A selective metabotropic glutamate receptor 7 agonist: Activation of receptor signaling via an allosteric site modulates stress parameters *in vivo*

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Metabotropic glutamate receptor (mGluR) subtypes (mGluR1 to mGluR8) act as important pre- and postsynaptic regulators of neurotransmission in the CNS. These receptors consist of two domains, an extracellular region containing the orthosteric agonist site and a transmembrane heptahelical domain involved in G protein activation and recognition of several recently synthesized pharmacological modulators. The presynaptic receptor mGluR7 shows the highest evolutionary conservation within the family, but no selective pharmacological tool was known. Here we characterize an mGluR7-selective agonist, N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082), which directly activates receptor signaling via an allosteric site in the transmembrane domain. At transfected mammalian cells expressing mGluR7, AMN082 potently inhibits cAMP accumulation and stimulates GTPγS binding (EC_{50}-values, 64–290 nM) with agonist efficacies comparable with those of L-2-amino-4-phosphonobutyrate (L-AP₄) and superior to those of L-glutamate. AMN082 ($\leq 10~\mu$M) failed to show appreciable activating or inhibitory effects at other mGluR subtypes and selected ionotropic GluRs. Chimeric receptor studies position the binding site of AMN082 in the transmembrane region of mGluR7, and we demonstrate that this allosteric agonist has little, if any, effect on the potency of orthosteric ligands. Here we provide evidence for full agonist activity mediated by the heptahelical domain of family 3 G protein-coupled receptors (which have mGluR-like structure) that may lead to drug development opportunities. Further, AMN082 is orally active, penetrates the blood–brain barrier, and elevates the plasma stress hormones corticosterone and corticotropin in an mGluR7-dependent fashion. Therefore, AMN082 is a valuable tool for unraveling the role of mGluR7 in stress-related CNS disorders.

G protein | mGluR

G protein-coupled receptors in vertebrates constitute a superfamily of 1,000–2,000 transmembrane heptahelical proteins that can be activated by a large number of extracellular signals such as photons, hormones, neurotransmitters, and growth and development factors. These receptors transduce and amplify cellular signals by the activation of G proteins, which in turn modulates cytoplasmic second-messenger and ion levels (1). Sequence comparison among the different G protein-coupled receptors revealed the existence of at least six receptor families (2). One of them, family 3, comprises receptors for extracellular calcium, pheromones, GABA, and L-glutamate. This receptor family is characterized by a large N-terminal extracellular domain that has been demonstrated by site-directed mutagenesis and x-ray crystallography to contain the binding site for orthosteric agonists (3–5). Metabotropic glutamate receptors (mGluRs) are members of family 3 and are activated by the major excitatory neurotransmitter of the mammalian brain, L-glutamate. The mGluRs act as important pre- and postsynaptic regulators of neurotransmission in the CNS, and there are at least eight subtypes (mGluR1 to mGluR8), which have been

divided into three groups (I–III) according to sequence homology, signal transduction mechanism, and agonist pharmacology (6, 7). Group I consists of mGluR1 and mGluR5, whereas mGluR2 and mGluR3 constitute group II. There are currently four members within group III: mGluR6, a postsynaptic mediator of L-glutamatergic neurotransmission in retinal ON bipolar cells, and mGluR4, mGluR7, and mGluR8, which are presynaptic receptors in the mammalian brain modulating release of neurotransmitters such as GABA, L-glutamate, and, possibly, monoamines (8). Pharmacological studies for group III mGluRs are most frequently performed with L-2-amino-4-phosphonobutyrate (L-AP₄), an unselective agonist for all four receptor subtypes (9); recently, a subtype-selective positive modulator, activating mGluR4, has become available (10, 11).

mGluR7 is of particular interest given that studies in mice with genetic ablation of mGluR7 indicate altered amygdala-dependent conditioned fear and aversion responses (12), and reduced anxiety- and stress-related behaviors (13). Moreover, mGluR7 ablation causes dysregulation of the hypothalamic–pituitary–adrenal axis and increases hippocampal BDNF protein levels (14). Taken together, these data may imply a potential role for mGluR7 in the modulation of stress-related psychiatric disorders.

To date, selective pharmacological probing of mGluR7 activation or inhibition in brain function was not possible because of a lack of suitable tools. Now we have identified N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082), which acts as an allosteric agonist of mGluR7. The selectivity, pharmacological mode of action, and presumable binding site of AMN082 are characterized extensively here, and we also report that the compound is orally active and brain-penetrable and that it modulates the release of the plasma stress hormones corticosterone and corticotropin (ACTH) in an mGluR7-dependent fashion; i.e., it does not occur in mGluR7-deficient mice.

Materials and Methods

Stable Cell Lines. Generation, culture, and pharmacological characterization of stable cell lines for mGluR1b, mGluR2, mGluR3, mGluR4, mGluR5a, mGluR6, mGluR7a, mGluR7b, mGluR8a, N-methyl-D-aspartate receptor (NMDAR) 1a/2A, NMDAR1a/2B, and GluR3i have been described (10, 15–18).

Construction of Chimeric Receptors and Corresponding Cell Lines. cDNAs encoding wild-type mGluR6 and mGluR7b were described previously (16, 19). cDNAs encoding chimeric mGluR6/7b and mGluR7/6 proteins were constructed by using the PCR overlap extension approach (3): the mGluR6/7b construct contains 570 aa derived from the N-terminal extracellular region of mGluR6 and the remaining C-terminal portion of mGluR7b, comprising the entire transmembrane region; mGluR7/6 is essentially the reverse chimera with the fusion point at amino acid 575. The four constructs were used to generate stably transfected cell lines by means of Flp recombinase-mediated integration (kit from Invitrogen) in CHO-C4 cells (Novartis Pharma AG, Basel), which contain one specific Flp recognition target (FRT) site; the identical host was used for all of the four new cell lines.

GTPγ^{35}S Binding Assays. Membranes were prepared from transfected mGluR2-, mGluR3-, mGluR4-, mGluR6-, mGluR7a-, mGluR7b-, mGluR8a-, mGluR6/7b-, and mGluR7/6-expressing cells, and GTPγ^{35}S binding assays were conducted by using the protocol described by Maj et al. (10).

Second-Messenger Assays. Measurements of cAMP accumulation were performed as previously described by using CHO cell lines stably expressing individual mGluR subtypes (15, 16). Measurement of [^{3}H]inositol phosphate formation was done according to Gasparini et al. (18), and calcium measurements were done as described by Maj et al. (10).

[^{3}H]LY341495 Binding Assay. Membrane fractions of CHO cells stably expressing mGluR7a (see above) were diluted in assay buffer (10 mM KH$_2$PO$_4$/100 mM KBr, pH 7.6), homogenized briefly by using a Polytron homogenizer (IKA Labortechnik, Staufen, Germany), and incubated for 10 min at 30°C. Assay mixtures were prepared in 96-well microtiter plates. The composition of the assay mixtures in a final volume of 200 μl per well was as follows: 10 mM KH$_2$PO$_4$/100 mM KBr (pH 7.6), 50 μg of pretreated membrane protein (IKA Labortechnik), 1.5 mg of wheat germ agglutinin scintillation proximity assay (WGA SPA) beads (Amersham Biosciences), 10 nM [^{3}H]LY341495, and the test compounds at the appropriate concentrations. Nonspecific binding was measured in the presence of 1 mM L-serine-O-phosphate (L-SOP). The samples were incubated for 60 min at room temperature (with shaking), before being counted in a TopCount (Packard). Data were analyzed by using nonlinear regression in the PRISM program (GraphPad, San Diego). IC$_{50}$ values were converted into K_i values by using the Cheng and Prusoff equation (20).

Animal Procedures, *in Vivo* AMN082 Administration. mGluR7$^{-/-}$ mice were generated as described in ref. 21 from E14 (129/Ola) embryonic stem cells. All of the mice in the studies reported here carried wild-type or mutant mGluR7 alleles on a 14th-generation (F$_{14}$) C57BL/6 genetic background. Age-matched groups of mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice were generated as described (14). Male animals were used in all experiments. Food pellets and tap water were available ad libitum. Male mGluR7$^{+/+}$ and littermate mGluR7$^{-/-}$ mice were injected orally (p.o.) with vehicle or 1–6 mg/kg AMN082. One hour later, mice (mGluR7$^{-/-}$ and mGluR7$^{+/+}$ mice in a randomized order) were decapitated rapidly (within 30 sec after first touching the cage), and trunk blood was collected ($n \geq 9$ per genotype). All animal experiments were subject to institutional review and conducted in accordance with the Veterinary Authority of Basel-Stadt.

Hormone Measurements. Plasma corticosterone and ACTH concentrations were measured by using commercially available RIA kits (ICN) as described (14). The inter- and intraassay coeffi-
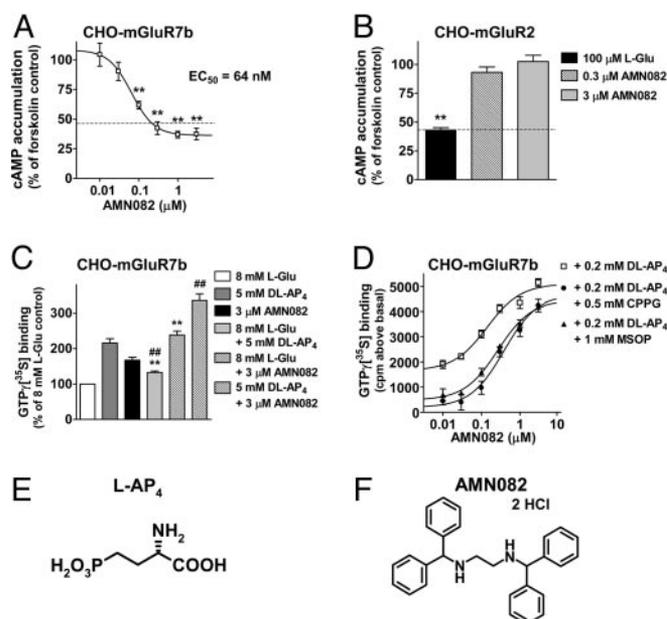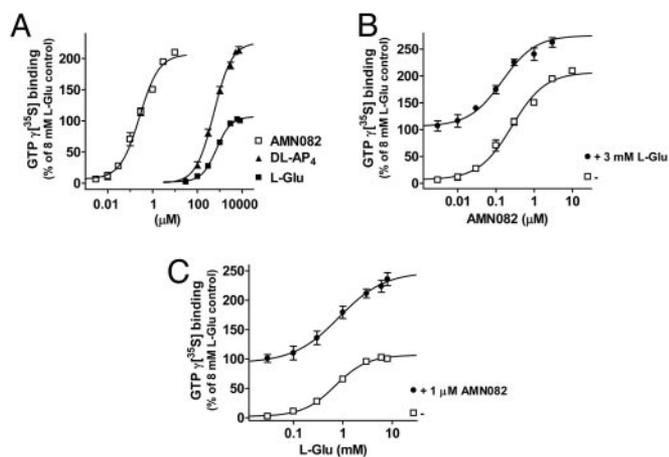

Fig. 1. AMN082 activates G protein signaling via mGluR7. (*A*) Effect of DL-AP$_4$ (4 mM, dotted line) and AMN082 (0.01–3 μM, solid line) on the inhibition of forskolin-stimulated cAMP accumulation in CHO cells stably expressing mGluR7b. (*B*) Effect of L-glutamate (L-Glu) and AMN082 on CHO mGluR2 cells. The results are normalized to control (30 μM forskolin-stimulated cAMP levels) and were pooled from at least six measurements obtained in three independent experiments and expressed as means with SEM. ∗∗, $P < 0.01$ vs. control; Dunnett's t test. (*C*) GTPγ^{35}S binding experiments using membranes from CHO mGluR7b cells. The indicated concentrations of AMN082, DL-AP$_4$, and L-glutamate were applied alone and in combination with each other. Three independent experiments were combined, and means with SEM are shown. Statistics symbols are shown for coapplication of drugs as follows: ∗∗, $P < 0.01$ vs. 8 mM L-glutamate (control); ##, $P < 0.01$ vs. 5 mM DL-AP$_4$ (Dunnett's t test). (*D*) Concentration–response curves of AMN082 in the presence of 0.2 mM DL-AP$_4$ with and without CPPG or MSOP, when using GTPγ^{35}S binding on CHO mGluR7b membranes; mean data ± SEM from a single representative experiment ($n = 3$). (*E* and *F*) Chemical structures of L-AP$_4$ and AMN082.

cients of variability for ACTH and corticosterone were similar to the manufacturer's reported values, with a detection limit of 5–10 pg/ml.

Materials. Tissue culture reagents were from Sigma or Life Technologies/GIBCO/BRL (Basel). L-Glutamate, MSOP, CPPG, DL-AP$_4$, L-AP$_4$, L-SOP, CNQX, LY341495, and tritiated LY341495 were purchased from Tocris (Bristol, U.K.). AMN082 and CGP40116 were synthesized at Novartis Pharma AG.

Results

Effect of AMN082 on Cloned mGluR7. AMN082 was identified by using high-throughput random screening of chemical libraries. The chemical structure of AMN082 (Fig. 1) is completely unrelated to the known mGluR7 ligands, which are all derived from the L-glutamate backbone. This compound elicited a concentration-dependent inhibition of forskolin-stimulated cAMP accumulation in CHO cells stably expressing human mGluR7b (Fig. 1*A*; EC$_{50}$, 64 ± 32 nM), comparable in efficacy with a saturating concentration of the orthosteric group III mGluR agonist DL-AP$_4$ (Fig. 1*A*, dotted line). Up to 3 μM, there was no effect of AMN082 in the same assay conducted with mGluR2-expressing CHO cells (Fig. 1*B*). Stimulation of GTPγ^{35}S binding was performed to evaluate further AMN082's pharmacological properties and mechanism of action. AMN082 (3 μM) produced 167 ± 8% stimulation relative to the maximal

Fig. 2. AMN082 and orthosteric L-glutamate (L-Glu) site ligands have little, if any, effect on each other's potency. All membranes were prepared from CHO cells stably expressing mGluR7b. (*A*) Concentration–response curves for AMN082, DL-AP$_4$, and L-glutamate. At least three independent experiments were pooled, and all values were normalized to the control stimulation of 8 mM L-glutamate (set to 100%); means with SEM are shown. (*B*) Concentration–response curves for AMN082 in the absence and presence of 3 mM L-glutamate concentrations. Three independent experiments were pooled, and all values were normalized as above; means with SEM are shown. (*C*) Concentration–response curves for L-glutamate in the absence and presence of 1 μM AMN082. Four independent experiments were pooled, and all values were normalized to control (as above). (*B* and *C*) □, no further chemicals were added. (*D*) The calculated parameters from at least three separate experiments, parts of which are shown in *B* and *C*, indicate that L-glutamate or AMN082 had little effect on AMN082 or L-glutamate potency, respectively. 95% CI, 95% confidence interval.

agonist activity of L-glutamate (set to 100%; Fig. 1*C*). The stimulating effects of AMN082 were almost additive with those of L-glutamate and DL-AP$_4$ (238 ± 11% and 336 ± 19%, respectively), whereas DL-AP$_4$ plus L-glutamate (both at maximally active concentrations) produced a smaller stimulation than DL-AP$_4$ alone (133 ± 4% vs. 216 ± 12%, Fig. 1*C*). Thus, L-glutamate and DL-AP$_4$ are likely to interact at the same receptor site, and the activity of the full agonist DL-AP$_4$ seems to be inhibited by the partial agonist L-glutamate. Next, the group III mGluR-selective antagonists MSOP and CPPG were tested against concentration–response curves of AMN082 conducted in the presence of submaximal DL-AP$_4$ (Fig. 1*D*): the DL-AP$_4$ component was completely abolished by the antagonists, whereas there was no inhibition of the AMN082-
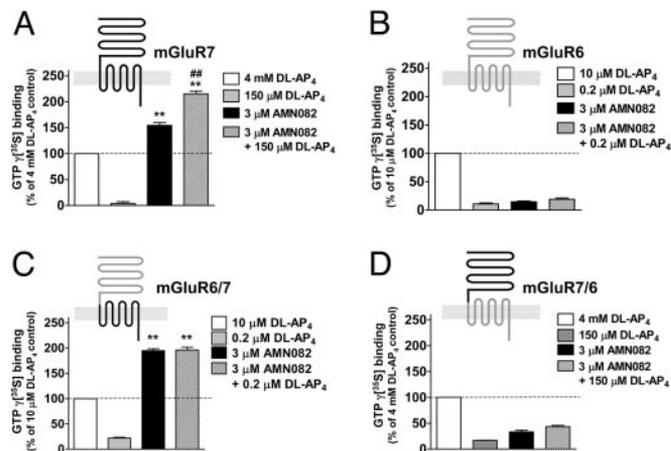
Fig. 3. AMN082 activates G protein signaling via a binding site in the transmembrane region of mGluR7. (*A* and *B*) The effect of AMN082 on GTPγ^{35}S binding in the absence and presence of DL-AP$_4$ when using membranes from CHO-C4 cells stably expressing mGluR7b (*A*) or mGluR6 (*B*). (*C* and *D*) The effect of AMN082 on mGluR6/7b (*C*) and mGluR7/6 (*D*) chimeric receptor cell membranes. All bars represent the mean ± SEM of at least three independent experiments (*n* = 3), and all values were normalized to the control stimulation of the respective agonist concentrations (4 mM DL-AP$_4$ for mGluR7 and mGluR7/6; 10 μM DL-AP$_4$ for mGluR6 and mGluR6/7b). **, Significant increases vs. maximal DL-AP$_4$ controls ($P < 0.01$); ##, $P < 0.01$ vs. additive effect of submaximal DL-AP$_4$ (gray bars) and 3 μM AMN082 (black bars); Dunnett's *t* test.

stimulated GTPγ^{35}S binding. Together, the data of Fig. 1 *C* and *D* suggest that AMN082 activates mGluR7 signaling most likely by binding to a different site than the orthosteric ligands L-glutamate, DL-AP$_4$, MSOP, and CPPG.

Concentration–response curves for AMN082, DL-AP$_4$, and L-glutamate are compared in Fig. 2*A*. AMN082 is far more potent than the orthosteric ligands, DL-AP$_4$ and L-glutamate; the EC$_{50}$ values (95% confidence intervals) are 260 nM (200; 360), 540 μM (440; 670), and 700 μM (580; 850), respectively. To analyze a potential cooperativity between L-glutamate site ligands and AMN082, concentration–response curves for AMN082 at different fixed concentrations of L-glutamate and, inversely, curves for L-glutamate vs. fixed concentrations of AMN082 were conducted (Fig. 2 *B–D*). The EC$_{50}$ of AMN082 varied between 140 and 290 nM with largely overlapping 95% confidence intervals (Fig. 2 *B* and *D*). Similarly, the EC$_{50}$ of L-glutamate was consistently between 640 and 830 μM, irrespective of the added concentration of AMN082 (Fig. 2 *C* and *D*). To address whether binding of AMN082 to mGluR7 affects the binding affinity of ligands for the L-glutamate site, we analyzed displacement of 10 nM [^{3}H]LY341495 (a competitive mGluR antagonist) binding from membranes prepared from CHO cells stably expressing mGluR7a; up to 30 μM AMN082 showed no displacement of this radioligand. In contrast, 10 mM L-AP$_4$, L-glutamate, or L-SOP abolished 100% of specific binding (data not shown). Moreover, we conducted [^{3}H]LY341495 displacement curves with L-SOP, L-AP$_4$, and L-glutamate, each in the absence and presence of 3 μM AMN082. The addition of AMN082 induced only a minor left-shift of each of the three curves; the calculated K_i values with 95% confidence intervals (in parentheses in μM) were as follows: L-SOP, 45 μM (39; 50); L-SOP plus 3 μM AMN082, 29 μM (24; 35); L-AP$_4$, 193 μM (165; 224); L-AP$_4$ plus 3 μM AMN082, 176 μM (144; 216); L-glutamate, 624 μM (446; 870); and L-glutamate plus 3 μM AMN082, 524 μM (436; 631) (data not shown).

AMN082 Directly Interacts with the Heptahelical Region of mGluR7. Next, we intended to localize the binding site of AMN082 to one discrete region of the mGluR7 protein and decided to use
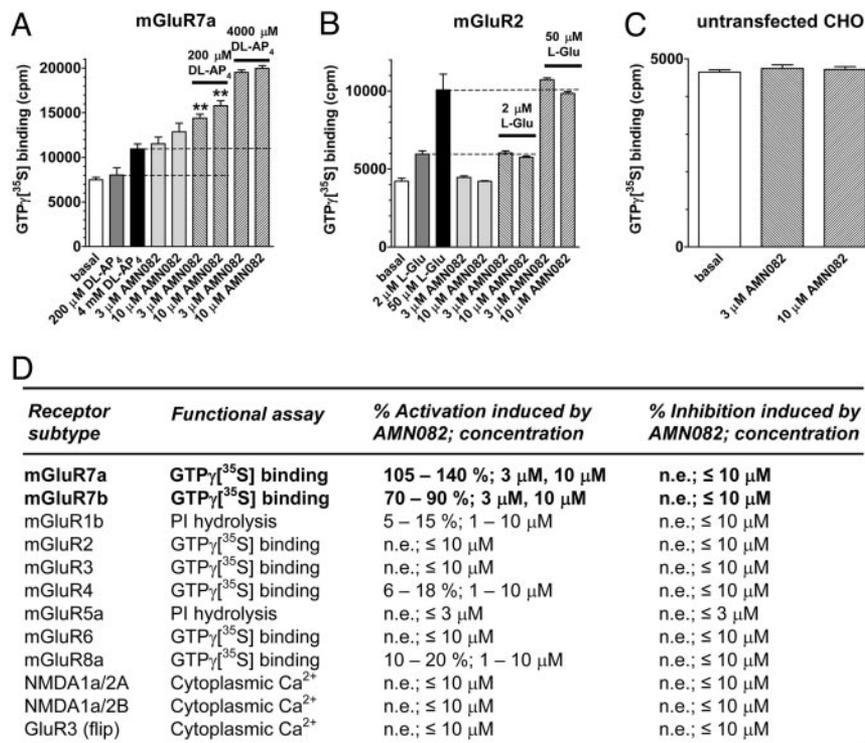
Fig. 4. mGluR7 selectivity of AMN082 across glutamate receptor subtypes. (*A*, *B*, and *D*). Phosphoinositol hydrolysis and GTPγ^{35}S binding were measured for group I mGluRs and groups II and III mGluRs, respectively, in mammalian cells stably expressing the individual, cloned mGluR subtypes. (*A* and *D*) Robust stimulation of both mGluR7 splice variants is demonstrated by GTPγ^{35}S binding. (*B* and *C*) No stimulation of GTPγ^{35}S binding by AMN082 was detected in CHO mGluR2 cells (*B*) or untransfected CHO cells (*C*). (*A*–*C*) The graphs represent typical experiments performed in triplicate and expressed as means with SEM. (*A*) All six groups that contained AMN082 showed significantly increased level of GTPγ^{35}S binding compared with the respective controls ($P < 0.01$ for each of those six groups; Dunnett's t test; symbols not shown). **, More than additive increases ($P < 0.01$) vs. the combined effect of 200 μM DL-AP$_4$ and the respective concentration of AMN082; Dunnett's t test. (*D*) Cytoplasmic calcium ion concentrations were measured for ionotropic GluRs, Ltk$^-$ cells, and HEK cells stably transfected with cloned NMDARs (subunit combination 1a/2A and 1a/2B) and a cloned α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, GluR3i (flip splice variant), respectively. Data from 2–4 independent experiments were averaged. n.e., no effect.

constructs of wild-type mGluR7b and mGluR6 as well as two chimeras: the mGluR6/7b construct contains the N-terminal extracellular region of mGluR6 and the C-terminal portion of mGluR7b comprising the entire transmembrane region; mGluR7/6 is the reverse chimera (see *Materials and Methods* and Fig. 3). When using the stimulation of GTPγ^{35}S binding, the activity of AMN082 on mGluR6/7b and mGluR7/6 chimeras was very similar to wild-type mGluR7b and mGluR6, respectively: AMN082 stimulated mGluR7b and mGluR6/7b by 150–200% relative to the maximal effect of DL-AP$_4$, but AMN082 produced only minor effects on mGluR6- and mGluR7/6-expressing membranes (10–25% relative to maximal DL-AP$_4$ stimulation) (Fig. 3). It is interesting to note that the stimulating effects of AMN082 in combination with DL-AP$_4$ were more than just additive on those mGluR7-expressing, but not on mGluR6/7b-expressing, membranes (Fig. 3 *A* and *C*). In addition, initial attempts using truncated mutants with deleted extracellular domains were made, but no activation with DL-AP$_4$ or AMN082 was observed (data not shown); it cannot be ruled out that their translated proteins were misfolded or incorrectly inserted into membranes.

Selectivity Profiling of AMN082. Before we addressed the activity of AMN082 at all eight known mGluRs and at three selected ionotropic receptors, we confirmed that there was no significant binding interaction of 1 μM AMN082 with 30 different nervous system targets using a radioligand displacement assay; this list included a selection of receptors for adrenaline, dopamine, GABA, histamine, acetylcholine, opiates, serotonin, and substance P plus selected neurotransmitter reuptake sites ($n = 2$–4 determinations per target; data not shown). Fig. 4 shows the effects of AMN082 on all eight mGluRs and on three ionotropic GluRs. The activating effect of 3 μM and 10 μM AMN082 was selectively seen at mGluR7a and mGluR7b with large efficacies of 70–140% (relative to maximal DL-AP$_4$ effects) when using the stimulation of GTPγ^{35}S binding (Fig. 4 *A* and *D*). Under an identical assay design, AMN082 (up to 10 μM) elicited little or no stimulating effects on membranes from mGluR2-, mGluR3-, mGluR4-, mGluR6-, or mGluR8a-expressing cells, and there was also no activation of GTPγ^{35}S binding in untransfected CHO cells (Fig. 4 *B*–*D*). Measurements of phosphoinositol hydrolysis were done to address whether AMN082 activates group I mGluR subtypes. AMN082 displays neither agonist-like nor positive modulatory activity at mGluR1b- or mGluR5a-expressing cells (Fig. 4*D*). Functional agonist and modulatory activities of AMN082 were also excluded for two NMDAR subtypes plus one α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subtype (NMDAR1a/2A, NMDAR1a/2B, and GluR3; Fig. 4*D*); this test was done via cytoplasmic calcium determinations by using stably transfected cell lines. Furthermore, up to 10 μM, AMN082 showed no antagonist-like effects at any of the tested mGluR or ionotropic GluR subtypes when using the functional receptor assay formats described above (Fig. 4).

***In Vivo* Activity of AMN082: Modulation of Stress Hormones in an mGluR7-Dependent Fashion.** Unlike all known L-glutamate site ligands for group III mGluRs, AMN082 readily passes the
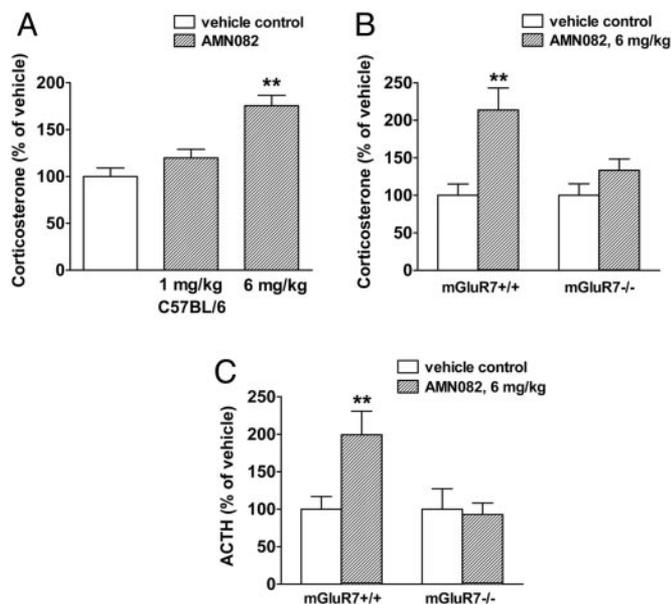
Fig. 5. AMN082 induces stress hormone increases in an mGluR7-dependent fashion. Vehicle or AMN082 was administered to mice orally (p.o.), and 1 hour later mice were decapitated and blood was collected. Plasma corticosterone and ACTH levels were measured with RIA kits. (*A*) Effect of 1 and 6 mg/kg AMN082 administered p.o. on plasma corticosterone level in naive C57BL/6 mice. (*B*) AMN082 (6 mg/kg administered p.o.) increased plasma corticosterone level to ≈200% of vehicle control in mGluR7$^{+/+}$, but not in mGluR7$^{-/-}$, mice. (*C*) AMN082 increased plasma ACTH level in mGluR7$^{+/+}$ mice to the same relative level as that shown for corticosterone, but no change was observed in mGluR7$^{-/-}$ mice. Data were normalized to each vehicle control and represent the mean ± SEM from 9–13 individual mice per group. **, $P < 0.01$ vs. vehicle control; two-way ANOVA followed by Fisher's post hoc test.

blood–brain barrier upon oral administration: 10 mg/kg oral AMN082 results in 0.29 μmol/kg in total brain tissue, and 14 mg/kg leads to 0.62 μmol/kg in rats and mice, respectively, 1 hour after oral administration (data not shown).

The role of mGluR7 in stress-related behavior is well documented (12–14). Therefore, we analyzed the effect of oral administration of AMN082 on serum levels of the stress hormones corticosterone and ACTH. Fig. 5*A* shows that AMN082 increases plasma corticosterone in a dose-dependent manner in a wild-type mouse strain (C57BL/6). Next, we used mGluR7-deficient mice (mGluR7$^{-/-}$) and their wild-type littermates (mGluR7$^{+/+}$). Again, oral administration of 6 mg/kg AMN082 elicited an increase of ≈200% of plasma corticosterone in mGluR7$^{+/+}$ animals, but no such rise was observed in mGluR7$^{-/-}$ mice (Fig. 5*B*). Similarly, blood levels of ACTH were also increased to 200% 1 hour after oral AMN082 administration in wild-type animals, but not in mGluR7-deficient mice (Fig. 5*C*).

Discussion

In these studies, we present the first selective mGluR7 agonist, AMN082. This compound acts via a previously undescribed site, is orally active, and selectively modulates the *in vivo* levels of two stress hormones, corticosterone and ACTH in wild-type, but not in mGluR7-deficient, mice, substantiating further a role for mGluR7 in stress physiology.

AMN082 elicits a full agonist response, comparable with L-AP$_4$, in the absence of glutamate site ligands. Our data with chimeric receptors strongly suggest the localization of an allosteric agonist site within the transmembrane domain of mGluR7. Definitive proof of this allosteric site awaits the development of an appropriate radioligand, which will enable determination of

physicochemical binding characteristics, such as K_d, K_{on}, and K_{off}. However, our chimeric receptor data, together with the absence of AMN082 activity at other G protein-coupled mGluRs expressed in identical host cells, as were used for mGluR7 functional studies, strongly argue that the agonist activity of AMN082 results from a direct compound interaction with the mGluR7 protein. In particular, this mGluR7-dependent agonist activity is seen in both the G protein assay (GTPγS binding) and in second-messenger determinations (cAMP accumulation), which drastically reduces the possibility of AMN082 interacting with components of the intracellular signaling cascades. In GTPγS binding, when using our previously described cell line for human mGluR7b (10, 16–18), the activities of AMN082 and L-glutamate site ligands were close to additive (Figs. 1 and 2). However, AMN082 and DL-AP$_4$ activities were significantly greater than additive on membranes from the new human mGluR7b cell line prepared for the present chimera studies (Fig. 3); significantly greater-than-additive effects of AMN082 and DL-AP$_4$ were also observed on mGluR7a-expressing membranes (Fig. 4*A*), although to a lesser extent. Similarly, the percent activation induced by AMN082 relative to DL-AP$_4$ differs reproducibly and significantly between the three cell lines. Further studies are required to understand these interesting differences. It may be possible that the mGluR7 receptor number in relation to the expression level of endogenous G proteins is quite different between the three cell lines; this possibility may potentially mask allosteric potentiation in one case but allow it in other cases. Moreover, it is unclear at present whether the alternative C termini of mGluR7a vs. mGluR7b could contribute to these differences. Interestingly, AMN082 was not inhibited by competitive glutamate site antagonists, and neither functional potency nor binding affinity of glutamate site agonists was significantly affected by AMN082 (Figs. 1 and 2). Accordingly, we propose that AMN082 binds to the transmembrane region of mGluR7 and favors directly the formation of the active receptor state, as postulated also for orthosteric agonists by the two-state model of receptor activation; orthosteric agonist binding seems to be not required for AMN082 interaction with mGluR7. Binding to the transmembrane region has also been postulated for the GABA$_B$ receptor modulator CGP7930, which was found to directly activate the receptor but with low efficacy (22). The mGluR5-positive modulator 3,3′-difluorobenzaldazine acted as a full agonist on mGluR5 deleted of its extracellular domain, but this compound did not act as an agonist itself on the nonmutated full-length receptor (23). A more recent mGluR5 positive modulator, CDPPB, displays partial agonist activity on wild-type mGluR5, and quite interestingly this agonist effect cannot be blocked by orthosteric antagonists (24), which is similar to AMN082. In many ways, AMN082 is also similar to ectopic agonists of M1 muscarinic receptors, such as AC-42, which shows 50–70% efficacy relative to full agonists; however, the effect of AC-42 is blocked by atropine (25, 26), whereas AMN082 is not blocked by orthosteric antagonists. To our knowledge, the present study of AMN082 provides the first evidence for full agonist activity mediated by the heptahelical domain of a wild-type family 3 G protein-coupled receptor.

Heretofore, the synthesis of subtype-selective group III mGluR agonists that significantly penetrate the blood–brain barrier has been unattainable, presumably because orthosteric receptor activation requires compounds with an α-amino acid moiety and a distal ionizable ω-phosphono group, which makes such molecules too hydrophilic for passing biological membranes. In contrast, the allosteric agonist AMN082 structurally is completely unrelated to amino acids. Its physicochemical properties allow oral administration, resulting in significant penetration of the blood–brain barrier, which may lead to drug development opportunities for harnessing pharmacological activity at its novel binding site.

A direct compound interaction of AMN082 with mGluR7 to elicit physiological efficacy *in vivo* is apparent because AMN082 elevates the plasma levels of the stress hormones corticosterone and ACTH in an mGluR7-dependent fashion; i.e., it does not occur in mGluR7-deficient mice (mGluR7$^{-/-}$). Importantly, 6 min of swim stress elevated corticosterone and ACTH in both mGluR7$^{+/+}$ (wild-type) and mGluR7$^{-/-}$ mice, to 400–600%, demonstrating that mGluR7-deficient mice are capable of rapid and robust stress hormone up-regulation, just like wild-type mice (14). However, the elevating effect of AMN082 on stress hormone levels is seen only in mice carrying the functional mGluR7 gene, and it is seen with oral doses that are likely to produce AMN082 brain levels, which are at EC$_{50}$ or above (see *Results*). L-glutamate has been implicated as a critical neurotransmitter in the regulation of neuroendocrine functions (27, 28). Whereas a component of glutamatergic control of neuroendocrine function is clearly ionotropic receptor-mediated (29–32), another component seems to act via the G protein-coupled glutamate receptor family, because the nonselective mGluR agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylate (ACPD) when administered intracerebroventricularly (i.c.v.) induced a significant increase in plasma corticosterone (33). An involvement of group I and possibly group II mGluRs has been reported (34–36). It also has been demonstrated that i.c.v. administration of the nonselective group III mGluR agonists L-AP$_4$ and L-SOP activates the hypothalamic–pituitary–adrenal axis (34). Our current data with AMN082 suggest that mGluR7, at least in part, modulates

this increase in stress hormones. The mechanism underlying this increase is currently unclear. Johnson *et al.* (34) suggested, based on an earlier model by Tasker *et al.* (37), that as group III mGluRs regulate the activity of GABA interneurons in the hypothalamus (38), agonists such as L-AP$_4$ and L-SOP might act by decreasing L-glutamate release from hippocampal–hypothalamic tracts. Thus, there would be a decreased tone driving GABAergic interneurons and a disinhibition on ACTH release factor-containing paraventricular nucleus (PVN) neurons of the hypothalamus. Alternatively, they speculate that group III agonists might be acting directly on presynaptic terminals of the GABAergic interneurons and activating heteroautoreceptors, and thus group III mGluR stimulation could directly limit the degree of inhibitory tone that is on the PVN cells. Both scenarios might explain the ability of AMN082 to induce a rise in serum corticosterone and possibly ACTH via mGluR7 activation, as has been seen in the present work.

In conclusion, we have identified a selective mGluR7 agonist, AMN082, that acts via an allosteric site and can serve as an invaluable tool for further unraveling the role of mGluR7 in stress-related CNS disorders.

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