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REVIEW

Volume-dependent osmolyte efflux from neural tissues: regulation by G-protein-coupled receptors

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Abstract

The CNS is particularly vulnerable to reductions in plasma osmolarity, such as occur during hyponatremia, the most commonly encountered electrolyte disorder in clinical practice. In response to a lowered plasma osmolarity, neural cells initially swell but then are able to restore their original volume through the release of osmolytes, both inorganic and organic, and the exit of osmotically obligated water. Given the importance of the maintenance of cell volume within the CNS, mechanisms underlying the release of osmolytes assume major significance. In this context, we review recent evidence obtained from our laboratory and others that indicates that the activation of specific G-protein-coupled receptors can markedly enhance the volume-dependent release of osmolytes from neural cells. Of particular significance is the observation that receptor activation significantly lowers the osmotic threshold at which osmolyte release occurs, thereby facilitating the ability of the cells to respond to small, more physiologically relevant, reductions in osmolarity. The mechanisms underlying G-protein-coupled receptor-mediated osmolyte release and the possibility that this efflux can result in both physiologically beneficial and potentially harmful pathophysiological consequences are discussed.

Keywords: ATP, glutamate, hyponatremia, regulatory volume decrease, taurine, thrombin.

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In a diverse range of tissues, the regulation of cell volume is essential for a number of physiological processes such as metabolism, apoptosis, and cell proliferation. However, this ancient homeostatic mechanism is of prime importance to the CNS because of the restricted volume of the skull and the importance of spatial relationships between cells in neural function. Neural cell swelling is observed in response to a number of pathological conditions, many of which result in hyponatremia, a frequently encountered clinical disorder in which both the Na^+ concentration and osmolarity of the plasma are significantly reduced. Here, we review the physiological significance of reductions in plasma osmolarity to the CNS and the mechanisms utilized by neural cells that enable them to adapt to such an environment. In addition, we emphasize recent evidence that highlights the potential importance that G-protein-coupled receptors (GPCRs) may play in volume regulation via their ability, under conditions of hypoosmolarity, to enhance the release of osmolytes and to enable neural cells to respond to reductions in osmolarity of the magnitude likely to be encountered *in vivo*. Under certain conditions, the ability of GPCRs to facilitate osmolyte release may be a two-edged sword as many of the organic osmolytes that are released in the CNS are neuroactive. Accordingly, the implications of GPCR-regulated osmolyte release under both physiological and pathophysiological

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Abbreviations used: $[Ca^{2+}]_i$, concentration of cytosolic Ca^{2+}; DCPIB, 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl) oxy]butanoic acid; DDF, 1,9-dideoxy-forskolin; ECS, extracellular space; GPCRs, G-protein-coupled receptors; KCC, K^+–Cl^- cotransporter; LPA, lysophosphatidic acid; mAChR, muscarinic cholinergic receptor; NPPB, 5-nitro,2(3-phenylpropylamino) benzoic acid; Oxo-M, oxotremorine-M; PAR-1, protease-activated receptor 1; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; RVD, regulatory volume decrease; S1P, sphingosine 1-phosphate; TK, tyrosine kinase; VSOAC, volume-sensitive organic osmolyte and anion channel.

conditions are discussed. Although the emphasis of the present review is on volume regulation in neural cells, for discussion of osmoregulation in additional cell types, the reader is referred to recent reviews (Nilius and Droogmans 2003; Stutzin and Hoffmann 2006; Hoffmann *et al.* 2007; Lang 2007).

Regulation of cell volume in the CNS under hypoosmotic conditions: physiological significance

Even modest increases in brain cell volume can have profound effects within the CNS as the spatial relationships between neurons, astrocytes and extracellular space (ECS) are compromised with attendant changes in neuronal excitability (Sykova 2004). The ECS is estimated to occupy approximately 20% of the brain volume and the reductions in this parameter will result in an increased lateral diffusion of neurotransmitters and higher extracellular concentrations per quantum of transmitter released (Thorne and Nicholson 2006). Although the brain is not a perfect osmometer, significant reductions in plasma osmolarity (10–20%), as may occur during hyponatremia, can result in increases in brain volume of the magnitude of 5–7%. This, in turn can lead to a compression of blood vessels, which generates episodes of anoxia and ischemia. As the intra-cerebral pressure increases, the brain parenchyma is displaced through the foramen magnum, which can lead to death via cardiac or respiratory arrest (Pasantes-Morales *et al.* 2002). Brain cells can swell either via changes in intracellular ion and water distribution (isotonic swelling or cellular/cytotoxic edema) or in plasma osmolarity (hypoosmotic swelling). Although neural cell swelling can be observed under physiological conditions, for example as a result of neurotransmission or intense neuronal discharge (Lux *et al.* 1986; Holthoff and Witte 1996; Darquie *et al.* 2001), larger changes are encountered during pathological conditions. The most common cause of hypoosmotic swelling is a condition known as hyponatremia, which is defined as a reduction in serum Na^+ concentration from a normal value of 145 to 136 mEq/L. Hyponatremia is the most commonly encountered electrolyte disorder in clinical practice and is conservatively estimated to occur in 2.5–5.0% of hospitalized patients, with the young and the elderly disproportionately affected (Bhardwaj 2006; Lien and Shapiro 2007). Although the prevalence of hyponatremia is similar in men and women, women have a much poorer prognosis in terms of death or permanent brain damage (Bhardwaj 2006). The majority of symptoms associated with hyponatremia are neurological in origin. Thus, when the plasma Na^+ concentration is between 125 and 135 mEq/L, symptoms include mild nausea, emesis, and headaches whereas below 120 mEq/L (a reduction in plasma osmolarity of approximately 20%), respiratory arrest, coma, and permanent brain damage may occur. Serum Na^+ concentrations of 110 mEq/L or below are associated with a high incidence of mortality. Hyponatremia can result from a number of pathological conditions such as congestive heart failure, nephrotic syndrome or hepatic cirrhosis. Water overloading, as occurs during the inappropriate secretion of anti-diuretic hormone (vasopressin), in some psychiatric disorders such as psychotic polydipsia, or in athletes and military personnel following excessive exercise, may also result in hyponatremia. Treatment of hyponatremia can include fluid restriction, slow administration of hypertonic saline, steroids, or vasopressin receptor antagonists (Bhardwaj 2006). Paradoxically, an overly rapid correction of hyponatremia by the administration of isotonic fluids can lead to cerebral dehydration, shrinkage, and myelinolysis, as cells that are chronically acclimated to hypotonicity respond via a rapid accumulation of both inorganic and organic osmolytes (McManus *et al.* 1995).

Osmolyte fluxes in response to hypotonic stress: regulatory volume decrease

Under steady-state conditions, an equilibrium exists between the concentrations of osmotically active solutes in the cytoplasmic and extracellular fluids. However, when plasma osmolarity is lowered, as occurs in hyponatremia, water flows down its concentration gradient and enters the brain via the capillary endothelial cells. The brain is relatively resistant to osmotic swelling and the increment in brain water is less than would be predicted on the basis of ideal osmotic behavior (Verbalis and Drutarosky 1988). Thus homeostatic mechanisms, which reduce the potential impact of increases in cell volume, appear to be rapidly activated upon the onset of hypoosmolarity. The first line of defense is an increase in flow of fluid from the interstitial space of the brain into the CSF and from there to the systemic circulation. A secondary, more sustained, mechanism whereby compensatory decreases in cell volume are affected is via the efflux of osmotically active solutes, primarily inorganic ions such as Cl^- and K^+ and small organic molecules, which then leads to the exit of obligated water. The normalization of cell volume that occurs as a result of osmolyte loss is referred to as regulatory volume decrease (RVD; Fig. 1). Conversely, under hypertonic conditions, neural cells actively accumulate organic and inorganic osmolytes in a process referred to as regulatory volume increase (Fig. 1). Although the loss of Cl^- and K^+ that occurs under hypoosmotic conditions contributes significantly to RVD, reductions in intracellular ion concentration may directly impact neural cell excitability. Thus, an additional class of compounds used by cells to counter changes in osmolarity is that of small organic molecules that have been considered to act as 'compatible' or 'non-perturbing' osmolytes, which function as osmoregulators without compromising cell function. However, the organic osmolyte, taurine, has been reported to regulate intracellular

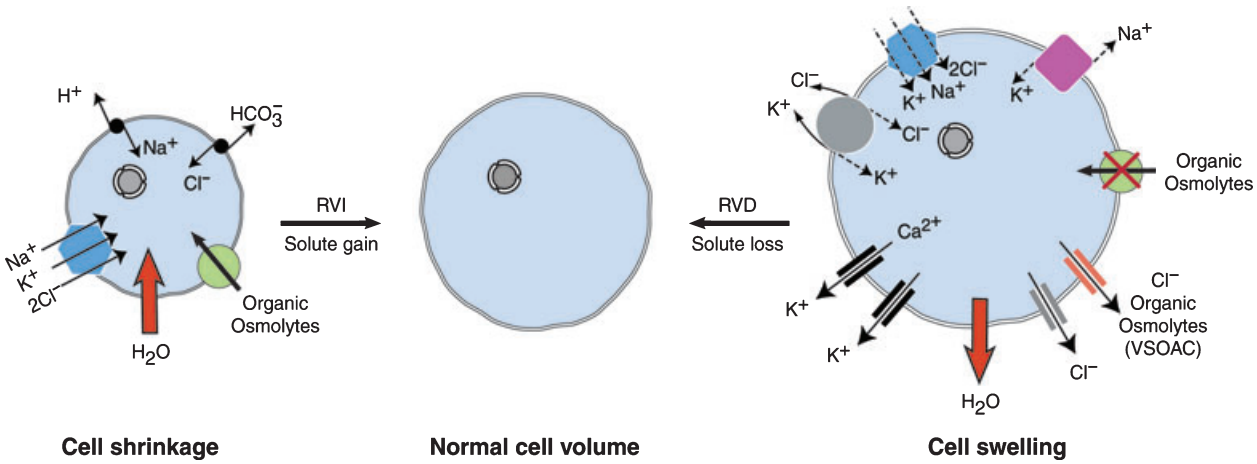

Fig. 1 Schematic illustration of some of the membrane carriers and channels that can be activated upon hypoosmotic or hypertonic challenge. During hypotonicity, KCl is potentially extruded (solid lines) via separate Ca^{2+}-dependent and -independent K^+ channels, Cl^- channels and a K^+–Cl^- symporter (KCC). To minimize the loss from cells under hypotonic conditions, K^+ can be re-accumulated (dashed lines) by the combined actions of the KCC (gray circle), Na^+–K^+–$2Cl^-$ symporter (NKCC; blue hexagon) and the Na^+–K^+ ATPase (purple square). The efflux of organic osmolytes (e.g. taurine, glutamate, and myo-inositol) may occur via a channel that primarily transports Cl^- ions and is termed the volume-sensitive organic osmolyte and anion channel (VSOAC). Under hypoosmotic conditions the re-uptake of organic osmolytes that occurs via Na^+-dependent transporters (green circle) may also be inhibited. The net efflux of osmolytes from cells leads to the exit of obligated water (red arrow) and regulatory volume decrease (RVD). Under conditions of hyperosmotic-induced cell shrinkage, increased NaCl influx can be mediated via the Na^+–H^+, Cl^-– HCO_3^- antiporters or by operation of the Na^+–K^+–$2Cl^-$ symporter (NKCC). Organic osmolytes are also transported into the cell via Na^+-dependent carriers, which increase in number. The influx of osmolytes results in an influx of water (red arrow) and regulatory volume increase (RVI).

processes (Warskulat *et al.* 2007; El Idrissi 2008) and, as discussed later, several organic osmolytes are neuroactive. Organic osmolytes, which potentially contribute approximately 30–35% to cell volume adaptation (Pasantes-Morales *et al.* 2002), are present in high concentrations in brain cytosol and can be grouped into three major classes: (i) polyols, such as myo-inositol or sorbitol; (ii) amino acids such as taurine, glutamate, aspartate, or glycine, and (iii) methylamines, such as betaine or glycerophosphorylcholine (Nilius *et al.* 1997; Lang *et al.* 1998; Pasantes-Morales *et al.* 2000a). In both neural and non-neural tissues there are developmental changes in the concentrations of individual organic osmolytes with the quantitatively major organic osmolytes present in the adult rat CNS being taurine, glutamate, glutamine, creatine, and myo-inositol (Miller *et al.* 2000). However, several other neuroactive amino acids or derivatives are also released from neural cells in response to hypotonic stress. These include GABA, glycine, aspartate, and *N*-acetyl-aspartate, all of which are present in mM concentrations in the CNS (McIlwain and Bachelard 1971).

Changes in the concentrations of osmolytes, both inorganic and organic, under conditions of chronic hyponatremia can be quite dramatic. Thus, when animals are rendered chronically hyponatremic, brain concentrations of myo-inositol, glutamate, and taurine decrease by 40–90%, whereas more modest percentage reductions (10–20%) are observed for Cl^- and K^+ (Thurston *et al.* 1989; Lien *et al.* 1991; Pasantes-Morales *et al.* 2002; Massieu *et al.* 2004). Proton magnetic resonance spectroscopy studies indicate that a pronounced loss of organic osmolytes (myo-inositol, glutamate, and glutamine) is also observed for the human brain under conditions of severe hyponatremia (Haussinger *et al.* 1994; Videen *et al.* 1995).

Although both astrocytes and neurons, when maintained in culture, are observed to swell in response to osmotic stress, glial cells make the major contribution to volume regulation in the CNS *in vivo* (Kimelberg 1995). As a consequence, their involvement in osmolyte release has been extensively documented (Mongin and Kimelberg 2002, 2005; Pasantes-Morales *et al.* 2006b; Ramos-Mandujano *et al.* 2007). Increases in Cl^- current, osmolyte release, and/or RVD in response to hypoosmolarity have also been monitored in neuronal preparations such as Purkinje cells (Nagelhus *et al.* 1993), sympathetic ganglia (Leaney *et al.* 1997), cerebellar granule cells (Moran *et al.* 1997; Morales-Mulia *et al.* 2001), mouse sensory trigeminal neurons (Viana *et al.* 2001), human NT2-N neurons (Novak *et al.* 2000), neuroblastoma (Altamirano *et al.* 1998; Loveday *et al.* 2003), and primary cultures of hippocampal and cortical neurons (Li and Olson 2004; Inoue *et al.* 2005). Hypoosmolarity has also been demonstrated to elicit a release of organic osmolytes from brain slices (Bothwell *et al.* 2001) and from isolated nerve ending preparations (Tuz *et al.* 2004; Tuz and Pasantes-Morales 2005). Although freshly isolated hippocampal neurons have been reported to lack volume regulation (Andrew *et al.* 1997; Aitken *et al.* 1998), it has been

suggested that loss of taurine during the tissue preparation may account for this discrepancy. Thus, RVD is observed in hippocampal slices only when supplemented with this amino acid (Kreisman and Olson 2003). However, unlike glia, pyramidal neurons in slices of cerebral cortex appear to be relatively resistant to a 15% reduction in osmolarity regardless of whether taurine was present or not (Andrew *et al.* 2007). It should also be noted that a previous *in vivo* study has demonstrated that, when rats are exposed to a 20% reduction in plasma osmolarity for 4 h, taurine (and other organic osmolytes) are released from cerebellar Purkinje neurons, but in the absence of a measurable increase in cell volume (Nagelhus *et al.* 1993). Although an explanation for these discrepancies is not yet apparent, it appears possible that individual populations of neurons may differ considerably in their susceptibility to osmotic swelling and that this could conceivably reflect differences in cytoskeletal rigidity, the relative absence of aquaporins or in rates of RVD (Kimelberg *et al.* 2006).

Volume-dependent osmolyte efflux from neural cells is mediated by anion/cation channels and transporters

The mechanism(s) by which an increase in cell volume is initially sensed by a putative 'osmotic sensor' remains an enigma. Changes in intracellular ionic strength (in particular Cl^-), macromolecular crowding or alterations in the cell cytoskeleton have been proposed to trigger alterations in cell volume (for reviews see Lang *et al.* 1998; Mongin and Orlov 2001). Experimentally, the release of both inorganic and organic osmolytes is most conveniently monitored following exposure of neural cells to a sudden and marked decrease in osmolarity. Under these conditions, cells rapidly swell (the extent of which is dependent upon the degree of hypotonicity and cell type), and osmolyte efflux constitutes part of the mechanism underlying RVD. However, under pathophysiological situations, small and gradual reductions in osmolarity (rather than a sudden decrease) would be expected. When hippocampal slices are subjected to such a gradual hypoosmotic challenge (~2.5 mOsM/min), cell swelling is not observed, even under conditions of 50% hypotonicity (Franco *et al.* 2000; Pasantes-Morales *et al.* 2000b). Under this condition, which is known as isovolumetric regulation, a slow efflux of osmolytes counters potential increases in cell volume. For both experimental paradigms, i.e. sudden and gradual reductions in osmolarity, the efflux of osmolytes from cells subjected to hypotonic stress is mediated via specific anion/cation channels and transporter molecules, as shown (Fig. 1).

Volume-sensitive organic osmolyte and anion channel

Swelling-activated Cl^- channels display very similar electrophysiological, biophysical, and pharmacological properties in both neural and non-neural cell types. These channels, which exhibit a conductance of 40–78 pS, are typically outward rectifiers and possess permeability characteristics ($I^- > NO_3^- > Br^- > Cl^- > F^-$) that correspond to Eisenman's anion permeability sequence (Nilius and Droogmans 2003). Swelling-activated Cl^- channels require ATP, but not its hydrolysis, as non-hydrolyzable analogs of ATP, ADP, or GTP can substitute (Jackson *et al.* 1994). The channel is blocked by a wide spectrum of anion channel inhibitors, such as 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid, 5-nitro,2(3-phenylpropylamino) benzoic acid (NPPB) and 1,9-dideoxy-forskolin (DDF). Despite extensive characterization of this class of channels and recent cloning of some that are osmosensitive, the identity of the swelling-activated Cl^- channel remains elusive. The ubiquity of the channel in cells and the absence of a specific high-affinity ligand are two factors that probably account for the paucity of information regarding the molecular properties of the channel (for review, see Okada 2006). The issue of whether this channel mediates the release of both Cl^- and organic osmolytes also remains uncertain. Evidence in favor of a shared channel is principally based upon two observations. First, organic osmolyte efflux and swelling-activated Cl^- current (or $^{125}I^-$ efflux, used as a tracer for Cl^-) exhibit remarkably similar pharmacological inhibitor profiles in the presence of the relatively non-specific anion channel inhibitors NPPB, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid, and DDF (Sanchez-Olea *et al.* 1996; Nilius *et al.* 1997; Kirk and Strange 1998; Junankar and Kirk 2000; Abdullaev *et al.* 2006). Recently however, an indanone compound, 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid (DCPIB), has been found to be a highly selective inhibitor of swelling-activated Cl^- channels but is without effect on other Cl^- channels (Decher *et al.* 2001). DCPIB can effectively attenuate both Cl^- current and inorganic/organic osmolyte efflux from astrocytes and SH-SY5Y neuroblastoma cells (Abdullaev *et al.* 2006; Cheema *et al.* 2007; Ramos-Mandujano *et al.* 2007). Second, in whole patch clamp studies, it has been demonstrated that volume-activated Cl^- channels in cultured cells are permeable to taurine, aspartate, and glutamate, when these amino acids are present in an anionic form (Banderali and Roy 1992; Jackson and Strange 1993; Boese *et al.* 1996). The relative ability of organic osmolytes to exit the cells is consistent with the predicted dimensions of the channel (minimum diameter between 5.4 and 8 Å, or ~0.6 nm; Okada 2006) and the molecular size of the individual osmolytes. The permeability ratios of (P_x/P_{Cl}^-) for the different organic osmolytes range from ~0.1 for glutamate or aspartate, ~0.2 for taurine and ~0.6 for the small amino acid, glycine. Conversely, the molecular size of the cyclical form of inositol (7.2 Å × 5.9 Å) is at the upper limit, thereby resulting in a relatively slow rate of efflux. The shared channel through which Cl^- and organic osmolytes are

released has been referred to as the 'volume-sensitive organic osmolyte and anion channel (VSOAC)' or, alternatively, 'volume-regulated anion channel'.

In contrast, marked differences with regard to pharmacological inhibitor profile for organic osmolyte and $^{125}I^-$ efflux have been observed for some non-neural cells (Lambert and Hoffmann 1994; Sanchez-Olea *et al.* 1995). Furthermore, in HeLa cells, the kinetics of activation and inactivation of taurine and $^{125}I^-$ efflux differed considerably, a result inconsistent with a single common pathway (Stutzin *et al.* 1999). There is also evidence from studies using tyrosine kinase (TK) inhibitors and/or anion channel blockers that individual organic osmolytes may efflux from brain slices and astrocytes via separate pathways (Mongin *et al.* 1999b; Franco *et al.* 2001; Bothwell *et al.* 2002; de La Paz *et al.* 2002). Taken collectively, these results leave open the possibility that more than one volume-sensitive Cl^- channel may contribute to the efflux of osmolytes, both inorganic and organic, during RVD. For additional discussion of the possibility that organic osmolytes and Cl^- efflux from cells by multiple channels, the reader is referred to a recent review by Shennan (2008).

Swelling-activated K^+ channels

Although the release of Cl^- and organic osmolytes from cells has been extensively studied, considerably less information is available regarding the efflux of K^+ (often monitored as $^{86}Rb^+$) from neural preparations. Following osmotic swelling, K^+ release from neural cells appears to be delayed (relative to Cl^-) and to often occur independently of changes in $[Ca^{2+}]_i$ (Moran *et al.* 1997; Quesada *et al.* 1999; Pasantes-Morales and Morales Mulia 2000; Tuz *et al.* 2001; Pasantes-Morales *et al.* 2006b). Evidence, mostly obtained from non-neural cells, suggests that a variety of different channels may mediate the efflux of K^+ under conditions of hypoosmotic stress. These include K^+ channels that are voltage gated, and those activated by stretch, swelling or an increase in the concentration of cytosolic Ca^{2+} $[Ca^{2+}]_i$ (Pasantes-Morales *et al.* 2006b). Few studies have been performed to identify the K^+ channels that are activated in neural cells in response to hypoosmolarity. However, in C_6 glioma cells, both Ca^{2+}-activated and Ca^{2+}-independent K^+ channels have been implicated (Ordaz *et al.* 2004). Moreover, it appears that at least two K^+ conductances with distinct pharmacological properties and osmotic thresholds are present in the latter cells.

K^+–Cl^- cotransporter

Recent evidence also points to a role for the K^+–Cl^- cotransporter (KCC) family of proteins in volume-dependent K^+ efflux from neural cells. Based upon the ability of *R*-(+)-[(2-*n*-butyl-6,7-dichlooro-2-cyclopentenyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid to inhibit RVD, it is estimated that KCC mediates 30–40% of osmolyte efflux in human glioma cells (Ernest *et al.* 2005). Smaller, but significant, contributions of KCC to K^+ efflux (10–20% of total) have also been reported for C_6 glioma cells (Gagnon *et al.* 2007). The addition of *R*-(+)-[(2-*n*-butyl-6,7-dichlooro-2-cyclopentenyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid results in a 20% inhibition of $^{86}Rb^+$ efflux from human SH-SY5Y neuroblastoma cells (Foster *et al.* 2008). Taken collectively, these results suggest that KCC may play a role, albeit limited, in K^+ efflux from some neural cells.

GPCR-mediated regulation of osmolyte efflux

Evidence is accumulating to suggest that the volume-dependent release of osmolytes can be significantly enhanced following the activation of specific cell-surface GPCRs (Fig. 2). At the outset, it should be noted that receptor regulation of osmolyte release has been observed for both neural and non-neural tissues (Table 1). However, the ability of GPCRs to potentiate osmolyte release, and thereby normalize cell volume, is of particular significance to neural cells given the widespread occurrence of these receptors in the CNS and the critical need to regulate neural cell volume within narrow limits. Furthermore, because many of the organic osmolytes released are neuroactive, the contribution that the efflux process may make to both physiological and pathophysiological events needs to be considered. The first indication that receptor activation may play a role in osmoregulation was obtained by Bender *et al.* (1993), who noted that when cultured astrocytes were exposed to hypotonic media, the ensuing volume correction could be accelerated by inclusion of specific ligands known to activate GPCRs, e.g. endothelin or norepinephrine. Indirect evidence for the potential involvement of GPCRs in osmolyte release was also obtained from experiments in which increases in $[Ca^{2+}]_i$, protein kinase C (PKC), or protein kinase A (PKA) activity were observed to potentiate volume-dependent efflux of osmolytes from astrocytes, C_6 glioma or differentiated NT2-N neurons (Strange *et al.* 1993; Novak *et al.* 2000; Moran *et al.* 2001). However, only in the last few years has the potential contribution of GPCRs in the regulation of osmolyte efflux from neural cells been systematically evaluated and mechanistic aspects examined.

Regulation of osmolyte release has been observed in response to a variety of distinct classes of neurotransmitters or neuromodulators including neuropeptides (vasopressin, endothelin, or oxytocin), cholinergic or adrenergic agonists, adenosine, ATP, thrombin, or lysophospholipids (Table 1a). The concentrations of these ligands required to elicit osmolyte release *in vitro* (nanomolar to micromolar) are well within the range of those found in the CNS (McIlwain and Bachelard 1971 and references therein; Das and Hajra 1989; Edsall and Spiegel 1999; Toman and Spiegel 2002; Hua *et al.* 2003a,b). Furthermore, the receptor subtypes identified to regulate osmolyte efflux are present within the

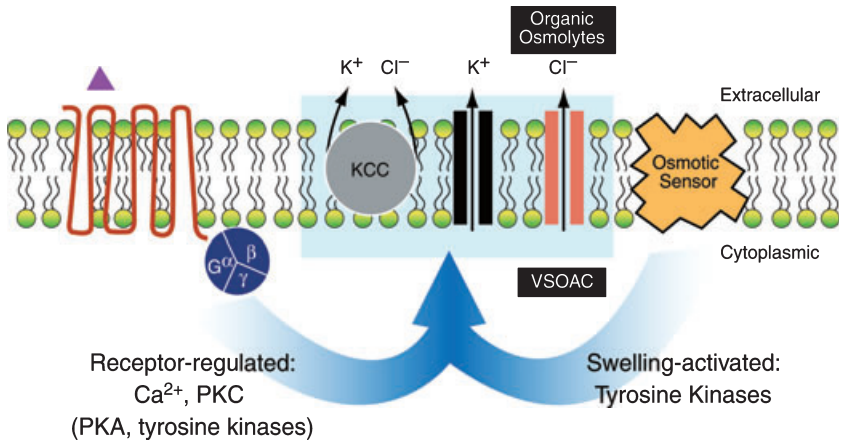

Fig. 2 Diagrammatic representation of swelling-activated (basal)- and G-protein-coupled receptor (GPCR)-mediated osmolyte release from neural cells. A reduction in osmolarity is proposed to trigger a hypothetical osmotic sensor which results in the tyrosine kinase (TK)-dependent activation of volume-sensitive organic osmolyte and anion channel (VSOAC), K^+ channels and KCC transporter, with the attendant efflux of K^+, Cl^-, and organic osmolytes. The release of these osmolytes can also be enhanced under hypoosmotic conditions by the activation of a GPCR (shown in orange) following the addition of an agonist (purple triangle). Receptor-mediated osmolyte release is often demonstrated to be dependent upon Ca^{2+} and protein kinase C (PKC) activity. The involvement of PKA and TKs in GPCR regulation of osmolyte efflux is less frequently documented, as is indicated by the parentheses. Although the release of osmolytes under basal- and receptor-stimulated conditions occurs via distinct mechanisms, the same (or similar) membrane channels and transporter molecules appear to mediate osmolyte efflux in both situations.

CNS. Although a diverse range of GPCRs has been implicated in osmoregulation in neural preparations, a number of common characteristics are apparent. First, many (but not all) of the receptor subtypes identified to increase volume-dependent osmolyte efflux are also those reported to couple to Ca^{2+} homeostasis and PKC activation, e.g. P_{2Y}, M_3 muscarinic cholinergic receptor (mAChR), protease-activated receptor 1 (PAR-1), and V_{1A} (Mongin and Kimelberg 2002; Heacock *et al.* 2004; Rosso *et al.* 2004; Cheema *et al.* 2005, 2007; Ramos-Mandujano *et al.* 2007). Second, activation of GPCRs facilitates the volume-dependent efflux of both inorganic and organic osmolytes (Abdullaev *et al.* 2006; Cheema *et al.* 2007; Foster *et al.* 2008). Third, receptor-stimulated efflux of organic osmolytes or Cl^- can be prevented by inclusion of VSOAC inhibitors such as NPPB, DDF, or DCPIB, a result that indicates the mediation of the same (or similar) anion channels under both swelling-activated and GPCR-regulated conditions (Abdullaev *et al.* 2006; Cheema *et al.* 2007; Ramos-Mandujano *et al.* 2007). Fourth, receptor activation elicits only a limited increase (if any) in the release of osmolytes under isotonic conditions and stimulated efflux is abolished under conditions of hyperosmolarity (Mongin and Kimelberg 2002; Heacock *et al.* 2004; Cheema *et al.* 2007). These latter observations are consistent with the concept that GPCRs serve principally to modulate the activity of channels that have been previously activated by changes in osmolarity. Fifth, and of most potential physiological significance, is the observation that the activation of GPCRs results in a lowering of the threshold osmolarity (set-point) at which osmolyte efflux occurs. This was first observed by Mongin and Kimelberg (2002) who demonstrated that limited reductions in osmolarity of the magnitude that might be encountered *in vivo*, i.e. 5–10%, elicited little or no increase in the release of D-aspartate from cortical astrocytes. However, inclusion of ATP, which activates P_{2Y} receptors, resulted in a five to 10-fold increase in aspartate release. Additional evidence that GPCR activation can alter the set-point for osmolyte release has been obtained for SH-SY5Y neuroblastoma cells (Heacock *et al.* 2004). Although these cells release taurine in response to hypotonicity, a statistically significant increase over that observed under isotonic conditions is not achieved until the osmolarity of the medium has been reduced by > 30% (Fig. 3b). In contrast, in the presence of a muscarinic cholinergic agonist, the efflux of taurine is significantly increased over basal even under isotonic conditions and a further enhancement of release occurs under conditions of limited reductions in osmolarity (6–16%: Fig. 3a). In contrast, mAChR-mediated taurine release is abolished under mildly hypertonic conditions. The ability of mAChR activation to potentiate osmolyte efflux is also observed when cells are exposed to the larger reductions in osmolarity that are routinely experimentally employed (> 30%; Fig. 3b). Thus in SH-SY5Y cells, activation of mAChRs lowers the osmotic threshold for taurine release from 230 to 340 mOsM. Similarly, mAChR activation reduces the set-point for $^{86}Rb^+$ efflux from 200 to 340 mOsM (Fig. 3c; Foster *et al.* 2008). This ability of GPCR activation to facilitate the

Table 1. Receptor-mediated osmoregulation in neural and non-neural cells

	Ligand	Cell type	Receptor subtype	Parameters monitored	References
(a) Neural tissues	Adenosine	Retina	A_1	Somal swelling	Uckermann *et al.* 2006; Wurm *et al.* 2008
		Pituicytes	A_{2B}	Taurine efflux	Pierson *et al.* 2007
	ATP	Cortical astrocytes	P_{2Y}	D-aspartate efflux, Cl^- current	Mongin and Kimelberg 2002, 2005; Abdullaev *et al.* 2006
		Cortical astrocytes	P_{2Y}	Cl^- current	Darby *et al.* 2003
		Pituicytes	ND	Taurine efflux	Rosso *et al.* 2004
		Hippocampal Neurons	ND	Cl^- current	Li and Olson 2004
		Retina	P_{2Y}	Somal swelling	Wurm *et al.* 2008
		Substantia nigra	ND	Taurine efflux	Morales *et al.*, 2007
	Bradykinin	Pituicytes	ND	Taurine efflux	Rosso *et al.* 2004
	Endothelin	Cortical astrocytes	ND	RVD	Bender *et al.* 1993
	Glutamate	Substantia nigra	AMPA	Taurine efflux	Morales *et al.*, 2007
	Lysophosphatidic acid	SH-SY5Y neuroblastoma	ND	Taurine, Rb^+ efflux	Heacock *et al.* 2006b; Foster *et al.* 2008
	Norepinephrine	Cortical astrocytes	ND	RVD	Bender *et al.* 1993
		Cortical astrocytes	β-adrenergic	Taurine efflux	Moran *et al.* 2001
	Oxotremorine-M	SH-SY5Y neuroblastoma	M_3 mAChR	Taurine, D-aspartate, inositol, Rb^+, I^- efflux	Loveday *et al.* 2003; Heacock *et al.* 2004; Cheema *et al.* 2007; Foster *et al.* 2008
	Oxytocin	Pituicytes	ND	Taurine efflux	Rosso *et al.* 2004
	Sphingosine 1-phosphate	SH-SY5Y neuroblastoma	ND	Taurine, Rb^+ efflux	Heacock *et al.* 2006b; Foster *et al.* 2008
	Thrombin	Cortical astrocytes	ND	RVD	Bender *et al.* 1993
		Cortical astrocytes	PAR-1	Taurine efflux	Cheema *et al.* 2005
		Cortical astrocytes	PAR-1	Glutamate efflux	Ramos-Mandujano *et al.* 2007
		1321N1 astrocytoma	PAR-1	Taurine efflux	Cheema *et al.* 2005
		SH-SY5Y neuroblastoma	PAR-1	Taurine, I^-, Rb^+ efflux	Cheema *et al.* 2007; Foster *et al.* 2008
	Vasopressin	Pituicytes	V_{1A}	Taurine efflux	Rosso *et al.* 2004
		Neocortex	V_{1A}	Intrinsic optical signals	Niermann *et al.* 2001
		Cortical astrocytes	V_{1A}	RVD	Sarfaraz and Fraser 1999
(b) Non-Neural tissues	Adenosine	Airway epithelia	ND	Taurine efflux	Musante *et al.* 1999
	Angiotensin	Ventricle	AT_1	Cl^- current	Ren *et al.* 2008
	ATP	MDA-MB-231 breast	ND	Rb^+ efflux	Gow *et al.* 2005
		Intestinal 407 epithelia	P_{2Y}	RVD	Dezaki *et al.* 2000
		HSG parotid	P_{2u}	RVD	Kim *et al.* 1996
		HTC hepatoma	P_{2Y}	Taurine, Cl^-, Rb^+ efflux	Junankar *et al.* 2002
		3T3 fibroblasts	ND	Taurine efflux	Franco *et al.* 2004
		Ehrlich ascites	ND	Taurine efflux	Falktoft and Lambert 2004
	Bradykinin	Ehrlich ascites	ND	Taurine efflux	Falktoft and Lambert 2004
	Ca^{2+}	Intestinal 407 epithelia	Ca^{2+}-R	Cl^- current, RVD	Shimizu *et al.* 2000
	Carbachol	HSY parotid	mAChR	RVD	Moran and Turner 1993
	EGF	3T3 fibroblasts	ND	Taurine, Cl^- efflux	Franco *et al.* 2004
		C127 mammary cells	ND	Cl^- current	Abdullaev *et al.* 2003
	Endothelin	Atrial cells	ET_A	Cl^- current	Du and Sorota 2000
	Histamine	Ehrlich ascites	H_1	Taurine efflux, RVD	Falktoft and Lambert 2004
	Leukotrienes	Ehrlich ascites	ND	RVD	Lambert 1989
	Lysophosphatidic acid	*Ltk* fibroblasts	ND	Taurine efflux	Heacock *et al.* 2006a

Table 1 *Continued*

Ligand	Cell type	Receptor subtype	Parameters monitored	References
Norepinephrine	Erythrocytes	α-adrenergic	Taurine efflux	Thoroed *et al.* 1995
	Myocytes	α-adrenergic	Cl^- current	Ellershaw *et al.* 2002
PGE_1	*Ltk* fibroblasts	EP_2	Taurine efflux	Heacock *et al.* 2006a
Sphingosine 1-phosphate	*Ltk* fibroblasts	ND	Taurine efflux	Heacock *et al.* 2006a
Thrombin	Arterial endothelial cells	ND	Cl^- current	Manolopoulos *et al.* 1997a
	BC_3H1, C_2C_{12} myoblasts	PAR-1	Taurine efflux	Manolopoulos *et al.* 1997b
	Ehrlich ascites	ND	Taurine efflux	Falktoft and Lambert 2004
	3T3 fibroblasts	PAR-1	Taurine efflux, Cl^- current, RVD	Vazquez-Juarez *et al.* 2008b

ND, not determined; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; EGF, epidermal growth factor; PGE_1, prostaglandin E_1; RVD, regulatory volume decrease.

release of osmolytes by lowering the set-point has also been observed in response to activation of PAR-1, sphingosine 1-phosphate (S1P), and lysophosphatidic acid (LPA) receptors in the SH-SY5Y cell line (Heacock *et al.* 2006b; Cheema *et al.* 2007) and for PAR-1 receptors in cortical astrocytes and 1321N1 astrocytoma cells (Cheema *et al.* 2005; Ramos-Mandujano *et al.* 2007). The ability of GPCRs to both increase the magnitude of osmolyte release and to lower the set-point for efflux may provide a physiological mechanism whereby neural cells are able to respond to small reductions in osmolarity – such as those encountered in hyponatremia, a condition in which a lowering of plasma osmolarity of > 20% is rarely observed.

Although the majority of studies have been conducted with cultured cells, there is evidence that receptor-mediated regulation of volume control under hypoosmotic conditions can also be demonstrated in more integrated preparations. Thus vasopressin, acting via V_{1A} receptors has been demonstrated to modulate activity-dependent water flux in neocortical slices (Niermann *et al.* 2001). Furthermore, activation of A_1 adenosine and P_{2Y} purinergic receptors have been linked to an inhibition of osmotic glial swelling in the intact retina (Uckermann *et al.* 2006; Wurm *et al.* 2008). Recently, evidence has been obtained for the involvement of both glutamatergic (α-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate) and purinergic receptors in the regulation of taurine release from osmotically stressed substantia nigra (Morales *et al.* 2007). Taken collectively, the ability of a diverse array of pharmacologically distinct GPCRs to both promote osmolyte efflux and lower the set-point for osmolyte release raise the possibility that tonic activation of receptors within the CNS may provide an important mechanism for volume regulation in neural tissues. For additional discussion of the potential role played by GPCRs in volume regulation, the reader is referred to a recent review by Vazquez-Juarez *et al.* (2008a).

Signal transduction mechanisms that mediate osmolyte release following activation of GPCRs

The signal transduction events that intervene between activation of the 'osmotic sensor' and volume correction under swelling-activated conditions are yet to be defined, and although several candidates have been suggested (Ca^{2+}, PKC, phospholipase A_2, or small molecular weight GTP-binding proteins), only TK activity appears to be a consistently observed attribute of volume-activated osmolyte release (for review, see Pasantes-Morales *et al.* 2006a). There is also a paucity of information regarding the identity of the intracellular signaling pathways that mediate GPCR-regulated osmolyte release from neural tissues. Moreover, the information currently available is largely restricted to the release of organic osmolytes. Notwithstanding these considerations, certain features of receptor-stimulated osmolyte release are apparent, most notably a marked dependence on the availability of Ca^{2+}, an observation consistent with the known ability of many of the same receptors to increase $[Ca^{2+}]_i$. In the neural cell types examined thus far (astrocytes and neurotumor cells), depletion of intracellular Ca^{2+} with either thapsigargin or 1,2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetra acetic acid (BAPTA-AM) significantly attenuates receptor-mediated release of taurine, glutamate, D-aspartate, or myo-inositol (Loveday *et al.* 2003; Cheema *et al.* 2005; Mongin and Kimelberg 2005; Heacock *et al.* 2006b; Ramos-Mandujano *et al.* 2007). However, no simple relationship exists between the magnitude of receptor-mediated increases in $[Ca^{2+}]_i$ and the extent of osmolyte release. For example, the rank order of efficacy for Ca^{2+} mobilization in SH-SY5Y neuroblastoma cells, oxotremorine-M (Oxo-M) > LPA > S1P, differs considerably from that for osmolyte release, S1P > Oxo-M > LPA. In the same cells, down-regulation of inositol trisphosphate receptors that results from prolonged exposure

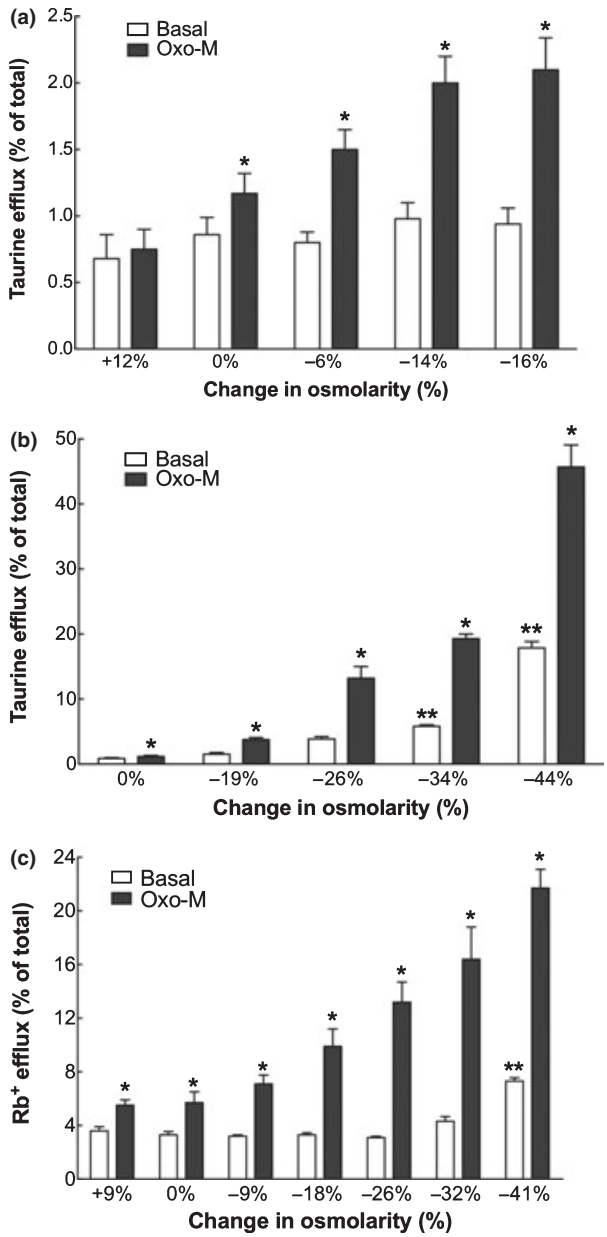

Fig. 3 Basal- and oxotremorine-M (Oxo-M)-stimulated efflux of taurine and $^{86}Rb^+$ as a function of osmolarity. SH-SY5Y cells pre-labeled with [^{14}C]taurine or $^{86}Rb^+$ were incubated in isotonic (340 mOsM), hypertonic (380 mOsM) or hypotonic (320–190 mOsM) buffer for either 5 min ($^{86}Rb^+$) or 20 min (^{14}C taurine) in either the absence or presence of 100 μM Oxo-M. Results are expressed as taurine or $^{86}Rb^+$ efflux (percent of total soluble radioactivity) and are the mean ± SEM for 4–13 separate experiments. Panel (a): taurine efflux under relatively small reductions or increase in osmolarity. Panel (b): taurine efflux under conditions of pronounced reductions in osmolarity. Panel (c): $^{86}Rb^+$ efflux under a range of osmolarities. *Different from basal release (minus Oxo-M), $p < 0.05$, by paired Student's *t*-test. **Different from basal efflux monitored under isotonic conditions, $p < 0.01$, by one-way ANOVA followed by Dunnett's multiple comparison test. Figure is constructed from data previously reported (Heacock *et al.* 2004; Foster *et al.* 2008).

to Oxo-M results in a 50–70% reduction in the ability of S1P and LPA to increase $[Ca^{2+}]_i$ but has no effect on the efficacy with which these two ligands can enhance osmosensitive taurine release (Heacock *et al.* 2006b). Furthermore, although the rise in $[Ca^{2+}]_i$ that results from the activation of GPCRs reflects both the influx of extracellular Ca^{2+} and mobilization of intracellular Ca^{2+}, a requirement for extracellular Ca^{2+} in osmolyte release is only observed for some receptors (e.g. mAChR in SH-SY5Y cells and PAR-1 receptors in astrocytes: Heacock *et al.* 2006b; Ramos-Mandujano *et al.* 2007). Taken collectively, these results suggest that, although Ca^{2+} availability appears to be a prerequisite for maximal receptor-stimulated osmolyte release, both the degree of dependence and source of Ca^{2+} utilized may differ, depending on the cell type and/or receptor involved. In terms of down-stream effectors for Ca^{2+}, a role for calmodulin is indicated from the ability of trifluoperazine, chlorpromazine or W7 to inhibit ATP- or thrombin-stimulated osmolyte release from astrocytes (Mongin and Kimelberg 2005; Ramos-Mandujano *et al.* 2007). However, the involvement of calmodulin kinase ll is less certain as inhibition of the enzyme with KN-93 or KN-62 results in little or no attenuation of receptor-stimulated osmolyte release (Cheema *et al.* 2005; Mongin and Kimelberg 2005). A correlation between receptor-regulated changes in osmoregulation and Ca^{2+} was first noted by Bender *et al.* (1993), who proposed a link between receptor-mediated increases in phospholipase C (PLC) activity and RVD. However, lysophospholipid receptors that mobilize Ca^{2+} and facilitate osmolyte efflux in SH-SY5Y cells do not elicit an increase in the activity of PLC (Heacock *et al.* 2006b). Furthermore, although PAR-1 receptors enhance osmolyte release from both 1321N1 astrocytoma and SH-SY5Y neuroblastoma cells, the addition of thrombin only increases PLC activity in the astrocytoma cell line (Cheema *et al.* 2005, 2007). Thus, it is now apparent that osmolyte release can be facilitated by Ca^{2+}-mobilizing receptors that operate via PLC-dependent or -independent mechanisms.

An additional prerequisite for maximal receptor-stimulated osmolyte release is that of PKC activity. For astrocytes and neurotumor cells, pre-incubation of cells with inhibitors of PKC, such as chelerythrine or bisindolylmaleimide, results in an attenuation of receptor-stimulated osmolyte efflux (Cheema *et al.* 2005; Mongin and Kimelberg 2005; Heacock *et al.* 2006b; Ramos-Mandujano *et al.* 2007). Down-regulation of PKC following overnight exposure of SH-SY5Y cells with phorbol 12-myristate 13-acetate, also severely attenuates mAChR-stimulated release of myo-inositol (Loveday *et al.* 2003). Recently, evidence for the involvement of two conventional isoforms of PKC, namely α and β1, in ATP-mediated release of D-aspartate from astrocytes has been obtained from experiments in which the activity of these enzymes was attenuated by either the use

of siRNA knock-down or inclusion of a cell-permeable pseudosubstrate inhibitory peptide. Of particular significance was the observation that the down-regulation of both PKCα and PKCβ1 was required for inhibition, suggesting a cooperative interaction of these two isoforms in mediating inhibition of osmolyte release (Rudkouskaya *et al.* 2008). Under conditions in which both PKC is inhibited and intracellular Ca^{2+} depleted, the ability of GPCRs to regulate organic osmolyte efflux is essentially abolished (Mongin and Kimelberg 2005; Heacock *et al.* 2006b). One unexpected finding is that PAR-1-mediated release of $^{125}I^-$ from SH-SY5Y cells (and to a lesser extent, that mediated by the mAChR) is relatively unaffected by either the depletion of intracellular Ca^{2+} or inhibition of PKC, even though the release of both taurine and $^{125}I^-$ occurs through pharmacologically similar membrane channels (Cheema *et al.* 2007). These results, which are in agreement with those previously obtained for P_{2Y} receptor activation of hepatoma cells (Junankar *et al.* 2002), suggest that distinct biochemical requirements may exist for the release of inorganic and organic osmolytes. It should be stressed that the swelling-activated release of organic osmolytes, in contrast to that observed under GPCR-stimulated conditions, also appears to be relatively independent of both Ca^{2+} and PKC in neural cells (Loveday *et al.* 2003; Mongin and Kimelberg 2005; Takano *et al.* 2005; Heacock *et al.* 2006b; Cheema *et al.* 2007; Rudkouskaya *et al.* 2008). These observations are consistent with the proposal that distinct mechanisms underlie basal- and receptor-mediated osmolyte efflux (Mongin and Kimelberg 2005). Because the receptor-regulated (but not swelling-activated) efflux of osmolytes is dependent on Ca^{2+} and PKC activity, it seems unlikely that this dependence reflects a direct activation of VSOAC itself. Other putative phosphorylation targets might include auxiliary proteins that either facilitate the opening of VSOAC, or recruit the channels to the cell surface. Alternatively, the hypothetical 'volume sensor' might be the target of phosphorylation, with a resultant change in the 'set-point' for osmolyte release.

There is general agreement that TK activity is required for the activation of VSOAC in neural tissues, based upon the ability of inhibitors such as genistein or tyrphostins to attenuate the efflux of organic osmolytes and Cl^-, and of inhibitors of tyrosine phosphatases to potentiate VSOAC activation (Sinning *et al.* 1997; Crepel *et al.* 1998; Mongin *et al.* 1999a; Deleuze *et al.* 2000; Morales-Mulia *et al.* 2001; Heacock *et al.* 2004; Cohen 2005). A significant body of evidence obtained from non-neural cells suggests that both receptor TKs (such as epidermal growth factor; Franco *et al.* 2004) and non-receptor TKs and may also play an important role in GPCR regulation of osmolyte release, with a member of the *src* family being the preferred candidate (see Vazquez-Juarez *et al.* 2008b). These observations are consistent with the known 'cross-talk' between GPCR and TK signaling pathways (Luttrell and Luttrell 2004). However, only a limited amount of information is available regarding the possible involvement of TK activity in GPCR regulation of osmolyte release in neural cells and some of this is conflicting. For example, although ATP-stimulated D-aspartate release from astrocytes is inhibited by 50–60% following inclusion of either the tyrphostin A51 or PP2, a *src* TK inhibitor, this is only observed under conditions of limited cell swelling (5% reduction in osmolarity). When cells are exposed to a larger reduction in osmolarity (30%), these inhibitors are without effect (Mongin and Kimelberg 2005). In contrast, in SH-SY5Y cells, mAChR-mediated stimulation of taurine and aspartate release, monitored under conditions of a 30% reduction in osmolarity, is inhibited by 60–70% by inclusion of the tyrphostin AG-18, whereas the inactive analog AG-9 is without effect. Further evidence for the involvement of TKs was obtained from experiments in which inhibitors of tyrosine phosphatase activity potentiated mAChR-mediated osmolyte release (Heacock *et al.* 2004).

There is also evidence from non-neural cells, particularly fibroblasts, for the involvement of the Rho family of monomeric G-proteins in osmolyte release (Vazquez-Juarez *et al.* 2008a). These small molecular weight GTP-binding proteins are involved in the reorganization of the actin cytoskeleton, a process that occurs during cell swelling. However, disruption of the actin cytoskeleton with cytochalasin does not attenuate osmolyte release from neural preparations (Kimelberg *et al.* 1990). Furthermore, in SH-SY5Y cells, pre-incubation with toxin B (an inhibitor of rac, rho, and cdc42) under conditions established to disrupt the cytoskeleton in these cells (Linseman *et al.* 1998, 2000, 2001) failed to attenuate lysophospholipid-stimulated taurine efflux (Heacock *et al.* 2006b).

Although non-receptor-mediated increases in cAMP have been reported to facilitate osmolyte efflux or swelling-activated Cl^- current in several tissues (Strange *et al.* 1993; Shimizu *et al.* 2000; Moran *et al.* 2001), there are currently only three documented examples of the ability of cyclase-linked GPCRs to enhance volume-dependent osmolyte release. Thus norepinephrine, acting via a β-adrenergic receptor, has been reported to increase taurine release from flounder erythrocytes and cortical astrocytes (Thoroed *et al.* 1995; Moran *et al.* 2001). In murine *Ltk* fibroblasts, prostaglandin E_1 stimulates the osmosensitive release of taurine via an EP_2 receptor. This effect was mimicked by the addition of cAMP analogs and attenuated in a mutated Ltk fibroblast cell line (RAB-10) in which the regulatory subunit of PKA exhibited a substantial reduction in its affinity for cAMP (Heacock *et al.* 2006a). In summary, it appears likely that GPCR regulation of osmolyte efflux from neural cells occurs via several distinct mechanisms and that Ca^{2+}, PKC, PKA and TK activity play a major role in this process.

Physiological and pathophysiological consequences of the receptor-stimulated efflux of organic osmolytes

When released in response to hypotonicity, several of the quantitatively major organic osmolytes in the CNS, (e.g. glutamate, aspartate, GABA, or glycine) could potentially activate their respective receptor subtypes present on nearby neurons and/or glia. Even taurine, which is often considered to be relatively inert, has been reported to regulate glycine, $GABA_A$, $GABA_B$, and *N*-methyl D-aspartate receptors (Albrecht and Schousboe 2005; Jia *et al.* 2008). Thus, in addition to providing a means whereby adaptations in cell volume are facilitated, the possibility that GPCR-mediated efflux of organic osmolytes may result in a number of downstream consequences, needs to be considered. In this context, three examples are discussed below:

Vasopressin regulation of osmosensitive taurine efflux from the neurohypophysis

In response to a reduction in plasma osmolarity, vasopressin release from the neurohypophysis ceases and as a consequence, an increased diuresis occurs at the kidney. Astrocytes present in the neurohypophysis are highly enriched in taurine (Pow *et al.* 2002) and the osmolyte is readily released in response to hypoosmolarity (Rosso *et al.* 2004). Once released, taurine then can activate glycine receptors present on nerve terminals that originate in the supraoptic nucleus and this results in an inhibition of vasopressin release (Hussy *et al.* 2001). Vasopressin, acting via V_{1A} receptors and a rise in $[Ca^{2+}]_i$, results in a further potentiation of the volume-dependent release of taurine. Thus, the ability of vasopressin, (along with co-released ATP) to stimulate taurine efflux provides a powerful negative feedback loop that, in a paracrine fashion, inhibits the further release of the neuropeptide (Rosso *et al.* 2004). Conversely, adenosine, a breakdown product of ATP, can inhibit the release of taurine from pituicytes through a mechanism involving the A_{2B} adenosine receptor and a rise in cAMP formation, thereby providing an additional means whereby vasopressin release can be regulated (Pierson *et al.* 2007).

ATP regulation of osmosensitive glutamate release from astrocytes

ATP is a major neurotransmitter in the CNS and is released from neurons during synaptic activity or, alternatively, from astrocytes where it functions either as a paracrine signaling molecule for nearby glia or as a glial-neuronal signaling molecule (Fields and Burnstock 2006). ATP addition significantly increases the ability of cortical astrocytes to release glutamate (often monitored as D-aspartate) in response to hypoosmolarity, an effect mediated via P_{2Y} purinergic receptors (Mongin and Kimelberg 2002, 2005; Takano *et al.* 2005). However, this situation is potentially complicated by the fact that, when substantially swollen, astrocytes themselves can release ATP, which then acts in an autocrine fashion to further enhance glutamate release (Darby *et al.* 2003). Although efficient inactivation mechanisms exist for removal of glutamate from the ECS, even modest increases in the concentration of glutamate could alter neuronal synaptic transmission and impact nearby glia. Paradoxically, as activation of metabotropic glutamate receptors on glial cells is reported to result in cell swelling (Hansson 1994), glutamate release may actually perpetuate cell swelling. Glutamate released upon cell swelling is reported to contribute to the rate of propagation of spreading depression, a slow wave of astrocyte and neuronal depolarization that leads to synaptic depression (Basarsky *et al.* 1999). A sustained increase in glutamate may also lead to neuronal death, so-called excitotoxicity.

Thrombin stimulation of osmolyte release from astrocytes and neurotumor cells

In the CNS, thrombin can exert neuroprotective effects, at low concentrations (50 pM to 100 nM), whereas higher concentrations of the protease can elicit degeneration and cell death (Xi *et al.* 2003). Addition of thrombin facilitates the osmosensitive release of glutamate and taurine from astrocytoma, neuroblastoma and cortical astrocyte preparations (Cheema *et al.* 2005, 2007; Ramos-Mandujano *et al.* 2007; Cheema and Fisher 2008). The effects of thrombin are mediated by the PAR-1 receptor subtype in all three tissues and require subnanomolar or low nM concentrations of thrombin for half-maximal activation of osmolyte release, results that are consistent with a neuroprotective role for the protease. However, it should be noted that under certain circumstances, low doses of thrombin may actually exacerbate rather than reduce brain injury. For example, injection of low doses of thrombin into the brain greatly enhances ischemia-, iron-, 6-hydroxydopamine-, or tissue plasminogen activator-induced brain injury (Figueroa *et al.* 1998; Hua *et al.* 2003a; Nakamura *et al.* 2005; Cannon *et al.* 2007). While the mechanism of this enhanced injury remains to be established, an enhanced efflux of glutamate via thrombin-induced VSOAC activation may play a role.

A role for VSOAC in osmolyte release under isotonic conditions?

Although VSOAC is strongly activated by reductions in osmolarity, it is now evident that even under conditions of isotonicity, the same channel can mediate the release of osmolytes, when activated by a GPCR. Thus, when cortical astrocytes are incubated under isotonic conditions, the addition of ATP results in an increased efflux of glutamate

and aspartate via a mechanism that is dependent upon intracellular Ca^{2+} and can be attenuated by inclusions of inhibitors of VSOAC such as NPPB, flufenamine, and gossypol (Jeremic *et al.* 2001; Takano *et al.* 2005). Although ATP addition elicits a smaller efflux of organic osmolytes than does hypotonicity, the profile of amino acids released is qualitatively similar (Takano *et al.* 2005). These results suggest that GPCRs can facilitate osmolyte release under isotonic conditions and that the release of neuroactive amino acids such as glutamate or aspartate may represent a mechanism for inter-cellular communication (Mulligan and MacVicar 2006).

If VSOAC is operational under conditions of isotonicity, what other factors might influence its activity? One possibility is that the activity of VSOAC, like that of other ion channels, is regulated by cholesterol availability. There is evidence that depletion of cholesterol from non-neural cells can facilitate VSOAC activity in response to hypotonicity (Levitan *et al.* 2000; Romanenko *et al.* 2004; Byfield *et al.* 2006). This has led to the proposal that cholesterol content regulates the equilibrium between the closed and open states of VSOAC, with cholesterol depletion favoring the open channel (Levitan *et al.* 2000). Although the loss of cholesterol also facilitates swelling-activated taurine release from SH-SY5Y cells, a more noteworthy effect of sterol depletion was a threefold increase in the ability of PAR-1 receptors to stimulate osmolyte release under isotonic conditions (Cheema and Fisher 2008). These results suggest that cholesterol availability (which is regulated by dietary, drug, and genetic factors) and GPCR activation may act in concert to promote the efflux of osmolytes under isotonic conditions.

Volume-sensitive organic osmolyte and anion channel may also mediate the release of neuroactive organic osmolytes that is triggered following episodes of ischemic insult under isotonic conditions (Mongin 2007). Anion channel blockers that prevent volume-dependent osmolyte release also inhibit the release of amino acids from cerebral cortex that occurs during global ischemia (Phillis *et al.* 1997, 1998). Tamoxifen, an inhibitor of VSOAC, has also been reported to offer neuroprotection following focal cerebral ischemia (Zhang *et al.* 2005). Recently, Kimelberg and colleagues have demonstrated that DCPIB, the most specific inhibitor of VSOAC currently available, reduces the infarct size following cerebral arterial occlusion (Zhang *et al.* 2008).

Can activation of GPCRs also regulate the influx of osmolytes?

Until recently, consideration of the possibility that osmotic stress results in a change in the transport of organic osmolytes into cells has largely been restricted to conditions of hypertonic stress, which have been demonstrated to result in the increased expression and activity of both the sodium-dependent myo-inositol transporter (Ibsen and Strange 1996; Fisher *et al.* 2002) and taurine transporter (Han *et al.* 2006). However, a recent study in hippocampal neurons has raised the possibility that hypotonic stress down-regulates taurine transport (Olson and Martinho 2006), a finding consistent with a previous study of organic osmolyte release from ascites tumor cells (Hoffmann and Lambert 1983). However, the mechanism underlying the regulation of the taurine transporter by hypoosmolarity remains to be determined. Under hypotonic conditions, the net loss of osmolytes from brain is the sum of changes in efflux and uptake. Although inorganic osmolytes are released from cultured neural cells to the same or greater extent than is observed for organic osmolytes under both basal (swelling-activated) and receptor-stimulated conditions (Abdullaev *et al.* 2006; Cheema *et al.* 2007), chronic hyponatremia results in a disproportionately greater percentage loss of organic

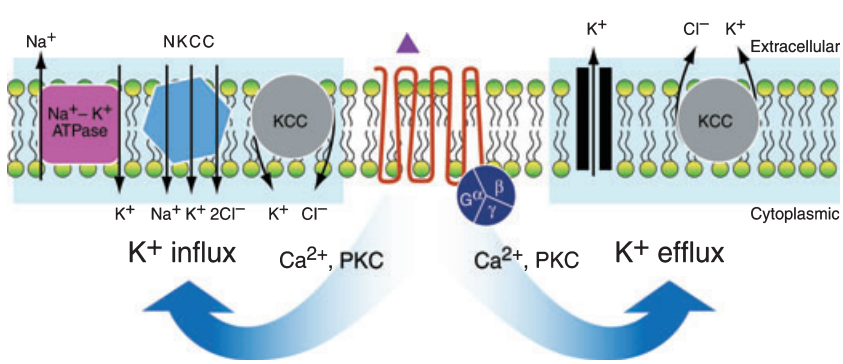

Fig. 4 Activation of G-protein-coupled receptors (GPCRs) in SH-SY5Y cells facilitates both the efflux and influx of K^+ under conditions of hypoosmolarity. Receptor-mediated K^+ efflux occurs via the activation of K^+ channels and K^+–Cl^- cotransporter (KCC), whereas GPCRs increase K^+ influx via a mechanism that involves Na^+–K^+ ATPase, NKCC, and KCC. Under mildly hypoosmotic conditions, receptor-mediated K^+ influx counters K^+ efflux such that little or no net loss of K^+ occurs. Under more pronounced reductions in osmolarity, receptor-mediated K^+ efflux exceeds that of influx, which leads to a loss of cellular K^+ (Foster *et al.* 2008).

osmolytes from the brain (Lien *et al.* 1991; Videen *et al.* 1995; Pasantes-Morales *et al.* 2002; Massieu *et al.* 2004). Furthermore, when astrocytes are cultured in hypoosmotic media, organic osmolytes, but not K^+, are lost from the cells (Olson 1999). One potential explanation for this apparent paradox is that, under hypoosmotic conditions, the volume-dependent efflux of inorganic osmolytes is accompanied by a compensatory uptake phase, as originally proposed by Mongin *et al.* (1994, 1996). Recently, it has been determined that the activation of mAChRs (and other GPCRs) facilitates both the efflux and influx of K^+ from human SH-SY5Y neuroblastoma cells in an osmosensitive manner (Foster *et al.* 2008). However, whereas K^+ channels and the KCC transporter mediate the efflux of K^+, GPCR-stimulated influx of K^+ is mediated via the Na^+–K^+–$2Cl^-$ cotransporter, KCC and ouabain-sensitive Na^+–K^+–ATPase activities (Fig. 4). Measurement of K^+ mass indicates that under conditions of either isotonicity or limited reductions in osmolarity (15%), GPCR-mediated efflux of K^+ is effectively countered by an influx of K^+, such that no net loss of cell K^+ occurs. Only under more pronounced reductions in osmolarity (30%) did the rate of K^+ efflux exceed that of influx and result in a net loss of K^+ (Foster *et al.* 2008). These results raise the intriguing possibility that receptor activation can dynamically regulate both the volume-dependent release and re-uptake of osmolytes.

Conclusions

Although volume regulation has previously been considered to be an intrinsic property of cells, it is now evident that many extracellular agonists, acting via GPCRs, can profoundly influence this process in both neural and non-neural cells. GPCRs represent the largest family of membrane proteins and approximately 300 such receptors are expressed in the adult mouse brain (Furuta *et al.* 2007). The observation that activation of a diverse array of GPCRs can facilitate volume-dependent osmolyte release points to a role for these receptors in osmoregulation in the brain, a tissue particularly vulnerable to osmotic disturbances. Furthermore, the ability of receptor activation to both increase osmolyte release and to lower the osmotic threshold at which efflux occurs raises the possibility that tonic activation of GPCRs on neural cells may provide a mechanism whereby cells are able to more efficiently respond to small reductions in osmolarity. Although the majority of receptors identified thus far appear to increase osmolyte release and thereby to facilitate volume correction, it is probable that other GPCRs are negatively coupled to osmolyte release, e.g. A_{2B} adenosine receptors in pituicytes (Pierson *et al.* 2007). The possibility that cross-talk between GPCRs could provide an additional means to fine-tune the volume-dependent output of osmolytes from neural cells, along with recent evidence indicating that GPCRs may also facilitate the uptake of osmolytes, further highlights the potential importance of this family of receptors in osmoregulation.

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