British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01510.x
www.brjpharmacol.org

Themed Section: Molecular Pharmacology of GPCRs

REVIEW

The orexin receptor OX_1R in colon cancer: a promising therapeutic target and a new paradigm in G protein-coupled receptor signalling through ITIMs

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Keywords
apoptosis; cancer therapy; caspase; colon cancer; G protein-coupled receptor; hypocretin; immunoreceptor tyrosine-based inhibitory motif; metastases; orexin; protein phosphatase SHP2; tyrosine-based motif; tumour

Received
21 March 2011
Revised
19 April 2011
Accepted
19 May 2011

An exciting aspect of the heptahelical orexin receptor 1 (OX_1R) has emerged recently, when it was shown that it drives apoptosis in human colon cancer cell lines. Here we review recent findings related to the role of OX_1R in colorectal cancers and the unexpected mechanism whereby this G protein-coupled receptor works. The OX_1R is aberrantly expressed at all steps of primary colorectal tumour progression and after local (lymph node) or distant (liver, lung) metastasis. No OX_1R is detected in normal colonic epithelial cells. Treatment of human colon cancer cells in culture with orexins promotes robust apoptosis and subsequent reduction of growth including in cells that are resistant to 5-fluorouracil, the most commonly used drug in chemotherapy. When human colon cancer cells are xenografted in nude mice, treatment with orexins dramatically slows tumour growth and even reverses the development of established tumours. Thus, OX_1R agonists might be novel candidates for colon cancer therapy. Activation of OX_1R drives apoptosis through G_q protein but independently of classical $G\alpha_q$ activation of phospholipase C. In fact, it is the freed $\beta\gamma$ dimer of G_q that plays a pivotal role by stimulating Src-tyrosine kinase. This results in phosphorylation of two immunoreceptor tyrosine-based inhibitory motifs (ITIM) in OX_1R and subsequent recruitment by OX_1R of the phosphotyrosine phosphatase SHP-2, which is activated thereby. Downstream events include release of cytochrome c from mitochondria and activation of caspase-3 and caspase-7. The role of ITIMs in OX_1R-driven apoptosis represents a new paradigm of G protein-coupled receptor signalling.

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Abbreviations

5-FU, 5-fluorouracil; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; $InsP_3$, inositol triphosphate; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; OX_1R, orexin receptor 1; OX_2R, orexin receptor 2; SHP-2, Src homology domain 2-containing protein tyrosine phosphatase 2

Introduction

Colon cancer is a common malignancy worldwide and causes considerable morbidity and mortality (Segal and Saltz, 2009). Molecular genetics has identified key genes, including tumour-suppressor genes and oncogenes, whose mutations or altered expression are associated with colorectal cancer (Markowitz and Bertagnolli, 2009). Colon cancer initiation

and progression are dependent on these genes but are also under the control of many growth factors or hormones present in primary tumour environment and acting at tyrosine kinase receptors or G protein-coupled receptors (GPCRs). Colon cancers do express a variety of GPCRs that fall into three categories. Many receptors are already present at similar levels in normal colon epithelial cells, others are simply overexpressed and some of them are aberrantly expressed in cancer cells, which means that they are not present in normal colonic epithelium (Laburthe *et al.*, 1978; Maoret *et al.*, 1994; Singh *et al.*, 2000; Darmoul *et al.*, 2003; Gratio *et al.*, 2009). The growth-promoting effects of peptide hormones such as gastrin (Singh *et al.*, 2000) or neurotensin (Maoret *et al.*, 1999), serine proteases such as thrombin (Darmoul *et al.*, 2003) or trypsin (Darmoul *et al.*, 2004) or lipids such as lysophosphatidic acid (Yang *et al.*, 2005) or prostaglandin E2 (Chell *et al.*, 2006) are mediated by GPCRs. Activation of these GPCRs promotes tumour cell growth through G protein transduction pathways and/or by transactivating the tyrosine kinase epidermal growth factor receptor (EGFR) for epidermal growth factor (Darmoul *et al.*, 2004; Lappano and Maggiolini, 2011). Due to its autoactivity and because it can be transactivated by GPCRs, EGFR is prominent in the growth of colon cancer and is already a therapeutic target for antibodies directed against its extracellular domain (Segal and Saltz, 2009).

While a large body of evidence shows that the environment of primary colon tumours is rich in growth factors (see earlier discussion), almost nothing was known until recently regarding the existence of growth inhibitory factors for colon cancer. In order to try to identify such inhibitory factors, we developed a very simple strategy consisting of screening the ability of a large series of peptide hormones and neuropeptides to inhibit colon cancer growth (Rouet-Benzineb *et al.*, 2004). We found that the neuropeptides orexins acting at the seven-transmembrane domain receptor orexin receptor 1 (OX_1R) are robust stimulants of apoptosis in colon cancer cells (Rouet-Benzineb *et al.*, 2004).

In this review, we aim to summarize current knowledge and recent findings on orexin receptors in colon cancer. Specifically, we will discuss two aspects: (i) the expression and pro-apoptotic role of OX_1R in primary colorectal tumours and metastases and in colon tumour cell lines. The recent data support the view that OX_1R represents an Achilles heel of colon cancer and is a new promising therapeutic target; (ii) the entirely novel mechanism by which the seven-pass transmembrane GPCR OX_1R triggers apoptosis. It involves phosphorylation of two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in OX_1R resulting in the recruitment of the phosphotyrosine phosphatase SHP-2, the activation of which is responsible for mitochondrial apoptosis. This mechanism clearly represents a new paradigm in GPCR signalling.

Orexins and orexin receptors: a short survey of general features

The two orexin neuropeptides were discovered in 1998 by two independent laboratories using subtractive cDNA cloning (de Lecea *et al.*, 1998) or as endogenous ligands for two orphan GPCRs (Sakurai *et al.*, 1998). They were referred to as orexin-A and orexin-B (Sakurai *et al.*, 1998) or hypocretin-1 and hypocretin-2 (de Lecea *et al.*, 1998). Similarly, the receptors were named OX_1R and OX_2R (Sakurai *et al.*, 1998) or Hctr1 and Hctr2 (de Lecea *et al.*, 1998). Both names are problematic because the regulation of feeding and appetite is probably not the major action of the neuropeptides, and they have no clear sequence homology with secretin. In the absence of recommendation by the International Union of Pharmacology and for the sake of clarity, we have used the names orexin and orexin receptor OXR in this review.

Several reviews on central or peripheral actions of orexins as well as on orexin receptors have been published during the last decade (Voisin *et al.*, 2003; Heinonen *et al.*, 2008; de Lecea, 2010; Kodadek and Cai, 2010; Laburthe *et al.*, 2010; Sakurai *et al.*, 2010; Scammell and Winrow, 2011). The main features are summarized in the following discussion. Orexin-A and orexin-B are encoded by the same gene and originate from a single precursor synthesized by hypothalamic neurons that project throughout the brain. They regulate sleep, wakefulness, breathing, reward system and drug addiction. Their strong impact on sleep–wakefulness is emphasized in human pathology, because orexin deficiency results in narcolepsy and cataplexy (Nishino *et al.*, 2000). Functions of orexins have been also described in peripheral tissues including digestive tract, pancreas, gonads and adrenal glands. The expression of orexins at the periphery (Johren *et al.*, 2001) needs to be clarified, and their presence in blood is still debatable (Voisin *et al.*, 2003; Heinonen *et al.*, 2008). The actions of orexins are mediated by two seven-pass transmembrane GPCRs OX_1R and OX_2R that recognize with poor selectivity the two closely related orexins that share 46% amino acid identity in humans. Classically, the activation of both orexin receptors induces cellular calcium transients through increase of intracellular inositol triphosphate ($InsP_3$), and the OX_1R has been also shown to be linked to calcium influx through transient receptor potential cation 3 channel (Peltonen *et al.*, 2009). Orexin receptors belong to the class A of GPCRs.

Discovery of orexins as pro-apoptotic peptides in colon cancer

The discovery of orexins as pro-apototic peptides came from the screening of peptide receptor agonists for their ability to inhibit colon cancer cell growth. We tested 26 peptide hormones and neuropeptides claimed to be expressed in the gut, and the screen was performed with the human colon cancer cell line Human tumour (HT)-29 grown in standard conditions in the presence of the robust growth-promoting effect of 10% fetal calf serum. Only two closely related peptides orexin-A and orexin-B were shown to inhibit HT29 cell growth (Rouet-Benzineb *et al.*, 2004). Orexins do not alter cell cycle and cell proliferation but promote cell apoptosis with typical externalization of plasma membrane phosphatidylserine, chromatin condensation and DNA fragmentation of nuclei (Rouet-Benzineb *et al.*, 2004; Voisin *et al.*, 2008). It appeared shortly that: (i) among the two orexin receptors

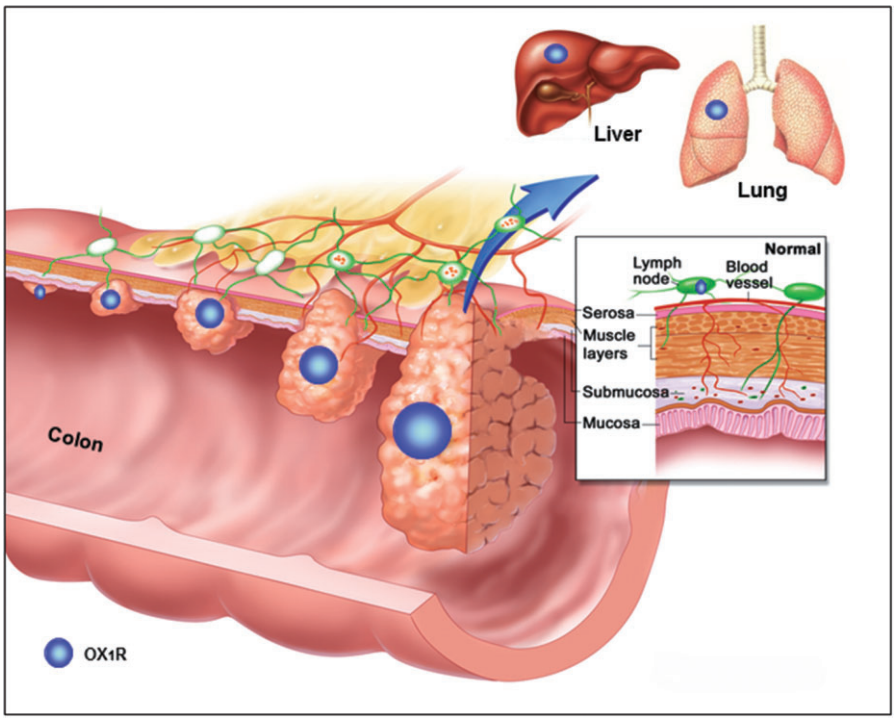

Figure 1

Expression of the orexin receptor OX_1R during colon cancer progression and metastasis. OX_1R (blue circles) is aberrantly expressed in primary tumours and metastases. The expression of OX_1R was detected very early during carcinogenesis and whatever the Dukes' stage of the primary tumours. After metastasis in the colon (lymph node) and in distant organs (liver, lung), OX_1R is still expressed. No OX_1R is present in normal colonic epithelial cells from which cancer cells derive. Adapted with permission from © 2005 Terese Winslow, US Government has certain rights.

OX_1R and OX_2R, only OX_1R is expressed by HT29 cells and is responsible for orexin-induced apoptosis; and (ii) orexins induce mitochondrial apoptosis with cytochrome c release from mitochondria to cytosol and activation of caspase-3 and caspase-7 (Rouet-Benzineb *et al.*, 2004).

Aberrant expression of OX_1R in colorectal tumours and metastases

In the early report on orexin receptors in colon cancer cells, it was already shown that OX_1R is expressed in the human colon cancer cell lines HT29, Caco-2, SW480 and LoVo but not in explant cultures of dissected human normal colonic mucosa (Rouet-Benzineb *et al.*, 2004). It is now known that the OX_1R is present in 100% of primary colorectal tumours tested by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and/or immunohistochemistry, including 21 tumours of the proximal colon and 17 tumours of the distal colon whatever their Dukes' stages (Voisin *et al.*, 2011). In sharp contrast, normal colonocytes adjacent to tumours as well as normal proximal and distal mucosae of patients with irritable bowel syndrome, taken as controls, are always negative for OX_1R expression. Thus, OX_1R is aberrantly expressed in epithelial cells during colon carcinogenesis (Voisin *et al.*, 2011). The molecular mechanisms whereby OX_1R is ectopically expressed during colon cancer progression are still unknown. The gene encoding OX_1R in humans maps to chromosomal region 1p33 (Sakurai *et al.*, 1998) within the 1p32-36 loci, which are known to undergo genetic changes in human colorectal tumourigenesis (Hanash, 1996). These genetic changes may hinder the repression of OX_1R gene that occurs in a normal colon. However, many other hypotheses could be raised including epigenetic alterations in colon cancer (Grady and Carethers, 2008) of the OX_1R gene.

The possibility to use OX_1R-targeted agonists in order to induce apoptosis of colon cancer cells (see further discussion) is more relevant in metastases than in the primary tumour, which can be resected. In this context, it is important to consider the fact that the status of a given receptor may be different in primary tumours and metastases. For example, the EGFR is expressed in most primary colorectal tumours, but a loss of EGFR expression is observed in a significant number of lymph node and liver metastases, an event that has evident implication for the treatment with EGFR-targeted antibodies (Scartozzi *et al.*, 2004; Bralet *et al.*, 2005). A large body of evidence indicates that OX_1R is present in colon tumour metastases. The receptor is expressed in all hepatic metastases tested as well as in human colon cancer cell lines established from lymph nodes, ascite and lung metastases (Voisin *et al.*, 2011). Figure 1 illustrates the expression of OX_1R in primary colorectal tumours, local metastases in lymph modes and distant metastases in liver and lung.

The orexin receptor OX_1R mediates apoptosis in colon cancer cells *in vitro* and *in vivo*

The expression of OX_1R by primary colorectal tumours as well as in metastases allows to induce apoptosis of cancer cells upon treatment with orexins. *In vitro*, the extent of orexin-induced apoptosis in various human colon cancer cell lines is clearly correlated to the level of OX_1R mRNA expression determined by RT-qPCR (Voisin *et al.*, 2011). Moreover, apoptosis can be induced in cell lines established from primary tumours and metastases as well (Voisin *et al.*, 2011). In view of the fact that failure of tumour cells to undergo apoptosis translates into tumour progression and chemotherapeutic resistance and given apoptosis emerges as a potential target for cancer treatment at various stages of tumour progression (Watson, 2004; 2006; Huerta *et al.*, 2006), the OX_1R becomes a new promising therapeutic target in colon cancer. However, two important issues had to be resolved for progress in this direction.

The first issue is related to colon cancer resistance to chemotherapy (Wolpin and Mayer, 2008; Segal and Saltz, 2009). The most commonly used drug, 5-fluorouracil (5-FU), is able to induce apoptosis in colon cancer, but development of drug resistance is a primary cause of failure of chemotherapy. In this context, whether OX_1R is expressed and is able to mediate orexin-induced apoptosis in 5-FU-resistant colon cancer cells are crucial questions. They have been addressed using the HT29-FU colon cancer cell line model in which a long-term 5-FU exposure selected cells resistant to the drug (Lesuffleur *et al.*, 1991). The OX_1R expression is similar in HT-29-FU cells and parental HT-29 cells (Voisin *et al.*, 2011). Moreover, treatment of HT-29-FU cells with orexins induces apoptosis and subsequent growth inhibition. This observation has important implications for colon cancer therapy and suggests that orexins promote apoptosis through a mechanism that is different from that of 5-FU and that persists in 5-FU-resistant cells (Voisin *et al.*, 2011).

The second issue is related to the efficacy of orexin treatment *in vivo*. This has been addressed using human colon cancer cells xenografted in nude mice. Upon subcutaneous inoculation of LoVo cells established from left supraclavicular metastases of colon cancer, a tumour develops rapidly at the site of inoculation. Daily i.p. injection of orexin-A beginning the day cancer cells are xenografted, dose-dependently reduces tumour volume. After a 15 day treatment, the tumour volume is decreased by ~80% with a dose as low as 1 μmol orexin-A/Kg (Voisin *et al.*, 2011). Similar data were reported with xenografts of HT-29 cells established from a primary colon tumour (Voisin *et al.*, 2011). Much more relevant in terms of therapy is the ability of orexins to rapidly and strongly reverse the development of established tumours as demonstrated by treatment of LoVo tumours in nude mice that is initiated 7 days after inoculation of cancer cells (Voisin *et al.*, 2011). It has been shown that orexins inhibit cultured colon cancer cell growth by inducing apoptosis (Rouet-Benzineb *et al.*, 2004). It is likely that orexins also reduce tumour growth *in vivo* by promoting apoptosis, because activation of caspase-3 occurs in tumours upon orexin treatment (Voisin *et al.*, 2011). The direct action of orexin injected in nude mice on OX_1R-bearing cancer cells in the tumour is supported by experiments with the only colon cancer cell line HCT116, which does not express OX_1R. Indeed, xenografts of HCT116 cells in nude mice result in development of tumours that are insensitive to orexin treatments (Voisin *et al.*, 2011). In conclusion, activation by exogenous orexins results in strong decrease of tumour development in mice xenografted with colon cancer cells *in vivo* without any adverse effect of orexins during treatment. Another interesting point is that long-term treatment of nude mice with orexins does not down-regulate OX_1R mRNA levels in tumours (Voisin *et al.*, 2011). It is thus unlikely that down-regulation of OX_1R in tumours upon orexin treatment may become a cause of resistance.

OX_1R: an Achilles heel of colon cancers?

The widespread expression of OX_1R in primary colorectal cancers and metastases, as well as the ability of exogenous orexins to promote colon cancer cell apoptosis and inhibition of tumour growth *in vivo*, raise several comments:

- OX_1R is expressed in primary colon tumours but not in normal colonic epithelial cells. Likewise, OX_1R is still expressed after migration of colon cancer cells in their main site of metastasis, for example, the liver, but not in normal liver cells (Voisin *et al.*, 2011). This represents an important feature in view of the possible use of OX_1R as a therapeutic target. In this context, it is interesting to consider the fact that besides OX_1R, which induces apoptosis through the intrinsic or mitochondrial pathway (Rouet-Benzineb *et al.*, 2004), colon cancer cells also express Fas receptors (O'Connell *et al.*, 2000), which, as death receptors, induce apoptosis through the extrinsic apoptosis pathway (Watson, 2004; 2006; Huerta *et al.*, 2006). Unfortunately, most colon cancer cell lines are somewhat resistant to Fas ligand-mediated apoptosis even if they are positive to Fas receptors (Huerta *et al.*, 2006). Moreover, normal colonic epithelial cells and hepatocytes are exquisitely sensitive to Fas-mediated apoptosis (Huerta *et al.*, 2006). This strongly limits the potential use of FasR agonists as possible candidates for chemotherapeutic intervention, because patients' cancer cells would remain relatively resistant to apoptosis, whereas normal colon and liver cells would be destined to commit suicide (Huerta *et al.*, 2006). Similarly, tumour necrosis factor (TNF)-related apoptosis ligand (TRAIL)-mediated resistance to apoptosis in colon cancer has been noted at multiple steps in the extrinsic pathway of apoptosis (van Geelen *et al.*, 2004) limiting the potential therapeutic use of TRAIL as an inductor of apoptosis in tumour cells. Attempts to use TNF and Fas ligand have also been thwarted by induction of NFκB-mediated inflammation and fulminant hepatic failure respectively (Watson, 2004). In the present state of our knowledge, OX_1R clearly does not suffer from any of the limits encountered with death receptors. Another remarkable property of OX_1R-mediated apoptosis in colon cancer cells is that it works in 5-FU-resistant cells (Voisin *et al.*, 2011) making

OX_1R a potential therapeutic target for OX_1R agonists that would be able to act in combination with classical chemotherapies in colon cancer (Wolpin and Mayer, 2008; Segal and Saltz, 2009).

- OX_1R in colon cancer might be considered as a new type of gene in cancer, because it is aberrantly expressed as a functional protein whose function, when activated by an agonist, is to promote apoptosis of the cancer cell. The colon cancer cells unexpectedly provide a new gate to promote their death, which was not present in the normal colonocytes from which they derive. In this context, an important question arises: is OX_1R activated in colorectal primary tumours or metastases *in vivo* by endogenous orexins? The answer is probably no for two main reasons: (i) colon tumours do not express the orexin precursor mRNA (Voisin *et al.*, 2011), ruling out the possibility that OX_1R in colon cancer cells might be activated by an intracrine pathway or by an autocrine loop; (ii) the major source of orexins is the brain where the orexinergic neurons are restricted to the hypothalamus (see earlier discussion). The sites of synthesis of orexins in the periphery are still debatable. Though early immunohistochemical studies detected orexins in the small intestine, stomach and pancreas in rodents (Kirchgessner and Liu, 1999), further RT-qPCR experiments identified orexin precursor mRNA in rat testis but not in most other peripheral tissues including the gut and liver (Johren *et al.*, 2001). This is in line with the absence of orexin precursor mRNA in normal human colonic mucosa and liver tissues. In this context, the OX_1R aberrantly expressed in colon cancer cells is most probably not activated by endogenous orexins in patients. On the other hand, there is no evidence suggesting that OX_1R exhibits constitutive activity in the absence of ligand, because transfection of OX_1R in CHO cells or HEK cells does not enhance basal apoptosis, whereas it confers the ability of orexins to promote apoptosis in those cells (Voisin *et al.*, 2008 and M. Laburthe and T. Voisin, unpubl. data). Therefore, it may be suggested that OX_1R in colorectal cancer constitutes a gate to apoptosis that probably remains unopened *in vivo* but could be openable by therapeutic administration of exogenous orexins or OX_1R agonists. In that respect, OX_1R might be considered as an Achilles heel of colon cancer, because targetting OX_1R with agonists leads to cancer cell death by apoptosis. The development of long-lived peptide agonists or non-peptide agonists of orexin receptors will represent thereby an important advance not only in neuroscience (Boss *et al.*, 2009) but also in colon cancer research. The OX_1R, orexins and forthcoming OX_1R agonists might be novel candidates for colorectal cancer therapy.

OX_1R-driven apoptosis: a novel mechanism for a GPCR involving ITIMs

At first sight, the OX_1R appears to be a classical G_q-coupled receptor the activation of which induces calcium transients (Voisin *et al.*, 2003). This classical G_q-mediated calcium response is certainly not sufficient to explain the OX_1R-driven apoptosis even though calcium participates in the onset of apoptosis (Rizzuto *et al.*, 2003). Indeed, several GPCRs in colon cancer cells do promote an increase in intracellular calcium but not only do not trigger apoptosis and rather stimulate cell proliferation, that is, muscarinic receptors (Medina and Rivera, 2010), neurotensin NT1 receptor (Maoret *et al.*, 1999), protease-activated receptor-2 (Darmoul *et al.*, 2001) or protease-activated receptor-1 (Darmoul *et al.*, 2004). Moreover, inhibition of intracellular $InsP_3$ increase abolishes OX_1R-mediated calcium transients but does nothing to OX_1R-driven apoptosis (Voisin *et al.*, 2008). Finally, promotion of apoptosis by orexins is an intrinsic property of OX_1R, because transfection of the receptor cDNA in cells devoid of endogenous OX_1R is sufficient to confer the ability of orexins to promote apoptosis as shown in Chinese hamster ovary CHO cells (Rouet-Benzineb *et al.*, 2004; Ammoun *et al.*, 2006; Voisin *et al.*, 2008; El Firar *et al.*, 2009) and mouse embryonic fibroblast (MEF) cells (Voisin *et al.*, 2008). Altogether, these observations prompted us to analyse the sequence of OX_1R for identification of new motifs that might be associated with its ability to trigger apoptosis. We identified two tyrosine-based motifs in OX_1R and demonstrated their crucial role in OX_1R-driven apoptosis (Voisin *et al.*, 2008; El Firar *et al.*, 2009). The first motif to be characterized (Voisin *et al.*, 2008) is a canonical ITIM present in the intracellular domain connecting the seventh transmembrane helix to the C-terminal tail of the OX_1R (Figure 2). The consensus ITIM sequence (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Ile-Leu-Val) is not considered to be a signature of GPCRs but represents a hallmark of immune inhibitory receptors on lymphoid and myeloid cells, the immunoglobulin G Fc-receptor FcγRIIB being prototypical of such receptors (Ravetch and Lanier, 2000; Daeron *et al.*, 2008). The second motif to be characterized (El Firar *et al.*, 2009) is an immunoreceptor tyrosine-based switch motif (ITSM) present in the intracellular domain connecting the first intracellular loop to the second transmembrane helix of the OX_1R (Figure 2). The consensus sequence of ITSM, Thr-X-Tyr-X-X-(Val,Ile) had never been identified previously in any GPCRs but was previously characterized in the signalling lymphocyte-activating molecule family of immunoreceptors (Ostrakhovitch and Li, 2006). This sequence is ITIM-like, and it may be suggested that ITIM and ITSM are very similar with a common permissive sequence (Ile/Leu/Val/Ser/thr)-X-Tyr-X-X-(Ile,Leu,Val). Indeed, ITIMs and ITSMs appear to function following the same paradigm because both motifs contain tyrosine residues that can be phosphorylated on activation of the corresponding immunoreceptors (see Sidorenko and Clark, 2003; Daeron *et al.*, 2008 for reviews).

The mechanism of OX_1R-driven apoptosis is schematized in Figure 2 and is the following. On activation of OX_1R by orexins, the tyrosine-based motifs ITIM and ITSM are tyrosine phosphorylated (Voisin *et al.*, 2008; El Firar *et al.*, 2009). This is a G_q-mediated event even though classical activation of phospholipase C is not involved (see earlier discussion). Indeed, transfection of OX_1R cDNA in G_q-deficient MEF cells does not confer the ability of orexins to promote apoptosis, whereas it does in G_q-bearing MEF cells (Voisin *et al.*, 2008). The activation of OX_1R allows the dissociation of the G_q protein into α_q and $\beta\gamma$ dimers. The freed $\beta\gamma$ dimers are known to activate Src-like tyrosine kinases (Gentili *et al.*, 2006), and experimental sequestration of $\beta\gamma$ dimers in cells (Koch *et al.*,

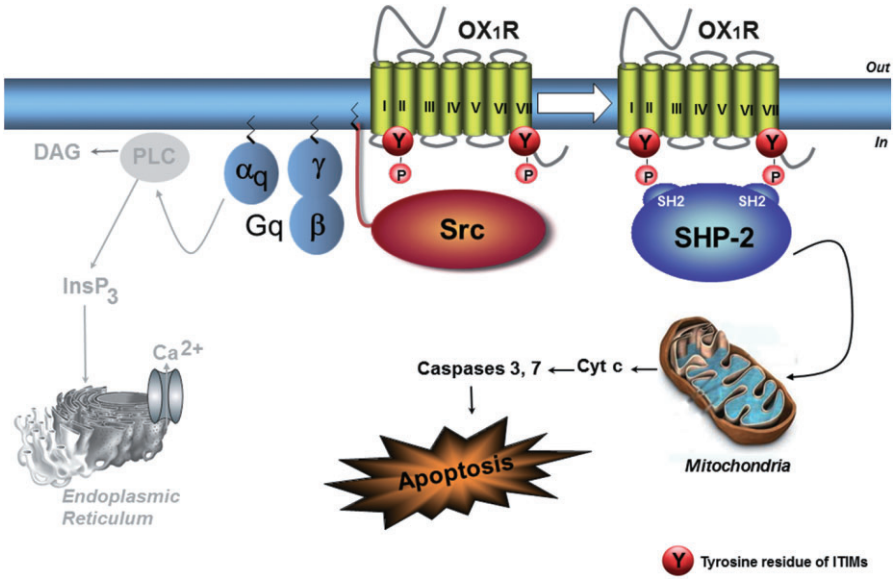

Figure 2

Mechanism of OX_1R-driven apoptosis. Activation of OX_1R by orexins promotes the dissociation of G_q protein into α_q and $\beta\gamma$ dimers. The classical pathway resulting in phospholipase C activation by α_q is not involved in OX_1R-mediated apoptosis and is shown in grey. Freed $\beta\gamma$ dimers stimulate the Src-tyrosine kinase resulting in the phosphorylation of the two ITIMs of OX_1R. The phosphotyrosine phosphatase SHP-2 is then recruited by OX_1R and activated thereby. Activated SHP-2 is mandatory for subsequent cytochrome c-mediated mitochondrial apoptosis. PLC, phospholipase C; Cyt c, cytochrome c; DAG, diacylglycerol; SHP-2, SH2 domain-containing phosphotyrosine phosphatase-2. See text for details.

1994) abolishes OX_1R-driven apoptosis (M. Laburthe and T. Voisin, unpubl. data). Next, activated Src kinases phosphorylate tyrosine within ITIM and ITSM of OX_1R as demonstrated using inhibitors of Src (Voisin *et al.*, 2008) and transfection of a dominant negative mutant of Src (M. Laburthe and T. Voisin, unpubl. data). The phosphorylation of the two motifs ITIM and ITSM is crucial, because mutation of tyrosine in either motif totally abolishes OX_1R-driven apoptosis (El Firar *et al.*, 2009). On tyrosine phosphorylation of ITIM and ITSM, the receptor recruits the phosphotyrosine phosphatase SHP-2 and thereby activates it (Voisin *et al.*, 2008; El Firar *et al.*, 2009). The activation of SHP-2 represents an early event in the initiation of apoptosis, and SHP-2 activation is mandatory in the process of orexin-induced apoptosis. This mechanism accounts for the dual participation of G_q and ITIMs in OX_1R-driven apoptosis and explains why the classical G_q-mediated pathway resulting in the activation of phospholipase C is not involved in the apoptotic response (Figure 2). This mechanism is novel in GPCRs, although it follows the general paradigm for ITIM function in immunoreceptors (Ravetch and Lanier, 2000; Daeron *et al.*, 2008). In that respect, it is interesting to consider the fact that ITIMs function in tandem in immunoreceptors, and the phosphoprotein phosphatases require their two SH2 domains to bind to adjacent ITIMs separated by a short connecting peptide within the receptor (Bruhns *et al.*, 1999). In OX_1R, the two sites ITIM and ITSM are far from each other in the primary sequence of the protein (Figure 2). A structural model of the human OX_1R has been developed showing that the distance between the two phosphorus atoms of phosphotyrosines in ITSM and ITIM compares well with the phosphopeptide binding sites in the SH2 domain of the protein phosphatase SHP-2 (El Firar *et al.*, 2009). It is thus suggested that ITIMs function in a spatial tandem in OX_1R (Laburthe *et al.*, 2010) contrasting with the linear tandems of two adjacent sites described in immunoreceptors (Daeron *et al.*, 2008).

The mechanism of OX_1R-driven apoptosis schematized in Figure 2 implies that the $\beta\gamma$ dimers of G_q are freed upon receptor activation and stimulate Src-tyrosine kinase, which in turn phosphorylates ITIMs initiating the apoptotic response thereby. Quite evidently, free $\beta\gamma$ dimers are also released during the activation of all GPCRs, and nevertheless, this does not result in apoptosis. The mechanisms that create selectivity in tyrosine phosphorylation of OX_1R ITIMs by Src are currently unknown. Two nonexclusive mechanisms can be suggested: (i) compartmentalization of the signalling complex may occur. Indeed, the G$\beta\gamma$ dimer, which is membrane associated due to the isoprenylation of the Gγ subunit (Marrari *et al.*, 2007), is likely to remain tethered to the activated receptor. Because Src can associate to the plasma membrane owing to its myristoylation and the presence of six basic residues at its amino terminus (Bjorge *et al.*, 2000), it could be activated at the vicinity of the activated OX_1R by the G$\beta\gamma$ dimer (Figure 3); (ii) a multi-step mechanism of activation of OX_1R may also occur (Figure 3). Binding of orexins to OX_1R leads to the G_q protein dissociation from the receptor to yield a Gα_q-GTP monomer (see Figure 2) and a G$\beta\gamma$ dimer, which activates Src-tyrosine kinase. A speculative further step of OX_1R activation would lead to another change in receptor conformation allowing exposure of tyrosine in ITIM and ITSM of OX_1R. This step would be necessary for phosphorylation of ITIM and ITSM in the receptor. We synthesized orexin analogs, which are able to promote intracellular calcium transients but are totally unable to promote apopto-

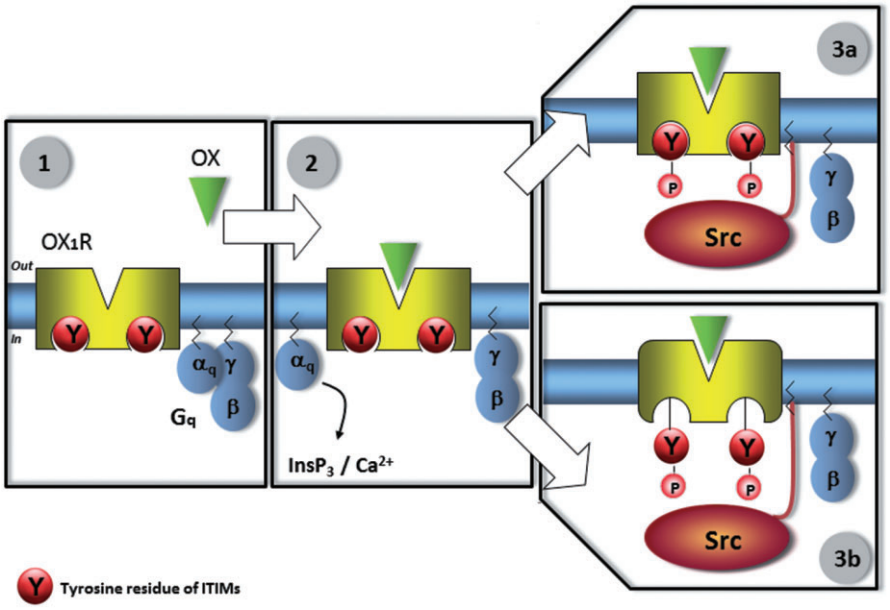

Figure 3

Proposed multi-step mechanisms of activation of OX_1R to account for the high specificity of the OX_1R-driven apoptosis. In step 1, OX_1R is not activated by orexins and the tyrosine residues of ITIMs are not phosphorylated (resting step). During step 2, binding of orexins to OX_1R leads to the G_q protein dissociation from the receptor to yield a $G\alpha_q$-GTP monomer and a $G\beta\gamma$ dimer, which are now free to modulate the activity of other intracellular protein. The $G\alpha_q$-GTP monomer activates phospholipase C leading to an increase in intracellular Ca^{++}, a process that is not involved in the apoptotic response. In Step 3a, the mechanism that could create selectivity in tyrosine phosphorylation of ITIMs by Src is compartmentalization of the signalling complex in which the $G\beta\gamma$ dimer activates Src at the vicinity of the activated OX_1R. Alternatively or additionally, a speculative Step 3b would lead to another ligand-induced change in receptor conformation allowing exposure of tyrosine in the two ITIMs of OX_1R. This step would be necessary for phosphorylation of ITIMs in the receptor. This scheme including Steps 3a and/or 3b would explain why all other GPCRs that are also able to free $G\beta\gamma$ dimers are not able to phosphorylate ITIMs in OX_1R and to promote apoptosis. After tyrosine phosphorylation of ITIMs, the OX_1R processes to further steps by recruiting SHP-2 resulting in the activation of the tyrosine phosphatase and the apoptotic response (see Figure 2).

sis (M. Laburthe and T. Voisin, unpubl. data). These data may fit with the sequential conformational changes of OX_1R suggested in Figure 3, although selection of distinct conformers consistent with ligand-directed signalling may also contribute. Whatever the mechanism(s), after tyrosine phosphorylation of ITIM and ITSM, the OX_1R recruits SHP-2 initiating the apoptotic response (see Figure 2).

Recruitment and activation of SHP-2 by OX_1R is such a proximal point of apoptosis regulation that many subsequent apoptosis-related events ending in release of cytochrome c from mitochondria (Rouet-Benzineb *et al.*, 2004) are currently under investigation in our group. At this stage, the key targets of SHP-2 leading to apoptosis remain elusive, as well as the targets of the tyrosine phosphatases SHPs in general (Neel *et al.*, 2003).

Conclusions and perspectives

The characterization of orexins as pro-apoptotic peptides in colon cancers and the entirely novel mechanism whereby the OX_1 receptor triggers apoptosis through phosphorylation of ITIMs open many questions or speculations and pave the way for promising perspectives. We would like to discuss four points:

- There is considerable interest in the development of small-molecule orexin receptor antagonists as a novel therapy for the treatment of insomnia and more generally of those medical and psychiatric conditions associated with disturbed vigilance (Boss *et al.*, 2009; Coleman and Renger, 2010; Kodadek and Cai, 2010; Scammell and Winrow, 2011). It is to be hoped that all these studies may also lead to the discovery of small-molecule agonists of orexin receptors, which may prove therapeutically useful in the treatment of colon cancer. This should be possible because orexin receptors are peptide receptors belonging to class A or rhodopsin-like GPCRs for which many nonpeptide agonists are now available. In this context, we do encourage pharmaceutical companies to screen chemical libraries not only for orexin receptor antagonist but also for agonists.
- The possibility of the expression of orexin receptors by other solid tumours in humans is an important issue and is currently under investigation. We have already shown that OX_1R is expressed in a neuroblastoma cell line that undergoes apoptosis upon orexin treatment (Rouet-Benzineb *et al.*, 2004). The OX_2R is present in a pancreatic carcinoma cell line in which orexin-induced apoptosis has been characterized (Voisin *et al.*, 2006). Orexin receptors have been also characterized in adrenocortical adenomas (Spinazzi *et al.*, 2005) but relation to apoptosis is not documented.

- Orexin neurons originating from the hypothalamus project throughout the brain where orexin receptors are widespread (see earlier discussion). The question thus arises of whether brain orexin receptors drive apoptosis in health and diseases. Normal adult brain neurons, which express orexin receptors and are stimulated by endogenous orexins, do not undergo apoptosis. This may be related to the high resistance of differentiated neurons to apoptosis induced by cytochrome c (Wright *et al.*, 2004), the mechanism whereby orexins induce apoptosis. In fact, mature neurons do not express detectable levels of Apaf-1 to which cytochrome c binds leading to the formation of the apoptosome (Johnson *et al.*, 2007). Because aberrant neuronal cell death is an outstanding feature of neurodegenerative diseases (Bredesen *et al.*, 2006), we previously made the hypothesis that orexin receptors may participate in the onset of apoptosis during neurodegeneration (Laburthe *et al.*, 2010). In this context, it is interesting to notice that a dual orexin receptor antagonist decreases amyloid-β plaque formation in a transgenic mice model suggesting that orexins may play a role in the pathogenesis of Alzheimer's disease (Kang *et al.*, 2009).
- A functional role of ITIMs in GPCRs has been described in very rare cases (see El Firar *et al.*, 2009). It is estimated that the human genome contains # 1000 genes that code for proteins of the GPCR structure with seven-transmembrane spanning domains. We previously carried out a non-exhaustive manual search for the presence of the permissive ITIM sequence, which revealed that ITIMs are much more frequent in GPCRs than initially thought (Laburthe *et al.*, 2010). We are currently mining automatically the GPCR databases with a new algorithm that is suitable to identify the small and degenerate sequence of permissive ITIM. These studies should provide new insights into the presence of ITIMs in all classes of GPCRs and during phylogenesis.

Acknowledgements

This work was supported by INSERM, CNRS and Université Paris-Diderot.

Conflict of interest

There is no conflict of interest.

References

Ammoun S, Lindholm D, Woortz H, Akerman KE, Kukkonen JP (2006). G-protein-coupled OX1 orexin/hctr-1 hypocretin receptors induce caspase-dependent and -independent cell death through p38 mitogen-/stress-activated protein kinase. J Biol Chem 281: 834–842.

Bjorge JD, Jakymiv A, Fujita DJ (2000). Selected glimpses into the activation and function of Src kinase. Oncogene 19: 5620–5635.

Boss C, Brisbare-Roch C, Jenck F (2009). Biomedical application of orexin/hypocretin receptor ligands in neuroscience. J Med Chem 52: 891–903.

Bralet MP, Paule B, Adam R, Guettier C (2005). Loss of epidermal growth factor receptor expression in lymph node and liver metastases of colon carcinoma. J Clin Oncol 23: 5844–5845.

Bredesen DE, Rao RV, Mehlen P (2006). Cell death in the nervous system. Nature 443: 796–802.

Bruhns P, Marchetti P, Fridman WH, Vivier E, Daeron M (1999). Differential roles of N- and C-terminal immunoreceptor tyrosine-based inhibition motifs during inhibition of cell activation by killer cell inhibitory receptors. J Immunol 162: 3168–3175.

Chell SD, Witherden IR, Dobson RR, Moorghen M, Herman AA, Qualtrough D *et al.* (2006). Increased EP4 receptor expression in colorectal cancer progression promotes cell growth and anchorage independence. Cancer Res 66: 3106–3113.

Coleman PJ, Renger JJ (2010). Orexin receptor antagonists: a review of promising compounds patented since 2006. Expert Opin Ther Pat 20: 307–324.

Daeron M, Jaeger S, Du PL, Vivier E (2008). Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol Rev 224: 11–43.

Darmoul D, Marie JC, Devaud H, Gratio V, Laburthe M (2001). Initiation of human colon cancer cell proliferation by trypsin acting at protease-activated receptor-2. Br J Cancer 85: 772–779.

Darmoul D, Gratio V, Devaud H, Lehy T, Laburthe M (2003). Aberrant expression and activation of the thrombin receptor protease-activated receptor-1 induces cell proliferation and motility in human colon cancer cells. Am J Pathol 162: 1503–1513.

Darmoul D, Gratio V, Devaud H, Laburthe M (2004). Protease-activated receptor 2 in colon cancer: trypsin-induced MAPK phosphorylation and cell proliferation are mediated by epidermal growth factor receptor transactivation. J Biol Chem 279: 20927–20934.

El Firar A, Voisin T, Rouyer-Fessard C, Ostuni MA, Couvineau A, Laburthe M (2009). Discovery of a functional immunoreceptor tyrosine-based switch motif in a 7-transmembrane-spanning receptor: role in the orexin receptor OX1R-driven apoptosis. FASEB J 23: 4069–4080.

van Geelen CM, de Vries EG, de Jong S (2004). Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. Drug Resist Updat 7: 345–358.

Gentili C, Boland R, Russo de Boland A (2006). Implication of G beta gamma proteins and c-SRC tyrosine kinase in parathyroid hormone-induced signal transduction in rat enterocytes. J Endocrinol 188: 69–78.

Grady WM, Carethers JM (2008). Genomic and epigenetic instability in colorectal cancer pathogenesis. Gastroenterology 135: 1079–1099.

Gratio V, Walker F, Lehy T, Laburthe M, Darmoul D (2009). Aberrant expression of proteinase-activated receptor 4 promotes colon cancer cell proliferation through a persistent signaling that involves Src and Erb-2 kinase. Int J Cancer 124: 1517–1525.

Hanash SM (1996). A role for chromosome 1 in colorectal cancer. Gastroenterology 111: 250–252.

Heinonen MV, Purhonen AK, Mäkelä KA, Herzig KH (2008). Functions of orexins in peripheral tissues. Acta Physiol (Oxf) 192: 471–485.

Huerta S, Goulet EJ, Livingston EH (2006). Colon cancer and apoptosis. Am J Surg 191: 517–526.

Johnson CE, Huang YY, Parrish AB, Smith MI, Vaughn AE, Zhang Q *et al.* (2007). Differential Apaf-1 levels allow cytochrome c to induce apoptosis in brain tumors but not in normal neural tissues. Proc Natl Acad Sci U S A 104: 20820–20825.

Johren O, Neidert SJ, Kummer M, Dendorfer A, Dominiak P (2001). Prepro-orexin and orexin receptor mRNA are differentially expressed in peripheral tissues of male and female rats. Endocrinology 142: 3324–3331.

Kang JE, Lim MM, Bateman RJ, Lee JJ, Smyth LP, Cirrito JR *et al.* (2009). Amyloid-β dynamics are regulated by orexin and the sleep-wake. Cycle Science 326: 1005–1007.

Kirchgessner AL, Liu M (1999). Orexin synthesis and response in the gut. Neuron 24: 941–951.

Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ (1994). Cellular expression of carboxyl terminus of a G protein-coupled receptor kinase attenuates G beta gamma-mediated signaling. J Biol Chem 269: 6193–6197.

Kodadek T, Cai D (2010). Chemistry and biology of orexin signaling. Mol Biosyst 6: 1366–1375.

Laburthe M, Rousset M, Boissard C, Chevalier G, Zweibaum A, Rosselin G (1978). Vasoactive intestinal peptide: a potent stimulator of adenosine 3′:5′-cyclic monophosphate accumulation in gut carcinoma cell lines in culture. Proc Natl Acad Sci U S A 75: 2772–2775.

Laburthe M, Voisin T, El Firar A (2010). Orexins/hypocretins and orexin receptors in apoptosis: a mini-review. Acta Physiol (Oxf) 198: 393–402.

Lappano R, Maggiolini M (2011). G protein-coupled receptors: novel targets for drug discovery in cancer. Nat Rev Drug Discov 10: 47–60.

de Lecea L (2010). A decade of hypocretins: past, present and future of the neurobiology of arousal. Acta Physiol (Oxf) 198: 203–208.

de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE *et al.* (1998). The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci U S A 95: 322–327.

Lesuffleur T, Kornowski A, Luccioni C, Muleris M, Barbat A, Beaumartin J *et al.* (1991). Adaptation to 5-fluorouracil of the heterogeneous human colon cancer tumor cell line HT-29 results in the selection of cells committed to differentiation. Int J Cancer 49: 721–730.

Maoret JJ, Pospai D, Rouyer-Fessard C, Couvineau A, Voisin T, Laburthe M (1994). Neurotensin receptor and its mRNA are expressed in many human colon cancer cell lines but not in normal colonic epithelium: binding studies and RT-PCR experiments. Biochem Biophys Res Commun 203: 465–471.

Maoret JJ, Anini Y, Rouyer-Fessard C, Gully D, Laburthe M (1999). Neurotensin and a non-peptide neurotensin receptor antagonist control human colon cancer cell growth in cell culture and in cells xenografted into nude mice. Int J Cancer 80: 448–454.

Markowitz SD, Bertagnolli MM (2009). Molecular basis of colorectal cancer. N Engl J Med 361: 2449–2460.

Marrari Y, Crouthamel M, Irannejad R, Wedegaertner PB (2007). Assembly and trafficking of heterotrimeric G proteins. Biochemistry 46: 7665–7677.

Medina VA, Rivera ES (2010). Histamine receptors and cancer pharmacology. Br J Pharmacol 161: 755–767.

Neel BG, Gu H, Pao L (2003). The 'Shp'ing news: SH2 domain-containing tyrosine phosphatase in cell signaling. Trends Biochem Sci 28: 284–293.

Nishino S, Ripley B, Overeem S, Lammers GJ, Mignot E (2000). Hypocretin (orexin) deficiency in human narcolepsy. Lancet 355: 39–40.

O'Connell J, Bennett MW, Nally K, Houston A, O'Sullivan GC, Shanahan F (2000). Altered mechanisms of apoptosis in colon cancer: Fas resistance and counterattack in the tumor-immune conflict. Ann N Y Acad Sci 910: 178–192.

Ostrakhovitch EA, Li SS (2006). The role of SLAM family of receptors in immune cell signaling. Biochem Cell Biol 84: 832–843.

Peltonen HM, Magga JM, Bart G, Turunen PM, Antikainen MS, Kukkonen JP *et al.* (2009). Involvement of TRPC3 channels in calcium oscillations mediated by OX(1) orexin receptor. Biochem Biphys Res Commun 385: 408–412.

Ravetch JV, Lanier LL (2000). Immune inhibitory receptors. Science 290: 84–89.

Rizzuto R, Pinton R, Ferrari D, Chami M, Szabadkai G, Magalhaes PJ *et al.* (2003). Calcium and apoptosis: facts and hypotheses. Oncogene 22: 8619–8627.

Rouet-Benzineb P, Rouyer-Fessard C, Jarry A, Avondo V, Pouzet C, Yanagisawa M *et al.* (2004). Orexins acting at native OX(1) receptor in colon cancer and neuroblastoma cells or at recomninant OX(1) receptor suppress cell growth by inducing apoptosis. J Biol Chem 279: 45875–45886.

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H *et al.* (1998). Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92: 573–585.

Sakurai T, Mieda M, Tsujino N (2010). The orexin system: roles in sleep/wake regulation. Ann N Y Acad Sci 1200: 149–161.

Scammell TE, Winrow CJ (2011). Orexin receptors: pharmacology and therapeutic opportunities. Annu Rev Pharmacol Toxicol 51: 243–266.

Scartozzi M, Bearzi I, Berardi R, Mandelosi A, Fabris G, Cascinu S (2004). Epidermal growth factor receptor (EGFR) status in primary colorectal tumors does not correlate with EGFR expression in related metastatic sites: implications for treatment with EGFR-targeted monoclonal antibodies. J Clin Oncol 22: 4772–4778.

Segal NH, Saltz LB (2009). Evolving treatment of advanced colon cancer. Annu Rev Med 60: 207–219.

Sidorenko SP, Clark EA (2003). The dual-function CD150 receptor subfamily: the viral attraction. Nat Immunol 4: 19–24.

Singh P, Dai B, Wu H, Owlia A (2000). Role of autocrine and endocrine gastrin-like peptides in colonic carcinogenesis. Curr Opin Gastroenterol 16: 68–77.

Spinazzi R, Rucinski M, Neri G, Malendowicz LK, Nussdorfer GG (2005). Preproorexin and orexin receptors are expressed in cortisol-secreting adrenocortical adenomas, and orexins stimulate in vitro cortisol secretion and growth of tumor cells. J Clin Endocrinol Metab 90: 3544–3549.

Voisin T, Rouet-Benzineb P, Reuter N, Laburthe M (2003). Orexin and their receptors: structural aspects and role in peripheral tissues. Cell Mol Life Sci 60: 72–87.

Voisin T, El Firar A, Avondo V, Laburthe M (2006). Orexin-induced apoptosis: the key role of the seven-transmembrane domain orexin type 2 receptor. Endocrinology 147: 4977–4984.

Voisin T, El Firar A, Rouyer-Fessard C, Gratio V, Laburthe M (2008). A hallmark of immunoreceptor, the tyrosine-based inhibitory motif ITIM, is present in the G protein-coupled receptor OX1R for orexins and drives apoptosis: a novel mechanism. FASEB J 22: 1993–2002.

Voisin T, El Firar A, Fasseu M, Rouyer-Fessard C, Descatoire V, Walker F *et al*. (2011). Aberrant expression of OX1 receptors for orexins in colon cancers and liver metastases: an openable gate to apoptosis. Cancer Res Published OnlineFirst March 17, 2011; doi:10.1158/0008-5472.CAN-10-3473.

Watson AJ (2004). Apoptosis and colorectal cancer. Gut 53: 1701–1709.

Watson AJ (2006). An overview of apoptosis and the prevention of colorectal cancer. Crit Rev Oncol Hematol 57: 107–121.

Wolpin BM, Mayer RJ (2008). Systemic treatment of colorectal cancer. Gastroenterology 134: 1296–1310.

Wright KM, Linhoff MW, Potts PR, Desmukh M (2004). Decreased apoptosome activity with neuronal differentiation sets the threshold for strict IAP regulation of apoptosis. J Cell Biol 167: 303–313.

Yang M, Zhong WW, Srivastava N, Yang J, Hoey T, An S (2005). G protein-coupled lysophosphatidic acid receptors stimulate proliferation of colon cancer cells through the β-catenin pathway. Proc Natl Acad Sci U S A 102: 6027–6032.