ATP modulates Hypothalamic Oxytocin Exocytosis via a novel Non-Purinergic receptor mechanism

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Abstract: The use of 56mM potassium chloride (KCl) to stimulate neurotransmitter or hormone release from central neurons is canonical but has been criticized for being nonphysiological. Recent data from this laboratory suggest that Adenosine triphosphate (ATP) is involved in a nonmetabolic way acting as a direct or indirect stimulant of oxytocin release from spinal cord synaptosomes.
Key Words: Mitochondria, ATP, Oxytocin, Purinergic receptor sub-family
Materials and Methods: We have examined the possibility that ATP has an effect in modulating the release of oxytocin from hypothalamic synaptosomes. To test this hypothesis, we used hypothalamic synaptosomes obtained from 30 day old Sprague-Dawley rats.
Results: Data obtained from these experiments demonstrated that ATP caused a 200 % increase of oxytocin (OT)release over control. ATP-induced release was not inhibited by pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), a P2X 1,2 or 3 purinergic receptor antagonist. The agonists of the P2Y receptor, ADP, and ATP-γ –S, the agonist of P2X4/6, did not stimulate oxytocin release over the control value. The use of GTP did not stimulate OT release above control, arguing against a simple metabolic role for ATP in this mechanism as suggested by data from experiments using PPADS, a selective P2 X1,2or 3 receptor antagonist.
Conclusion: Studies with known agonists and antagonist of the purinergic receptor family suggest that ATP stimulated release of OT from hypothalamic synaptosomes could be mediated by a novel receptor/protein other than a member of the purinergic receptor family.

Introduction

Magnocellular neurons of the hypothalamus found in the supraoptic nucleus and the paraventricular nucleus are the primary producers of Oxytocin (OT). The majority of this secretion travels via axons of the infundibulum to the posterior pituitary where it is released into the pituitary portal system as a hormone [1].

OT and OT receptors (OTRs) are found in many locations in the central nervous system. Recently, OT release has been identified in visceral organs such as the stomach, small intestine, kidney and heart (unpublished data of Robert Parks and Jaya Haldar 2010). The extensive anatomical presence of OT and OTRs indicates that OT might play a significant role in many physiological systems.

The classic hormonal activities of OT, eg. parturition, and milk ejection , are well known. But, OT mediates higher order

behavioral activities. Maternal behavior is closely associated with OT release in cortical synapses. Complex social interactions, such as monogamy have been related to OT and OTR activity [2]. Interactions between OT and digestive hormones, such as gherlin and leptin indicate a linkage with feeding behaviors such as satiety or the initiation of feeding [3].

Previous results from our laboratory suggests that ATP and the mitochondria may be important in the regulatory mechanism that controls the release of OT as a neurotransmitter in the spinal cord of rats (Chowdhury,N. Ph.D thesis 2004). Rotenone (RT) and potassium cyanide (KCN), both classical inhibitors of the electron transport chain, strongly inhibited OT release from spinal cord synaptosomes. These observations suggested that ATP has some role in OT release.

There are two families of purinergic receptors the P2X family and the P2Y family. Both receptors regulate calcium transport. The P2X group is made up of ionotrophic (directly gated) Ca^{++} receptor /channels. They are designated P2X 1, 2, 3 , 4 , 6, and a heterodimer of the 4 and 6 isoforms has also been reported. The preferred ligand is ATP. The antagonist for type P2 X1, X2, and X3 is PPADS (Pyridoxal-phosphate-6-azophenyl-2', 4'-disulfonate) and the agonist for the X4 and X6 is ATPgS.

The P2Y family members are metabolic (indirectly gated) receptors. They are members of the seven trans-membrane receptor group or G protein coupled receptors (GPCR). The second messenger system employed is usually diphospoglycerate (DPG), which associates it with the PKC pathway. The CCK receptor is also a member of this group of receptors. The preferred agonist of P2Y is ADP.

Studies by Song and Sladek (2007), done with hypothalamic/pituitary preparations suggest a important regulatory role for either or both purinergic receptor systems in the direct stimulation of antidiuretic hormone (ADH) and OT release. In these preparations, release was inhibited by micromolar concentrations of the P2X antagonist PPADS (Song 2007).

MATERIALS AND METHODS

Dissection

For each experiment, three one month old, 75-100 gram Sprague-Dawley male rats were sacrificed by decapitation. The brains were quickly removed from the skull, excess blood washed from the brain with ice cold phosphate buffered saline (PBS) . Four incisions were made in the brain: i) anteriorly to remove the frontal cortex up to the optic chiasm, ii) posteriorly to remove the brainstem and cerebellum, iii) laterally on right and left to remove the temporal cortex, and iv) superiorly, to remove the parietal cortex. A block of tissue remained which contained the following: i) the optic chiasm, anteriorly, ii) the posterior end of the third ventricle, iii) the medial eminence on the inferior border, and iv) superior end of the block contains the superior portion of the third ventricle. The resulting dissected tissue contained the hypothalamic neurons of interest, namely the paraventricular and supraoptic nuclei that contain large numbers of oxytocinergic neurons (Figure 1). The tissue from all 3 animals were pooled, weighed and sucrose gradient buffer (SGB) as a volume at 5 times the weight of the pooled tissue was added. The composition of SGB is 320mM sucrose, 1mM EDTA and 0.25mM dithieothreitol (DTT). The tissue was homogenized with 8-

10 strokes of a Dounce homogenizer. Sample

Synaptosome preparation by differential centrifugation

All centrifugations were performed at 4^0 C. The homogenized tissue was centrifuged at 3600 x g for 10 minutes. The supernatant fluid was collected into a cold centrifuge tube. The pellet was resuspended in 2 ml of SGB and centrifuged again for 10 minutes at 3600 x g. This supernatant was added to the supernatant fluid. The combined supernatant fluid was carefully layered onto a column of sucrose gradient consisting of 3%, 12%, 15%, and 23% sucrose from top to bottom. The sample was centrifuged at 11,000 x g for 11 minutes . The purest synaptosomal fraction was found in a band between the 15% and 23% sucrose. This band was collected with a Pasteur pipette and washed twice with cold Krebs buffer and collected by centrifugation at 1500 x g [4,5,6] and visualized by transmission electron microscopy (Figure 2).

Incubation

After washing, the entire yield of synaptosomes were resuspended in 3.3ml of incubation media (1540 mM NaCl, 56 mM KCl, 10 mM $MgCl_2$, 0.25% BSA, 100 mM glucose, 100 mM HEPES), the suspension is mixed on vortex and divided into equal portions of 500 ul. In all experiments samples were incubated for 3 minutes. The remaining volume was saved for determination of protein content [7]. Antagonists were always added 1 min. prior to the stimulant, except for the mitochondrial Ca^{++} channel blocker CGP 37157, that was added three minutes prior to stimulation [8,9].

Quantification of OT release by Radioimmunoassay (RIA)

The OT molecule contain one disulfide bridge between the Cys in positions 1 and 6. RIA uses the Tyr in position 2 for attachment of an I^{125} label, which is essential for quantification of OT. The low molecular weight (1007) of OT makes its detection difficult by electrophoretic methods. Thus, the use of RIA becomes important, due to the fact that this assay has very high sensitivity. The RIA can detect quantities as low as 1 pg of OT. At the end of three minute incubation period, tubes were spun in a microcentrifuge for one minute and the supernatant fluid was collected from each sample. The sample from each incubation experiment was assayed using RIA. The unknowns were incubated with anti-OT antibody for 5 days followed by incubation with I^{125} OT for 2 days. The unknowns were compared to a standard curve and the quantities of OT released from each experiment was expressed in picograms (pg). After Biorad protein determination for each experiment, the pg OT release was standardized to pg/mg protein for preparation of charts.

Materials

Percoll for sucrose gradient purchased from Fischer Bioscience.
Antibody used in RIA was a polyclonal anti-OT antibody created and donated by Dr. Robert Feldman.

Hypothalamic synaptosomes were prepared from one month-old, 75-100 gram Sprague-Dawley male rats. Three animals per experiment were obtained from Taconic labs, Elmont, N.Y. The animals were quickly sacrificed by cervical dislocation and decapitation. This was done as quickly

as possible and every effort was made to minimize suffering or stress to the animals. Protocols for animal care and use were strictly adhered to as prescribed by the committee on Animal care and Use of St. John's University, Queens, N.Y. All animal protocols were done with strict adherence to the Guide.

RESULTS

Cholecystokinin (CCK8) has been shown to be a good physiologic stimulant of OT release from synaptosomes derived from spinal cord and other brain areas (Chowdhury & Haldar, unpublished data). To determine whether CCK 8 is an effective stimulant for hypothalamic synaptosomes as well, we used different concentrations of CCK8. Dose response curve obtained with CCK8 (Fig. 2) demonstrates that 50nM CCK8 gave the strongest and most consistent release of OT from the hypothalamic synaptosomes. Therefore, in all following experiments 50nM CCK-8 has been used as the positive control . These data in figure 2 represent an average of five experiments.

Similar to CCK8 experiments, ATP also causes OT release from hypothalamic synaptosomes (Fig. 3). The 50nM dose of ATP strongly stimulated OT release from hypothalamic synaptosomes. These data represent an average of four experiments. Experiments were done to identify if a purinergic receptor system was responsible for the transduction of ATP mediated OT release. Previous reports have suggested a relationship between P2X or P2Y receptors in endocrine release. (Song 2007,Hegg 2003,Denda 2012,Custer 2012, Knott 2008,2005, Troadec 2002,Buller 1996, Chen 1995, Day 1993).
PPADS with a dose of 1 uM was unable to inhibit ATP induced OT release in hypothalamic synaptosome . The histogram in figure 4 consisted of treatment of synaptosomes with PPADS for 1 minute followed by 50nM ATP added on top of the PPADS. These data strongly suggest that ATP–induced OT release was not mediated by a P2X purinergic receptor. Many authors have reported an important role for a purinergic receptor in the mediation of hormone release in the pituitary, hypothalamus and other tissues. However, in our system , even with a ratio of 50 nM ATP and 1uM PPADS there was no inhibition.

In an attempt to examine these data, agonist to other P2X and P2Y receptors were used. ADP is a well documented agonist of the P2Y receptor. In experiments done with ADP at similar concentration and time there was no significant increase in OT release over the control value.

ATP –γ- S, the agonist of the P2X 4/6 receptor, was used as a stimulant ligand at the standard incubation conditions. Here again there was no increase in OT release over the control value. (Figure 5)

Lastly, GTP was used to test if this purine nucleotide would effectively stimulate OT release like ATP. GTP did not stimulate OT released to an equal or greater extent to ATP, again yielding a OT concentration that was close to the control value. These data also suggest that the nucleotide is not acting in a simple metabolic way but is a tranducer in a signal pathway that results in OT exocytosis from the synaptosome preparations. (data not shown)

DISCUSSION

Two families of receptors use adenosine nucleotides as their preferred ligand.

The P2Y receptor (P2YR) is a member of the GPCR group of receptors.

These receptors commonly employ protein kinase pathways to transduce the message for the release of Ca++ . They are commonly found on plasma membranes. In experiment using ADP as a stimulant there was no OT release over the control value (Figure 5)

The P2X receptor (P2XR) is a ligand-gated Ca^{++} channel. It is found on organelle membranes and plasma membranes. When it is present on organelle membranes, such as ER or mitochondria, it functions as a directly (ligand) gated efflux calcium channel. The presence of a P2X receptor in this system would simply explain the role of ATP and Ca^{++} in OT release.

ATP consistently stimulates OT release from synaptosomes (figure 4) but the use of PPADS, a selective P2X receptor antagonist, consistently failed to inhibit ATP mediated release of OT in this system(Figure 5) . The P2X 1,2and 3 subtypes of this receptor family has been shown to be more sensitive to PPADS than other subtypes suggesting that a different P2 receptor could be active in our system and modulate the release of OT from hypothalamic preparations (Song 2007, Troadec 2002 and Hegg 2003). This suggests two possible P2X receptors, P2X4 or P2X6 or a heterodimer of the two. This negative data might suggest that in our system P2X4 or P2X6 are working rather than P2X1,2 or 3 subtypes. Both of these receptors are only mildly affected by PPADS. They are commonly found associated with the CNS. The agonist of P2X4 and P2X6 receptors is ATP γ -S. The preferred ligand of P2YR is ADP (Abbracchio et al. (2006). In experiments with ATP γ–S there was no increase in the stimulated release of OT by ATP- γ -S when compared to ATP(figure 5). Experiments with ADP demonstrated weak stimulation as well. (figure 6)

To test if the other purine nucleotide, Guanosine, could be playing a role in this system, GTP was used as a stimulant in release experiments. GTP stimulation at the same concentration and time showed no increase in OT release when compared to ATP or control values (Figure 8). These data also suggest that the stimulatory effect of ATP on OT release from synaptosomes is not a simple energetic or metabolic effect.

In conclusion, PPADS , ATPγS, and ADP experiments argue against a P2 mediated activation . Opening the possibility that a novel receptor / protein is transducing the activation of mitochondrial calcium efflux with resultant OT release from the hypothalamus. Three possible mechanisms include ATP acting as an intracellular , autocrine or paracrine stimulus. Future research will be aimed at divining these possibilities.

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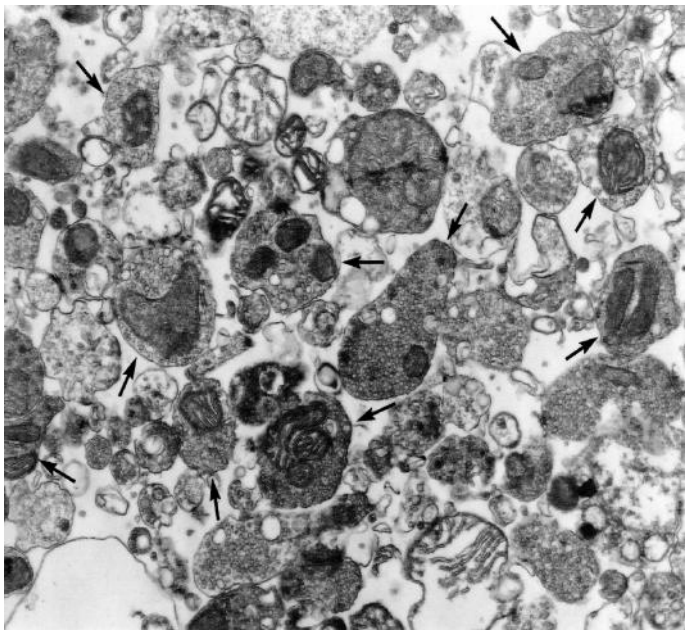


Fig. 1. Transmission electron micrograph of synaptosome preparation. In this TEM the synaptosomes can be seen (arrows). A fragment of a mitochondrion can Be seen in lower right of this figure.

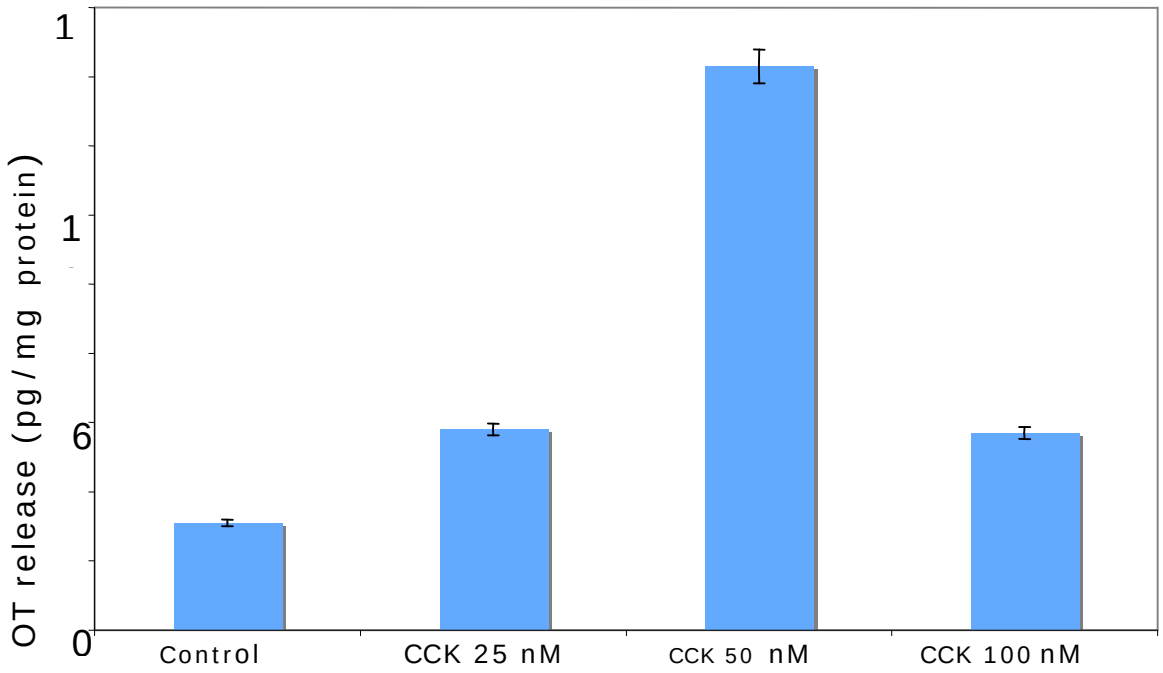


Fig.2. The effect of CCK on oxytocin release from synatosomes. The data represent the average of five experiments and the error bars are the standard error of the mean (SEM).

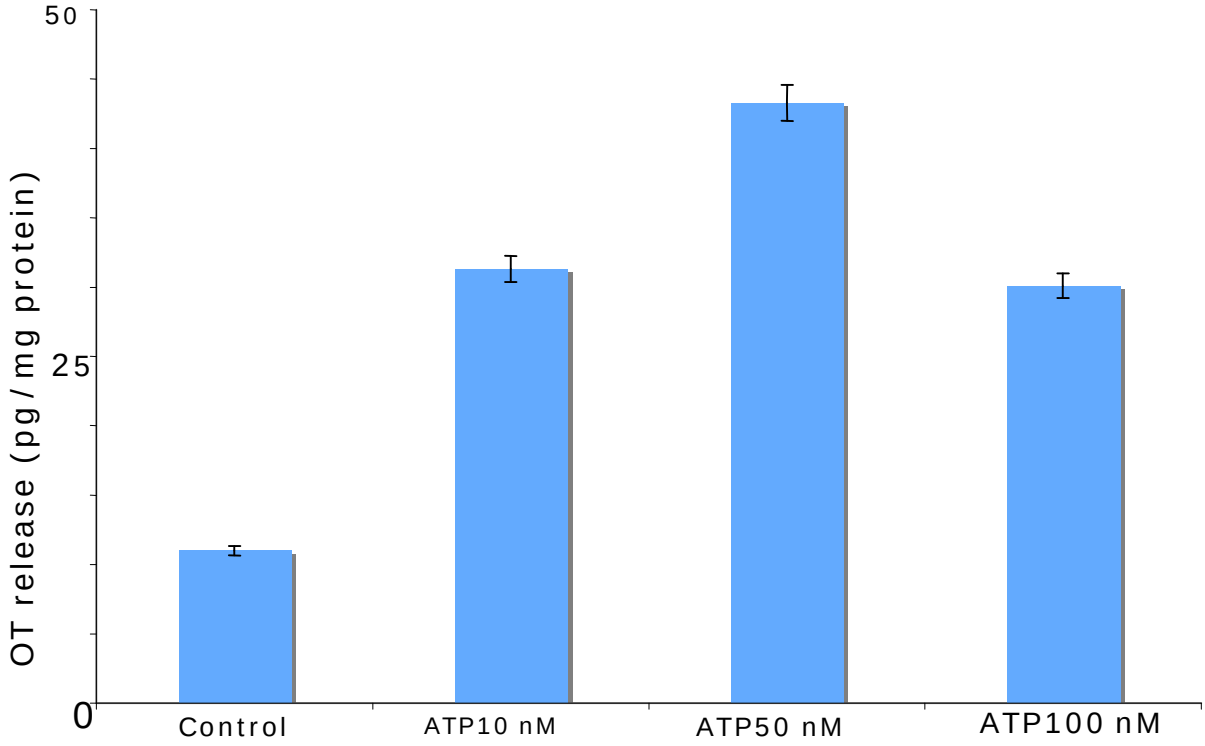


Fig 3. Effect of ATP on oxytocin release from synaptosomes. The data represent the average of five experiments and the error bars are the standard error of the mean (SEM).

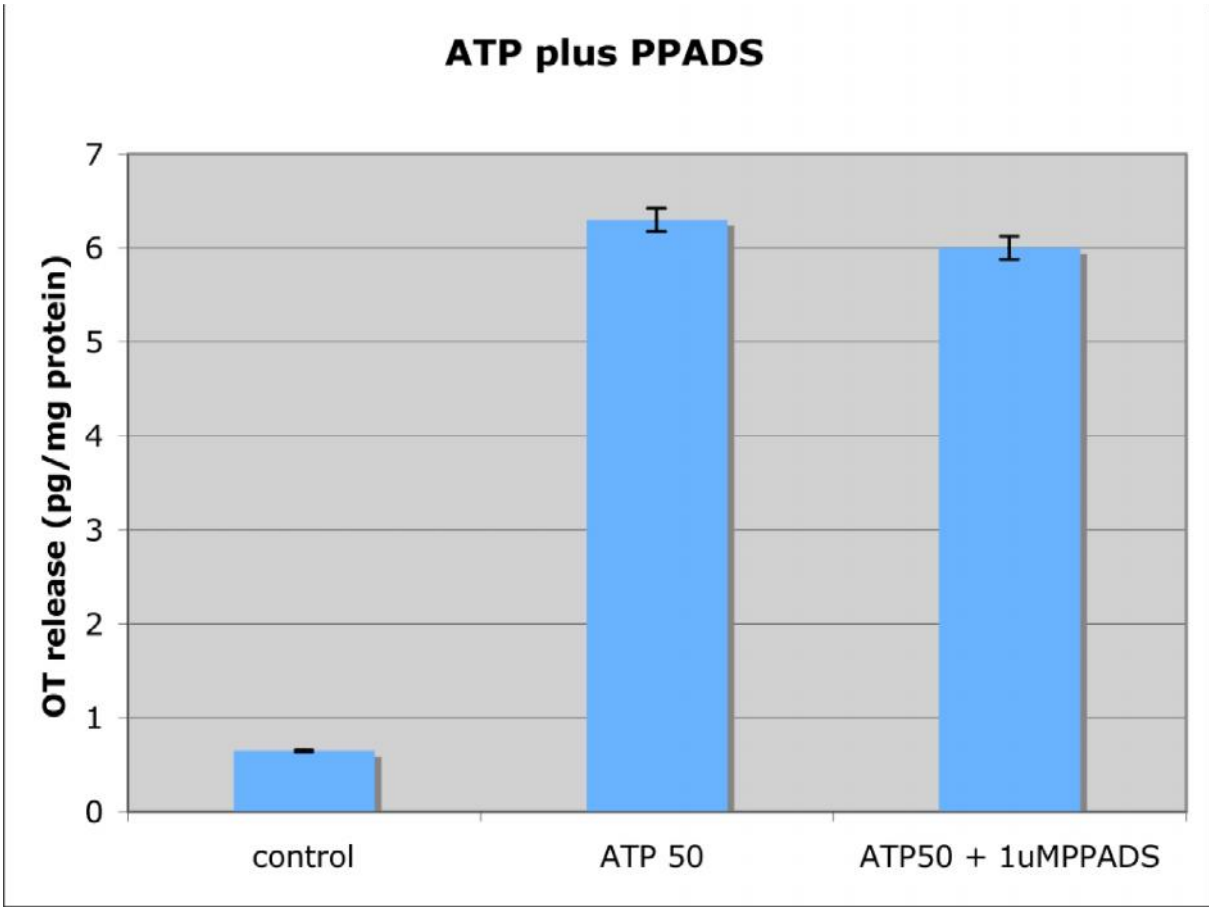
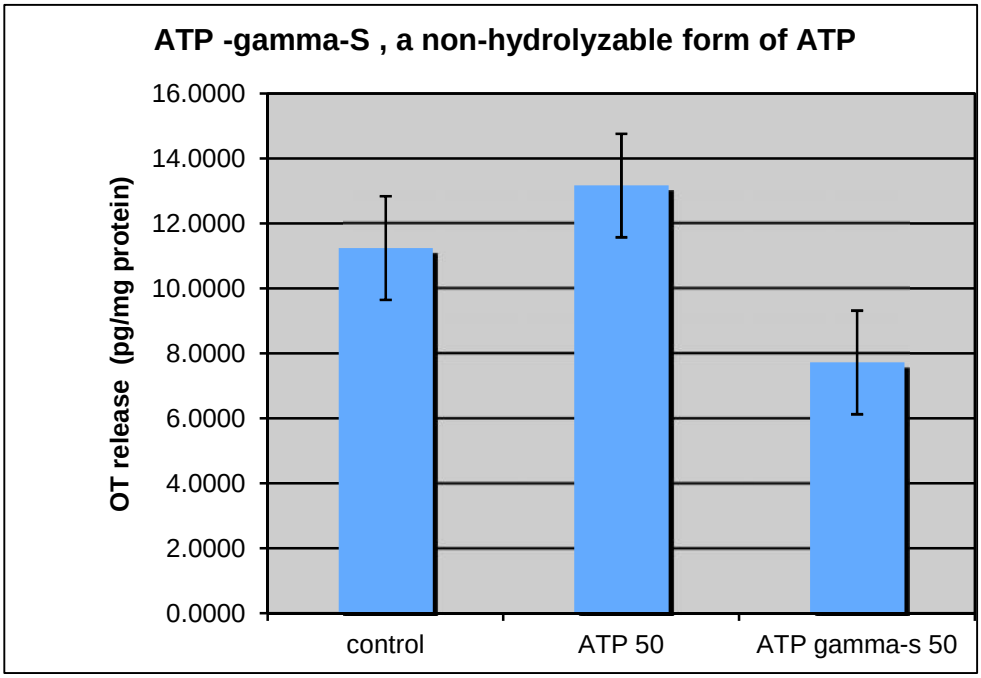


Fig. 4.
PPADS with a dose of 1 uM was unable to inhibit ATP induced OT release. the data in this figure includes 9 separate experiments. Each experiment consists of three variable samples (ATP 50nm + 1uM PPADS) one control (unstimulated) and one positive control (ATP 50nM). PPADS was incubated for 1 min. followed by ATP added on top of PPADS for 3 min. Error bars are SEM. In additional trials, concentrations of up to 1 mM PPADS were used with no change in result (figure not shown) These data argue against a P2X1, 2 or 3 receptor system being active in this system.

Fig.5 ATP γ S

In these incubation experiments The time and concentration was consistent with all other test. The consitiutive release , negative control , was larger in these samples than in prior tests.



Regardless, the concentration of secreted OT in the ATP-γ-s samples was much smaller than the 50 nM ATP samples indicating very weak stimulation of OT from the synaptosomes. ATP –γ-s is a known agonist of the P2X 4/6 receptor . This receptor system is reported to be common in the CNS, particularly the hypothalamus. ATP γ s is a weakly hydrolysable form of ATP . The weak performance of this stimulant not only argues against the P2x4/6 receptor but could suggest that ATP needs to be hydrolysed to some extent to stimulate OT. Data from ADP and GTP (figures not shown) further weaken this suggestion that hydrolysis is a factor.