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NAADP is an unusual second messenger thought to mobilize acidic Ca^{2+} stores such as lysosomes or lysosome-like organelles that are functionally coupled to the endoplasmic reticulum. Although NAADP-sensitive Ca^{2+} stores have been described in neurons, the physiological cues that recruit them are not known. Here we show that in both hippocampal neurons and glia, extracellular application of glutamate in the absence of external Ca^{2+} evoked cytosolic Ca^{2+} signals that were inhibited by preventing organelle acidification or following osmotic bursting of lysosomes. The sensitivity of both cell types to glutamate correlated well with lysosomal Ca^{2+} content. However, interfering with acidic compartments was largely without effect on the Ca^{2+} content of the endoplasmic reticulum or Ca^{2+} signals in response to ATP. Glutamate but not ATP elevated cellular NAADP levels. Our data provide evidence for the agonist-specific recruitment of NAADP-sensitive Ca^{2+} stores by glutamate linking the actions of NAADP to a major neurotransmitter in the brain.

INTRODUCTION

Changes in the cytosolic Ca^{2+} concentration regulate a multitude of cellular processes [1]. In the central nervous system, Ca^{2+} is vitally important for neurotransmitter release, neuronal excitability, synaptic plasticity and neuronal growth [2]. Ca^{2+} homeostasis is perturbed during ageing and in age-related diseases such as Alzheimer's disease [3]. Understanding how neuronal Ca^{2+} signals are precisely composed is thus vital for a full understanding of neuronal function during both health and disease.

Influx of Ca^{2+} across the plasma membrane through voltage and ligand-gated ion changes is a major mechanism whereby neuronal Ca^{2+} levels are increased. However intracellular Ca^{2+} stores are also an important source of Ca^{2+} for generating Ca^{2+} signals and are often mobilised by a variety of neurotransmitters [4]. A major class of intracellular Ca^{2+} channels responsible for store release are the receptors for the second messenger D-myo-inositol 1,4,5-trisphosphate (IP_3) [5;6]. These Ca^{2+}-regulated channels are located on the endoplasmic reticulum, are activated upon agonist-evoked production of IP_3 and have been implicated in neuronal functions including long-term depression in the cerebellum [7]. The second major family of intracellular Ca^{2+} channels comprise ryanodine receptors [8]. Like IP_3 receptors, they are located on the endoplasmic reticulum and like IP_3 receptors, they are both activated and inhibited by Ca^{2+} at low and high concentrations, respectively [8]. Indeed, Ca^{2+}-induced activation of ryanodine receptors appears particularly important in amplifying Ca^{2+} signals deriving from other sources such as voltage-sensitive Ca^{2+} channels [9]. Ryanodine receptors can be modulated by the NAD metabolite cyclic

ADP-ribose [10]. Cyclic ADP-ribose regulation of pre-synaptic ryanodine receptors has been proposed as a possible mechanism contributing to long-term depression in the hippocampus [11].

More recently, a novel mechanism of intracellular Ca^{2+} release activated by nicotinic acid adenine dinucleotide phosphate (NAADP) has been characterised primarily in non-neuronal cells [12]. NAADP sensitive Ca^{2+} channels are unique in several respects. Surprisingly, NAADP-induced Ca^{2+} release is not directly regulated by cytosolic Ca^{2+} [13-15]. Release of Ca^{2+} by this mechanism might therefore be better suited for triggering rather than amplification of Ca^{2+} signals [16-18]. Indeed, in intact cells NAADP-induced Ca^{2+} release is attenuated by inhibitors of IP_3 and ryanodine receptors [16]. In contrast, in broken preparations such as the extensively studied sea urchin egg homogenate, these inhibitors have little effect on NAADP responses [19]. Such observations have led to the hypothesis that NAADP targets a channel distinct from IP_3 and ryanodine receptors [20] possibly TRPML1 [21] and that in intact cells the resulting NAADP evoked Ca^{2+} signal is propagated via IP_3 and ryanodine receptors through Ca^{2+}-induced Ca^{2+} release [16-18]. It should be noted however that direct activation of ryanodine receptors [22-24] and Ca^{2+} influx channels [25;26] by NAADP have also been proposed as alternative mechanisms [24].

Another defining feature of NAADP receptors is their location. Although in some cell types, NAADP appears to mobilize Ca^{2+} directly from endoplasmic reticulum [27;28], this is not the case in a majority of the other cell types that have been studied to date. Thus, early studies in sea urchin eggs demonstrated that Ca^{2+} stores sensitive to NAADP can be distinguished from endoplasmic reticulum Ca^{2+} stores both physically by centrifugation [19;29] and pharmacologically by their insensitivity to thapsigargin [14]. More recent studies suggest that NAADP-sensitive Ca^{2+} stores in these cells are reserve granules which are acidic organelles [30]. Indeed, in several different mammalian cell types, the stores targeted by NAADP are likely the functionally related lysosomes or lysosome-like organelles [31-35]. Importantly, inhibition of NAADP-mediated Ca^{2+} signalling (either by desensitizing NAADP receptors or depleting the Ca^{2+} stores upon which they reside) inhibits Ca^{2+} signals in response to certain extracellular stimuli thought traditionally to exclusively couple to IP_3 production including cholecystokinin [32;36], T cell receptor/CD3 agonist [37], endothelin-1 [33;34;38], histamine [35] and glucagon-like peptide-1 [39]. These data suggest a messenger role for NAADP in response to these cues. Recent direct measurements of NAADP levels have confirmed that NAADP is indeed a Ca^{2+} mobilizing messenger in sea urchin gametes [40] and other cells [33;35;41-43]. Detailed kinetic analyses of NAADP changes reveal a rapid transient increase upon agonist binding which in pancreatic acinar cells precedes changes in levels of cyclic ADP-ribose and global cytosolic Ca^{2+} increases [42]. Such an increase may well underlie a trigger release of Ca^{2+} via NAADP receptors which in turn recruits IP_3 and ryanodine receptors resulting ultimately in complex Ca^{2+} signals.

Relatively little is known concerning the actions of NAADP in the nervous system. In *Aplysia*, presynaptic injection of NAADP at a cholinergic synapse stimulates evoked acetylcholine release [44]. NAADP also increases the frequency of spontaneous excitatory post synaptic potentials at the frog neuromuscular junction [45]. Indeed, as in non-neuronal cells, the effects of NAADP appear to be thapsigargin-independent [45]. Moreover, the combined effects of NAADP and IP_3 or NAADP and cyclic ADP-ribose are more than additive [46] consistent with functional coupling of NAADP receptors and Ca^{2+} induced Ca^{2+} release channels [47]. Regarding mammalian preparations, Galione and colleagues have demonstrated that NAADP mobilizes Ca^{2+} from rat brain microsomes [15]. Specific binding sites for NAADP have also been identified in the rodent brain [48]. Autoradiographic analysis, demonstrates that NAADP receptors are present throughout the adult brain and that their distribution differs significantly from that of IP_3 and ryanodine receptors [48]. Despite this biochemical data, functional evidence for NAADP signalling in mammalian neurons is scant. In addition to possible extracellular effects of NAADP [49], we have shown intracellular delivery of NAADP in to cortical neurons and PC12 cells mediates Ca^{2+} signals that are inhibited by acid store blockers but readily demonstrable following depletion of endoplasmic reticulum Ca^{2+} stores with thapsigargin [50;51]. We also confirmed the presence

of NAADP receptors in the former preparation using a novel cell permeable acetoxymethyl ester of NAADP [52]. Importantly, NAADP was shown to potentiate neurite extension in primary neuronal cultures by a mechanism involving IP_3 and ryanodine receptors [50]. Remarkably in PC12 cells, NAADP-mediated Ca^{2+} signals (which differed kinetically from those mediated by IP_3) were sufficient to cause neuronal differentiation [51]. We have also recently characterized NAADP-mediated Ca^{2+} signals in medullary neurons and provided evidence for coupling of NAADP-sensitive Ca^{2+} channels to non-selective cation channels on the plasma membrane [53]. Thus, NAADP receptors are likely expressed, functional and physiologically important in the nervous system however endogenous cues that recruit them have yet to be identified.

In the present study, we examined the effect of interfering with acidic compartments on mobilization of intracellular Ca^{2+} stores from hippocampal neurones and glia by the neurotransmitters glutamate and ATP. We also measured NAADP levels in response to these stimuli. We provide evidence that glutamate but not ATP is an NAADP-linked agonist coupled to acidic Ca^{2+} stores.

EXPERIMENTAL

Hippocampal cell culture

Mixed cultures of hippocampal neurons and glia were prepared from 2 day old rat pups (Sprague Dawley, University College London breeding colony) as previously described [54]. Briefly, hippocampi were dissected and placed in ice-cold HEPES-buffered saline (HBS) composed of 156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.35). The tissue was minced, treated with Trypsin-EDTA for 7-8 min at 37°C and triturated in Neurobasal medium supplemented with 2% B27 and 2 mM L-glutamine (all from Invitrogen). The cells were then plated onto 25 mm circular cover glasses (thickness # 1) coated with poly-D-lysine (0.1mg/ml) and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The media was changed every 3 - 4 days and the cultures were used within 7-10 days of preparation.

Localization of acidic organelles

Acidic compartments in hippocampal cultures were labelled with Lysotracker Green (Molecular Probes) by incubating the cells with the dye (200 nM) in HBS for 60 min either in the absence or presence of bafilomycin-A1 (1 µM). The latter was included to prevent organelle acidification in order to determine the specificity of staining. The cells were then washed with Ca^{2+}-free HBS which contained EGTA (1 mM) in place of Ca^{2+} and confocal fluorescence images captured following excitation at 488 nm (emission = 505 nm - 530nm) using a 63X objective. In some experiments, images were acquired in real time following acute addition of 200 µM glycyl-L-phenylalanine-ß-naphthylamide (GPN) in order to effect osmotic lysis of lysosomes.

Measurement of cytosolic Ca^{2+} concentration

Cells were loaded with the fluorescent Ca^{2+} indicator Fura-2 by incubation of the cultures for 30 min with HBS supplemented with 2.5 µM Fura-2 AM and 0.005% w/v pluronic acid (Molecular Probes). The cells were then rinsed three times in HBS and incubated for an additional 30 minutes to allow de-esterification. The cover glass on which the cells were attached was then mounted in to a chamber (1 ml volume) and placed on the stage of an Olympus IX71 inverted microscope. Cells were viewed with a 20X objective. Neurons were easily distinguishable from glia as they appeared phase bright and lay just above the focal plane of the glia. Fura-2 fluorescence (emission > 440 nm) was captured by a cooled CCD camera following sequential illumination at 340 and 380 nm using a monochromator light source (T.I.L.L. Photonics). Image pairs were captured every 3 s and 340/380 fluorescence ratios derived from user-defined regions of interest corresponding to individual cells using *TILLvisION* software. All data were corrected for background fluorescence but no attempt was made to calibrate the fluorescence ratios to Ca^{2+} concentration due to inherent problems associated with the use of esterified Ca^{2+} indicators [55].

Basal fluorescence ratios were determined by averaging data over the first 30 s of image acquisition. Capture was halted (see breaks in traces) whilst the medium was replaced with Ca^{2+}-free HBS. The cells were then stimulated by substituting half the bath volume with agonist-containing solution. Where indicated, bafilomycin-A1 (1 µM) was added 1 h prior to recording and present in all solutions throughout. In some experiments, the cells were depolarized by elevating extracellular K^+ (via Na^+ substitution) to a final bath concentration of 39 mM.

Cells were considered responsive to a particular agonist or GPN if the change in the fluorescence ratio was 20% or more than that of the basal ratio. For thapsigargin, the threshold was set to 10 % because of the modest nature of the cytosolic Ca^{2+} increase. The magnitude of the response was calculated by subtracting the basal ratio from the peak ratio obtained in response to the stimulus (ΔR) and presented as mean ± standard error of the mean (s.e.m.) for either all cells in the population or the responding cells only as indicated.

Measurement of NAADP levels

Brains were removed from adult (200-300 g) female wistar rats (Harlan, UK) and transferred to Hanks' Balanced Salt Solution supplemented with a protease inhibitor cocktail (Roche Diagnostics, UK) and gassed with 95% O_2/5% CO_2. Tissue slices (650 µm), prepared using a tissue chopper (Mickle Engineering, Gomshall, Surrey, UK), were triturated by pipetting and the resulting suspension washed by centrifugation (800 xg for 3 min at 4 °C). Cells were incubated at 36°C for 15 min prior to measurement of NAADP. The suspensions were then maintained in Hanks' Balanced Salt solution or stimulated with either glutamate or ATP (10 µM). Aliquots from each suspension were removed just before stimulation and, at the times indicated, quenched by the addition of an equal volume of 1.5 M $HClO_4$ followed by sonication (Jencons Vibracell amplitude 60). The samples were then incubated on ice for a further 15 min and the denatured protein pelleted by centrifugation (9000 xg for 10 min). Supernatant fractions containing the NAADP were neutralized with an equal volume of 2 M $KHCO_3$ and the samples centrifuged (9000 xg for 10 min) to remove the resulting $KClO_4$ precipitate. NAADP content was determined using a radioreceptor assay based on binding sites for [^{32}P]NAADP in sea urchin egg homogenates [52;56]. Briefly the homogenates were incubated with the radioligand either in the absence or presence of the extracts and bound radioactivity measured following rapid filtration. The NAADP content was then quantified by comparison of the reduction in radioligand binding by known concentrations of authentic NAADP. Data were normalized to the total protein content of the samples determined using bichinconinic acid (Sigma).

RESULTS

To examine the role of acidic Ca^{2+} stores in neurotransmitter-evoked release of intracellular Ca^{2+} stores in cells of the central nervous system, we first localized acidic compartments in live mixed neuronal and glial primary cultures derived from the rat hippocampus. Brief incubation of the cultures with the fluorescent weak base, Lysotracker green which accumulates in acidic compartments resulted in the labelling of vesicular structures throughout the cytoplasm of both neurones (Fig 1A, left) and glia (Fig. 1B, left). Labelling above background fluorescence was not apparent in separate batches of cells from the same culture that were instead pre-incubated for 1h with 1 µM bafilomycin-A1 (Fig. 1A,B, right), a selective inhibitor of vacuolar-type ATPases [57]. Vacuolar-type ATPases are ATP-dependent proton pumps responsible for the acidification of several organelles including lysosomes and endosomes. Labelling was also disrupted following acute treatment with the lysosomotropic agent GPN. GPN causes osmotic lysis of lysosomes by acting as a substrate for the lysosomal exopeptidase, cathepsin C [58]. As shown in the middle panels of Fig. 1A-B, GPN addition initiated a progressive loss in Lysotracker fluorescence in both cell types. These data indicate that bafilomycin-A1 and GPN can be used to disrupt acidic vesicles *in vitro*.

Because both bafilomycin-A1 and/or GPN abolish NAADP-mediated Ca^{2+} signals in several cell types [28;30;32-35;38;39;50;51], we examined their effects on neurotransmitter-evoked release of intracellular Ca^{2+} stores in order to identify potential NAADP-linked agonists. To isolate metabotropic receptor responses, cells were stimulated with a sub-maximal concentration of agonist in the absence of extracellular Ca^{2+}. Under these conditions, Ca^{2+} influx through ionotropic receptors would be abolished.

Cytosolic Ca^{2+} concentration was determined by loading cells with the fluorescent Ca^{2+} indicator fura-2 and measuring the ratio of fluorescence upon excitation of the dye at 340 nm and 380 nm (see Methods). Prior to agonist stimulation, the basal fluorescence ratio was 0.31 ± 0.003 (n = 211 cells) and 0.34 ± 0.003 (n = 143 cells) for neurons and glia, respectively (n = 4 independent preparations). The typical response of an individual control neuron and glial cell to glutamate (10 μM), the first of the neurotransmitters tested, is shown in Figures 2A and B, respectively. The magnitude of the glutamate responses, i.e the difference in fluorescence ratio at its peak from that prior to stimulation (ΔR) in the responding cells was similar in both cell types with ΔR values of 0.3 ± 0.02 (n = 102) for neurons and 0.46 ± 0.03 (n = 80 cells) for glia. Strikingly, when cultures were pre-treated with bafilomycin-A1 (1 μM), their responsiveness to glutamate was substantially reduced. Examples of cells that failed to respond to glutamate after incubation with bafilomycin-A1 are shown in Figures 2C-D. Pooled data from several similar experiments revealed that bafilomycin-A1 decreased the percentage of cells responsive to glutamate by more than two-fold (Fig. 2E). Population ΔR values for neurons and glia (i.e. the magnitude of the responses in both responding and non-responding cells) was also significantly decreased following bafilomycin A-1 treatment (Fig. 2F). These data are consistent with a requirement for acidic Ca^{2+} stores in initiating glutamate-mediated Ca^{2+} signals.

We next examined the effects of GPN on cytosolic Ca^{2+} signals in response to glutamate. In control experiments, glutamate mediated the expected responses in both neurones and glia (Fig. 3A-B). Addition of GPN to cultures in Ca^{2+}-free medium also mediated a Ca^{2+} signal presumably due to osmotic lysis of lysosomes (Fig. 3C-D). Analysis of 8 independent cultures indicated that GPN evoked a Ca^{2+} response in 47 ± 7 % of the neurons and 62 ± 5 % of the glia with ΔR values of 0.37 ± 0.02 (n = 189) and 0.27 ± 0.02 (n = 133), respectively in the responding cells. Ca^{2+} signals in response to GPN were heterogeneous consisting of either a monotonic increase or an oscillatory one. Additionally, these signals were slow to develop (see inset in Fig. 3C) taking an average of 2.1 ± 0.1 min (range = 0.7 – 5.6; n= 61 cells) and 2 ± 0.1 min (range = 0.6 – 5.8; n = 52 cells) to peak in neurons and glia, respectively (n = 3 independent preparations). Importantly, as with bafilomycin-A1, a majority of the cells failed to respond to glutamate after GPN treatment (Fig. 3C-D). Reduced agonist sensitivity (Fig. 3E) and population ΔR values (Fig. 3F) was observed in both the neuronal and glial populations in the presence of GPN. These data suggest that the acidic stores recruited by glutamate are lysosomes or lysosome-like organelles.

Because lysosomes are known to reform upon removal of GPN, we assessed the sensitivity of the cultures to glutamate following washout of GPN. The traces in Fig 4A show the response of 2 exemplar neurons from different cultures that were stimulated with GPN, allowed to "recover" in Ca^{2+} -containing medium and re-stimulated with GPN. Although both neurons responded to the first GPN addition, only one of them (trace i) responded to GPN a second time. These data highlight the heterogeneity in the sensitivity of the cultures to GPN re-stimulation which ranged from 3 – 51 % of the neurons examined. Importantly, when the same cultures were stimulated with glutamate after GPN wash-out, their sensitivity mirrored that to GPN re-challenge. This is shown in Fig. 4B, where the neuron that responded to glutamate after GPN stimulation and wash-out (trace i) was from the same culture as the neuron in Figure 4A which responded to GPN a second time. A plot of the percentage of neurons and glia that responded to glutamate (Fig. 4C) and the corresponding population ΔR values (Fig. 4D) against GPN in this experimental paradigm reveals a clear correlation. Thus, although lysosome re-formation as evidenced by GPN sensitivity was not a consistent observation, our data show that lysosomes can reform and that sensitivity to glutamate in different cultures is correlated with lysosomal Ca^{2+} content.

In a set of related experiments, we examined the sensitivity of the cultures to glutamate after GPN challenge and wash-out except that cells were maintained in Ca^{2+} free medium throughout the recovery period. Following this treatment, the cells consistently failed to respond to glutamate. Results from a typical neuron are shown in Fig. 4E. This reduction in sensitivity of the cultures was not due to passive "leak" of the Ca^{2+} stores due to the protracted incubation in the absence of extracellular Ca^{2+} since control neurons which were not stimulated with GPN but processed identically reproducibly responded to glutamate (Fig. 4F). Results from several of these experiments are shown in Fig. 4G-H demonstrating near complete lack of sensitivity and population response of both neurons and glia to glutamate. Since maintaining cells in the absence of extracellular Ca^{2+} after lysosome disruption more than likely prevents Ca^{2+} uptake upon lysosome reformation, these data again suggest a tight correlation between glutamate sensitivity and lysosomal Ca^{2+} content.

Metabotropic glutamate receptor-mediated elevations in cytosolic Ca^{2+} concentration are generally ascribed to mobilization of endoplasmic reticulum-based Ca^{2+} stores via the production of inositol trisphosphate [59]. Given the clear sensitivity of glutamate responses to bafilomycin-A1 and GPN, it was necessary to examine what effect these inhibitors had on the Ca^{2+} content of the endoplasmic reticulum. Endoplasmic reticulum Ca^{2+} content was estimated by measuring cytosolic Ca^{2+} responses following inhibition of sarco-endoplasmic reticulum ATPases with thapsigargin. In control experiments, addition of thapsigargin evoked a slow increase in cytosolic Ca^{2+} concentration in both neurons and glia consistent with passive depletion of Ca^{2+} from the endoplasmic reticulum (Fig. 5A-B). Bafilomycin-A1 pretreatment had little effect on these signals in both cell types (Fig. 5C-D). Thus, both the proportion of cells that responded to thapsigargin (Fig. 5G) and the population ΔR values (Fig. 5H) in the presence of bafilomycin-A1 were similar to those from control cultures although we did note a modest increase in the latter that was statistically significantly in glia only (Fig. 5H). We also examined the effects of GPN on thapsigargin-evoked mobilisation of endoplasmic reticulum Ca^{2+} stores. The effects depended on the cell type as highlighted by the typical responses in Fig. 5E-F. In neurons, there was a modest (but statistically insignificant) reduction in the percentage of cells that responded to thapsigargin after GPN (Fig. 5G). In glia, however there was a substantial reduction in both the sensitivity of the cultures to thapsigargin (Fig. 5G) and the corresponding population ΔR value (Fig. 5H). Collectively, the data in Fig. 5 suggests that neuronal endoplasmic reticulum Ca^{2+} stores were largely replete following disruption of acidic Ca^{2+} stores with bafilomycin-A1 or GPN but that GPN appeared to deplete endoplasmic reticulum Ca^{2+} stores in glia but not neurons.

The above data provides strong evidence that glutamate recruits acidic Ca^{2+} stores. To determine whether mobilization of these Ca^{2+} stores was a general phenomenon of Ca^{2+}- linked neurotransmitters or specific to glutamate, we examined the effects of bafilomycin-A1 and GPN on cytosolic Ca^{2+} signals in response to a second agonist, ATP. ATP, like glutamate evoked transient Ca^{2+} elevations in the absence of extracellular Ca^{2+} in a majority of the neurons and glia analysed (Fig. 6A-B). In stark contrast to glutamate however, ATP responses were unaffected by prior treatment of cultures with bafilomycin-A1 (Fig. 6 C-D). Pooled data from several experiments is shown in Fig. 6G-H. The response of the cultures to ATP following GPN treatment also differed to those evoked by glutamate. Again, as with bafilomycin-A1, GPN failed to affect the percentage of cells that responded to ATP in neurons and glia (Figures 6E-G). We did however note a significant decrease in population ΔR response in both cell types (Fig. 6H). This was ascribable to reduced ΔR values in the responding cells which decreased in 4 independent preparations from 0.46 ± 0.03 (n = 108 cells) to 0.223 ± 0.02 (n = 71 cells) in neurons and from 0.55 ± 0.03 (n = 118 cells) to 0.26 ± 0.03 (n = 55 cells) in glia in the absence and presence of GPN, respectively.

In the final set of experiments, we made direct measurements of cellular NAADP levels using a sensitive radioreceptor assay that we have developed [56;60]. NAADP was readily detectable in the whole brain cell suspensions at a resting level of 4 ± 2 pmol/mg protein (n = 14). In control experiments, NAADP levels remained relatively constant over a 10 min period (Fig. 7A, C). However, stimulation of the cells with glutamate resulted in a remarkable increase in NAADP content (Fig. 7B). Inspection of the

individual time-courses revealed variability in both the magnitude and the kinetics of NAADP changes between experiments (Fig. 7B). The increase in NAADP levels in response to glutamate for example ranged from 12 – 36 fold (mean = 24 ± 6, n = 4). In 3 of 4 experiments, NAADP levels had peaked by the time the first measurement had been made (10 s). In general, the NAADP levels returned to basal levels within 1 min although in one preparation the increases were more sustained (Fig. 7B, blue trace). In stark contrast to the effects of glutamate, ATP failed to increase cellular NAADP levels (Fig. 7D). Pooled data of absolute NAADP levels at their peak in control and stimulated cells are summarized in Figure 7E. These results provide direct evidence for agonist-specific generation of NAADP in the brain.

DISCUSSION

Several studies including our own using central neurons [50] have shown that NAADP evokes release of intracellular Ca^{2+} stores, and that these responses are inhibited by prior incubation of cells with bafilomycin-A1 and/or GPN [28;30;32-35;38;39;50;51]. Such data provide strong evidence that NAADP receptors are located on lysosome-like organelles (but see [28]). A major finding of the present contribution is that these same inhibitors reduce cytosolic Ca^{2+} responses to glutamate. Because glutamate challenge was performed in the absence of external Ca^{2+}, the resulting signals are more than likely due to metabotropic glutamate receptor activation. Thus our study provides the first evidence for the recruitment of acidic Ca^{2+} stores via this class of glutamate receptors.

Importantly, inhibition of neurotransmitter-evoked Ca^{2+} signals by the bafilomycin-A1 was clearly agonist-specific. Thus, whereas Ca^{2+} responses to glutamate were inhibited by this compound (Fig. 2), those to ATP were unaffected (Fig. 6). Bafilomycin-A1 also did not inhibit Ca^{2+} responses to thapsigargin (Fig. 5) suggested that endoplasmic reticulum Ca^{2+} stores were intact. These finding provides strong evidence for a specific effect of bafilomycin-A1 on acidic stores. Such sensitivity of only certain agonist mediated Ca^{2+} responses to this acid store blocker has previously been reported in pancreatic [32] and smooth muscle cells [33;35;38]. This is the first report in neurons and glia.

By directly monitoring cytosolic Ca^{2+} concentration during osmotic lysis of lysosomes by GPN, we show that that the target organelles in both neurons and glia are indeed substantial stores of Ca^{2+} (Fig. 3 - 6). These data are consistent with recent demonstrations of GPN-sensitive Ca^{2+} stores in neurons [61] as well as several other non-neuronal mammalian cell types [28;35;38;39;62-66]. Like bafilomycin-A1, GPN also inhibited glutamate responses in both neurons and glia with out affecting thapsigargin responses in neurons. We did however observe reduced thapsigargin responses in glial cells. One possibility to explain this inhibition is that GPN may act as an NAADP mimetic i.e the robust release of Ca^{2+} from lysosomes may cause Ca^{2+}-induced Ca^{2+} release from the endoplasmic reticulum in a manner akin to the proposed action of NAADP (see below).

An additional difference between GPN and bafilomycin-A1 reported here is the effect of these compounds on responses to ATP. Although GPN did not affect the responsiveness of the cells to ATP, consistent with the results with bafilomycin-A1 (Fig. 6G), the Ca^{2+} responses were smaller in both cell types relative to both control and bafilomycin-A1-treated cultures (Fig. 6H). Thus, the initiation of ATP-mediated Ca^{2+} responses was unaffected by GPN but the magnitude of the resulting responses is reduced albeit to a lesser extent than with glutamate (compare Figs. 5F and 6H). In glia, this inhibition may be due to the depletion of endoplasmic reticulum Ca^{2+} stores by GPN but such an effect can not explain the results in neurons where endoplasmic reticulum stores were unperturbed (Fig. 5). Of potential concern is the concomitant release by GPN of hydrolytic enzymes in to the cytosol in our experiments causing damage of for example endoplasmic reticulum Ca^{2+} channels. It should be pointed out however that lysosomal enzymes have acidic pH optima and therefore likely inactive at the neutral pH of the cytosol upon their translocation. Moreover, we show that the effects of GPN on glutamate-mediated Ca^{2+} signals were reversible (see below). If inhibition were due to, for example, proteolysis then the effects would not be expected to recover over the relatively short time course of the experiments performed here. Guse and colleagues have argued that GPN acts in a non-specific manner in T lymphocytes since it blocked not only NAADP-mediated Ca^{2+} release but also Ca^{2+} release in response to IP_3 and cyclic ADP-ribose [28]. It should be noted however that in this cell type, desensitization of NAADP-sensitive Ca^{2+} channels by micromolar NAADP concentrations also abolished IP_3- and cyclic ADP-ribose-evoked Ca^{2+} release [37]. Thus, GPN-mediated inhibition of Ca^{2+} release by IP_3 and cyclic ADP-ribose might well be due to selective depletion of acidic NAADP-sensitive Ca^{2+} stores presumably as a result of tight functional coupling of intracellular Ca^{2+} release channels. What effect GPN has on messenger evoked Ca^{2+} release in glial cells remains to be established.

Interestingly, the recovery of the response to glutamate following bursting of acidic stores and subsequent incubation in the presence of external Ca^{2+} correlated well with reformation of lysosomes (as evidenced by parallel recovery of the Ca^{2+} response to GPN) (Fig. 4). These data are significant as they

indicate that lysosomal Ca^{2+} content dictates sensitivity of cells to glutamate. In marked contrast, no recovery of glutamate responses was apparent if cells were maintained in the absence of extracellular Ca^{2+} following GPN challenge suggesting that loading of lysosomes requires Ca^{2+} entry across the plasma membrane. At present, there is scant information available concerning the mechanism whereby lysosomes fill with Ca^{2+} although endocytosis of Ca^{2+} from the extracellular space is an attractive candidate [65] (but see [63;67]). An alternative explanation for the lack of reversibility in the absence of extracellular Ca^{2+} may be that lysosomes do not reform under these conditions. Indeed evidence that release of luminal Ca^{2+} is required for endosome-lysosome fusion has been obtained [68].

Both glutamate and ATP are known to elevate IP_3 levels through the activation of phospholipase C resulting in release of Ca^{2+} from the endoplasmic reticulum [2]. Our data suggest that in addition to this pathway, glutamate can also elevate cellular NAADP levels (Fig. 7). This is the first direct identification of an NAADP-linked agonist in neuronal cells. Little is known concerning the metabolic pathway for NAADP generation. What is clear is that members of the ADP-ribosyl cyclase family of multi-functional enzymes can produce several molecules involved in Ca^{2+} homoeostasis including NAADP *in vitro* [69]. What is not clear, is whether these enzymes catalyze NAADP generation *in vivo* (see [35]). In this context, it is interesting to note that several neurotransmitters, including glutamate [70] can regulate ADP-ribosyl cyclase activity in membrane preparations derived from neuronal cells and cell lines (reviewed in [71]). Indeed, metabotropic glutamate receptor-mediated responses in dopaminergic neurons are only inhibited when both IP_3 and cADPR/RyR receptors are blocked suggesting that glutamate receptors are indeed capable of coupling to multiple Ca^{2+} signalling pathways within the same cell [72].

The kinetics of agonist-evoked NAADP generation we report here (Fig. 7) are strikingly similar to those reported in pancreatic acinar cells [42], T lymphocytes [43] and myometrial cells [35]. The very rapid transient increase is entirely consistent with the trigger hypothesis [16] according to which cytosolic Ca^{2+} increases in response to certain agonists result from an initial release of Ca^{2+} from NAADP-sensitive stores which then initiates further release from the endoplasmic reticulum by Ca^{2+}-induced Ca^{2+} release. It is important to stress the requirement for Ca^{2+}-induced Ca^{2+} release in this scheme and that NAADP receptors act upstream of IP_3 and/or ryanodine receptors. Thus, previous studies showing blockade of agonist-evoked Ca^{2+} signals by blockers of endoplasmic reticulum Ca^{2+} channels signalling do not exclude a requirement for NAADP in triggering them.

In summary, based on our data, we propose that glutamate couples to both NAADP and IP_3 signalling pathways whereas ATP couples exclusively to the latter (Fig. 7F). Such heterogeneity in coupling may constitute a mechanism for generating agonist-selective Ca^{2+}-dependent effects in the brain.

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FIGURE LEGENDS

Figure 1 Disruption of acidic vesicles in primary hippocampal cultures.
Confocal images of Lysotracker green fluorescence from two neurones (A) and a glial cell (B). Images were captured either before (left) or at 1 min intervals after the addition of GPN (middle). The images on the right were taken from cells that had been pre-treated with bafilomycin-A1 (1 μM) for 1 h prior to labelling. Transmitted light images of the cells are shown on the far right. Dotted lines show the outline of the glial cells. Scale bar = 10 μm.

Figure 2. Inhibition of V-type ATPases attenuates glutamate-mediated Ca^{2+} signals.
A-D Typical cytosolic Ca^{2+} responses of individual neurons (A, C) and glia (B, D) stimulated with glutamate (10 μM) in the absence of extracellular Ca^{2+}. In control experiments (A - B), cultures were first depolarised by increasing extracellular K^+ to confirm the identity of the neurons which responded with an elevation in cytosolic Ca^{2+}. Bafilomycin-A1 (1 μM) was added to the cultures in C and D 1 h prior to recording. E -F, Pooled data quantifying the proportion of neurons and glia that responded to glutamate in the absence and presence of bafilomycin-A1 (E; mean ± s.e.m. of 3 independent cultures) and the corresponding ΔR values in all cells from each population (F; mean ± s.e.m from 72 – 146 cells). * $p<0.05$, ** $p<0.01$.

Figure 3. Osmotic busrting of lysosomes attenuates glutamate-mediated Ca^{2+} signals.
A-D Typical cytosolic Ca^{2+} responses of individual neurons (A, C) and glia (B, D) stimulated with glutamate (10 μM) in the absence of extracellular Ca^{2+}. Cells were from control cultures (A, B) or cultures acutely stimulated with GPN (200 μM; C - D). The inset in C shows an expanded time course of the fluorescence intensity recorded from the cell at the indicated excitation wavelengths upon GPN addition. E -F, Pooled data quantifying the proportion of neurons and glia that responded to glutamate in the absence and presence of GPN (E; mean ± s.e.m. of 4 independent cultures) and the corresponding ΔR values in all cells from each population (F; mean ± s.e.m from 111 – 211 cells). ** $p<0.01$.

Figure 4. Glutamate sensitivity correlates with the Ca^{2+} content of lysosomes.
Cultures were stimulated with 200 μM GPN in the absence of extracellular Ca^{2+}, GPN removed and the cells allowed to recover in either the presence (A - B) or absence (D) of Ca^{2+}. The cells were then stimulated again with 200 μM GPN (A) or 10 μM glutamate (B, D) in Ca^{2+}-free medium. A-B, Responses of two neurones from separate cultures (i, ii) following washout of GPN in the presence of extracellular Ca^{2+} showing variable sensitivity to GPN re-challenge (A) and glutamate (B). Results from 3 cultures are presented in C where the proportion of neurons (closed circles) and glia (open circles) that responded to glutamate is plotted against the proportion of cells from the same culture that responded to GPN a second time. Corresponding ΔR values for each population is shown in D. E - H, Typical results from neurons demonstrating consistent lack of response of cultures to glutamate following washout of GPN in the absence of extracellular Ca^{2+} (E) compared to a control cultures that were not stimulated with GPN (F). G – H, Pooled data quantifying the proportion of neurons and glia that responded to glutamate following recovery in Ca^{2+}-free medium (G; mean ± s.e.m. of 3 independent cultures) and the corresponding ΔR values in all cells from each population (H; mean ± s.e.m from 45 – 105 cells). ** $p<0.01$.

Figure 5 Thapsigargin–sensitive Ca^{2+} stores are largely unaffected by acidic store blockers.
Typical cytosolic Ca^{2+} responses of individual neurons (A,C,E) and glia (B,D,F) stimulated with the SERCA inhibitor thapsigargin (1 μM) in the absence of extracellular Ca^{2+}. Cells were from control cultures (A, B), cultures incubated with 1 μM bafilomycin-A1 (C, D) or cultures acutely stimulated with 200 μM GPN (E, F). G – H, Pooled data quantifying the proportion of neurons and glia that responded to thapsigargin in the absence and presence of the inhibitors (G; mean ± s.e.m. of 3 independent cultures) and the corresponding ΔR values in all cells from each population (H; mean ± s.e.m from 66 – 161 cells). ns = $p>0.05$, ** $p<0.01$.

Figure 6. Recruitment of acidic Ca^{2+} stores is agonist-specific.
Typical cytosolic Ca^{2+} responses of individual neurons (A,C,E) and glia (B,D,F) stimulated with ATP (10 μM) in the absence of extracellular Ca^{2+}. Cells were from control cultures (A, B), cultures incubated with 1 μM bafilomycin-A1 (C, D) or cultures acutely stimulated with 200 μM GPN (E, F). G – H, Pooled data quantifying the proportion of neurons and glia that responded to thapsigargin in the absence and presence of the inhibitors (G; mean ± s.e.m. of 4 independent cultures) and the corresponding ΔR values in all cells from each population (H; mean ± s.e.m from 81 – 206 cells). ns = $p>0.05$, ** $p<0.01$.

Figure 7. Agonist-specific generation of NAADP.
Cellular NAADP content of untreated whole brain cell suspensions (A, C) and suspensions stimulated with either 10 μM glutamate (B) or ATP (D). Each trace was obtained from an independent preparation. E, Pooled data (mean ± s.e.m) from 4 such experiments quantifying the peak increase in NAADP levels in response to the indicated cue. ns = $p>0.05$, * $p<0.05$. F, Proposed model whereby glutamate and ATP recruit different complement of intracellular messengers and Ca^{2+} stores to mediate elevations in cytosolic Ca^{2+} (black circles). Both messengers elevate IP_3 (and possibly cyclic ADP-ribose) to release Ca^{2+} from the endoplasmic reticulum (square) via IP_3/ryanodine receptors (oval structures). In addition, glutamate elevates NAADP which activates NAADP–sensitive Ca^{2+} channels (cylinders) in the lysosome (open circle). This process provides a local trigger release of Ca^{2+} that is subsequently propagated by the endoplasmic reticulum via Ca^{2+}-induced Ca^{2+} release (+). Sites of action of the pharmacological agents used in this study are indicated.

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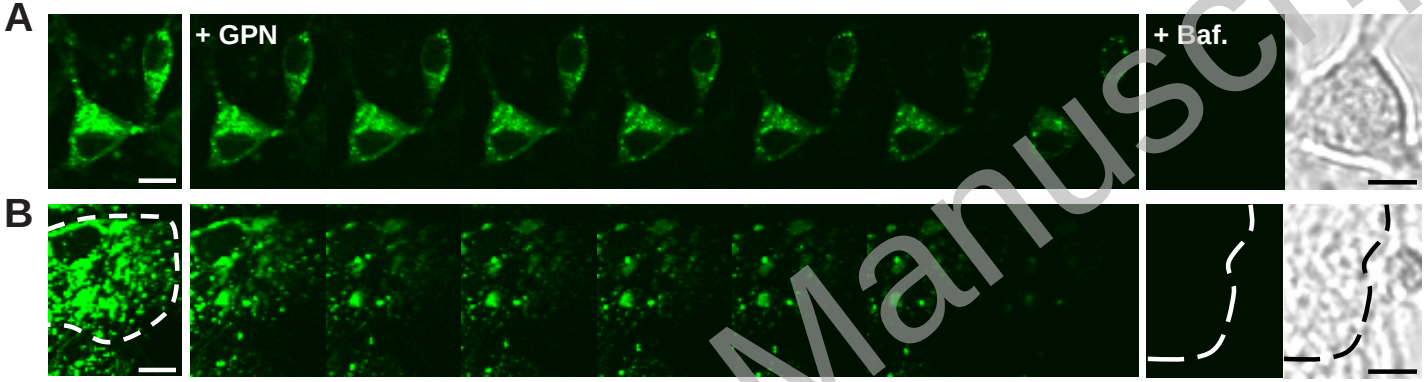
Figure 1



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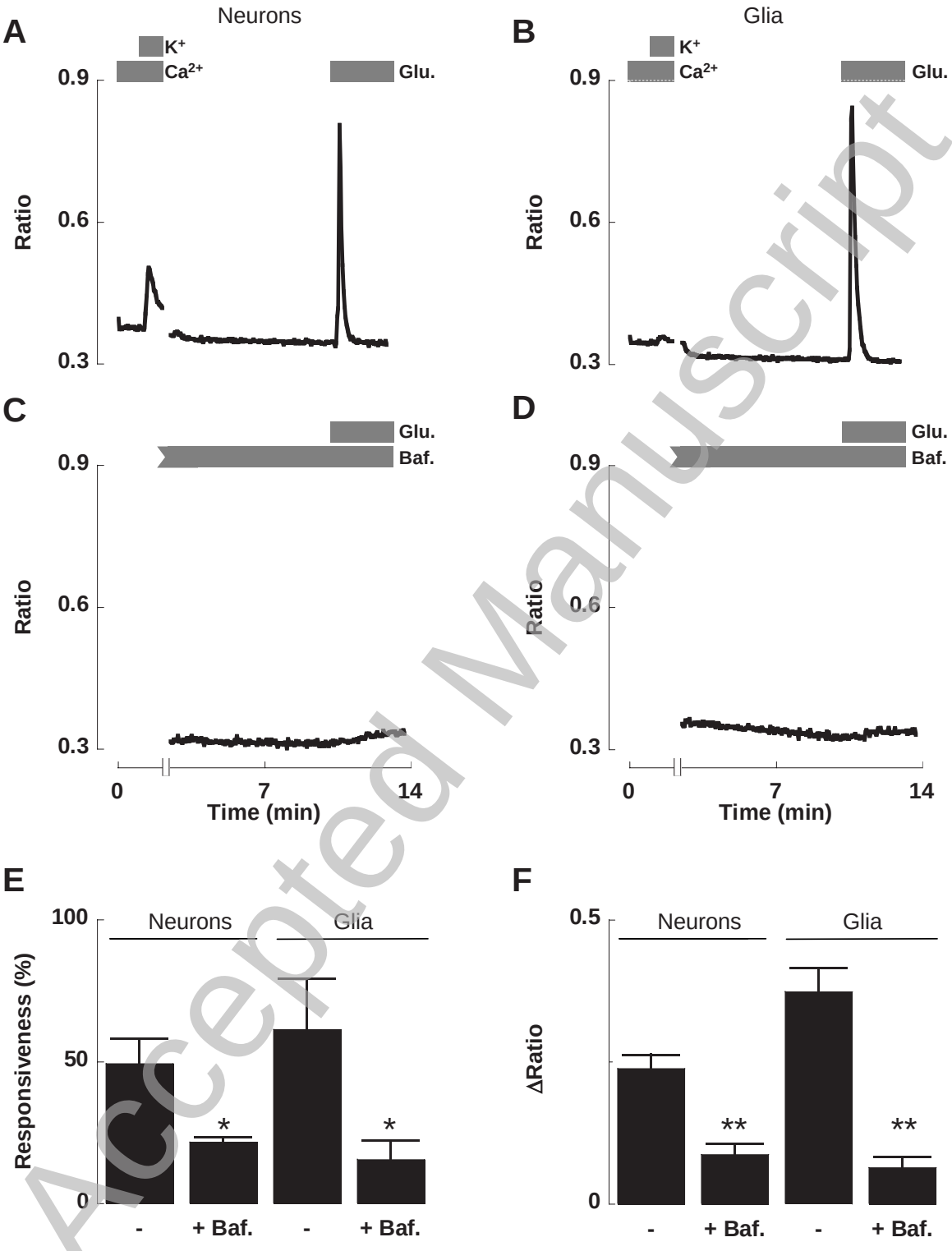

Figure 2

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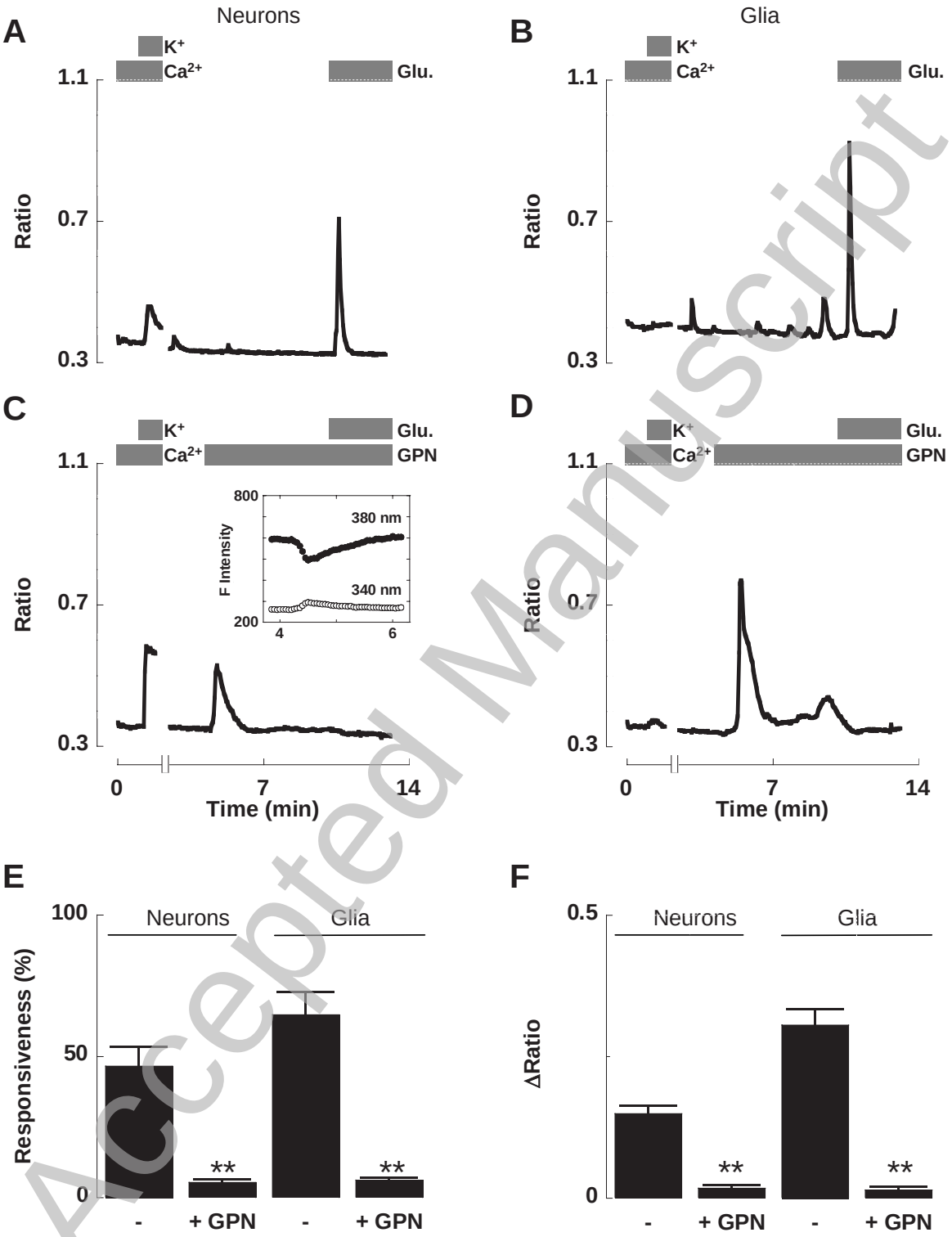

Figure 3

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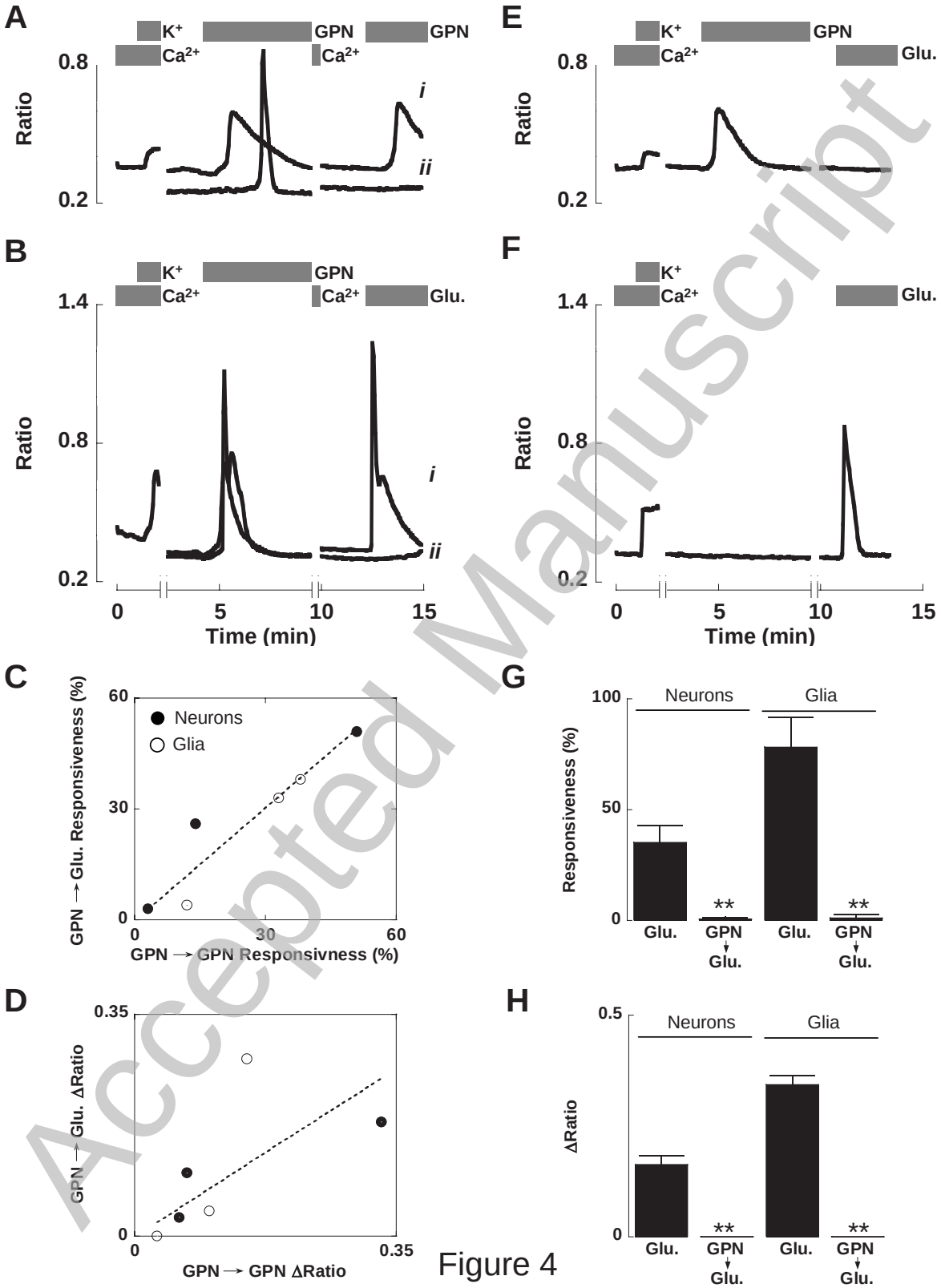

Figure 4

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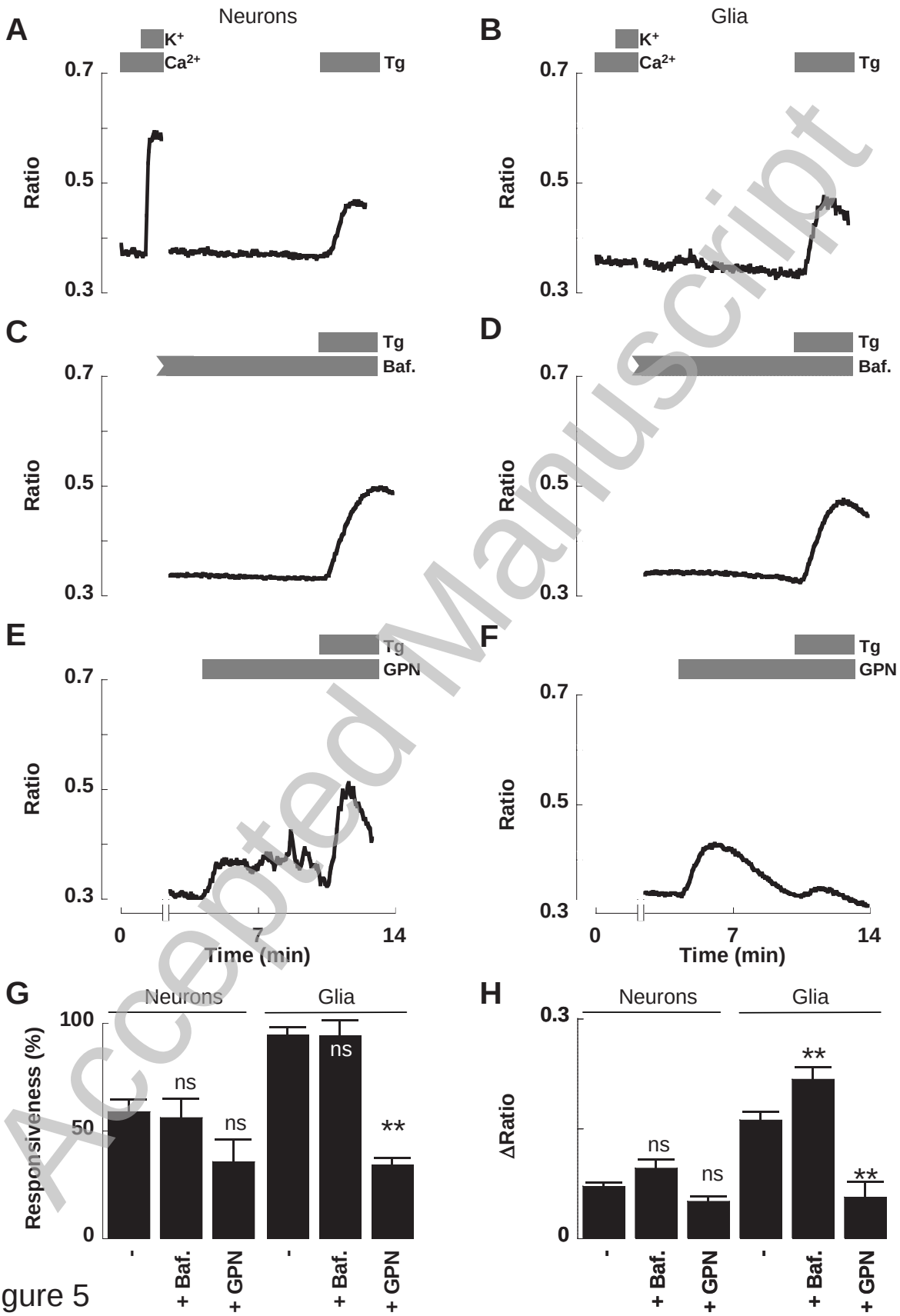

Figure 5

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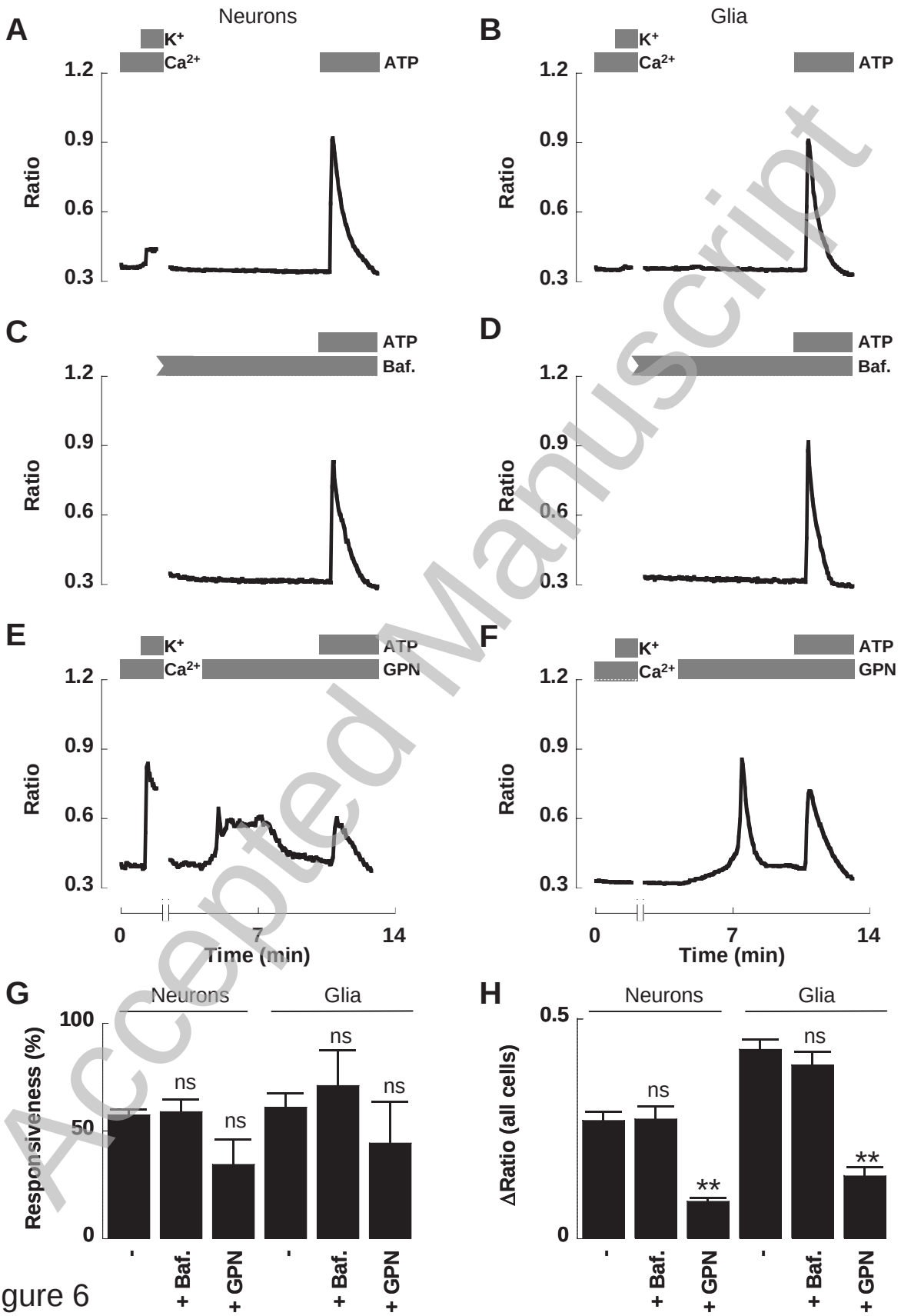

Figure 6

Biochemical Journal Immediate Publication. Published on 23 Jun 2009 as manuscript BJ20090194

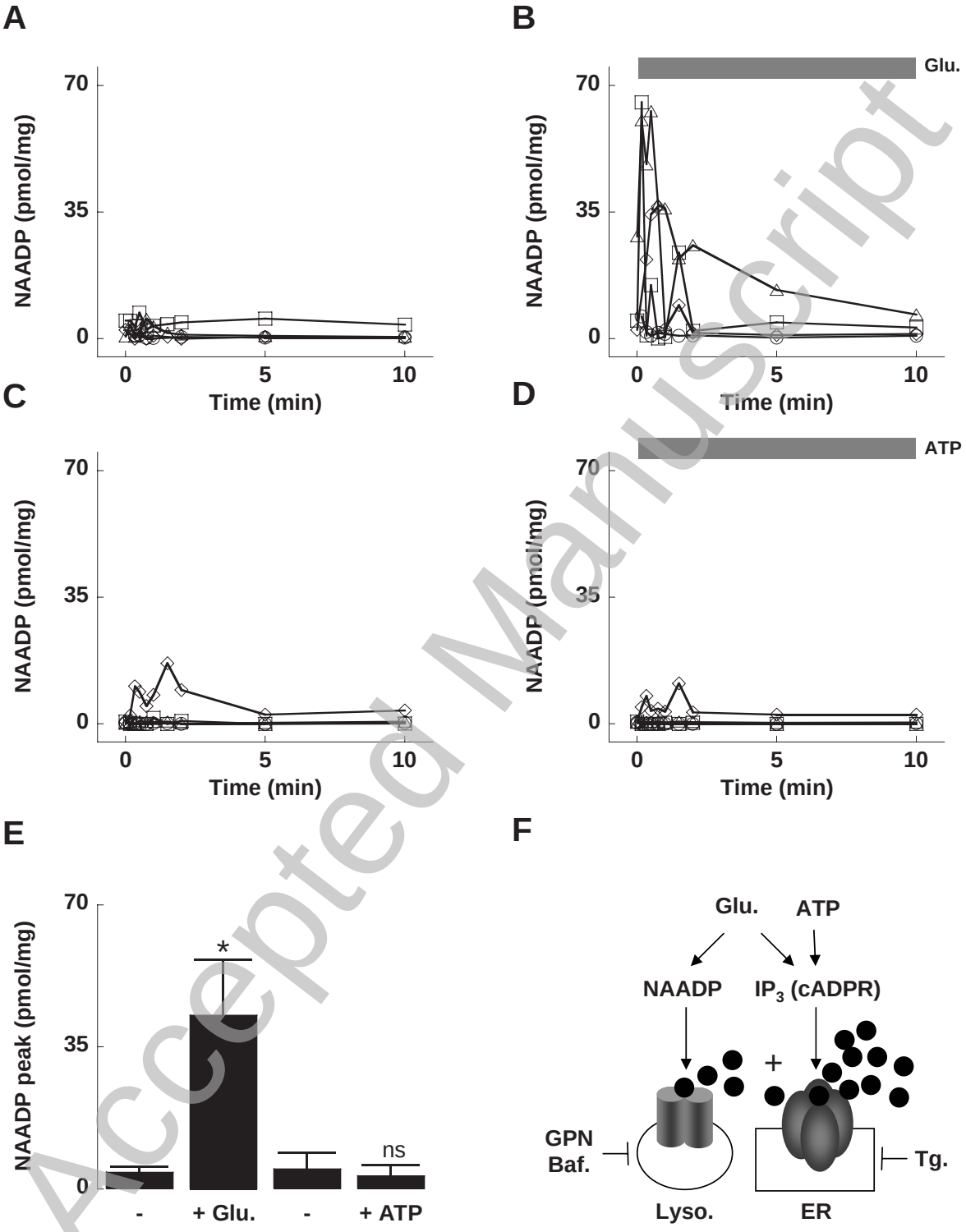

Figure 7