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**The subpopulation of microglia sensitive to neurotransmitters/
hormones is modulated by stimulation with LPS, Interferon- γ and IL-4**

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Main Points

We found that subpopulations of acutely isolated or cultured microglia express distinct neurohormone and neurotransmitter receptors. We observed that these subpopulations change in cultured microglial cells when activated indicating that microglia comprise a highly heterogeneous population of cells with respect to their chemosensitivity.

Key Words

Neurotransmitter receptor, neurohormone receptor, microglia, IFN-gamma, LPS, IL-4

Abstract

Neurotransmitters/-hormones have recently been identified as factors controlling the function of microglia, the immune competent cells of the central nervous system. In this study we compared the responsiveness of microglia to neurotransmitters/-hormones. We freshly isolated microglia from healthy adult C57Bl/6 mice and found that only a small fraction (1 - 20 %) responded to the application of endothelin, histamine, substance P, serotonin, galanin, somatostatin, angiotensin II, vasopressin, neurotensin, dopamine or nicotine. In cultured microglia from neonatal and adult mice, a similarly small population of cells responded to these neurotransmitters/-hormones. To induce a pro-inflammatory phenotype, we applied LPS or IFN- γ to the cultures for 24 h. Several of the responding populations increased, but there was no uniform pattern when comparing adult with neonatal microglia or LPS with IFN- γ treatment. IL-4 as an anti-inflammatory substance increased the histamine, substance P and somatostatin sensitive populations only in microglia from adult, but not in neonatal cells. We also found that the expression of different receptors was not strongly correlated indicating that there are many different populations of microglia with a distinct set of receptors. Our results demonstrate that microglial cells are a heterogeneous population with respect to their sensitivity to neurotransmitters/-hormones and that they are more responsive in defined activation states.

Introduction

Microglial cells are the resident immune cells of the brain. It is well established that microglial cells are activated by any pathologic event in the brain. They transform from a ramified morphological phenotype into an amoeboid form. Activated microglia typically increase their phagocytic activity, release pro-inflammatory cytokines, migrate to the site of injury or increase the expression of the MHCII molecule for antigen presentation. Despite the notion that the morphological transformation is a common indicator of their activation, the functional changes can be quite distinct with respect to the type of pathology and the pathological progression (Hanisch and Kettenmann 2007). We have recently demonstrated that (morphologically activated) microglial cells in a mouse model of Alzheimer's disease decrease their phagocytic activity coincident with the appearance of plaques (Krabbe et al. 2013).

Microglial activation is controlled by a plethora of different factors. One can distinguish between 'on signals', which are newly appearing signals such as bacterial cell wall components during infection, and 'off signals' which disappear or are down-regulated (Biber et al. 2007). Neurotransmitters can act as such 'off signals' for microglia (Pocock and Kettenmann 2007). A series of studies has demonstrated that microglial cells express a large variety of transmitter and hormone receptors, which control distinct microglial functions. Functional receptors for glutamate, GABA, noradrenalin, dopamine, ATP and serotonin have been identified and have been shown to modulate distinct microglial activities (Kettenmann et al. 2011). Activation of the P2Y₁₂ purinergic receptor induces microglial chemotaxis (Haynes et al. 2006) while activation of serotonin receptors promotes process motility but decreases phagocytosis (Krabbe et al. 2012).

In the present study we tested the functional expression of 7 neurohormone receptors (endothelin, substance P, galanin, somatostatin, angiotensin II, vasopressin and neurotensin), and 4 neurotransmitter receptors (histamine, serotonin, dopamine and the nicotinic acetylcholine agonist nicotine) in microglia freshly isolated from adult mice and in cultured neonatal and adult microglia. In order to mimic pro- and anti-inflammatory phenotypes in cultured microglia, we also treated neonatal and adult cultures with LPS, interferon gamma (IFN γ) and IL-4. Since most of the receptors which we studied, with the exception of nicotine, trigger InsP3-mediated release of calcium from internal stores, we employed calcium imaging to investigate their function during physiological and pathological conditions.

Methods

Preparation of acutely isolated microglia

Microglia from adult C57BL/6 mice (P49-56) were acutely isolated and purified for calcium imaging using magnetic activated cell sorting (MACS). Microglia were isolated from cell suspensions using magnetic CD11b microbeads from Miltenyi Biotec (Bergisch Gladbach, Germany), according to the manufacturer's instructions and as previously described (Nikodemova and Watters 2012). In brief, adult mice were sacrificed by cervical dislocation and the brain was removed. After removal of the cerebellum and olfactory bulbs, the remaining cortex and midbrain was cut into small pieces (1mm³) and dissociated using the Miltenyi Biotec Neural Tissue Dissociation Kit (Trypsin). After dissociation using a glass Pasteur pipette, the suspension was passed through a 40µm cell strainer followed by centrifugation for 10mins at 300g and 4°C to obtain a single cell suspension. The cell suspension was then incubated for 15mins with Miltenyi Biotec myelin removal beads, followed by magnetic separation using LS columns, with 2 columns used per brain. Following myelin removal, the myelin free flow-through was incubated with CD11b beads for 15mins, and the cell suspension was applied to an MS column (Miltenyi Biotec) and washed three times with HBSS/0.5% BSA/2mM EDTA. Labelled CD11b positive cells (microglia) within the column were then flushed out and plated onto glass coverslips, followed by 15 minutes incubation in DMEM medium to allow adherence. Calcium imaging was then carried out as described below.

Preparation of cultured microglia

Primary microglia cultures were prepared from the cerebral cortex and midbrain of newborn C57BL/6 mice (P0-P3) as described previously (Giulian and Baker 1986). The microglia were seeded at $1-1.5 \times 10^5$ cells per glass coverslip (14 mm diameter). Cultures usually contained >95% microglia as detected by isolectin B4 (*Griffonia simplicifolia*) and were used for experiments within 1 day after plating.

Microglia were also cultured from adult mice (P49-56) as previously described in detail (Scheffel et al. 2012). Briefly, cortical and midbrain tissue was freed of blood vessels and meninges in Hank's balanced salt solution (HBBS), mechanically dissociated into 1mm^3 pieces and trypsinized in 1% trypsin and 0.05% deoxyribonuclease for 5 min at room temperature, as described for neonatal microglia elsewhere (Giulian and Baker 1986). Digested tissue was dissociated using a fire-polished pipette and washed twice in HBBS. Cells were then plated on a confluent monolayer of P0 astrocytes in 75cm^2 flasks. The feeder layer of astrocytes was depleted of neonatal microglial cells using clodronate (200 $\mu\text{g}/\text{ml}$) before the adult microglia were added. The adult mixed glial cultures received fresh complete DMEM medium every other day and were treated with 33% L929-conditioned medium after 7 days or once cells became confluent. Microglia were shaken off 1 week later and were used for experiments within 1 day of plating.

Activation of cultured microglia

To induce a pro-inflammatory phenotype, microglia cultured from neonatal and adult cells were incubated with 100 ng/ml lipopolysaccharide (LPS) (Enzo, New York, NY, USA) or 20 U/ml IFN- γ (R&D systems, Minneapolis, MN,

USA). An anti-inflammatory phenotype was induced by incubating with 30 ng/ml IL-4 (Peprotech, Rocky Hill, NJ, USA). Stimulating agents were added to cultures 5-7 hours after plating microglia on glass coverslips. Incubation was carried out overnight for 18-24 hours.

Calcium imaging

Freshly isolated cells were loaded with 5 μ M fluo-4/AM (Invitrogen, Carlsbad, CA, USA) in the presence of the detergent 0.02% Pluronic F-127 in HEPES buffer which contained (in mM) NaCl 150.0, KCl 5.4, MgCl₂ 1.0, CaCl₂ 2.0, HEPES 10.0, glucose 10.0, adjusted with NaOH to pH 7.4 supplemented with 1% tomato lectin conjugated Alexa Fluor®594 for 40 min at room temperature. After staining, coverslips were washed for 10 min in HEPES buffer. Cultured microglia were stained with 5 μ M fluo-4/AM for 40 min at room temperature followed by a 10 min wash in HEPES buffer without the addition of tomato lectin. Coverslips were then transferred to a recording chamber and buffer flow was adjusted to 3-4 ml/min. Fluorescence pictures were taken every 3 s at 488 nm excitation wavelength produced by a Polychrome II monochromator (Till Photonics, Martinsried, Germany) using 40x Achromplan water immersion objective (NA 0.75, Zeiss, Jena, Germany). A perfusion system was installed to change the solution within seconds. Before calcium recording from freshly isolated cells, tomato lectin-positive microglia were identified by fluorescence excitation at 560 nm and emission observed at 630 nm. In order to determine whether cells responded to the application of the test substance, an algorithm was used. This algorithm calculated the average baseline of the calcium signal 1min before application of the test substance and the peak of the fluorescence signal during application. When

the peak of the fluorescence signal was at least 4 standard deviations higher than the average baseline, this was considered a positive response. The peak was only counted as positive if it occurred at least 10 s after the start of application, in order to account for the delay in the concentration change in the chamber. If there was significant bleaching, which resulted in a shift of the baseline, this was corrected for.

Statistical analysis

All data represent the percentage of cells responding from at least 3 mice for freshly isolated microglia or 3 preparations for cultured microglia. The total number of cells responding to ATP was calculated for all experiments for each condition. The total number of cells responding to a given substance in a given condition was then calculated as a percentage of the ATP responding population. Based on the ability of cells to respond with an elevation of intracellular Ca^{2+} to the application of neurotransmitters/hormones, every individual cell was categorized as responding or non-responding; subsequently data were collected as categorical variables and the proportions of responding cells were compared between different experimental conditions (adult freshly isolated, untreated adult cultured and neonatal cultured as well as treatment of cultured cells with LPS, IFN or IL4). Since our data are categorical, we cannot use ANOVA with post-hoc correction tests, which are used to analyze normally distributed quantitative variables. Instead we applied chi-squared test for comparison of multiple proportions (R software, stats, chisq.test function) followed by the post-hoc Marascuillo procedure to test for the significance of differences between pairs of proportions (Marascuillo

1966). Lower and upper limits of the 95% confidence interval for a proportion was calculated according to the procedure described by E. B. Wilson (Wilson 1927) using R software (stats, prop.test function) (Verzani 2005), The differences were considered statistically significant at $p < 0.05$.

Results

Subpopulations of freshly isolated microglia respond to defined neurotransmitters/-hormones

We isolated microglia from adult mouse cortex and midbrain by MACS using CD11b microbeads to specifically purify microglia. The cells were loaded with the Ca^{2+} -sensor Fluo-4 for 40 min and labelled in parallel with 1% tomato lectin conjugated Alexa Fluor®594 to verify that the cells were microglia. Each neurotransmitter/-hormone was applied for one minute and after a 5 min wash, ATP (500 μM) was applied for 30 seconds. We selected only those cells which showed a clear response to ATP which were, in most experiments, all cells in the selected field. Of those cells which responded to ATP, 1% of cells responded to endothelin application, 10% to histamine, 26% to substance P, 9% to serotonin, 14% to galanin, 11% to somatostatin, 7% to angiotensin II, 5% to vasopressin, 8% to neurotensin, 6% to dopamine and 6% to nicotine with a transient increase in Fluo-4 fluorescence corresponding to an increase in Ca^{2+} . Figure 1A-K shows examples of a positive response for each substance and figure 1L shows the percentage of freshly isolated adult microglia responding to each substance. Table 1 describes the mean integral and amplitude of the calcium responses for each substance compared to the ATP response, as well as the duration of the calcium transients. The mean amplitudes and integral (which combines the data of amplitude and duration) were typically smaller when compared to the ATP response, while the duration was calculated as the mean number of seconds from the beginning of the increase in fluorescence, which was four standard deviations higher than the baseline, to the end of the signal. Figure 2A shows images of freshly isolated, as well as neonatal and adult cultured microglia,

during calcium imaging with neurotensin and ATP as a positive control. Figure 2B shows the corresponding calcium traces for highlighted cells from each preparation.

Subpopulations of cultured neonatal and adult microglia respond to neuropeptides/-hormones

Endothelin-1, histamine, substance P, serotonin, galanin, somatostatin, angiotensin II, vasopressin, neurotensin, dopamine and nicotine were all applied to cultured neonatal (postnatal day 1) microglia using the same calcium imaging protocol described above for freshly isolated cells. ATP was used as a positive control. Of those cells which responded to ATP, 6% of cells responded to endothelin application, 2% to histamine, 9% to substance P, 22% to serotonin, 4% to galanin, 6% to somatostatin, 5% to angiotensin II, 8% to vasopressin, 13% to neurotensin, 4% to dopamine and 5% to nicotine with a transient increase in Fluo-4 fluorescence corresponding to an increase in Ca^{2+} (Figure 3).

Microglia from adult brain were cultured for 1 week, then shaken off and plated onto glass coverslips after 1 week and tested for functional receptor expression using calcium imaging as before. The cells responding to endothelin (13%; $p < 0.001$) and nicotine (17%; $p < 0.001$) application was significantly higher than the percentage of responders in freshly isolated adult cells (Figure 1L). The population responding to histamine (3%; $p < 0.001$), to substance P (1%; $p < 0.001$) and to galanin (6% $p < 0.001$) was significantly lower than the percentage of freshly isolated cells. The population responding to serotonin (6%), to somatostatin (12%), to angiotensin II (11%), to

vasopressin (2%), to neurotensin (6%), and to dopamine (7%) did not significantly differ when compared to freshly isolated adult cells (Figure 4).

Distinct neurotransmitter/-hormone sensitive populations can be increased by treatment with IFN- γ , LPS and IL-4

A pro-inflammatory phenotype was induced in cultured neonatal microglia by 18h treatment with 100 ng/ml LPS or 20 U/ml IFN- γ . An anti-inflammatory phenotype was induced through overnight treatment with 30 ng/ml IL-4. LPS treatment resulted in a significantly higher population of endothelin, histamine, somatostatin and vasopressin sensitive cells (Figure 3), while IFN- γ treatment resulted in a significantly larger histamine, galanin, somatostatin, angiotensin II, vasopressin and dopamine sensitive population and a significant decrease in the serotonin sensitive population (Figure 3). IL-4 treatment did not result in an increase in any of the neurotransmitter/-hormone sensitive populations but led to a significant decrease in the substance P, serotonin, neurotensin and nicotine sensitive populations (Figure 3).

Incubation of cultured adult microglia with 100 ng/ml LPS resulted in a significantly higher population of endothelin, histamine, substance P, serotonin, galanin, somatostatin, angiotensin II and vasopressin sensitive populations (Figure 4). Treatment of adult microglia with 20 U/ml IFN- γ resulted in a significant increase in the histamine and neurotensin sensitive populations, and a significant decrease in the serotonin, somatostatin, angiotensin II and nicotine sensitive populations. Responses to all other substances were not significantly different to untreated adult microglia (Figure 4). Treatment of adult microglia with 30 ng/ml IL-4 resulted in a significant increase in the substance P sensitive population and a significant decrease in

the angiotensin II and dopamine sensitive populations (Figure 4). Table 2 summarises all the results for calcium imaging experiments from freshly isolated and cultured microglia with fold change and significance.

Consecutive application of three neurotransmitters/hormones shows heterogeneity in the expression pattern

We addressed the question of whether different transmitter receptors are expressed in a coordinated fashion or at random. We therefore applied galanin, somatostatin and angiotensin II to IFN- γ stimulated neonatal microglia since a high amount of cells responded under these conditions. When tested alone, 24, 30 and 45% of the cells responded to application of these substances, respectively. We consecutively applied galanin, somatostatin and angiotensin II to one given cell, with a 5 minute washout in between each substance and a 30 second application of ATP as a positive control at the end of the experiment (Figure 5). We found that 31% of cells responded to only one ligand, 8% to two and 2% to all three. 59% of cells did not respond at all (except to ATP). Fourteen percent responded to galanin, 3% to somatostatin and 14% to angiotensin II alone. The order of application was also changed. We subsequently tested somatostatin, followed by angiotensin II and then galanin; we found that 62% of cells did not respond to any of the ligands, 18% to only one, 16% to two and 3% to all three. Similarly, when the order of application was switched to angiotensin II – galanin – somatostatin, 67% did not respond, 28% to 1 ligand, 4% to 2 and 1% to all three. The data are summarized in Table 3. Based on the percentage of cells responding to two given transmitters we calculated the probability that they co-respond and compared it to the measured value. These values were fairly

similar indicating that the expression of the different receptors is independently regulated. When calculating the probability that a cell responds to all three different transmitters, we found that the measured value was higher than the calculated value indicating that there is a low cooperativity of expression for triple sensitive cells. The only exception was seen during the somatostatin – angiotensin – galanin application, where there were an increased percentage of cells to respond to both angiotensin and galanin than to a single response to somatostatin, angiotensin or galanin (Figure 5E). The measured probability was higher at 0.123 compared to the calculated probably at 0.051 (Table 3). This demonstrates that there may be some receptor cooperativity which is not random.

A similar series of experiments was carried out on adult cultured microglia treated with LPS (Figure 6). We consecutively applied endothelin, histamine and substance P since a large amount of LPS stimulated cells responded under these conditions. We also tested the sequence histamine – substance P – endothelin, and substance P – endothelin –histamine. We made the same observations as for the neonatal IFN- γ stimulated cells. Table 4 contains a summary of the data. Measured probabilities were calculated by dividing the percentage response by 100. Calculated probabilities are shown in Table 3 and 4 in italics.

Discussion

Microglia comprise a heterogeneous population with respect to their responsiveness to neurotransmitter/-hormones

We have studied the microglial Ca^{2+} response to endothelin, histamine, substance P, serotonin, galanin, somatostatin, angiotensin II, vasopressin, neurotensin, dopamine and nicotine in three different preparations, namely freshly isolated adult cells, cultured adult and neonatal cells. We found that only a subpopulation of less than 20% of cells responded to any of these ligands, suggesting that small heterogeneous subpopulations of microglia exist that are characterized by distinct functional receptor expression. We found that all three preparations showed a similar functional expression pattern.

In our isolation procedure to obtain freshly isolated microglia, we strip the cells of their processes. If the receptors are predominantly located on the processes, the cells might lose their sensitivity. While we can not exclude that possibility, a study on virally transduced microglia did not provide any evidence that Ca^{2+} signals are stronger in processes as compared to the soma (Seifert et al. 2011). In addition, we found that cultured microglia both from adult and neonatal tissue also show a similar response pattern as the freshly isolated cells. We thus assume that our data reflect the response pattern of microglia in the tissue.

So far, Ca^{2+} imaging for the substances we tested has not been performed on naive, resting microglial cells. We previously recorded Ca^{2+} responses to ATP, endothelin-1, substance P, histamine and serotonin in microglia which were transduced with a retrovirus encoding a calcium sensor, after triggering microglial proliferation by a stab wound in vivo. About half of the ATP-

sensitive population responded to these four ligands and this population was not altered 42 days after the injection (Seifert et al. 2011). In this preparation one has to take into account that these microglial cells have undergone an activation process and that they may be distinct from naïve microglia. A different approach was used by Eichhoff et al. by delivering the Ca^{2+} indicator dye Oregon green BAPTA 1 to microglial cells *in vivo* with an electroporation technique (Eichhoff et al. 2011). They found that 20% of the cells responded to glutamate, but none to carbachol. The electroporation technique, however, might also have an impact on the properties of the cells.

Other transmitter receptors were studied with the patch-clamp technique in acute slices. About half of the microglial cells in rat or mouse responded to the GABA_B receptor ligand SKF 97541 with the induction of an outwardly rectifying K^+ conductance (Kuhn et al. 2004). The use of GABA_B receptor-specific antibodies indicated that the population of GABA_B receptor-expressing microglia was lower than 50% and this discrepancy can be explained by the fact that patch-clampers make an individual selection of cells. In the facial nerve lesion model, activated microglia show an increase in GABA_B receptor expression. Subpopulations of microglial cells also responded to dopamine and adrenergic receptor agonists when studied with the patch-clamp technique in acute slices from postnatal day 8 animals; about a third of the cells responded to dopamine with a change in K^+ conductance, and a fourth to adrenergic agonists (Farber et al. 2005). Despite differences in the preparation and activation state of the microglia, all of these studies show that only a subpopulation of microglia expresses functional receptors for a given neurotransmitter/neurohormone. Regardless of age, isolation procedure and stimulation, we have been able to support this data and thus conclude

that microglia constitute a rather heterogeneous cell population with respect to their response to neurotransmitters and neurohormones.

Manipulation of cultured microglia with LPS, IFN- γ nor IL-4 alters the functional expression pattern of neurotransmitter/-hormones

LPS, IFN- γ and IL-4 triggered defined changes in the functional expression pattern of the receptors that we studied. The histamine sensitive population increased in both adult and neonatal microglia after treatment with both LPS and IFN- γ . We also observed differences between adult and neonatal cultures such as the increase in the angiotensin II sensitive population in adult, but not neonatal LPS treated microglia or the increase in the angiotensin II sensitive population only in neonatal cells after IFN- γ treatment. Thus we could not recognize a uniform pattern in the change in chemosensitive microglial populations in different activation paradigms.

Microglia do not show a correlated expression of receptors

One could speculate that there is one small population of microglia which expresses all the receptors and a large population which does not respond at all. Alternatively, each cell could express a distinct subset resulting in a large number of distinct subpopulations. We addressed this question experimentally and found evidence for the latter. We selected galanin, somatostatin and angiotensin II in IFN- γ treated neonatal cells, and endothelin, histamine and substance P application in LPS treated adult cells, since under these conditions the percentage of responding cells was much higher as compared to control cells. The probability of a given cell to respond to two transmitters could be calculated by multiplying the probability of the population to respond

to each of the two, assuming that the expression is not correlated. The actual determined probability value matched that of the predicted value confirming that the expression of galanin, somatostatin and angiotensin II or endothelin, histamine and substance P is not correlated. One exception to this rule, however, might be angiotensin and galanin, since a high percentage of cells responded to both of these substances when applied in the order somatostatin – angiotensin – galanin. Apart from this observation, only the cell population responding to all three of the tested ligands was slightly higher than that of an uncorrelated expression. This indicates that there is an immense diversity of microglia with respect to their neurotransmitter/-hormone sensitivity.

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Figure Legends

Figure 1 A subpopulation of freshly isolated adult microglia respond to neurotransmitters and neurohormones

Representative examples of intracellular calcium transients induced in freshly isolated adult microglia cells by 60 s application of endothelin (A), histamine (B), substance P (C), serotonin (D), galanin (E), somatostatin (F), angiotensin II (G), vasopressin (H), neurotensin (I), dopamine (J) and nicotine (K) at the concentration as indicated. After a 5min washout, 500 μ M ATP was applied for 30 s as a positive control. L shows the percentage of freshly isolated adult microglia to respond to each neurotransmitter/hormone. Data are shown as the proportion of responding cells \pm margin of error for 95% confidence interval.

Figure 2 Neonatal and adult cultured and freshly isolated adult microglia respond to neurotensin with an increase in intracellular calcium

Fluo-4 fluorescence image of neonatal cultured microglia (top row), adult cultured microglia (middle row) and freshly isolated microglia (bottom row) shown at baseline calcium, during application of 100nM neurotensin and during application of 500 μ M ATP (A). 2B shows the corresponding calcium traces for the regions of interest as indicated by circle in A.

Figure 3 Treatment of neonatal cultured microglia with LPS, IFN- γ and IL-4 has distinct effects on the transmitter and hormone responses

Percentage of cultured neonatal cells which responded to endothelin (A), histamine (B), substance P (C), serotonin (D), galanin (E), somatostatin (F), angiotensin II (G), vasopressin (H), neurotensin (I), dopamine (J) and nicotine (K). Untreated neonatal microglia (control) are compared to cultures treated with LPS (100ng/ml), IFN- γ (20U/ml) and IL-4 (30ng/ml). Data are presented as the percentage of cells which responded to a given substance (the ATP responsive population is taken as 100%) from at least 10 investigated microscopic fields from at least 3 independent cell preparations and statistical significance is shown as *** for $p \leq 0.001$, ** for $p \leq 0.01$ and * for $p \leq 0.05$. Data are shown as the proportion of responding cells \pm margin of error for 95% confidence interval.

Figure 4 Treatment of adult cultured microglia with LPS, IFN- γ and IL-4 has distinct effects on the receptor repertoire

Percentage of cultured adult cells which respond to endothelin (A), histamine (B), substance P (C), serotonin (D), galanin (E), somatostatin (F), angiotensin II (G), vasopressin (H), neurotensin (I), dopamine (J) and nicotine (K). Untreated adult microglia (control) are compared to cultures treated with LPS (100ng/ml), IFN- γ (20U/ml) and IL-4 (30ng/ml). Data are presented as the percentage of cells which responded to a given substance (the ATP responsive population is taken as 100%) from at least 10 investigated microscopic fields from at least 3 independent cell preparations and statistical significance is shown as *** for $p \leq 0.001$, ** for $p \leq 0.01$ and * for $p \leq 0.05$. Data are shown as the proportion of responding cells \pm margin of error for 95% confidence interval.

Figure 5 Consecutive application of galanin, somatostatin and angiotensin II shows heterogeneity in the expression pattern

Calcium imaging for these substances was carried out on IFN- γ treated neonatal microglia. An example trace for each experimental sequence shows (A) the cell responds to both galanin and angiotensin II after the sequence galanin – somatostatin – angiotensin II, (B) a response to somatostatin only after the application sequence somatostatin – angiotensin II – galanin, (C) a response to angiotensin II and somatostatin after the sequence angiotensin II – galanin – somatostatin. (D, E, F) Bar charts show the probability that a cell will respond to each given substance, alone and in combination with a response from one or more other substances. The probability changes depending on the order of application.

Figure 6 Consecutive application of endothelin, histamine and substance P shows heterogeneity in the expression pattern

Calcium imaging for these substances was carried out on LPS treated adult microglia. An example trace for each experimental sequence shows (A) the cell responds only to endothelin after the sequence endothelin – histamine – substance P, (B) a response to histamine and endothelin after the application sequence histamine – substance P – endothelin, (C) a response to all 3 ligands after the sequence substance P – endothelin – histamine. (D, E, F) Bar charts show the probability that a cell will respond to each given

substance, alone and in combination with a response from 1 or more other substances. The probability changes depending on the order of application.

Table 1

Mean integral, amplitude and duration of neurotransmitter/-hormone induced Ca^{2+} transients for freshly isolated adult microglia.

Table 2

A summary of fold change and statistical significance (decoded as greyscale) of microglial populations responsive to the specific ligands in LPS, IFN- γ and IL-4 treated neonatal and adult cultured cells compared to controls.

Table 3

Probability of a single or a combination of responses to galanin, somatostatin and angiotensin II depending on the order of application. Measured probabilities were calculated by dividing the percentage response by 100. Calculated probabilities are shown in italics.

Table 4

Probability of a single or a combination of responses to endothelin, histamine and substance P depending on the order of application. Measured probabilities were calculated by dividing the percentage response by 100. Calculated probabilities are shown in italics.

Figure 1

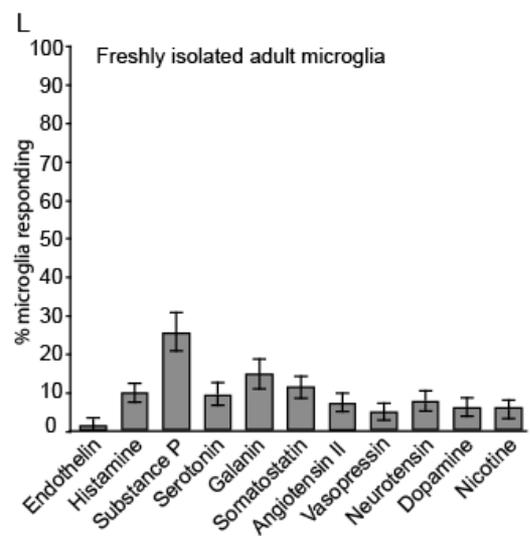
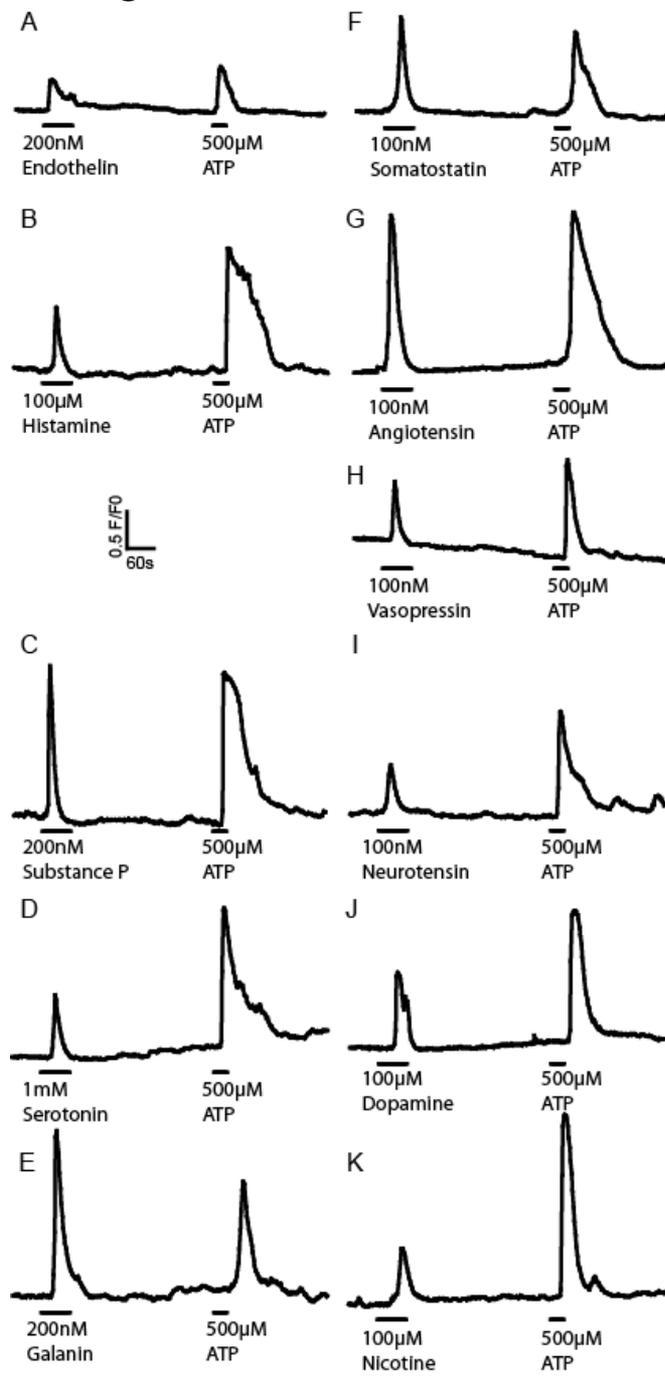


Figure 2

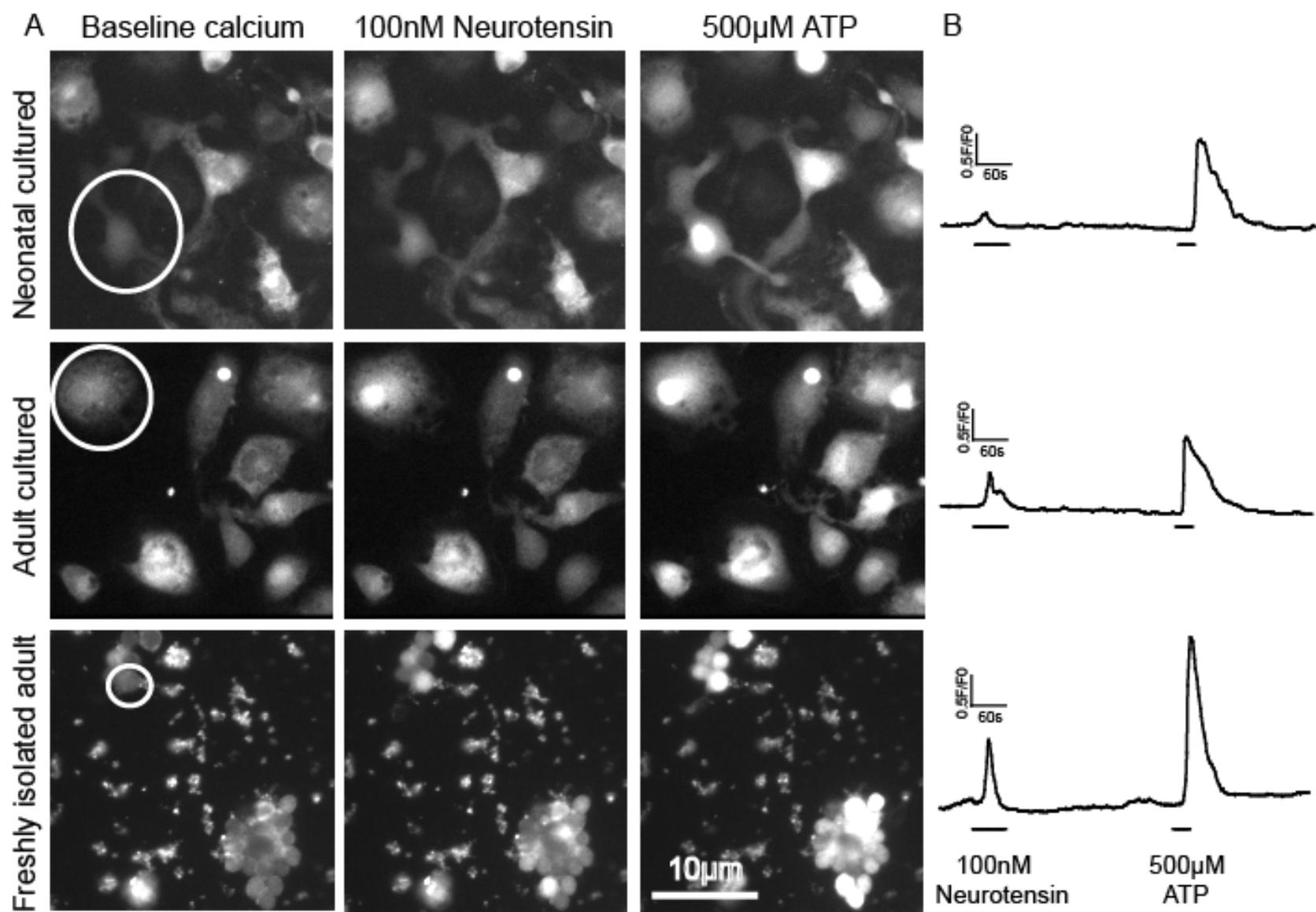


Figure 3

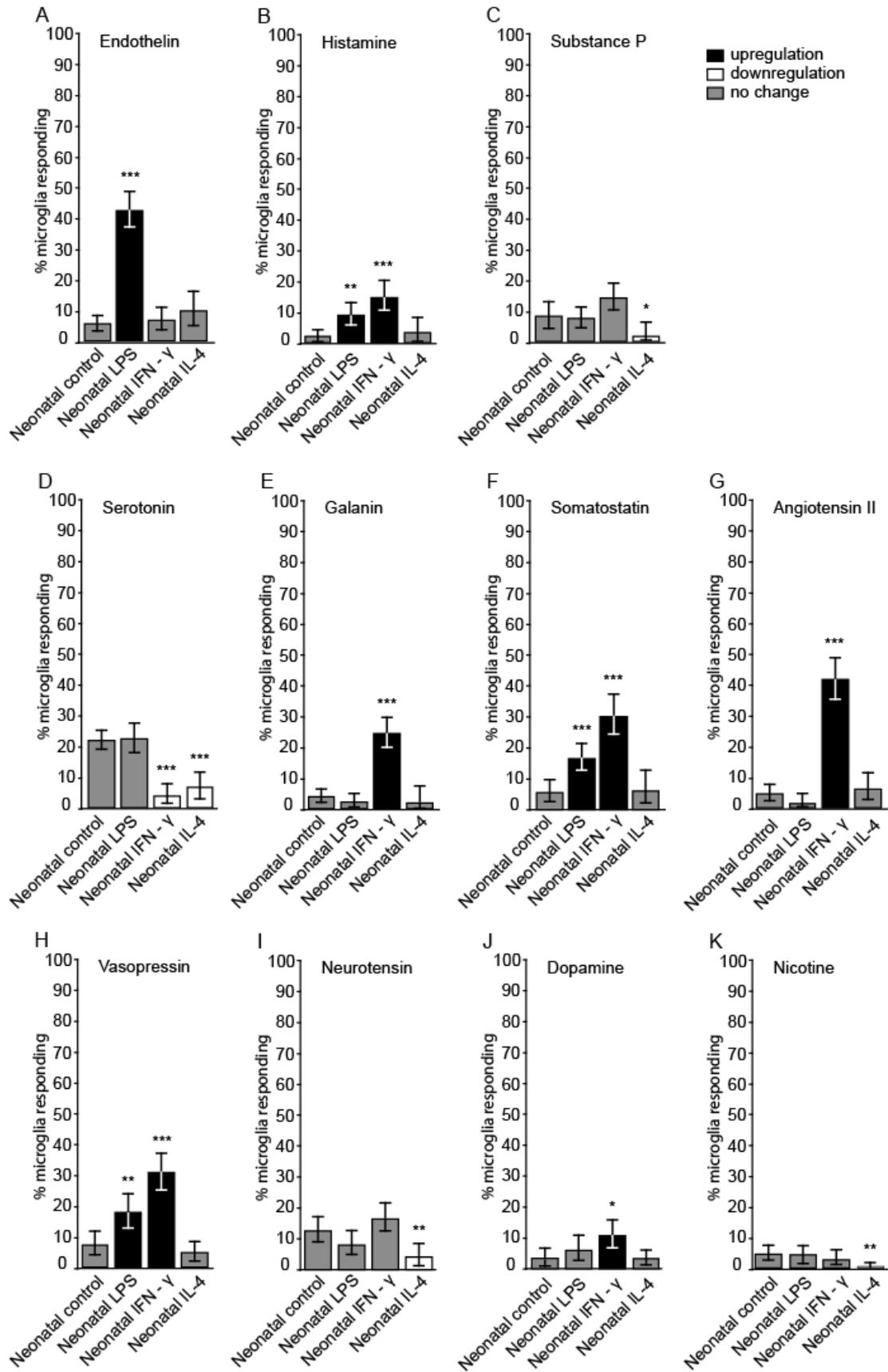


Figure 4

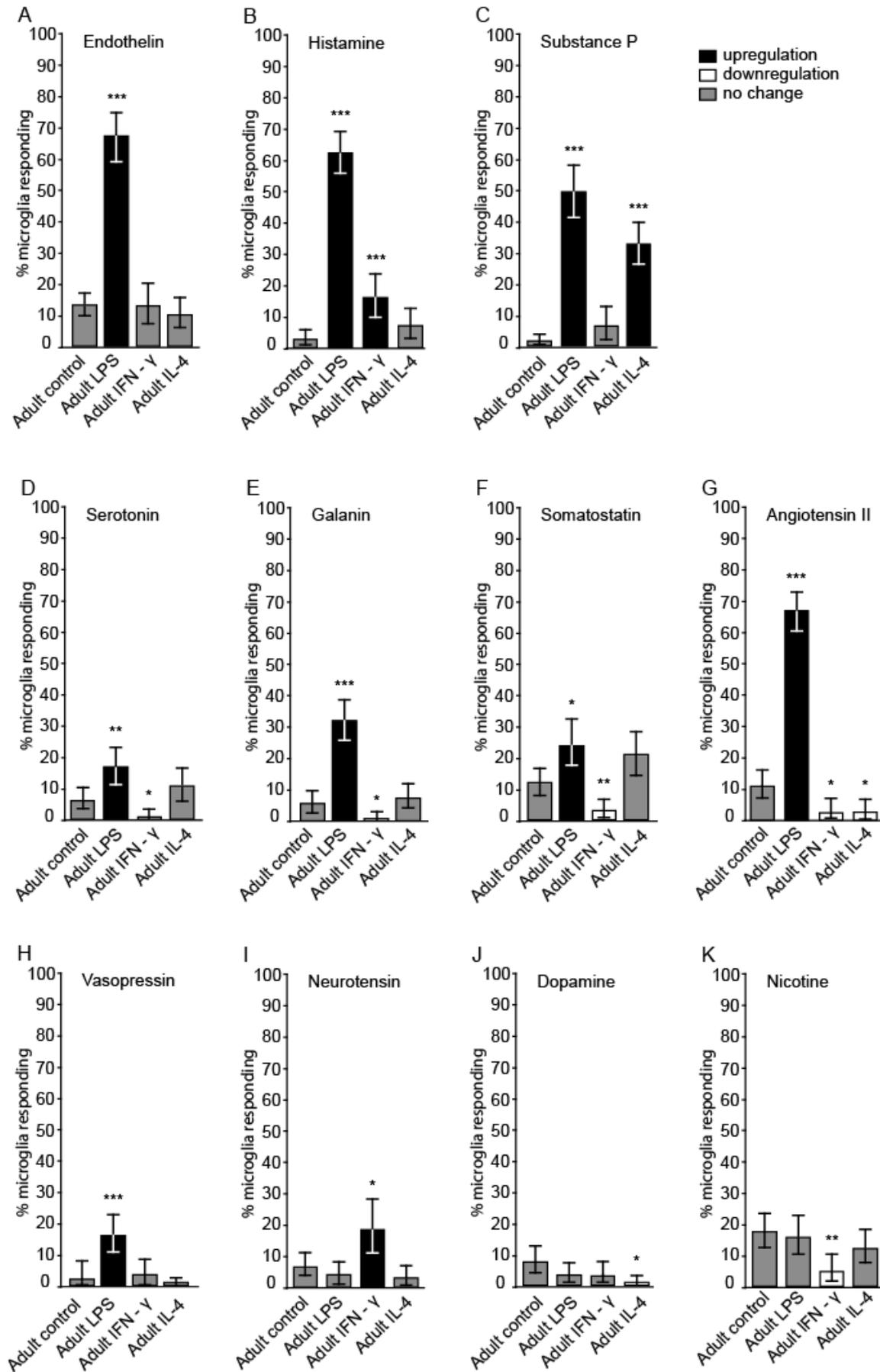


Figure 5

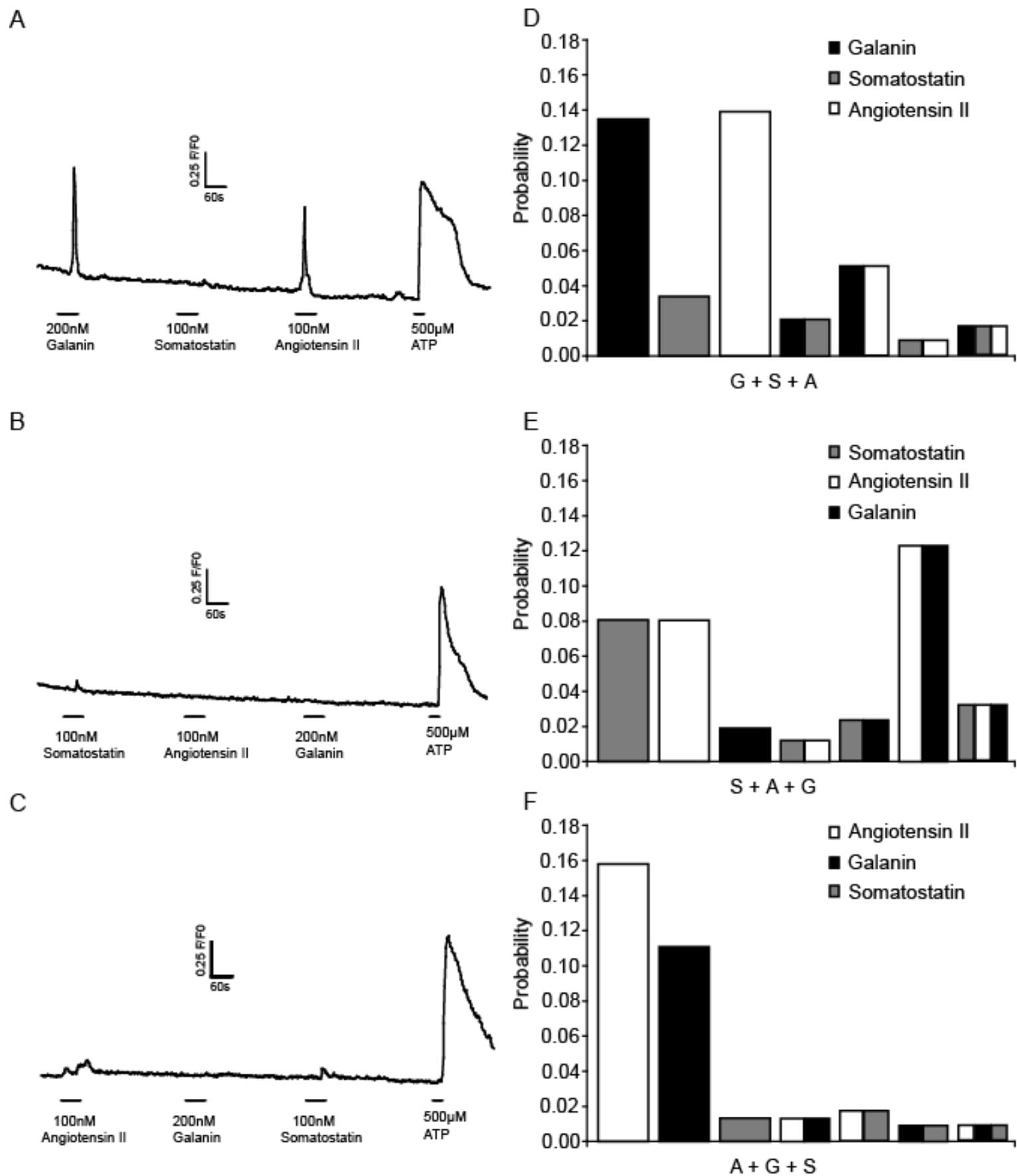


Figure 6

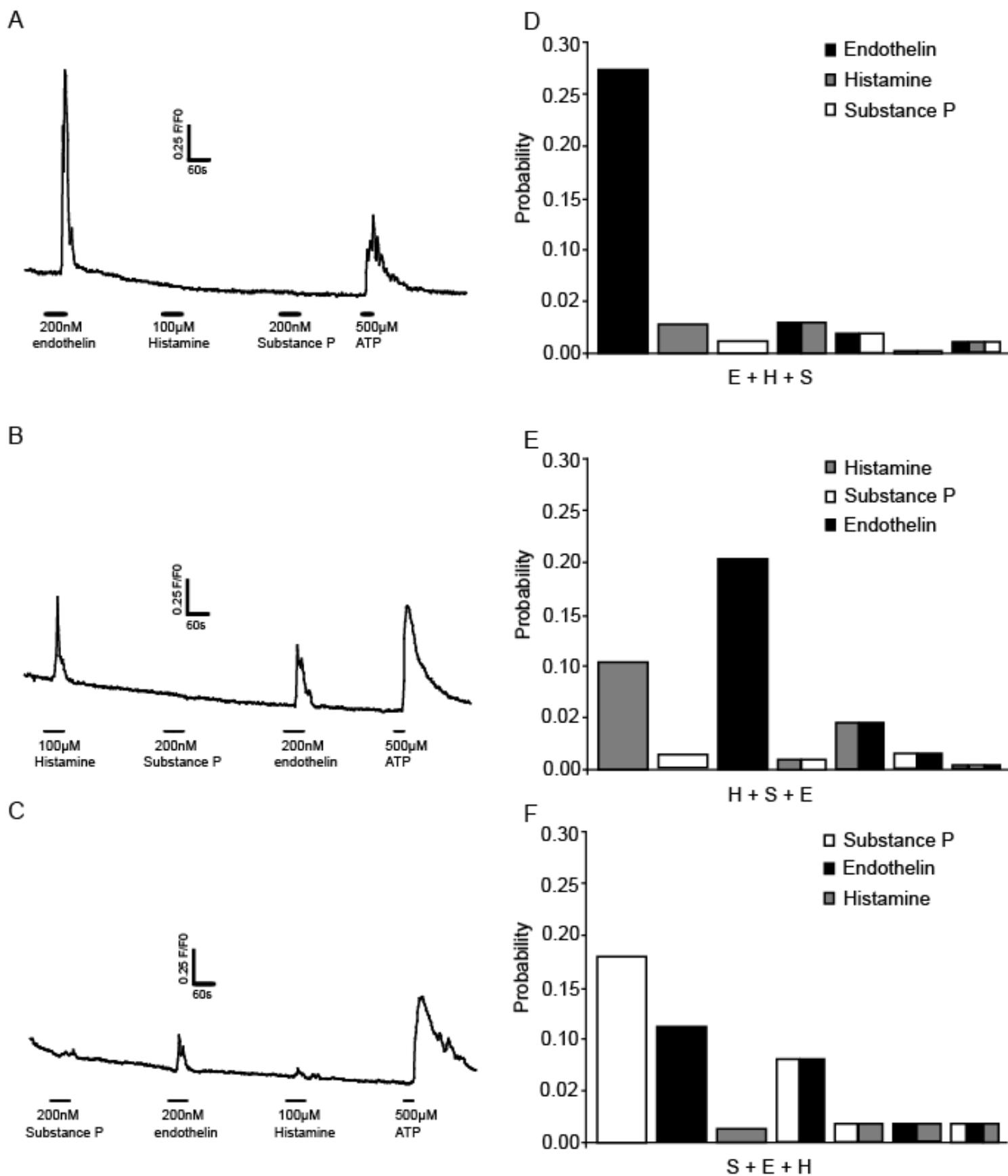


Table 1 Mean integral, amplitude and duration of calcium transients in freshly isolated adult microglia

	Mean integral of fluorescence change as a % of ATP response (\pm SEM)	Mean amplitude as a % of ATP response (\pm SEM)	Mean duration (s) (\pm SEM)
Endothelin	57 (18)	85 (4)	53 (16)
Histamine	58 (9)	61 (5)	84 (9)
Substance P	51 (7)	93 (7)	58 (7)
Serotonin	41 (8)	74 (6)	72 (9)
Galanin	80 (11)	100 (6)	90 (6)
Somatostatin	56 (6)	70 (4)	85 (8)
Angiotensin II	48 (5)	69 (3)	76 (5)
Vasopressin	67 (7)	71 (4)	69 (7)
Neurotensin	52 (6)	66 (5)	73 (6)
Dopamine	60 (7)	68 (6)	82 (6)
Nicotine	56 (9)	62 (6)	83 (7)

Table 2 Upregulation and downregulation of the response compared to control with fold change

	Neonatal cultured			Adult cultured		
	LPS	IFN- γ	IL-4	LPS	IFN- γ	IL-4
Endothelin	7.3	1.1	1.6	5.3	1.0	0.8
Histamine	3.7	6.0	1.4	24.9	6.5	3.0
Substance P	0.9	1.6	0.2	34.4	4.7	22.4
Serotonin	1.0	0.2	0.3	2.8	0.2	1.7
Galanin	0.6	5.6	0.4	5.9	0.3	1.3
Somatostatin	2.7	4.9	1.0	2.0	0.3	1.7
Angiotensin II	0.3	8.8	1.3	6.2	0.3	0.2
Vasopressin	2.3	4.0	0.6	7.5	1.9	0.6
Neurotensin	0.6	1.3	0.3	0.8	3.0	0.5
Dopamine	1.6	2.8	0.8	0.5	0.5	0.2
Nicotine	0.9	0.6	0.2	1.0	0.3	0.7

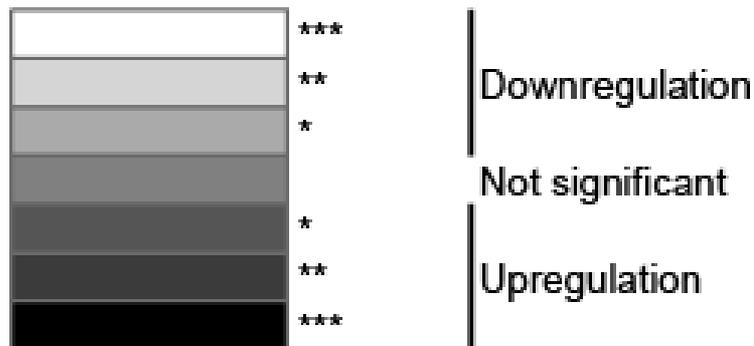


Table 3 Probability of single or combination response to galanin, somatostatin and angiotensin II

	Somatostatin only	Galanin only	Angiotensin II only	Angiotensin II + Somatostatin	Angiotensin II + Galanin	Somatostatin + Galanin	Angiotensin II + Somatostatin + Galanin	All cells responding to -		
								Somatostatin	Galanin	Angiotensin II
G + S + A	0.034	0.135	0.139	0.008 (0.017)	0.051 (0.048)	0.021 (0.018)	0.017 (0.0039)	0.080	0.224	0.215
S + A + G	0.081	0.021	0.081	0.013 (0.038)	0.123 (0.051)	0.025 (0.031)	0.034 (0.0078)	0.153	0.203	0.250
A + G + S	0.014	0.112	0.158	0.019 (0.010)	0.014 (0.029)	0.009 (0.007)	0.009 (0.0015)	0.051	0.144	0.200

Table 4 Probability of single or combination response to endothelin, histamine and substance P

	Endothelin only	Histamine only	Substance P only	Endothelin + Histamine	Endothelin + Substance P	Histamine + Substance P	Endothelin + Histamine + Substance P	All cells responding to -		
								Endothelin	Histamine	Substance P
E + H + S	0.275	0.026	0.011	0.030 (0.024)	0.019 (0.015)	0.004 (0.003)	0.011 (0.0011)	0.335	0.071	0.045
H + S + E	0.202	0.103	0.013	0.045 (0.043)	0.013 (0.011)	0.009 (0.007)	0.004 (0.0017)	0.265	0.161	0.040
S + E + H	0.112	0.013	0.179	0.018 (0.015)	0.080 (0.067)	0.018 (0.02)	0.018 (0.0045)	0.228	0.067	0.295