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Novel Insights Into M₃ Muscarinic Acetylcholine Receptor Physiology and Structure

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Abstract

Recent studies with M₃ muscarinic acetylcholine receptor (M3R) mutant mice suggest that drugs selectively targeting this receptor subtype may prove useful for the treatment of various pathophysiological conditions. Moreover, the use of M3R-based designer G protein-coupled receptors (GPCRs) has provided novel insights into how G_q-coupled GPCRs can modulate whole-body glucose homeostasis by acting on specific peripheral cell types. More recently, we succeeded in using X-ray crystallography to determine the structure of the M3R bound to the bronchodilating drug tiotropium, a muscarinic antagonist (inverse agonist). This new structural information should facilitate the development of orthosteric or allosteric M3R-selective drugs that are predicted to have considerable therapeutic potential.

Keywords

Muscarinic acetylcholine receptor; G protein-coupled receptor; X-ray structure; Mutant mouse models; Muscarinic receptor physiology

Introduction

The M₃ muscarinic acetylcholine receptor (M3R) is a prototypic member of the class A family of GPCRs (Wess, 1996; Caulfield and Birdsall, 1998). The M3R is one of the five GPCR subtypes (M₁–M₅) through which acetylcholine exerts many of its important physiological actions (note that acetylcholine also activates nicotinic acetylcholine receptors, which are ligand-gated ion channels). For more than two decades, we have used the M3R as a model system to better understand how class A GPCRs function at the molecular level. The M3R, like the M₁ and M₅ muscarinic receptors, is selectively linked to G proteins of the G_q family (Wess, 1996). Like most of the other muscarinic receptor subtypes, the M3R is widely expressed in different peripheral and central tissues (Wess,

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1996; Caulfield and Birdsall, 1998; Wess et al., 2007). However, at present, ligands endowed with a high degree of M3R selectivity are not available.

To learn more about the physiological roles of the M3R, we generated and analyzed whole-body M3R KO mice (Yamada et al., 2001). These studies showed that the M3R regulates many important peripheral and central physiological functions. Consistent with pre-existing pharmacological data, we found that M3R activation stimulates smooth muscle contractions, promotes glandular secretion, and triggers the dilation of peripheral blood vessels (reviewed by Wess et al., 2007). Studies with M3R KO mice also revealed several unexpected phenotypes. For example, whole-body M3R KO mice showed a decrease in body fat mass, associated with a reduction in food intake, central sympathetic outflow, and whole-body energy expenditure (Yamada et al., 2001; Gautam et al., 2006a). Experiments with M3R KO mice also support the concept that M3R activity plays role in tumor formation in the gastrointestinal tract (Raufman et al., 2008, 2011).

To gain more detailed insight into M3R physiology, we started to apply Cre-loxP technology to generate mutant mice that lack M3Rs only in specific cell types or tissues. For example, we found that mice that selectively lacked M3Rs in pancreatic β -cells showed a decrease in insulin release and impaired glucose tolerance, indicating that β -cell M3Rs play a critical role in maintaining whole-body glucose homeostasis (Gautam et al., 2006b). Consistent with this concept, transgenic mice that selectively overexpressed M3Rs in pancreatic β -cells exhibited greatly improved glucose tolerance and increased insulin release (Gautam et al., 2006b). Moreover, these transgenic mice were resistant to diet-induced glucose intolerance and hyperglycemia. Taken together, these data strongly suggest that approaches aimed at enhancing signaling through β -cell M3Rs might become therapeutically useful for the treatment of type 2 diabetes (T2D).

Interestingly, brain-specific M3R KO mice showed a dwarf phenotype, associated with a pronounced hypoplasia of the anterior pituitary gland, suggesting that central M3Rs may represent a potential drug target for the treatment of human growth disorders (Gautam et al., 2009). Moreover, these mutant mice showed an increase in central sympathetic outflow, associated with reduced bone mass, indicating that the activity of central M3Rs promotes bone formation (Shi et al., 2010). In a recent study, Arteaga-Solis et al. (2013) demonstrated that leptin decreases signaling through M3Rs expressed by airway smooth muscle cells by reducing parasympathetic tone, thus normalizing lung function in obese mice. This observation may be relevant for developing new therapeutic strategies for the management of obesity-associated asthma.

We also generated mutant mice that lacked M3Rs selectively in hepatocytes (Li et al., 2009) or in specific neuronal subpopulations of the hypothalamus known to be involved in the regulation of food intake and whole-body energy homeostasis (Y. Cui and J. Wess, unpublished observations). Despite extensive phenotyping studies, these mice did not exhibit any significant metabolic changes.

Taken together, the systematic analysis of several M3R mutant mouse strains has revealed that M3Rs are involved in a wide spectrum of physiological and, potentially,

pathophysiological functions. Importantly, these studies strongly suggest that the M3R represents an attractive target for the treatment of various human diseases. However, as mentioned earlier, ligands exhibiting a high degree of M3R selectivity are not available at present. To facilitate the design of such ligands, we recently initiated work to obtain a high-resolution X-ray structure of the M3R.

The High-resolution X-Ray Structure of the M3R in Complex With Tiotropium

In 2012, we published the first high-resolution X-ray structure of the M3R (Kruse et al., 2012). We decided to crystallize the rat M3R since we had subjected this receptor subtype to extensive mutagenesis studies in the past. However, it should be noted that the human and rat M3Rs show a very high degree of amino acid identity (92%), particularly within the transmembrane (TM) receptor core, which is almost perfectly conserved (Wess, 1996). At the same time, Haga et al. (2012) reported the X-ray structure of another muscarinic receptor subtype, the human M₂ muscarinic receptor (M2R). The two structures represent the inactive states of the two receptors in complex with a muscarinic antagonist (inverse agonist). The ligands used were the atropine derivative tiotropium (for the M3R) and R-(−)-3-quinuclidinyl benzilate (QNB; for the M2R), respectively (Fig. 1a). The two ligands have very high affinity for all muscarinic receptor subtypes. Since they dissociate from their target receptors only very slowly, they proved to be particularly useful to stabilize the two receptors during the purification and crystallization process.

In the following, we will briefly summarize the key features of the M3R-tiotropium complex. Tiotropium is used in the clinic as a bronchodilating agent and is highly effective in the treatment of chronic obstructive pulmonary disease (Barnes, 2000, 2004). It binds to M3Rs with subnanomolar affinity and dissociates from the M3R only very slowly, with a dissociation half-life of ~30 hr at room temperature (Barnes, 2000).

By fusing a FLAG epitope tag to the amino terminus of the M3R and a hexa-histidine tag to the carboxy terminus of the receptor, we were able to obtain pure M3R-tiotropium preparations of sufficient quantity and homogeneity for crystallographic studies (Kruse et al., 2012). Using the lipidic cubic phase crystallization approach, we eventually succeeded in obtaining crystals that yielded strong diffraction with a microfocus X-ray source. We compiled a dataset to a final resolution of 3.4 Å, allowing confident placement of the receptor backbone, amino acid side chains, and the bound ligand tiotropium. The overall structure of the M3R is similar to that of other biogenic amine GPCRs that have been crystallized during the past few years (Kruse et al., 2012). The tiotropium binding pocket is buried within the TM receptor core (Fig. 1a), similar to the binding mode of other biogenic amine ligands. However, the M3R structure also displays several features that appear to be unique for the muscarinic receptor family, as we will discuss in more detail below.

Fig. 1b shows an extracellular view of the tiotropium binding pocket. The amino acids that interact with the ligand are located on TM helices 3–7 (TM3–TM7). Importantly, these amino acids are perfectly conserved among all five muscarinic receptor subtypes (Wess, 1996), explaining why it has been so challenging to develop subtype-selective muscarinic

ligands targeted at this so-called orthosteric binding site. Fig. 1c summarizes some of the key M3R-tiotropium interactions. A TM6 asparagine side chain (N507^{6.52}) forms critical hydrogen bonds with the acid moiety of the ligand (note that the superscript indicates the amino acid position according to the Ballesteros-Weinstein GPCR numbering system). The negatively charged side chain of a TM3 aspartate residue (D147^{3.32}) forms a salt bridge with the positively charged ammonium group of the ligand. In fact, this type of interaction is conserved among all biogenic amine GPCRs. This ion pair is surrounded by a cage of three conserved tyrosine residues highlighted in orange in Fig. 1c (Y148^{3.33}, Y506^{6.51}, and Y529^{7.39}).

Fig. 1d depicts a cross-sectional side view of the M3R-tiotropium complex. This representation shows that the three conserved tyrosine residues that make contact with the ligand form a lid above tiotropium, a feature not previously observed in other biogenic amine GPCR structures. Clearly, this structural feature interferes with the ability of the ligand to dissociate from the receptor. Thus, the dissociation of tiotropium from the M3R must involve a conformational rearrangement of this lid structure. The nature of these structural changes remains unclear at present.

Moreover, Fig. 1d reveals another important M3R feature, a relatively large, solvent-accessible vestibule, which is separated from the orthosteric binding pocket by the tyrosine lid. The amino acids lining this outer receptor cavity are less well conserved among the five muscarinic receptor subtypes than are the residues lining the orthosteric binding site (Wess, 1996; Kruse et al., 2012). Thus, this extracellular vestibule represents an attractive target for the development of subtype-selective allosteric muscarinic drugs and bitopic agents that can interact with both the orthosteric and allosteric sites simultaneously. In fact, during the past few years, M₁, M₄, and M₅ muscarinic receptor-selective allosteric agents have been developed that are predicted to interact with this extracellular binding cavity (Digby et al., 2010; Keov et al., 2011).

A comparison of the X-ray structures of the M3R and the M2R indicates that the two receptors bind their respective ligands in a strikingly similar fashion involving a conserved set of amino acids (Kruse et al., 2012; Haga et al., 2012). Like the M3R, the M2R also features a large solvent-accessible vestibule, suggesting that this structural feature is conserved among all muscarinic receptors.

The M3R and the M2R couple to different functional classes of G proteins. Whereas the M3R preferentially couples to G_q-type G proteins, the M3R is selectively linked to G_i portions of the G_i family (Wess, 1996). We found that the orientations of the cytoplasmic end of TM5 and the second intracellular loop (i2 loop) differ between the M3R and the M2R. It is possible that these structural differences are related to the ability of the two receptors to couple to different sets of heterotrimeric G proteins (Kruse et al., 2012).

Expression of M3R-Based Designer GPCRs in Transgenic Mice

Background

In 2007, Bryan Roth and his coworkers first described a set of muscarinic receptor-based designer GPCRs, which they referred to as DREADDs ('designer receptors exclusively activated by a designer drug'; Armbruster et al., 2007). These designer receptors are unable to bind the endogenous muscarinic receptor agonist, acetylcholine, due to two single point mutations present in TM3 and TM5, respectively (Y^{3.33}C and A^{5.46}G in the rat M3R sequence; Guettier et al., 2009). Importantly, DREADDs can be efficiently activated by a compound called clozapine-N-oxide (CNO). CNO is a metabolite of clozapine, an antipsychotic drug, and has no known pharmacological actions. The initial series of DREADDs represented GPCRs that were able to selectively activate G proteins of the G_q or G_i family, respectively (Armbruster et al., 2007).

The work by Armbruster et al. (2007) prompted us to generate additional DREADDs endowed with different coupling properties. For example, by replacing the second and third intracellular loops of an M3R-based DREADD with the corresponding β_1 -adrenergic receptor sequences, we generated a DREADD that was able to selectively activate G_s (Guettier et al., 2009).

More recently, we described the development of an M3R-based DREADD that is unable to couple to G proteins but retains arrestin-dependent signaling (Nakajima and Wess, 2012). This novel DREADD differs from the M3R-based G_q DREADD only in an R to L point mutation (R165L^{3.50}) at the bottom of TM3 within the highly conserved DRY motif. A large body of evidence suggests that arrestin-2 and -3, following their recruitment by activated GPCRs, can act as scaffolding proteins to initiate novel intracellular signaling pathways (Rajagopal et al., 2010; Shukla et al., 2011). Thus, this newly developed, arrestin-biased DREADD should prove highly useful to dissect the physiological roles of arrestin-dependent signaling pathways in different tissues and cell types *in vivo*.

In the following, we will briefly summarize two recent studies that demonstrate the usefulness of DREADD technology to explore the physiological relevance of G_q-dependent signaling pathways in the regulation of glucose homeostasis. Specifically, we generated transgenic mice that express an M3R-based DREADD that is selectively linked to G proteins of the G_q family in pancreatic β -cells (Guettier et al., 2009; Jain et al., 2013) or hepatocytes (Li et al., 2013). For the sake of simplicity, we refer to this particular G_q DREADD as Rq, as we did in past studies.

Studies with transgenic mice expressing an M3R-based designer receptor (Rq) selectively in pancreatic β -cells

Several years ago, we reported that acute CNO treatment of transgenic mice selectively expressing the Rq designer receptor in pancreatic β -cells (β -Rq mice) resulted in striking metabolic changes *in vivo*, including increased insulin release, reduced blood glucose levels, and greatly improved glucose tolerance (Guettier et al., 2009). Very recently, we subjected β -Rq mice to additional studies in order to explore the *in vivo* metabolic effects caused by chronic activation of a G_q-coupled GPCR expressed by pancreatic β -cells (Jain et al., 2013).

We found that chronic CNO treatment of β -Rq mice led to pronounced increases in pancreatic insulin content and β -cell mass by simulating β -cell proliferation and transcriptional processes that result in increased insulin synthesis (Jain et al., 2013). Moreover, qRT-PCR studies demonstrated that the expression of Pdx1 and several other key β -cell transcription factors was significantly increased in islets prepared from β -Rq mice chronically treated with CNO. Most notably, Pdx1 plays a key role in β -cell differentiation and replication, and the maintenance of proper β -cell function (Cerf, 2006; Bernardo et al., 2008; Kaneto et al., 2008). In agreement with previous findings that Pdx1 stimulates the expression of many functionally critical β -cell genes (Zangen et al., 1997; Hart et al., 2000), we observed that the expression of the *Ins2*, *Pcks1*, *Pcks2*, and *Glut2* genes were significantly elevated in islets of β -Rq mice chronically treated with CNO (Jain et al., 2013).

Most strikingly, chronic activation of Rq signaling in pancreatic β -cells also led to greatly elevated *IRS2* transcript and protein levels, triggering an increase in the activity of Akt and ERK1/2. These kinases mediate many of the important cellular functions of *IRS2*, including the stimulatory effects of *IRS2* on β -cell replication (Taniguchi et al., 2006). It is therefore likely that enhanced signaling through the *IRS2*-Akt-ERK1/2 cascade plays an important role in mediating the beneficial effects on β -cell function caused by chronic activation of Rq.

To explore the molecular mechanisms responsible for the increase in *IRS2* expression mediated by activation of a β -cell G_q -coupled receptor, we carried out a series of studies using an M3R-expressing insulinoma cell line and islets from β -Rq and wild-type (WT) control mice. The data that we obtained were consistent with the concept that activation of $G_{q/11}$ signaling in pancreatic β -cells stimulates the PLC β /PKC pathway (as expected), resulting in the activation of ERK1/2 which in turn stimulates the activity of the *Irs2* promoter (also see Udelhoven et al., 2010). Additional studies with *IRS2*-deficient mice (Withers et al., 1998) demonstrated that the Rq-mediated beneficial effects on β -cell function required the presence of *IRS2* (Jain et al., 2013).

To test the potential clinical relevance of these findings, we generated a mouse model of diabetes by injecting mice, both β -Rq and WT control mice, with a relatively low dose of streptozotocin (STZ) over several days, a protocol which causes a severe but not complete loss of β -cell mass (Jain et al., 2013). As a result, this treatment led to severe diabetes in WT mice as well as non-CNO-treated β -Rq mice, including hyperglycemia, low plasma insulin, glucose intolerance, and impaired glucose-induced insulin release. Strikingly, chronic CNO treatment of the β -Rq mice was able to prevent all STZ-induced metabolic deficits. For example, Fig. 2 illustrates that STZ-treated β -Rq mice showed normal fasting blood glucose levels when chronically consuming CNO drinking water (Jain et al., 2013).

Additional studies demonstrated that that chronic CNO treatment of the STZ-exposed β -Rq mice led to significant increases in pancreatic insulin content and β -cell mass, accompanied by the enhanced expression of many genes critical for β -cell function and growth, including *Irs2* and various β -cell transcription factors (Jain et al., 2013). Taken together, these findings clearly indicate that chronic activation of a designer G_q -coupled receptor selectively expressed in pancreatic β -cells can prevent STZ-induced diabetes, most likely by stimulating pathways that promote β -cell function and proliferation. We obtained very similar results

when we studied β -Rq and WT control mice maintained on a high fat diet which is known to cause hyperglycemia, glucose intolerance and insulin resistance (Jain et al., 2013).

In summary, studies with β -Rq mutant mice led to the identification of a novel β -cell signaling pathway that has multiple beneficial metabolic effects under physiological and pathophysiological conditions. Our findings should stimulate the development of novel classes of antidiabetic drugs that exert their beneficial actions on β -cell function by activating G_q -coupled receptors or downstream signaling pathways.

Characterization of transgenic mice expressing an M3R-based designer receptor (Rq) selectively in hepatocytes

Changes in hepatic glucose fluxes are known to play a major role in the pathophysiology of T2D. Many studies have shown that an increase in the rate of hepatic glucose production (HGP) is the major contributor to fasting hyperglycemia in T2D (Taylor, 1999; Postic et al., 2004; Lin and Accili, 2011). Thus, a better understanding of the signaling pathways that regulate hepatic glucose fluxes is of great potential clinical relevance.

Hepatic glucagon receptors play a central role in maintaining normoglycemia under fasting conditions. Activation of these receptors by the hormone glucagon triggers increases in blood glucose levels in response to hypoglycemia (Jiang and Zhang, 2003). The glucagon receptor is a class B GPCR that is selectively coupled to the stimulatory G protein, G_s . In T2D, plasma glucagon levels are unphysiologically high, suggesting that increased signaling through liver glucagon receptors is a key feature of T2D (D'Alessio, 2011; Unger and Cherrington, 2012).

Hepatocytes also express various G_q -coupled GPCRs, including different muscarinic, vasopressin, and α_1 -adrenergic receptor subtypes (Exton et al., 1981; Clair et al., 2003; Li et al., 2009). These receptors are not only found in the liver but are also expressed by many other peripheral and central tissues (Regard et al., 2008). The potential roles of these G_q -linked receptors in regulating hepatic glucose fluxes under physiological and pathophysiological conditions are not well understood.

To shed light on this issue, we recently generated a transgenic mouse line that expresses the Rq designer receptor selectively in hepatocytes (Li et al., 2013). For the sake of simplicity, we refer to these mice as Hep-Rq mice. CNO treatment of primary hepatocytes prepared from Hep-Rq mice caused a pronounced increase in intracellular calcium levels. This effect was absent in primary hepatocytes prepared from WT mice (Li et al., 2013), clearly indicating that Rq is functional in hepatocytes of Hep-Rq mice. Thus, Hep-Rq mice serve as an excellent model system to explore the in vivo metabolic effects following ligand (CNO)-induced activation of a G_q -coupled receptor selectively expressed in hepatocytes.

We demonstrated that acute treatment of Hep-Rq mice with CNO caused pronounced, dose-dependent increases in blood glucose levels, without any concomitant changes in plasma insulin levels (Li et al., 2013). In addition, CNO-injected Hep-Rq mice showed impaired glucose tolerance and significantly increased blood glucose levels in a pyruvate challenge test (Li et al., 2013), consistent with an Rq-mediated increase in HGP.

When we injected Hep-Rq mice with maximally effective doses of glucagon and CNO, we made the surprising observation that CNO and glucagon caused increases in blood glucose levels that were similar in magnitude. To study liver glucose fluxes in greater detail, we also carried out isotope-labeling experiments using chronically catheterized conscious Hep-Rq mice. The outcome of these experiments demonstrated that hepatocyte Rq receptors mediated the stimulation of both gluconeogenesis and glycogenolysis (Li et al., 2013). Taken together, these data raise the possibility that hepatocyte GPCRs that signal through G_q-type G proteins play important roles in regulating blood glucose levels. Since enhanced HGP is one of the characteristic features of T2D, these findings are of considerable potential pathophysiological relevance.

By using GPCR array technology, we identified several G_q-linked GPCRs that are expressed by mouse hepatocytes (Li et al., 2013). These receptors include GPR91 (succinate receptor 1), the AT_{1a} angiotensin II receptor, the V_{1a} and V_{1b} vasopressin receptors, and the α_{1b}-adrenergic, PAR₁ thrombin, S1P₂ lysophospholipid, and M3R receptor subtypes. Interestingly, previous studies have shown that activation of α₁-adrenergic receptors and other G_q-linked GPCRs can stimulate hepatic glycogenolysis via Ca²⁺-dependent allosteric activation of phosphorylase kinase (Exton et al., 1981; Blackmore et al., 1982; Reinhart et al., 1984). It is therefore likely that a similar mechanism contributes to the ability of CNO to stimulate glycogenolysis in Hep-Rq mice.

About 90% of the increase in HGP above basal observed in T2D is thought to be due to an enhanced rate of gluconeogenesis (DeFronzo, 2004). In agreement with this finding, hepatic gluconeogenesis is also greatly increased in animal models of diabetes, including the *ob/ob* mouse (Turner et al., 2005). Interestingly, qRT-PCR studies demonstrated that the expression levels of several G_q-coupled receptors are significantly increased in the liver of *ob/ob* mice, as compared to lean littermates (Li et al., 2013). Among all GPCR genes analyzed, the V_{1b} vasopressin receptor showed the most robust increase in receptor transcript levels (Li et al., 2013). We therefore speculated that enhanced signaling through the V_{1b} receptor and other G_q-coupled receptors might contribute to maintaining high HGP in this mouse diabetes model. To test this hypothesis, we injected *ob/ob* mice and lean littermates with either the gluconeogenic substrate pyruvate alone or in combination with the selective V_{1b} receptor antagonist, SSR149415 (Serradeil-Le Gal et al., 2002; Griebel et al., 2005). In the absence of co-injected SSR149415 (pyruvate treatment only), the *ob/ob* mice showed significantly greater elevations in blood glucose levels than lean littermates (Fig. 3), consistent with the concept that hepatic gluconeogenesis is enhanced in *ob/ob* mice (Turner et al., 2005). Strikingly, after co-injection of pyruvate with SSR149415, *ob/ob* mice showed greatly reduced blood glucose excursions that were similar in magnitude to those observed with lean littermates (Li et al., 2013; Fig. 3). This finding raises the possibility that pharmacological blockade of V_{1b} vasopressin receptors may represent a novel approach to reduce HGP in T2D. An important issue that remains to be addressed is whether similar changes in hepatic GPCR expression levels are found in humans.

Recent studies suggest that the Rq designer receptor can also activate G protein-independent signaling cascades including arrestin-dependent pathways, similar to the M3R (Alvarez-Curto et al., 2011; Nakajima and Wess, 2012). Thus, future studies need to address the

question whether G protein-independent signaling networks also contribute to the metabolic phenotypes displayed by the Hep-Rq mice. Another important issue that remains to be explored is to which extent chronic CNO treatment of Hep-Rq mice affects liver glucose fluxes and whole-body glucose homeostasis. Such studies could pave the way for the development of novel therapeutic strategies aimed at targeting hepatic, G_q-linked GPCRs for therapeutic purposes.

Concluding Remarks

During the past few years, major technological advances have led to unprecedented novel insights into M3R physiology and structure. The new information gained from these studies should greatly aid the development of novel classes of muscarinic drugs. Specifically, based on the published M3R structure, it should be possible to design negative or positive allosteric modulators that selectively act on the M3R. As has been discussed elsewhere (Digby et al., 2010; Keov et al., 2011), such allosteric agents might cause fewer unwanted side effects as compared to orthosteric muscarinic ligands.

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Fig. 1.

Key features of the rat M3R-tiotropium high-resolution X-ray structure. **a** Chemical structures of tiotropium and QNB. **b** Extracellular view of the M3R-tiotropium binding pocket. **c** Scheme highlighting key interactions between distinct amino acid side chains of the M3R and the tiotropium ligand. **d** Cross-sectional side view of the M3R-tiotropium complex. The receptor protein is shown in black, the ligand as yellow and red spheres, and the receptor surface in green. This image illustrates that the three conserved tyrosine residues that make contact with the ligand (see panels b and c) form a lid above the tiotropium ligand present in the orthosteric binding site. This figure was generated based on data published by Kruse et al. (2012).

Fig. 2.

Chronic CNO treatment of β -Rq mice prevents streptozotocin (STZ)-induced hyperglycemia. Mice of the indicated genotypes were injected with STZ for five consecutive days (50 mg/kg i.p. once per day). Where indicated, mice received CNO via the drinking water (0.25 mg/ml) for 10 weeks (CNO treatment was started 1 week prior to the first STZ injection; for more experimental details, see Jain et al., 2013). This figure shows fasting blood glucose levels measured at the end of the CNO treatment period. Data are expressed

as means $\pm$ SEM (n=6 per group; *** $P < 0.001$, as compared to the three control groups).
This figure was modified based on published work (Jain et al., 2013).

Fig. 3.

The selective V_{1b} vasopressin receptor antagonist SSR149415 restores WT-like blood glucose excursions in *ob/ob* mice in a pyruvate challenge test. *Ob/ob* and WT lean control mice received an i.p. injection of 1.5 mg/g of sodium pyruvate, either alone or in combination with SSR149415 (20 mg/kg i.p.), a selective V_{1b} receptor blocker, and blood glucose levels were measured at the indicated time points. Data are given as means $\pm$ SEM ($n=4$ per group; $*P < 0.05$ or $**P < 0.01$, as compared to the three control groups). This figure was modified based on published work (Li et al., 2013).