were identified by FRIL on the lateral margins of pinealocytes (not shown). In these dual-double-labelled samples, where Cx36 was labelled by 10- and 30-nm gold beads and Cx43 was labelled by 5- and 20-nm gold beads, 10-nm and 30-nm gold beads were only on pinealocyte gap junctions, whereas 5-nm and 20-nm gold beads were on gap junctions between several identified cell types, including interstitial cells, vascular endothelial cells, and pia mater surrounding the pineal, as will be described in a separate report (Vanderpool, Rash, Yasumura and Nagy, in preparation).

Discussion

The present results confirm previous reports describing the expression of Cx36 protein and mRNA in the pineal gland of rat (Belluardo *et al.*, 2000; Condorelli *et al.*, 1998, 2000), and demonstrate for the first time its cellular localization to mouse and rat pinealocytes that were identified by their immunolabelling for either TrypH or 5-HT. The presence of Cx36 also in the pineal stalk is consistent with the known distribution of pinealocytes in this structure (Calvo & Boya, 1985; Rath *et al.*, 2016). Although gap junctions linking pinealocytes to glial cells have been described (Cieciura & Krakowski, 1991), we found no association (not shown) between Cx36 localized to pinealocytes and Cx43 reported elsewhere to be localized to glial cells and interstitial cells (Tsao *et al.*, 2017), consistent with the non-permissiveness of gap junction formation between these two connexins (Teubner *et al.*, 2000; Li *et al.*, 2008). FRIL double labelling for Cx36 and Cx43 showed no overlap of these connexins in

any gap junctions, and no cells were labelled for both connexins. Rather Cx36 was found only on pinealocytes, as supported by the present immunofluorescence observations. Ultimately, however, electrophysiological or dye-coupling approaches would need to be conducted to determine whether functional heterologous coupling occurs between different cell types in the pineal.

It was previously reported that Cx26 is expressed in the rat pineal, where it was localized to pinealocytes and was considered to support the demonstrated electrical and dye-coupling between these cells (Saez *et al.*, 1991). However, in contrast to the high density of immunofluorescence labelling for Cx36 observed in the present study, labelling of Cx26 previously reported in the pineal appeared to be relatively sparse (Saez *et al.*, 1991) in relation to the tightly packed nature of pinealocytes. In this regard, our recent work showed a total absence of Cx26 expression in the pineal despite robust labelling for this connexin in surrounding leptomeningeal tissues (Tsao *et al.*, 2017), as demonstrated using a set of well-characterized anti-Cx26 antibodies (Nagy *et al.*, 2001, 2011). Further complicating this issue is that intercellular communication mediated by gap junctions between pinealocytes was demonstrated by observations of dye-transfer following single cell injections of Lucifer Yellow (LY) (Saez *et al.*, 1991), which is poorly permeable across Cx36-containing gap junction channels (Teubner *et al.*, 2000; Bukauskas, 2012; Ek-Vitorin & Burt, 2012). Our localization of Cx36 to pinealocytes demonstrated here and lack of Cx26 detection in the pineal as reported elsewhere (Tsao *et al.*, 2017), together with demonstrations of LY-coupling between these cells, raise inconsistencies, the basis for which remain to be determined. However, we cannot exclude the possibilities that Cx36-containing pinealocyte gap junctions are sufficiently dense to support some degree of LY-coupling and/or that pineal cells express an additional connexin that was not examined here.

Gap junctions composed of Cx36 are widely distributed throughout the brain, and have been localized to subpopulations of neurons where these junctions form electrical synapses (Pereda, 2014; Pereda *et al.*, 2013). Intracellular immunofluorescence labelling for Cx36 within neuronal cytoplasm was not detectable, whereas surface labelling had either a punctate appearance, presumably reflecting the localization of Cx36 to gap junctions, as deduced from correlations of the subcellular locations and distribution of Cx36-puncta with that of ultrastructurally-identified Cx36-containing gap junctions (Rash *et al.*, 2007a,b; Li *et al.*, 2008; Curti *et al.*, 2012). The Cx36-puncta observed in the pineal, with a similar absence of intracellularly detectable Cx36 also reflects the localization of Cx36 to gap junctions, as seen by FRIL. Moreover, the distinct immunofluorescence Cx36-strands found in rat are unusual, corresponding to immunogold labelled string and ribbon gap junctions found by FRIL. These patterns also correspond to the immunofluorescence labelling observed for ZO-1 (but not separately examined by FRIL). We have previously shown that ZO-1 is co-localized with Cx36 at gap junctions in brain and that these two proteins engage in direct molecular interaction (Li *et al.*, 2004). However, additional short and long ZO-1-strands (*i.e.*, labelling for ZO-1 not co-localized with Cx36 and presumably reflecting ZO-1-positive tight junctions in other cell types) closely adjacent to Cx36-strands or in continuity with these strands is also consistent with freeze-fracture ultrastructural features of associations between gap junctions and tight junctions of rat pinealocytes, where linear networks of gap junction particles show continuity with and transitions to tight junction networks (Taugner *et al.*,

1981). However, we have not as yet detected such continuity of string gap junctions with tight junction strands, presumably reflecting the rarity of such transitions.

Our results add to the list of neuroendocrine cell types that have been found to express Cx36, including pancreatic β -cells, adrenal medullary chromaffin cells, and hypothalamic neuroendocrine cells (Michon *et al.*, 2005). Normal secretion of insulin in response to physiological stimuli was shown to be dependent on Cx36-mediated cell-cell coupling between β -cells (Ravier *et al.*, 2005; Head *et al.*, 2012), and normal catecholamine secretion in response to afferent nerve activity was shown to be impaired in Cx36-deficient chromaffin cells (Desarmenien *et al.*, 2013). Further, an upregulation of Cx36 expression and an increased occurrence of synchronized calcium transients between chromaffin cells *ex vivo* have been described in studies of acute adrenal slices from stressed rats (Colomer *et al.*, 2008). In these and other neuroendocrine systems (Michon *et al.*, 2005), intercellular communication mediated by Cx36-containing gap junctions is critically required for synchronization of electrical coupling, coordination of Ca^{2+} -mediated actions, and regulation of neurosecretory activities among ensembles of coupled cells (Martin *et al.*, 2001; Ravier *et al.*, 2005; Head *et al.*, 2012). In pancreatic β -cells, Cx36-mediated coupling is also important for maintaining the physiologically relevant pulsatile nature of insulin secretion (Head *et al.*, 2012). Like other neuroendocrine cells, pinealocytes are electrically active; they display action potentials (Reuss, 1987; Schenda & Wollrath, 1998, 1999) and express a variety of ion channels (Zemkova *et al.*, 2011; Yu *et al.*, 2015). In addition, clusters of pinealocytes, perhaps corresponding to those described here having a high concentration of Cx36-puncta at a confluence of their initial processes and exhibit distinct patterns of rhythmic activity (Schenda & Vollrath, 1997, 1998), where a large subset of these cells generate spontaneous Ca^{2+} oscillations synchronized with periodic membrane depolarizations (Mizutani *et al.*, 2014). Further, although pinealocytes appear not to store but rather directly release melatonin immediately after its synthesis, there is nevertheless evidence that melatonin release occurs in a pulsatile fashion (Pang & Yip, 1988; Simonneaux *et al.*, 1989; Chan *et al.*, 1990a,b).

Conclusions

Gap junction-mediated coupling is a defining characteristic of endocrine-neuroendocrine organ function *in vivo* (Desarmenien *et al.*, 2013). Accordingly, it is likely that intercellular coupling of pinealocytes via Cx36-containing gap junctions serves to support the above synchronous and rhythmic activities of these cells. This conjecture may now be tested using transgenic mice having Cx36 gene deletion.

Acknowledgments

This work was supported by grants from the Canadian Institutes of Health Research and the Canadian Natural Sciences and Engineering Research Council to J.I. Nagy, and by National Institutes of Health (NIH) grants NS31027, NS44010, and NS44395 to J. E. Rash. We thank B. McLean for excellent technical assistance.

Abbreviations

Cx26 connexin26

Cx36	connexin36
Cx43	connexin43
FRIL	freeze-fracture replica immunogold labelling
GFAP	glial fibrillary acidic protein
5-HT	5-hydroxytryptamine
LY	Lucifer Yellow
TBS	50 mM Tris-HCl, pH 7.4, containing 1.5% sodium chloride
SPB	Sorenson's phosphate buffer
TBST	TBS containing 0.3% Triton X-100
TrypH	tryptophan hydroxylase
ZO-1	zonula occludens-1

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Fig. 1. Immunofluorescence labelling of Cx36 in the pineal of adult mouse (C57BL/6) and rat. (A,B) Sections of mouse whole pineal double-labelled for TrypH and Cx36, showing TrypH-positive pinealocytes (A1, arrows) and, in the same field, the distribution of Cx36 in clusters of puncta (A2, arrows) in the pineal of adult wild-type mouse. Similar images of TrypH-labelled pinealocytes (B1, arrows) from a Cx36 null mouse shows, in the same field, absence of labelling for Cx36 (B2). (C) Immunofluorescence labelling of Cx36 in rat pineal using two different antibodies against Cx36, Ab39-4200 (C1) and in the same field Ab51-6300 (C2), showing complete co-localization of labelling in overlay (C3, arrows). (D) Overview of labelling for Cx36 in rat pineal, showing relative density and size variation of labelled structures. (E) Higher magnification of Cx36 immunofluorescence in mouse pineal,

showing labelling to consist of clusters of fine Cx36-puncta (arrows). (F)
Immunofluorescence labelling of Cx36 along the pineal stalk of adult rat. Low magnification transverse section shows dense labelling around the pineal stalk (F1, arrows) surrounding a lumen (asterisk), and higher magnification sagittal section shows patches of labelling (F2, arrows) that consist of clusters of Cx36-puncta (F2, inset).

Fig. 2.

Confocal double immunofluorescence labelling of TrypH and Cx36 in the pineal of adult mouse (CD1) and rat. (A,B) Overlays of labelling for TrypH and Cx36 in mouse pineal, showing punctate appearance of labelling and dense collections of Cx36-puncta (A, arrows) among clusters of pinealocytes and at a confluence of their processes (A, arrowheads), and localization of fine Cx36-puncta to pinealocyte soma (B, arrow) and along pinealocyte processes (B, arrowheads). (C–D) Overlays of labelling for TrypH and Cx36 in rat pineal, showing high concentrations of densely packed Cx36-puncta among pinealocytes processes (C, arrows), at sites of apparent close contact between pinealocyte processes (D, arrow), and unusual strand-like labelling along pinealocyte somata and their processes (E, arrows).

Fig. 3.

Confocal double immunofluorescence labelling of 5-HT and Cx36 in the pineal of adult rat and mouse (CD1). (A) Images of fluorescence Nissl-stained cells (pseudocolored red) (A1) and, in the same field, labelling for 5-HT (A2) in rat pineal, showing that most Nissl-positive cells are 5-HT-positive pinealocytes, as shown in overlay (A3) (arrows). Additional labelling for 5-HT is seen in sympathetic fibers (arrowheads). (B) Confocal images from rat pineal, showing 5-HT-positive pinealocyte processes (B1, arrows) and, in the same field, strand-like labelling for Cx36 localized along those processes (B2, arrows). (C–F) Overlay images of double labelling for Cx36 and 5-HT in mouse pineal, showing dense collections of Cx36-puncta (C, arrows) localized to pinealocyte initial processes (C, arrowheads), Cx36-puncta along fine processes (D, arrowheads) including points of their intersection (D, arrows), Cx36-puncta localized to pinealocyte cell bodies (E, asterisks) including points of apparent apposition (E, arrows), and pinealocyte cell bodies (F, asterisks) with a confluence of their intermingled initial processes (F, arrowheads) displaying clusters of Cx36-puncta (F, arrow).

Fig. 4. Confocal double immunofluorescence labelling of Cx36 and ZO-1 in adult rat pineal. (A) Labelling of Cx36 (A1) and in the same field ZO-1 (A2), showing co-localization of labelling at both cluster of Cx36-puncta (arrowheads) and at Cx36-strands (arrows). (B) Higher magnification of a similar configuration of labelling as in (A), showing either complete co-localization of ZO-1 with Cx36 (arrow) or strand-like ZO-1-positive structures (arrowheads) that lack labelling for Cx36, but display close proximity to Cx36-strands. (C) Overlay image of double labelling for Cx36 and ZO-1, showing areas of co-localization (arrowheads), and distinct labelling for ZO-1 at long arrays of presumptive tight junctions (arrows).

Fig. 5. FRIL images of pinealocyte gap junctions labelled for Cx36. (A) Low magnification showing multiple pinealocytes (purple overlay) forming gap junctions labelled for Cx36 (red overlays) at the margin of a lumen (L) within a lobule. L, lumen; M, mitochondria. (B) Higher magnification of the inscribed boxes in A, shown as stereoscopic image of a largely P-face (P) reticular gap junction (red overlay) labelled for Cx36 by 11 10-nm gold beads and five 30-nm gold beads. Arrow denotes connexon E-face pits of the apposed cell. Barred circles represent “noise” for Cx43. (C) Additional rotation and tilt of this periluminal area revealed extensive E-face (E) gap junction strands labelled for Cx36. Calibration bars are 0.1 μm unless otherwise noted.

Fig. 6. FRIL images revealing E-face of Cx36-labelled pinealocyte gap junction. (A) Low magnification overview of large reticular gap junction between two pinealocytes bordering the lumen (L). M, mitochondria. (B) Higher magnification stereoscopic image of the inscribed box in A, showing an E-face gap junction (red overlay) labelled for Cx36 by 27 to 33 10-nm gold and by 14 to 26 30-nm gold beads. (For documentation of the basis for “cryptic” labelling of intact connexons remaining beneath E-face pits, see (Fujimoto, 1995; Kamasawa *et al.*, 2006). Barred circle represents “noise” for Cx43 on the wrong side of the replica. Calibration bars are 0.1 μm unless otherwise noted.