Expression and role of cocaine-amphetamine regulated transcript (CART) in the proliferation of biliary epithelium

Arianna Casini,[1] Giorgio Vivacqua,[2] Rosa Vaccaro,[1] Anastasia Renzi,[3] Stefano Leone,[1] Luigi Pannarale,[1] Antonio Franchitto,[4] Paolo Onori,[1] Romina Mancinelli,[1] Eugenio Gaudio[1]

[1]Department of Anatomical, Histological, Forensic Medicine and Orthopaedics Sciences, Sapienza University of Rome, Italy
[2]Integrated Research Center (PRAAB), Campus Biomedico University of Rome, Italy
[3]Department of Pathology, Akershus University Hospital, Lorenskog, Norway
[4]Department of Movement, Human and Health Sciences, University of Rome Foro Italico, Italy

ABSTRACT

Cholangiocytes, the epithelial cells that line the biliary tree, can proliferate under the stimulation of several factors through both autocrine and paracrine pathways. The cocaine-amphetamine-regulated-transcript (CART) peptide has several physiological functions, and it is widely expressed in several organs. CART increases the survival of hippocampal neurons by upregulating brain-derived neurotrophic factor (BDNF), whose expression has been correlated to the proliferation rate of cholangiocytes. In the present study, we aimed to evaluate the expression of CART and its role in modulating cholangiocyte proliferation in healthy and bile duct ligated (BDL) rats *in vivo*, as well as in cultured normal rat cholangiocytes (NRC) *in vitro*. Liver samples from both healthy and BDL (1 week) rats, were analyzed by immunohistochemistry and immunofluorescence for CART, CK19, TrkB and p75NTR BDNF receptors. PCNA staining was used to evaluate the proliferation of the cholangiocytes, whereas TUNEL assay was used to evaluate biliary apoptosis. NRC treated or not with CART were used to confirm the role of CART on cholangiocytes proliferation and the secretion of BDNF. Cholangiocytes proliferation, apoptosis, CART and TrkB expression were increased in BDL rats, compared to control rats. We found a higher expression of TrkB and p75NTR, which could be correlated with the proliferation rate of biliary tree during BDL. The *in vitro* study demonstrated increased BDNF secretion by NRC after treatment with CART compared with control cells. As previously reported, proliferating cholangiocytes acquire a neuroendocrine phenotype, modulated by several factors, including neurotrophins. Accordingly, CART may play a key role in the remodeling of biliary epithelium during cholestasis by modulating the secretion of BDNF.

Key words: liver; biliary epithelium; cholangiocyte proliferation; cocaine-amphetamine regulated transcript (CART).

Correspondence: Romina Mancinelli, Department of Anatomical, Histological, Forensic Medicine and Orthopaedics Sciences, Sapienza University of Rome, via Alfonso Borelli 50, 00161 Rome, Italy. Tel. +39.06.49918063.
E-mail: romina.mancinelli@uniroma1.it

Contribution: AC, GV, worked on the study design, performed morphological and immunohistochemical experiments and wrote the paper; SL, AR, captured pictures, interpreted the results and wrote the paper, formatting the manuscript as an article; GV, AC, RV, worked in the preliminary research reports about CART and BDNF; LP, helped in the experimental and revisional aspects; AF, PO, monitored the experiments and performed a critical revision of the manuscript; EG, RM, coordinated the work, interpreted the results and performed a critical revision of the manuscript. All the authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Introduction

Cholangiocytes are the cells of the biliary epithelium and represent the target of various diseases called cholangiopathies. In these liver diseases, a crucial step is represented by the maintenance of biliary homeostasis by modulating the balance between cholangiocyte proliferation and apoptosis/senescence.[1,2] Bile duct ligation (BDL) is a widely used model for studying the growth/loss of the biliary epithelium in rodents, since it mimics some of the features of human chronic cholestatic liver diseases,[3-5] in which proliferating cholangiocytes acquire a neuroendocrine phenotype, modulated by several factors including NTs, neuropeptides and gastrointestinal hormones.[1,3,6-9] The arcuate nucleus of the hypothalamus contains two different populations of neurons that are implicated in the regulation of feeding behavior and that could be also involved in the modulation of the biliary epithelium.[10-12] The first population produces neuropeptide Y that inhibits biliary hyperplasia in cholestatic rats by paracrine and autocrine mechanisms.[13,14] The second population of neurons produces cocaine-amphetamine regulated transcript (CART), the anorexigenic regulatory peptide highly expressed in the brain's appetite control centers.[15,16] CART regulates various physiological functions, including stress and response to drugs.[17,18] Studies on CART distribution indicated that the CART system is distributed in neuronal and non-neuronal populations in the rat central nervous system, pituitary gland, adrenal gland, and digestive tract.[19-21] In the digestive tract, CART has been detected in numerous populations of neurons of the submucosa and myenteric plexuses as well as in epithelial and neuroendocrine cells such as pancreatic islets, gastrin-producing G cells of the antral mucosa, duodenal mucosa, and Brunner glands.[20-24] Recent studies have shown that CART acts as a neuroprotective agent and activates extracellular signal-regulated kinase (ERK) in both primary cortical neurons and neuronal cell lines[25,26] In addition, CART is a regulator of neuronal survival and promotes the differentiation of primary hippocampal neurons by upregulating brain derived neurotrophic factor (BDNF) expression.[27] The effects of BDNF, in turn, are mediated by its high affinity for tyrosine-kinase receptor kinase B (TrkB)[28] and the p75 neurotrophin receptor (p75NT).[29] In pancreatic beta cells CART is necessary for normal islet function and insulin secretion by glucose.[30] It induces the phosphorylation of CREB, MAPK, PKB and FoxO1 that are all key mediators of cell survival and proliferation, whereas it prevents pancreatic beta cell apoptosis induced by glucotoxicity.[31] Since no data exist regarding the expression of CART by the biliary epithelium and concerning its potential role in cholangiocytes proliferation, in the present research we aimed at assessing the expression/role of CART in the biliary epithelium and its possible role in biliary homeostasis.

Materials and Methods

Reagents

All reagents were obtained from Dako-Agilent (Santa Clara, CA, USA) unless otherwise stated. The antibodies for cytokeratin-19 (CK-19) and for the receptor TrkB were obtained from Abcam (Cambridge, UK). While those targeting p75NTR, together with the TUNEL assay kit (Apoptag) were purchased from Millipore (Temecula, CA, USA). The antibodies for proliferating cell nuclear antigen (PCNA) and for cocaine-amphetamine-regulated transcript (CART), together with Ultracruz aqueous mounting medium with DAPI were produced by Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the specific secondary antibodies AlexaFluor were obtained from Invitrogen, Life Technologies Ltd. (Paisley, UK). CART for the *in vitro* stimulations was bought by Peptide Institute Inc, Japan. Enzyme-linked immunosorbent assay (ELISA) kits to measure BDNF levels in biliary supernatants were obtained from Abnova (Taipei, Taiwan), whereas cell proliferation assay kit from Merck Millipore (Burlington, MA, USA).

In vivo studies

The studies were performed in Fischer male rats (225-250 gm, purchased from Charles River Laboratories, Wilmington, MA, USA) that were subjected to sham (n=5) or BDL (n=5) for 1 week. The ligation of the extrahepatic bile duct was performed as previously described.[3] Before each experimental procedure the animals were anesthetized with euthasol (200-250 mg/kg BW). The animals were maintained in a temperature-controlled environment (20-22°C) with 12:12-h light-dark cycles. All animal experiments were performed following the regulations of the protocols approved by Baylor Scott & White Institutional Animal Care and Use Committee (#2011-010).

Histology and immunohistochemistry

After collection, rat liver specimens were fixed in 4% paraformaldehyde for 24 h. Then, liver samples were embedded in paraffin and cut into 3 µm sections. For immunohistochemistry (IHC) after deparaffinization and rehydration, sections were pretreated for 20 min at room temperature with 1x PBS containing H_2O_2, to inactivate the endogenous peroxidase activity. Antigens were retrieved, as indicated by the manufacturer's instructions, by applying Proteinase K (code S3020; Dako-Agilent) for 10 min at room temperature. Then, sections were incubated for one hour at room temperature with antibodies for: i) rabbit anti-Cytokeratin 19 (ab133496; Abcam) 1: 200 to evaluate and quantify the intrahepatic biliary mass (IBDM); ii) mouse anti-PCNA (sc-56, Santa Cruz Biotechnology) 1:50; or iii) goat anti-CART (sc-18068, Santa Cruz Biotechnology) 1:500, or iv) rabbit anti-TrkB (ab 51190, Abcam) 1:100; or v) rabbit anti p75NTR (AB1554, Millipore) 1:100 overnight at 4°C. Negative controls with the omission of the primary antibody (replaced with normal serum from the same species) were included for all samples. Then, they were rinsed twice with 1x PBS for 5 min, incubated for 20 min at room temperature with secondary biotinylated antibody (LSAB+ System-HRP, code K0690; Dako-Agilent, Glostrup, Denmark) and then with Streptavidin-HRP (LSAB+ System-HRP, code K0690, Dako-Agilent). Diaminobenzidine (DAB, Dako-Agilent) was used as substrate, and sections were counterstained with hematoxylin for 5 min at room temperature.

TUNEL analysis

Cholangiocyte apoptosis was evaluated in paraffin-embedded liver sections (4–5 µm thick) by a quantitative terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) kit (Apoptag; Millipore). The percentage of TUNEL-positive cholangiocytes was counted in six non-overlapping fields (magnification 40x) for each slide and the data were expressed as the percentage of TUNEL-positive cholangiocytes.

Immunofluorescent staining for TrkB and p75NTR receptors

The expression of TrkB and p75NT receptors was evaluated by immunofluorescence (IF) in cell smears of isolated cholangiocytes from normal and BDL rats. Biliary cells were isolated by immunoaffinity separation with a specific monoclonal antibody against an antigen expressed by all intrahepatic rat cholangiocytes. Cell count and viability (~97%) were measured by trypan blue exclusion. Purity (98-99%) of cholangiocytes was assessed by

γ-GT histochemistry. Then, cholangiocytes were transferred to the slides, allowed the smear to air dry completely and fixed in acetone for 5 min. For IF, non-specific antigen detection was blocked by 5% normal goat serum. Specimens were incubated for 1h at room temperature with primary antibodies as following: rabbit anti-TrkB (ab 51190, Abcam) 1:100 or rabbit anti p75NTR (AB1554, Millipore) 1:100. Then, specimens were washed and incubated for 1h at room temperature with labeled isotype-specific secondary antibodies (anti-rabbit AlexaFluor-488, Invitrogen, Life Technologies Ltd.) and counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Ultracruz aqueous mounting medium with DAPI, sc-24941, Santa Cruz Biotechnology) for visualization of the cell nuclei. Images were visualized using Leica Microsystems DM 4500 B Fluorescence Microscopy (Weltzlar, Germany) equipped with a JenoptikProg Res C10 Plus Videocam (Jena, Germany).

In vitro studies

In separate experiments, *in vitro* studies were performed on our normal rat cholangiocytes (NRC).[8] Cells were maintained at 37°C in a 5% CO_2 incubator in a culture medium composed of DMEM-F-12 supplemented with: 4 μg/mL forskolin, 3.4 μg/mL 3,3′,5-triiodo-L-thyronine, 0.4 μg/mL dexamethasone, 5 μg/mL gentamicin, and 50 μg/mL trypsin inhibitor, plus 5% Nu-Serum IV, 5% FBS, 25 ng/mL EGF, 20 mM L-glutamine, 1% glyceryl monostearate, and 0.1 mM MEM non-essential amino acid solution. After trypsinization, cells were seeded into 96-well plates (10,000/well) in a final volume of 200 μL of medium and allowed to adhere to the plate overnight. NRC were incubated at 37°C with 0.2% BSA (basal), or CART 100 nM (4351-s, Peptide Institute Inc., Osaka, Japan)[32] for 48 h before evaluation of proliferation through cell proliferation assay kit based of activity of the mitochondrial dehydrogenases in the sample (2210, Millipore).[33] In addition, to evaluate how CART influences the expression of BDNF in IMCL, BDNF secretion was evaluated with the ELISA Kit (KA0330, Abnova) according to the manufacturer's instructions in supernatant collected from IMCL treated with 0.1% bovine serum albumin (BSA) or 100 nM CART for 48 h.

Data analysis

The percentage of cholangiocytes that were immunoreactive for CK19, PCNA and CART was measured on 5 tissue sections per animal randomly selected from each experimental group, using a specific computer-assisted image analyzer (IAS Delta Sistemi, Rome, Italy). The percentage of cells positive to PCNA (assumed as proliferating) or to TUNEL (assumed as apoptotic) on the total number of cholangiocytes was evaluated by counting positive cells in different microscopic fields and computing, for each experimental group, the ratio between proliferation and apoptosis. The percentage of PCNA- and TUNEL-positive cells from different microscopic fields was statistically analyzed by ANOVA as well as the paired Student's *t*-test ($p<0.005$).

Results

Proliferation and apoptosis

To determine the balance between the proliferative and the apoptotic activity of cholangiocytes, we first quantified intrahepatic bile duct mass (IBDM) by measuring CK19 positive cells (Figure 1A). Subsequently, we measured the number of PCNA-positive cholangiocytes to determine the proliferation (Figure 1B) and apoptotic cholangiocytes by TUNEL analysis (Figure 2). Like previous studies,[34-36] the data demonstrated a significant increase in IBDM as well as in the number of PCNA-positive cholangiocytes in BDL rats compared to control rats (Figure 1 A,B). Cholangiocytes from control rat livers were virtually negative for TUNEL staining, whilst the number of TUNEL-positive cholangiocytes increased by 5.92 % following BDL (Figure 2). In control rat liver, we found a physiologic balance between proliferation and apoptosis of cholangiocytes, whereas a prevalence of proliferation was detected in 1-week BDL rats.

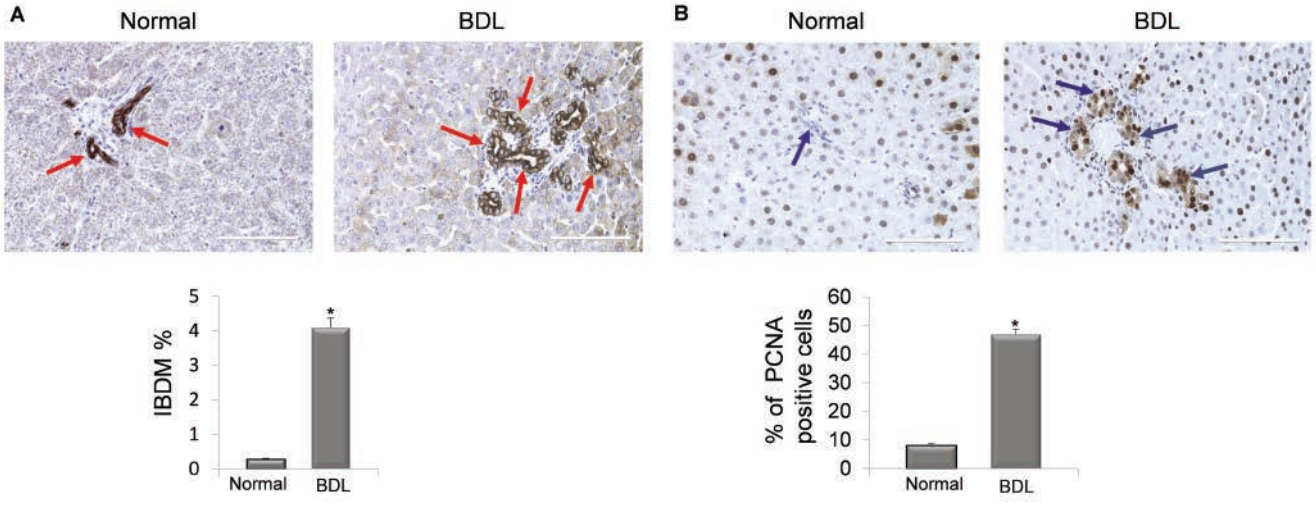

Figure 1. Representative immunohistochemistry for cytokeratin 19 (CK19) and proliferating cell nuclear antigen (PCNA). **A**) Images of immunoreactivity for CK19, a specific marker of biliary epithelium, in liver sections; in 1-week BDL rats IBDM increased in comparison with normal rats. The differences among experimental groups were significant ($p<0.005$). **B**) Immunoreactivity for PCNA in liver sections from normal and 1-week BDL rats. PCNA was expressed by few proliferating cholangiocytes (and in rare hepatocytes) in healthy rat liver; in 1-week BDL rats the number of proliferating cholangiocytes increased compared to normal rats. Data are reported as mean of cumulative five values from the evaluation of 5 randomly selected portal areas. The differences among experimental groups were significant. *$p<0.005$ *vs* cholangiocytes from control rats; scale bar: 200 μm, original magnification 20x.

CART expression

The percentage of CART-positive cholangiocytes was neglectable in control rat, but CART expression was significantly increased in cholangiocytes from BDL rats (Figure 3). There were no significant differences regarding CART expression among the different cellular subtypes of the biliary epithelium (HPCs, small cholangiocytes and large cholangiocytes).

TrkB and p75NTR expressions

It has been shown that the interactions between BDNF and its receptors p75NTR and TrkB can have different effects.[37,38] In fact, the Trk receptor family frequently promotes cell survival, while p75NTR might be able to induce apoptosis. For these reasons, we aimed at better investigate whether these receptors are differentially expressed in our experimental models.[39] By using IF,

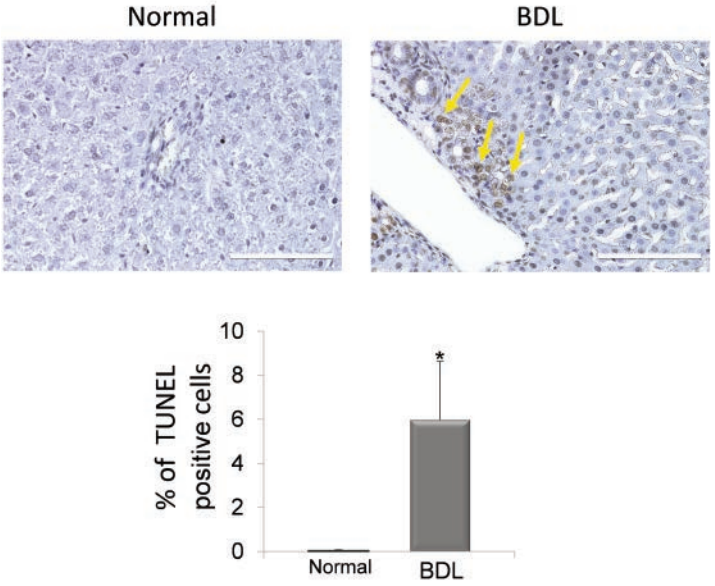

Figure 2. Evaluation of cholangiocyte apoptosis through TUNEL assay. In the intrahepatic cholangiocytes of normal rats, very few apoptotic cholangiocytes were detected. In BDL there was a significant increase in apoptotic cholangiocytes; the nuclei and the cytoplasm of apoptotic cholangiocytes were condensed. The differences among experimental groups were significant. *p<0.005 *vs* cholangiocytes from control rats; scale bar: 200 µm, original magnification 20x.

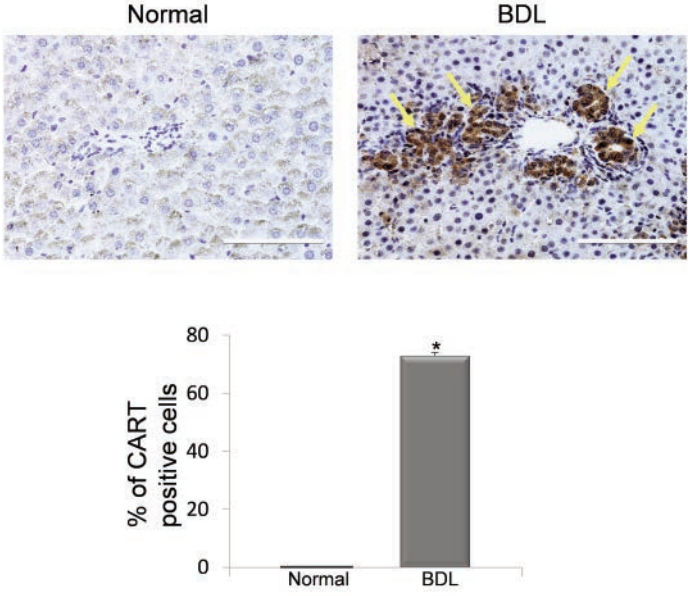

Figure 3. By immunohistochemistry CART expression was virtually negative in normal rats, while CART immunopositivity increased in BDL samples. The differences among experimental groups were significant. *p<0.005 *vs* cholangiocytes from control rats; scale bar: 200 µm, original magnification 20x.

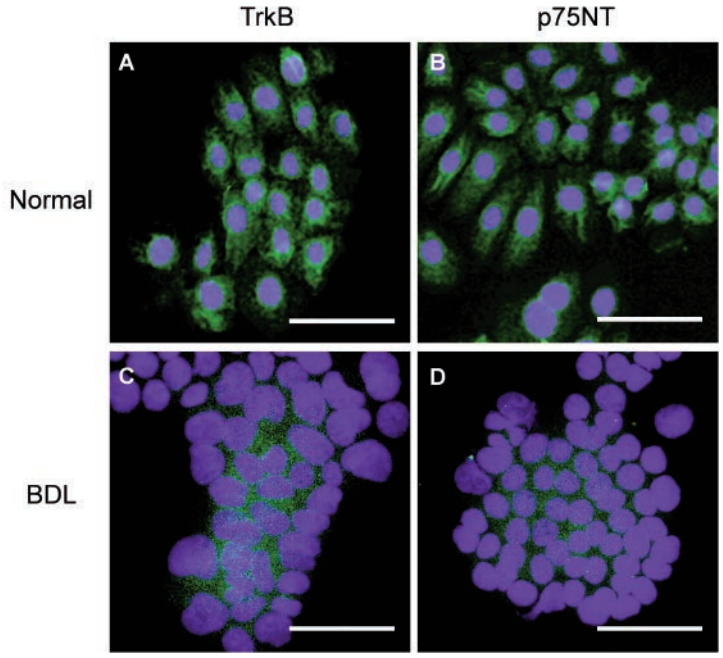

Figure 4. By immunofluorescence, cholangiocytes from normal rats express both BDNF receptors: TrkB (**A**) and p75NTR (**B**). Cholangiocytes from BDL liver samples have shown the expression of both receptors TrkB (**C**), and p75NTR (**D**). Specific immunoreactivity for TrkB and p75NTR is shown in green with representative field of isolated cholangiocytes from normal and BDL rats; cell nuclei were stained in blue with DAPI. Scale bar: 200 μm, original magnification 20x.

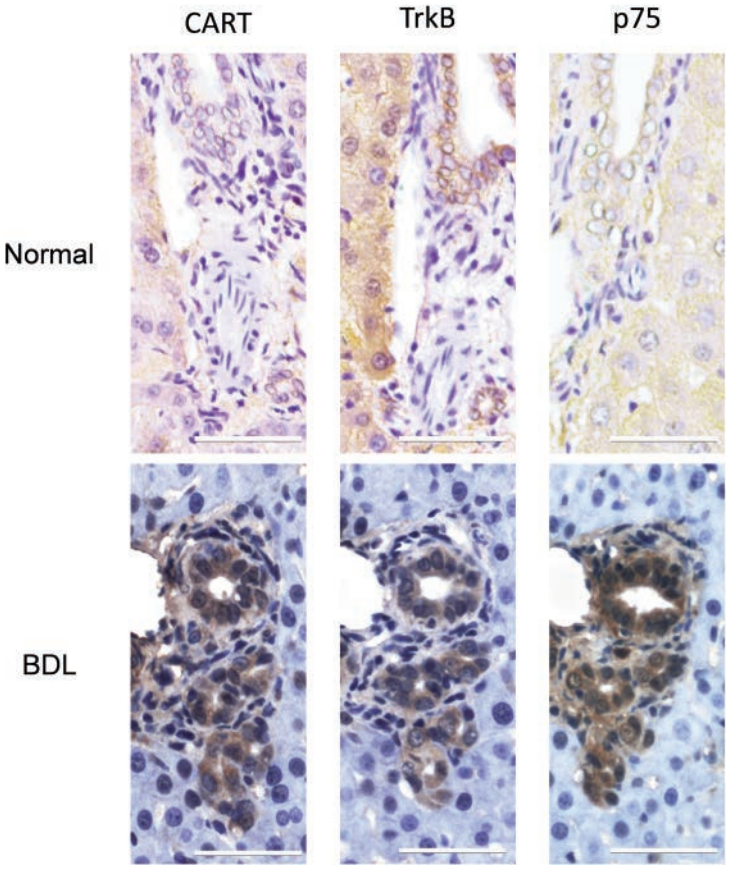

Figure 5. Representative seriated immunohistochemistry of CART, TrkB and p75NTR in normal and BDL 1-week models. In control livers, we showed a low and broad expression in both hepatocytes and cholangiocytes. After 1-week of BDL, we found a higher presence of CART and both BDNF receptors in the typical proliferating biliary epithelium. Scale bar: 200 μm, original magnification 20x.

we demonstrated the expression of both TrkB and p75NTR in control rat cholangiocytes (Figure 4 A,B). In isolated cholangiocytes from 1-week BDL rats, we found a persistent expression of TrkB and p75NTR (Figure 4 C,D) which could be possibly linked to the coactivation of TrKB and p75NT, after one weeks of BDL, highlighting their combined role in sustaining cellular survival and proliferation of the biliary epithelium. The immunostaining in consecutive sections for CART, TrkB and p75NTR have shown increased co-expression between CART and the two receptors in 1-week BDL rats compared with control rats (Figure 5).

In vitro CART effects on cholangiocyte growth and BDNF levels

We used NRC to detect the role of CART in driving cell proliferation and expression of BDNF in cholangiocytes. By using cell proliferation assay, we detected a significant increase in the proliferation of NRC after treatment with CART for 48 h compared to the corresponding untreated cells (Figure 6A). Furthermore, by measuring the levels of BDNF by ELISA in the cell medium of cultured cholangiocytes, we found that treatment with CART caused a significantly increased secretion of BDNF by NRC when compared to the corresponding basal values (Figure 6B).

Discussion

In the present study we have used *in vitro* and *in vivo* models of cholestasis to detect the role of CART in the balance between apoptosis and proliferation of the biliary epithelium. With the *in vivo* study we have demonstrated the expression of CART in the biliary epithelium following 1 week of BDL and we have shown that it correlates with increased levels of BDNF and increased rate of proliferation, detected by PCNA staining. Furthermore, by the *in vitro* study we found that CART induces the enhancement of the endogenous expression of the neurotrophin BDNF from cholangiocytes as well as the increased expression of BDNF receptors.

Both our *in vivo* and *in vitro* findings support the hypothesis that CART could be associated with the proliferation of the biliary epithelium. In fact, the *in vivo* study focused on the expression of CART in experimental models of cholestasis, showing, for the first time, the expression of CART in the growing biliary epithelium. On the other hand, the *in vitro* study demonstrated the effects of CART on the proliferation of the biliary epithelium, which may be mediated by the induction of BDNF expression. In accordance, we have also found an increased co-expression of CART, TrkB and p75NTR in the seriated sections of 1-week BDL rats, which suggests the action of BDNF in sustain cell proliferation in BDL rats. Further supporting the view that CART mediates the hyperexpression of BDNF, which in turn mediates the proliferation of the biliary epithelium in BDL rats, through the activation of TrkB receptors, we have also detected a neglectable expression of CART in control rat livers, whereas cholangiocytes from 1-week BDL rats overexpressed CART together with the proliferation marker PCNA. It has been shown that CART promotes the generation, survival, and differentiation of neurons probably by modulating the expression of several growth factors and neurotrophic factors.[27,40,41] CART has a protective role in the central nervous system through complex interactions with the immune and inflammatory system.[42-44] Pleiotropic functions of CART are also known in endocrine cells.[45-48] To better pinpoint the role of CART in the proliferation and remodeling of the biliary epithelium, we performed *in vitro* studies in control rat cholangiocytes and we further demonstrated that the effects of CART on the proliferation of biliary epithelium are mediated by enhanced cholangiocyte BDNF secretion. NTs, a family of dimeric proteins working as growth factors, include NGF, NT-3, NT-4, BDNF.[49] The latter is a neurotrophin expressed in the nervous system as well as in different types of epithelial and mesenchymal progenitor cells, including the biliary epithelium.[50-53] Neurotrophin-mediated interactions through p75NTR and the Trk receptors frequently have antagonist effects on the neuronal cells during development and in pathological conditions. For instance, the binding to Trk receptors almost

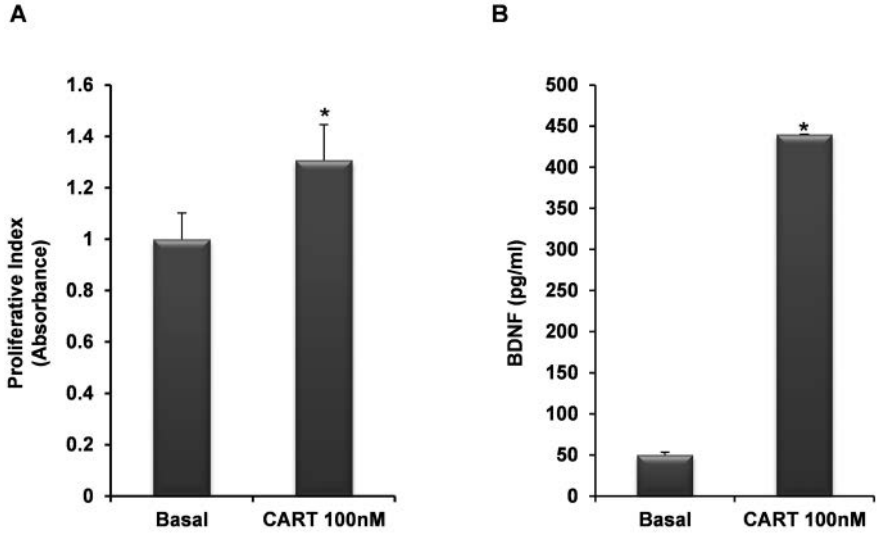

Figure 6. Graphs showing the proliferation and the BDNF levels in NRCs. **A**) In the cell proliferation assay, after treatment with CART (100nM) for 48 h, there was an increase of cholangiocyte proliferation if compared to basal condition. **B**) ELISA analysis demonstrated that after a similar treatment with CART (100 nM) there was an increase of BDNF levels in the cell conditioned medium of treated biliary cells compared to the cells treated with vehicle. *p<0.005 *vs* basal treatment.

always promotes neuronal survival and differentiation,[54] whereas targeting of p75NTR frequently promotes apoptosis.[55,56] Vivacqua *et al*. have detected the expression of BDNF and its two receptors (TrkB and p75NTR) in normal and BDL rat livers at different stages and they have shown that increased expression of TrkB receptors correlates with increased percentage of proliferating cholangiocytes, whereas, when the levels of p75NTR receptor are raised, apoptosis prevails.[12] The activation of the TrkB receptor by BDNF induces an increase in the intracellular levels of cAMP and the recruitment of the MAPK/Erk pathway, with a consequent activation of cellular proliferation and cellular differentiation. On the other hand, the activation of the p75NTR receptor induces the transcription factor NFκB, involved in cellular growth and trophism as well as the JNK/c-Jun pathway, responsible for the transcription of pro-apoptotic genes.[57,58] Our *in vitro* study also showed the expression of both TrkB and p75NTR receptor in normal rat cholangiocytes in accordance with a previous study.[12] Moreover, we have confirmed the expression of both receptors and the co-expression with CART in 1-week BDL liver, through IHC with an enhanced presence of both TrkB and p75NTR in comparison with normal rats. For these reasons and together with the significantly increased proliferation rate of cholangiocytes after CART administration, we hypothesize that NTs signaling in the liver might be driven by CART through the increased secretion of BDNF and the upregulation of the TrkB and p75NTR receptors, which favor, in turn, the proliferation of the biliary tree. Further studies are needed to investigate the expression of CART in cholangiocytes at different stages of BDL, considering that after three weeks of BDL apoptosis prevails on proliferation and this may be due to the activation of a pro-apoptotic intracellular pathway through activation of p75NTR without coactivation of TrkB.

In conclusion, our observation that CART induces a significant increase of BDNF secretion in NRC provides further support for this new function of CART in the proliferation and remodeling of the biliary epithelium. This is in line with previous studies showing that CART is able to stimulate the production of BDNF[27,43] and cAMP,[20] activating the MAPK/Erk cascade[25] and promoting cell survival and differentiation in neurons.[25,26] Therefore, we propose that CART regulates cholangiocyte proliferation *via* BDNF-dependent signaling mechanisms. Further studies are necessary to better understand possible beneficial roles and prospective of CART and/or BDNF administration in the control of proliferation and remodeling of the biliary epithelium in NRC and cholestatic liver diseases. Our finding suggests that the CART peptide, as it can promote neural differentiation in the brain, similarly, it could intervene in the restoration of liver damage, probably as a potential modulator of the BDNF neurotrophic factor.

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