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Ion dependence of ligand binding to metabotropic glutamate receptors

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Abstract

The ionic requirements for ligand binding to metabotropic glutamate receptors were carried out on secreted truncated receptors containing only the extracellular ligand binding domains of the receptors. The influence of ions on agonist binding was examined in mGluR1, mGluR3, and mGluR4 representing Group I, II, and III metabotropic glutamate receptors, respectively. [^{3}H]Quisqualic acid binding to mGluR1 required the presence of calcium (or magnesium) ions but not sodium or chloride ions while [^{3}H]DCG-IV binding to mGluR3 was dependent upon both cations and anions. [^{3}H]L-AP4 binding to mGluR4 required chloride ions but not monovalent or divalent cations. The EC_{50} for chloride facilitation of L-AP4 binding to mGluR4 was 63 mM; this value is approximately one-half of the normal resting extracellular chloride concentration. These results demonstrate that metabotropic glutamate receptor subtypes require different complements of ions for ligand binding and suggest that natural physiological fluctuations in synaptic ion concentrations may regulate receptor binding and activation.

Keywords: G-protein coupled receptor; Chloride; Calcium; Binding pocket; Family 3; Venus flytrap domain

Many neurotransmitter receptors, including G-protein coupled receptors, have specific ionic requirements for the optimal binding of ligands and drugs. Several receptors in subfamily 3 (also known as Family C) of the G-protein coupled receptor superfamily are activated by or are modulated by cations. For example, the calcium-sensing receptor, which regulates calcium homeostasis in the body, is activated by calcium and magnesium. The functional responses of other Family 3 receptors such as the metabotropic glutamate receptors (mGluRs) and the $GABA_B$ receptor are modulated by divalent cations. Calcium ions modulate the binding of some agonists but not antagonists to the $GABA_B$ receptor. In the absence of calcium, the affinity of GABA (but not the agonist baclofen) is reduced 10-fold [1]. The observation that mutation of serine 269 in the $GABA_B$ R1 subunit eliminates the enhancing effects of calcium indicates that calcium and GABA may share overlapping binding sites [1].

The responsiveness of Group I mGluRs to agonists is also modulated by calcium. Studies with the cloned mGluR1a receptor subtype have shown that calcium directly activated the receptor [2,3] or enhanced agonist-stimulated activity [4]. Other investigations showed that protracted stimulation of heterologously expressed mGluR1 by glutamate required extracellular calcium ions [5,6]. In cerebellar Purkinje neurons, calcium stimulated the mGluR1-mediated response and enhanced the dynamic range of an mGluR1-mediated cation current and intracellular calcium mobilization [7,8].

Relatively little information is available on the requirements or effects of other ions on the other subtypes of mGluRs. Chloride ions have been shown to stimulate the binding of the Group III mGluR agonist [^{3}H]L-AP4 to mGluR4 [9], and both chloride and bromide ions have been shown to facilitate the binding of the mGluR antagonist [^{3}H]LY341495 to Group II [10] and Group III mGluRs [11]. In the case of [^{3}H]LY341495, binding to Group II mGluRs [10], and to a lesser extent with the Group III mGluRs [11], nitrate anions also stimulated binding to the full-length human receptors.

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In the present study, we conducted a side-by-side comparative analysis of the effects of various ions on the binding of high affinity agonists to the mGluRs. Three radiolabeled probes including [^{3}H]quisqualic acid, [^{3}H]DCG-IV, and [^{3}H]L-AP4 were used to study the ionic requirements of agonist binding to truncated forms of mGluR1, mGluR3, and mGluR4 representing Group I, II, and III mGluRs, respectively. The results demonstrate that each mGluR has a unique profile of ion requirements for ligand binding. The observation that the EC_{50} value for chloride facilitation of agonist binding to mGluR4 is only about 2-fold lower than the resting extracellular chloride concentration suggests the possibility of an endogenous regulatory role for this ion in modulating the activation of Group III mGluRs in the brain.

Experimental procedures

Materials. Tris, NaCl, and Hepes were from the EM Science (Gibbstown, NJ, USA). $MgCl_2{\cdot}6H_2O$, $Ca(NO_3)_2$, $Mg(NO_3)_2$, $NaNO_3$, NaBr, L-glutamate, and choline chloride were purchased from Sigma–Aldrich Co. $CaCl_2{\cdot}2H_2O$, KCl, and sodium acetate were obtained from BDH Inc. (Toronto, Canada). $NaHCO_3$ was from ACP Chemicals Inc. (Montreal, Canada). L-Serine-*O*-phosphate, [^{3}H]L-AP4 (specific activity 58 Ci/mmol), and [^{3}H]DCG-IV (specific activity 18.3 Ci/mmol) were purchased from Tocris Cookson Ltd. [^{3}H]Quisqualate (specific activity 26 Ci/mmol) was from Amersham Pharmacia Biotech UK Ltd. (Buckinghamshire, England).

Expression constructs. The full-length rat mGluR1a cDNA in the pCis vector was modified as follows to obtain the untagged truncated receptor containing the Venus flytrap domain from the start codon to serine 522. A PCR was performed on mGluR1 in pCis using a forward primer (5′-CGG GATCCGAGAGGGGCAGTAGTGGAGGCAGAG-3′) and a reverse primer (5′-GCTCTAGATCGTACCATTCCGCTTTTGTTCATC-3′). Restriction sites for *Bam*HI and *Xba*I were incorporated in the primer sequences. The PCR product was subjected to restriction digestion by *Bam*HI and *Xba*I to yield a 1.6-kb fragment which was subcloned into the pcDNA3.0 mammalian expression vector (Invitrogen Corp.).

The truncated rat mGluR3 receptor encompassing the Venus flytrap domain from the start codon to tyrosine 437 was produced from the full-length mGluR3. The full-length mGluR3 in pcDNA3.0 *c-myc* tagged downstream of the signal peptide as previously described [12,13] was used as the template for a PCR with a forward primer: 5′-AGGGGTACCGA GCTCGGATCCACTAGTAAC-3′ and a reverse primer: 5′-AACCGCT CGAGTCAATAGGGCTCACAGGGGATACAG-3′. *Kpn*I and *Xho*I restriction sites were incorporated into the primer sequences. The PCR products were then digested with *Kpn*I and *Xho*I, and ligated into the pcDNA3 to produce the final construct *c-myc*-tagged extracellular domain of mGluR3 in pcDNA3. The construction of the *c-myc*-tagged Venus flytrap domain of mGluR4 in pcDNA3 was described previously [14].

Transient transfections. Plasmid DNAs for transfections were prepared using QIAspin Miniprep Kit (Qiagen) and transiently transfected into HEK-293 or HEK-293 TSA-201 cells in 100 mm plates using a calcium phosphate method as described previously [14]. Twenty-four hours after transfection, the cell culture medium containing minimal essential medium (Life Technologies, Inc.) supplemented with 6% fetal bovine serum (Hyclone, Inc.) and antibiotics was replaced with 7 ml of OptiMEM (Life Technologies, Inc.). Forty-eight hours after transfection, the culture media containing the secreted truncated receptors were collected and processed as described below.

Soluble protein preparation and radioligand binding assays. The normal binding buffers were as follows: mGluR1, Hepes 40 mM, and $CaCl_2$ 2.5 mM, pH = 7.5, [15]; mGluR3, Tris 50 mM, $MgCl_2$ 2 mM, and $CaCl_2$ 2 mM, pH = 7.0, [13]; mGluR4, Hepes 30 mM, NaCl 110 mM, $MgCl_2$ 1.2 mM, KCl 5 mM, and $CaCl_2$ 2.5 mM, pH = 8.0, [9,16] containing 0.1 mM phenylmethylsulfonyl fluoride. The cell culture medium containing the secreted receptor protein was collected and centrifuged at 48,400*g* for 20 min at 4 °C. The supernatant was dialyzed at 4 °C in 1000 ml of normal binding buffer (changed 3× over a 24 h period). The samples were then centrifuged at 48,400*g* for 45 min at 4 °C. One hundred microliters of the dialyzed media overlaying transfected HEK cells was mixed with 100 μl of binding buffer, and 25 μl of 10× competing drug (for blanks to define non-specific binding) or 25 μl of assay buffer (for total binding), and 25 μl of 10× radioligand in all binding assays in a final total assay volume of 250 μl.

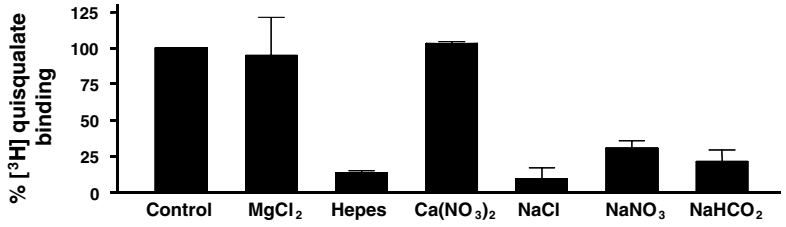
The binding assay for mGluR1 was carried out by incubating the sample with [^{3}H]quisqualic acid (20 nM) for 40 min at room temperature followed by an incubation on ice for 15 min; non-specific binding was defined by the presence of 500 μM of L-glutamate. The mGluR3 binding assay was conducted using 25 nM [^{3}H]DCG-IV while non-specific binding was defined by the presence of 500 μM of L-glutamate. The [^{3}H]DCG-IV assay was carried out as described above for the mGluR1 binding assay. The binding assay for truncated mGluR4 was performed as described previously [14] using 30 nM [^{3}H]L-AP4 and non-specific binding was defined by the presence of 300 μM of L-SOP. In all three binding assays, following the appropriate incubation times and conditions, 340 μg of γ-globulin and 200 μl of 30% cold polyethylene glycol (PEG) were added to each sample. The mixture was incubated on ice for 4 min and centrifuged at 13,200*g* for 4 min at 4 °C. The supernatant fraction was aspirated. The pellet was washed with 500 μl of cold 15% PEG and solubilized in 500 μl of 1 M NaOH for overnight. The samples were counted using a Canberra Packard TriCarb 2100 liquid scintillation counter.

For ion replacement, the normal binding buffers were replaced via dialysis with individual buffers containing different compositions of ions. The ion substituted or modified buffers used in the dialysis were also used in the radioligand binding assays. The chloride stimulation curves for mGluR4 were generated using 30 nM [^{3}H]L-AP4 and increasing concentrations of choline chloride and Hepes buffer (30 mM, pH = 8.0) were used for both dialysis and binding. Radioligand binding data were analyzed using GraphPad Prism 3.0 (GraphPad Software, Inc.).

Results

The effects of ions on the binding of [^{3}H]quisqualic acid to truncated rat mGluR1

[^{3}H]Quisqualic acid is a high affinity selective agonist for Group I mGluRs (mGluR1 and mGluR5 [15]). No specific binding was detected in the media collected from mock transfected HEK cells while a high level of binding was consistently observed in the media collected from cells transfected with truncated rat mGluR1a (5.8 ± 0.9 pmol bound/mg protein). The base (control) buffer consisted of Hepes buffer containing 2.5 mM $CaCl_2$. Ion depletion and substitution experiments indicated that the level of binding was reduced by 90% in the absence of $CaCl_2$ (Hepes only condition, Fig. 1). Magnesium chloride and calcium nitrate could completely substitute for $CaCl_2$ and restore binding to control levels. The replacement of $CaCl_2$ with an equimolar concentration of sodium chloride produced a low level of binding that was similar to that seen with Hepes only, while the addition of either sodium nitrate or sodium bicarbonate produced only small increases in binding above the level observed in the Hepes only condition. These results indicated that the binding of [^{3}H]quisqualic acid to the extracellular domain of mGluR1



	Control	$MgCl_2$	Hepes	$Ca(NO_3)_2$	NaCl	$NaNO_3$	$NaHCO_2$
Hepes (40 mM)	V	V	V	V	V	V	V
$MgCl_2 \cdot 6H_2O$ (2.5 mM)	X	V	X	X	X	X	X
NaCl (5 mM)	X	X	X	X	V	X	X
$NaNO_3$ (5 mM)	X	X	X	X	X	V	X
$NaHCO_3$ (5 mM)	X	X	X	X	X	X	V
$CaCl_2 \cdot 2H_2O$ (2.5 mM)	V	X	X	X	X	X	X
$Ca(NO_3)_2$ (2.5 mM)	X	X	X	V	X	X	X
pH = 7.5 with NaOH	V	V	V	V	V	V	V

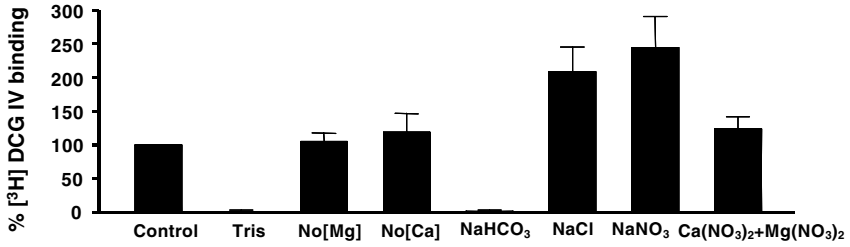
Fig. 1. Ion dependency of [^{3}H]quisqualic acid binding to truncated mGluR1. Assays were performed using 20 nM [^{3}H]quisqualic acid. The results are means ± SEM of 2 and 3 experiments conducted in triplicate on the truncated soluble receptor. The normal (control) binding buffer consisted of 40 mM Hepes with 2.5 mM $CaCl_2$ (pH = 7.5). The table below the figure lists the ion ingredients of each buffer tested (V, present; X, absent).

requires divalent cations such as calcium or magnesium but not the monovalent cation sodium or chloride anions.

The effects of ions on the binding of [^{3}H]DCG-IV to truncated rat mGluR3

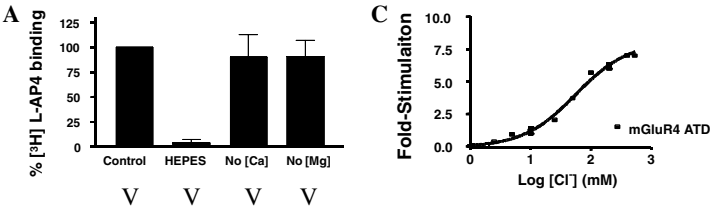
DCG-IV is a selective high affinity agonist for Group II mGluRs (mGluR2 and mGluR3 [17]). No specific [^{3}H]DCG-IV binding was detected in membrane fractions from mock transfected HEK cells [12] or in the soluble fraction (media) overlaying mock transfected HEK cells (data not shown). In HEK cells transfected with truncated mGluR3, a high level of binding was observed (5.9 ± 0.6 pmol/mg protein). The base buffer consisted of 50 mM Tris with 2 mM $MgCl_2$ and 2 mM $CaCl_2$. When both calcium chloride and magnesium chloride were excluded from the binding buffer, virtually no specific binding was detected (Fig. 2). However, when only one of these salts was replaced with NaCl to retain the same chloride ion concentration, binding was maintained at a level that was similar to that seen in control conditions (i.e., with base buffer).

Several additional conditions were tested; replacement of $CaCl_2$ and $MgCl_2$ with the corresponding nitrate salts maintained the levels of binding seen with the chloride salts (Fig. 2). Replacement of $CaCl_2$ and $MgCl_2$ with NaCl or $NaNO_3$ stimulated [^{3}H]DCG-IV binding to mGluR3 above the level seen in the control condition. In contrast, the replacement of $CaCl_2$ and $MgCl_2$ with sodium bicarbonate eliminated detectable specific binding (Fig. 2). These results indicate that [^{3}H]DCG-IV binding to mGluR3 requires either divalent or monovalent cations along with either chloride or nitrate but not bicarbonate anions.

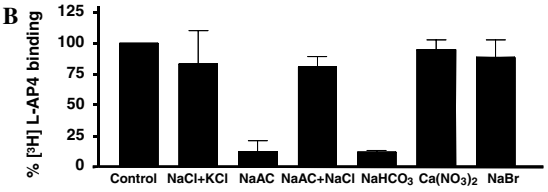


	Control	Tris	No[Mg]	No[Ca]	$NaHCO_3$	NaCl	$NaNO_3$	$Ca(NO_3)_2$+$Mg(NO_3)_2$
Tris (50 mM)	V	V	V	V	V	V	V	V
$MgCl_2 \cdot 6H_2O$ (2 mM)	V	X	X	V	X	X	X	X
$Mg(NO_3)_2$ (2 mM)	X	X	X	X	X	X	X	V
NaCl (4 mM)	X	X	V	V	X	2 times	X	X
$NaHCO_3$ (8 mM)	X	X	X	X	V	X	X	X
$NaNO_3$ (8 mM)	X	X	X	X	X	X	V	X
$CaCl_2 \cdot 2H_2O$ (2 mM)	V	X	V	X	X	X	X	X
$Ca(NO_3)_2$ (2 mM)	X	X	X	X	X	X	X	V
pH = 7.0 with HAC	V	V	V	V	V	V	V	V

Fig. 2. Ion dependency of [^{3}H]DCG-IV binding to truncated mGluR3. Assays were performed using 25 nM [^{3}H]DCG-IV. The results are means ± SEM of 2 and 3 experiments conducted in triplicate on the truncated receptor. The normal (control) binding buffer contained 50 mM Tris, 2 mM $MgCl_2$, and 2 mM $CaCl_2$ (pH = 7.0). The table below the figure lists the ion ingredients of each buffer tested (V, present; X, absent).



Hepes (40 mM)	V	V	V	V
$MgCl_2 \cdot 6H_2O$ (1.2 mM)	V	X	V	X
NaCl (110 mM)	V	X	V	V
$CaCl_2 \cdot 2H_2O$ (2.5 mM)	V	X	X	V
KCl (5 mM)	V	X	V	V
pH = 7.5 with NaOH	V	V	V	V



Hepes (40 mM)	V	V	V	V	V	V	V
$MgCl_2 \cdot 6H_2O$ (1.2 mM)	V	X	X	X	X	X	X
NaCl (110 mM)	V	V	X	V	X	X	X
NaAC (110 mM)	X	X	V	V	X	X	X
$NaHCO_3$ (110 mM)	X	X	X	X	V	X	X
NaBr (110 mM)	X	X	X	X	X	X	V
$CaCl_2 \cdot 2H_2O$ (2.5 mM)	V	X	X	X	X	X	X
$Ca(NO_3)_2$ (2.5 mM)	X	X	X	X	X	V	X
KCl (5 mM)	V	V	X	X	X	X	X
pH = 7.5 with NaOH	V	V	V	V	V	V	V

Fig. 3. Ion requirements of [^{3}H]L-AP4 binding to truncated mGluR4. Assays were performed using 30 nM [^{3}H]L-AP4. In each panel, the results are means ± SEM of three experiments conducted in triplicate. (A) Ion dependency of [^{3}H]L-AP4 binding to truncated mGluR4. (B) Additional ion substitutions for [^{3}H]L-AP4 binding to mGluR4. (C) Chloride-dependency curve for [^{3}H]L-AP4 binding to truncated mGluR4. Choline chloride was added in the concentrations indicated. The EC_{50} value generated from the curves was 63 ± 4 mM.

The effects of ions on the binding of [^{3}H]L-AP4 to truncated rat mGluR4

The agonist [^{3}H]L-AP4 can be used to study the pharmacological properties of the Group III mGluRs, mGluR4, and mGluR8 [9,16,18]. As seen with mGluR1 and mGluR3, in the absence of added ions, only a minimal level of [^{3}H]L-AP4 binding was observed in HEK cells expressing rat mGluR4. However, the pattern of ion dependency of [^{3}H]L-AP4 binding to truncated mGluR4 was different from that observed with [^{3}H]quisqualic acid binding to mGluR1 and [^{3}H]DCG-IV binding to mGluR3. Chloride containing buffers were required for [^{3}H]L-AP4 binding to mGluR4 (Fig. 3). However, bromide and nitrate appeared to be viable substitutes for chloride anions (Fig. 3B). In contrast, buffers containing acetate and bicarbonate as the anions showed only low levels of specific binding. Divalent cations were not essential for [^{3}H]L-AP4 binding to mGluR4 because buffers excluding both calcium and magnesium (e.g., NaCl + KCl, sodium acetate + NaCl, or sodium bromide, Fig. 3B) were capable of maintaining binding at control levels.

The chloride-dependency of [^{3}H]L-AP4 binding to mGluR4 was examined further by generating EC_{50} values for chloride. In these experiments, increasing concentrations of choline chloride were incubated in the [^{3}H]L-AP4 binding assay (Fig. 3C). The EC_{50} value calculated was 63 ± 4 mM ($N = 3$).

Discussion

To circumvent potential complications from the transmembrane and intracellular domains of the proteins, radioligand binding studies were carried out on soluble truncated mGluRs that contained only the Venus flytrap domains of mGluR1, mGluR3, and mGluR4. Each of the three mGluRs examined in this study showed a unique pattern of ion dependency for agonist binding. Our results demonstrate that divalent cations but not the monovalent cation sodium or chloride anions are required for the binding of [^{3}H]quisqualic acid to mGluR1.

The requirement for calcium in the activation of Group I mGluRs coupled to phosphoinositide turnover and release of intracellular calcium has been somewhat controversial. Evidence that calcium is required for the activation of mGluR1 and that a serine in the binding pocket mediates the binding of calcium has been presented by Kubo et al. [2], while Francesconi and Duvoisin [3] also

showed that mGluR1 was activated by Ca^{2+} in the absence of glutamate and this activation was antagonized by Mg^{2+}. However, the results from Nash et al. [6] suggested that calcium enhances agonist binding to mGluR1 and is necessary for the continuous stimulation of the receptor but is not absolutely obligatory for receptor activation. In another study, it was reported that [^{3}H]quisqualic acid binding to the extracellular domain of mGluR1 was unaffected by the presence or absence of calcium [19]. However, in light of the present results demonstrating that magnesium can substitute for calcium, it is possible that magnesium ions present in the binding assay in the study by Selkirk et al. could have maintained [^{3}H]quisqualic acid binding in the absence of calcium.

The ionic requirements for [^{3}H]DCG-IV binding to mGluR3 appeared to be more complex than the ion dependency of mGluR1 or mGluR4. The presence of cations was necessary for the detection of [^{3}H]DCG-IV binding to mGluR3; however, this requirement could be fulfilled by the presence of either monovalent or divalent cations. Zinc is another cation that was previously reported to modulate Group II mGluR binding in recombinant expression systems and in brain tissue sections. With full-length mGluR3 expressed in HEK-293 cells, zinc induced a complex biphasic effect on the binding of the Group II agonist [^{3}H]LY354740, showing a reduction in binding at lower zinc concentrations and an enhancement at higher concentrations. In contrast, using the same radioligand, zinc ions exerted biphasic inhibition on binding to mGluR2 [20]. Histidine 56 in mGluR2, which is located at the entrance of the ligand binding pocket, was identified as a major determinant of the zinc effect, and it was proposed that zinc likely affected orthosteric agonist binding via an allosteric mechanism [20].

In our experiments, we found that anions were also required to support [^{3}H]DCG-IV binding to mGluR3; chloride or nitrate but not bicarbonate anions facilitated [^{3}H]DCG-IV binding to mGluR3. Binding of the antagonist [^{3}H]LY341495 to full-length mGluR3 was also reported to be enhanced by both chloride and bromide ions [10,11]. Unlike the effects we observed with [^{3}H]L-AP4 binding to mGluR4 (see below), [^{3}H]DCG-IV binding to mGluR3 could not be further increased with increasing concentration of chloride ions (data not shown).

Chloride ions but not cations were obligatory for [^{3}H]L-AP4 binding to mGluR4. The chloride-dependency of mGluR4 suggests a potentially important role for this ion in the functional activity and physiological properties of this receptor, and possibly with other Group III mGluRs (mGluR6, mGluR7, and mGluR8). The experimentally determined affinity for chloride in modulating agonist binding to mGluR4 (63 mM) is approximately one-half the resting extracellular chloride concentration in the adult CNS. Therefore under normal conditions in the synapse, the chloride site on mGluR4 would be saturated.

In the mammalian CNS, mGluR4 is located presynaptically on both glutamatergic and GABAergic synapses [21–23] where it acts to inhibit neurotransmitter release [24,25]. Electrophysiological studies [26] and immunocytochemical analyses [23] have shown that mGluR4 is present on GABAergic interneurons and may regulate GABA release from these cells, suggesting the possibility of a regulatory role for Group III mGluRs on GABAergic transmission. In immature neurons, activation of postsynaptic $GABA_A$ receptors results in the efflux of intracellular chloride ions into the synaptic cleft whereas the flow of chloride ions is reversed in mature cells [27]. It is conceivable that under conditions of high neuronal activity, the $GABA_A$ receptor channel and/or other chloride channels or transporters such as the potassium-chloride co-transporter 2 (KCC2) [28–30] may mediate fluctuations in the level of chloride ions at synapses where these proteins are highly expressed.

Our findings suggest the possibility that a transient reduction in the synaptic chloride concentration could reduce agonist binding to mGluR4 and perhaps other Group III mGluRs. Reduced binding to Group III receptors would have the net effect of increasing neurotransmitter release. At GABAergic synapses, a dynamic level of synaptic chloride may modulate the binding of L-glutamate released from adjacent terminals (neurotransmitter spillover) to activate presynaptic Group III mGluRs; this arrangement could operate as a feedback mechanism for regulating GABAergic transmission.

Acknowledgments

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