

**Improving emotional face perception in autism with diuretic
bumetanide: a proof-of-concept behavioral and functional brain
imaging pilot study**

**Authors: Nouchine Hadjikhani^{1,2,3*}, Nicole R Zürcher^{1,2}, Ophelie Rogier²,
Torsten Ruest², Loyse Hippolyte², Yehezkel Ben-Ari⁴ and Eric Lemonnier⁵.**

Affiliations:

**¹MGH/HST/HMS Athinoula A. Martinos Center for Biomedical Imaging, Boston,
USA**

²Brain Mind Institute, EPFL, Lausanne, Switzerland

³ Gillberg Neuropsychiatric Center, University of Gothenburg, Sweden

⁴INMED, Marseilles, France

⁵ Laboratoire de Neurosciences, Université de Brest EA4645, France

Short title: Bumetanide improves emotional perception in autism

Corresponding author:

Nouchine Hadjikhani

A. A. Martinos Center for Biomedical Imaging

149 13th Street

Charlestown, MA 02129

Tel: +1 617 724 5625

Fax: +1 530 309 4976

Email: nouchine@nmr.mgh.harvard.edu

Abstract

Clinical observations have shown that GABA-acting benzodiazepines exert paradoxical excitatory effects in autism, suggesting elevated intracellular chloride (Cl^-)_i and excitatory action of GABA. In a previous double blind randomized study, we have shown that the diuretic NKCC1 chloride importer antagonist bumetanide, that decreases (Cl^-)_i and reinforces GABAergic inhibition, reduces the severity of autism symptoms. Here, we report results from an open-label trial pilot study in which we used functional magnetic resonance imaging and neuropsychological testing to determine the effects of 10 months bumetanide treatment in adolescents and young adults with autism. We show that bumetanide treatment improves emotion recognition and enhances the activation of brain regions involved in social and emotional perception during the perception of emotional faces. The improvement of emotion processing by bumetanide reinforces the usefulness of bumetanide as a promising treatment to improve social interactions in autism.

Keywords

Autism spectrum disorders, GABA, bumetanide, fMRI, emotion, face perception, treatment.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental, life-long condition characterized by deficits in social interactions and communication, and by the presence of repetitive behaviors, that affects approximately 1% of the population ((CDC) and Baio, 2012: 1; American_Psychiatric_Association, 2013). Genetic mutations that impact synapse operation (Jamain et al., 2003; Bourgeron, 2009; Giannandrea et al., 2010; Weiss, 2009; Tabuchi et al., 2007) as well as environmental factors during pregnancy (Croen et al., 2011b; Croen et al., 2011a; Patterson, 2009; Kemper and Bauman, 1998; Dossche, 2005) contribute to the emergence of ASD. Research on the genetic basis of ASD has identified hundreds of possible genetic mutations, but how brain malformations are induced and how they lead to neurological sequelae is still not understood. The development of autism seems to already start in utero (Courchesne et al., 2011; Bauman and Kemper, 1985; Ploeger et al., 2010).

GABAergic signaling is affected in ASD, resulting in an imbalance between excitation and inhibition (Chao et al., 2010; Pizzarelli and Cherubini, 2011; Gogolla et al., 2009; Dossche, 2005). ASD patients have reduced gamma oscillations (Grice et al., 2001; Brown et al., 2005; Wilson et al., 2007), which are generated by GABAergic neurons (Lewis et al., 2005; Lisman and Buzsaki, 2008; Pizzarelli and Cherubini, 2011) and are instrumental in sensory binding and higher cognitive functions

(Singer, 1993; Lisman and Idiart, 1995; Murthy and Fetz, 1992). Interestingly, the GABA acting benzodiazepines that enhance GABAergic inhibition exert paradoxical actions on autistic children augmenting agitation and other symptoms (Marrosu et al., 1987). This paradoxical reaction has been shown to result from elevated intracellular chloride ($[Cl^-]_i$) that shifts the polarity of GABA from excitation to inhibition (Nardou et al., 2011b). Indeed, in epilepsies, but also spinal cord insults, the levels of $[Cl^-]_i$ are elevated leading to excitatory GABA actions that are further enhanced by GABA-acting benzodiazepines or phenobarbital (Nardou et al., 2011b; Nardou et al., 2011a). This observation has raised considerable interest for the use of diuretics in order to reestablish the hyperpolarization of GABAergic signals and hence to reinforce its inhibitory potency, and has led to therapeutic assays in epilepsy treatment.

The increase of $[Cl^-]_i$ in pathology has a dual origin: an internalisation of the chloride exporter KCC2 – leading to a failure of neurons to export excessive chloride - and a persistent or enhanced activity of the chloride importer NKCC1 leading to exacerbated accumulation of chloride (Dzhala et al., 2005; Nardou et al., 2011b). KCC2 is at present an unlikely target for drug treatments because it is labile, readily internalized, highly activity-dependent, and because there are currently no selective agonists available. In contrast, NKCC1 is stable and antagonists have been identified, notably the diuretic and highly specific NKCC1 antagonist bumetanide. Bumetanide

has been extensively utilized since 1975 in adults and since 1986 in children to treat acute and long-term conditions including hypertension, broncho-pulmonary dysplasia, nephritic syndrome or congestive heart failure (Sullivan et al., 1996). Bumetanide has a short half-life (between 1h30 and 3h) and (poorly) crosses the blood-brain barrier (Li et al., 2011) thanks to an active transporter (SLC16A50) (Murakami et al., 2005). The use of bumetanide is safe provided that it is accompanied with regular controls of kaliemia and kidney functions in patients to determine possible adverse effects.

Relying on the observation of paradoxical actions of benzodiazepines on ASD patients (Marrosu et al., 1987), the effects of chronic bumetanide treatment were recently tested in a double blind randomized study (Lemonnier et al., 2012). In this trial, conventional measures of behavioral and clinical evaluation of autism in children were used, including Childhood Autism Rating Scale (CARS), Clinical Global Impression (CGI) and Autism Diagnostic Observation Schedule (ADOS), and a significant amelioration of clinical symptoms was found. However, it remained important to determine the effects of this treatment on some core symptoms of autism, notably on the processing of facial expressions and recognition of emotions.

The goal of the present study was therefore to examine potential brain mechanisms underlying the action of bumetanide.

Here, using quantitative behavioral testing (experiment 1) and functional MRI (experiment 2) in an open-label trial design, we tested the effect of bumetanide treatment on performance in emotion recognition. Using fMRI, we assessed changes in brain activation in response to the perception of dynamic movies of facial expressions in two separate sessions before and after treatment. We tested the hypothesis that bumetanide treatment would improve performance for emotion recognition, and lead to increased activation of brain areas involved in emotion processing.

Materials and Methods

Ethics Statement: The study was approved by the Committee of Persons Protections CPP west 6-570-6/4/2009, and by the French Health Products Safety Agency (AFSAPS -A90936-66 4/12/2009, NCT01078714). The behavioral and fMRI protocols were approved by Hospital Ethical Committee. All adult participants gave written consent before the start of the study. Minor participants gave their assent and

one of their parents gave written consent. All procedures followed the Declaration of Helsinki.

Seven high-functioning males with ASD took part in the study. All participants underwent repeated scanning after 10 months of bumetanide treatment (1mg/day). They were 19.3 ± 4.6 (mean \pm SD) years old at the first testing session (range: 14.8-28.5).

Participants were diagnosed by an experienced clinician according to DSM IV-TR (APA, 2000) criteria and the Autism Diagnosis Observation Schedule (ADOS)(Lord et al., 2000) and the Autism Diagnostic Interview-Revised (ADI-R)(Lord et al., 1994). All participants met criteria for ASD and were diagnosed with Autism (n=2) and Asperger Syndrome (N=5) based on their language development history. They were also asked to complete the Autism Quotient (AQ) (Baron-Cohen et al., 2006) and Empathy Quotient (EQ) (Baron-Cohen and Wheelwright, 2004) self report questionnaires. The performance IQ (PIQ) was assessed using Wechsler non-verbal scales (WASI, 1999; Wechsler and Naglieri, 2006). In addition, the Toronto Alexithymia Scale (TAS-20) was assessed before and after treatment in seven of the nine participants (Bagby et al., 1994).

Participants' characteristics are given in **Table 1**.

During the treatment period, patients underwent clinical controls as well as monitoring of electrolytes (including potassium and sodium), kidney and liver functions and blood sugar at days 7, 14, 30, 60 and then every six months. We frequently observed increased urinary output, but this was never accompanied by signs of dehydration (no weight reduction, no increase in Na⁺ levels). No side effects such as orthostatic hypotension, cramps, weakness, diarrhea, myalgia, arthralgia, dizziness or nausea were observed. In one of the patients, we observed hypokaliemia after one month of treatment that was readily corrected by oral potassium supplement.

Experiment 1: Behavioral testing for emotional labeling

Stimuli and task: In this task, participants had to recognize a low-intensity and therefore ambiguous facial expression. Four expressions were used: happy, fearful, angry and neutral. Dynamic morphs were created from the NimStim Emotional Face Stimuli database (<http://www.macbrain.org/faces/index.htm#faces>) between NEUTRAL and each EMOTIONAL expression using Morph Age Pro (<http://www.creaceed.com/morphage/>), and still images were created at 40% intensity level between neutral and the full emotional expressions. Participants were

then presented with one still image depicting happy, fearful or angry at 40% intensity or NEUTRAL on the left side of the computer screen, while on the right still Ekman stimuli (Ekman and Friesen, 1976) representing each of the four full emotional expressions were shown. The location of a particular facial expression presented on the right was counterbalanced across trials to avoid habituation and control for location. For each type of emotion, four trials were delivered, totaling to 16 stimuli shown in pseudorandom order. The test was non-verbal: participants had to indicate using a button box which of the four facial expressions presented on the right matched the best with the expression seen on the left. The images remained on screen until a response was given. Performance measures consisting of reaction time (RT) and accuracy were recorded. To control that the concept of classification was understood and that effects were specific to faces, we designed stimuli showing 4 OBJECT categories: instruments, fruits, clothes, and animals. In total, 16 different exemplars were shown. As for the experiment above, participants had to indicate on a button box which image on the right was the best match for the picture on the left. No feedback on accuracy was given in any of these non-verbal tests, so that participants could not learn the task during the session. A pairwise Wilcoxon rank test was used to compare emotion/object category recognition performance before and after treatment.

Experiment 2: Functional brain imaging

fMRI data acquisition

Anatomical and functional MR images of brain activity were collected in a 3T high-speed echoplanar-imaging device (Tim Trio, Siemens, Erlangen) using a 12-channel matrix coil. Participants lay on a padded scanner couch and wore foam earplugs. Foam padding stabilized the head. High-resolution (1.0 x 1.0 x 1.0mm) structural images were obtained with a multi-echo magnetization-prepared rapid acquisition gradient echo (ME-MPRAGE) sequence (176 slices, 256x256 matrix, echo time (TE1)=1.64 ms, (TE2)=3.5 ms, (TE3)=5.36 (TE4)=7.22 ms; repetition time (TR)=2530 ms; flip=7°). MR images of brain activity were then collected. Functional sessions began with an initial sagittal localizer scan. Slices were automatically positioned using AutoAlign Head LS (Landmark Survey) from Siemens. The co-registered functional acquisition (TR=3,000 ms, 46 AC-PC 3mm thick slices, TE=30ms, flip angle 90°, matrix=64x64) lasted 417 seconds. Other anatomical and functional sequences were also acquired during this session but are not described in the present report.

Stimuli and task for the fMRI experiment

During the functional scan, dynamic face stimuli were presented. We used a series of 24 short movies created from the NimStim database, representing morphs of facial expressions from neutral to fearful, happy or angry. In order to control for emotional expression and movements, morphs were also created for the NEUTRAL condition, using NEUTRAL faces and their mirror images. Each movie lasted for 5 seconds, with a dynamic morph starting from NEUTRAL and going to full emotional expression, lasting 3 seconds, followed by 2 seconds of the final full emotional expression. Stimuli were presented in a block design. There were 8 blocks in total, 2 for each facial expression. Eight stimuli were presented per block. So a total of 16 morphs were presented for each facial expression. A red fixation cross was presented for 1 second between movies and for an additional 3 seconds between blocks. Four times in the run a blue fixation cross was presented. To ensure that participants were paying attention to the stimuli, a button box was used to record participants' responses to the presence of the blue cross between stimuli presentation. Participants were instructed to look attentively at the faces, and to press a button every time they saw a blue cross. Functional data from one participant for this paradigm could not be acquired during the first session due to technical problems with the scanner, and therefore his data were only included for the behavioral experiment.

fMRI data Analysis

FSL (FMRIB Software Library) package and techniques were used in data preparation and processing. Brain extraction of high-resolution anatomical images was carried out using Christian Gaser's VBM8 toolbox for SPM8 (Ashburner et al., 2000) and fed into FEAT. fMRI data processing was performed using FEAT (fMRI Expert Analysis Tool) version 5.98 (Smith et al., 2004; Woolrich et al., 2009; Worsley, 2001). Each functional run was first motion-corrected with MCFLIRT (Cox, 1996) and spatially smoothed with full width at half maximum of 8 mm. First level analyses were carried out for each subject to compute the contrast of interest, i.e. [EMOTION > NEUTRAL]. Subsequently, treatment effects [after > before] were assessed by submitting these contrasts to a paired higher-level mixed effects GLM analysis using FLAME 1+2 (FMRIB's Local Analysis of Mixed Effects).

Mixed effect variance is the sum of fixed-effects variance (the within-session across-time variances estimated in the first-level analyses) and "random-effects" (RE) variance (the "true" cross-session variances of first-level parameter estimates). Mixed effect analysis was chosen because it models the session and subject variability and

therefore allows inferences to be made to a wider population from which the subjects were drawn (<http://fsl.fmrib.ox.ac.uk/fsl/fsl4.0/feat5/detail.html>).

Clusters were formed using FSL's cluster tool, and data are reported with a threshold of $z > 2.3$ and a minimum cluster size of 300 voxels.

Results

Effects of bumetanide treatment on behavior: Emotional face recognition

As shown in **Fig. 1**, bumetanide treatment significantly improved overall accuracy in emotion matching of faces with 40% intensity to their 100% intensity counterpart (overall mean accuracy [% correct] \pm SD before: 62.5 ± 21.0 ; after: 75.0 ± 13.5 ; $p=0.04$). Bumetanide treatment also significantly improved overall reaction time for face emotion matching (overall mean reaction time [seconds] \pm SD before: 8.18 ± 3.42 ; after: 5.82 ± 1.54 ; $p=0.04$). This effect was only seen for emotional matching of faces. (**Fig. 1**). Object matching was not significantly different between sessions (overall mean accuracy [% correct] \pm SD before: 100 ± 0 ; after: 98.2 ± 3.0 ; $p=0.16$, overall mean reaction time [seconds] \pm SD before: 2.25 ± 0.61 ; after: 2.02 ± 0.62 ; $p=0.24$).

Effects of bumetanide treatment on behavior: Alexithymia

Alexithymia was assessed with the TAS-20. The TAS-20 uses a cut-off scoring: Scores equal or less than 51 are considered as non-alexithymia, and scores equal or greater than 61 are considered as alexithymia, with a grey zone between 52 to 60. Mean score before treatment was 60.8 ± 10.9 , and significantly improved to a score of 55.4 ± 12.0 after treatment ($p=0.03$). These results indicate that bumetanide treatment may improve the ability to identify and describe one's own emotions.

Effects of bumetanide treatment on brain activation

The results presented here show the comparison between EMOTIONAL and NEUTRAL faces, and the modulatory effect of emotion on brain activation PRE and POST treatment with bumetanide.

After 10 months of treatment, significantly increased activation was observed for EMOTIONAL compared to NEUTRAL faces (see Table 2, Figure 2). Bumetanide treatment increased brain activation for EMOTIONAL faces in early visual areas, as well as in face processing areas including the inferior occipital cortex and the fusiform cortex. In addition, increased activation was seen in cortical and subcortical areas involved in emotional processing, including the nucleus accumbens and the amygdala, as well as the orbitofrontal cortex and the temporal pole; in areas involved in social processing, including the superior temporal cortex; in areas involved in

attentional processing (intraparietal sulcus, superior parietal lobule); in the lobule and vermis VI of the cerebellum, as well as in crus I, involved in emotional and cognitive processing.

Discussion

Our study is an open-label trial with only 7 treated patients, necessarily limiting the scope of the conclusions. However, this is the first such study comparing behavioral performance for emotion recognition and brain activation in response to dynamic emotional faces before and after bumetanide treatment.

The imaging data showed a striking increase of brain activation in the face and social/emotional processing network between sessions. EMOTIONAL faces elicited significantly more activation in face encoding areas, including the inferior occipital cortex, and the fusiform cortex, key face processing regions. Increased activation after treatment was also observed bilaterally in the superior temporal cortex, involved in the processing of dynamic, expressive aspects of faces (Allison et al., 2000). The right superior temporal cortex region plays a key role in facial emotion recognition and is involved in social perception. Previous studies have demonstrated that selective attention to facial emotion specifically enhances activity of the right superior

temporal cortex compared with attention to the face per se (Narumoto et al., 2001); increased activation was also present in the parietal cortex, involved in attentional aspects of emotion processing (Narumoto et al., 2001).

Increased activation was also observed in areas involved in reward, motivation and emotion, including the nucleus accumbens, the amygdala, and the orbitofrontal cortex, possibly indicating increased interest for emotional faces after treatment, and increased emotional processing. Finally, increased activation was observed in cerebellum lobule VI and crus I, both involved in emotional processing (Schmahmann and Sherman, 1998; Buckner et al., 2011). In a recent study, we showed that neurotypical controls showed more activation than high-functioning individuals with ASD during Thatcherized face processing in the cerebellum (Zurcher et al., 2013). The present data, showing increased cerebellar and cortical activation, suggest normalization in brain activation during face perception after bumetanide treatment.

Social interaction impairments are at the core of difficulties encountered by individuals with ASD, and a previous study has shown that oxytocin, given intranasally, can improve social behavior and increase gazing time in the eye-region of faces in a group of 13 participants with autism (Andari et al., 2010). However this

effect is punctual and is limited to the window of action of oxytocin, which is about 1-2 hours. Interestingly, recent studies performed by Ben-Ari and colleagues have shown that delivery in rodents is associated with an abrupt and dramatic reduction of $[Cl^-]_i$ that exerts a neuro-protective role and analgesic action on the newborn's brain (Tyzio et al., 2006; Mazzuca et al., 2011). The analgesic actions are mimicked by bumetanide that, like oxytocin, reduces activity in pain pathways by reducing intracellular chloride (Mazzuca et al., 2011). Collectively, these observations suggest that the behavioral improvement observed with bumetanide and oxytocin may share common mechanisms.

Of further interest, NKCC1 is up-regulated in epilepsy and other disorders in which $[Cl^-]_i$ are elevated and GABA excitatory (Zhu et al., 2008; Cohen et al., 2002). These alterations therefore appear to be common responses of neurons to insults and suggest that elevated activity of the co-transporter occurs in adult neurons in a variety of pathological conditions.

The present study contains several limitations, as it is an open label trial, with a limited number of participants, scanned in two sessions that were separated by long time interval (~ 10 months) and in which all the participants had normal intelligence (so we do not know whether the same behavioral and brain activation would also be

observed in patients with intellectual deficiencies). In addition, the data of this open-label trial could be interpreted as the result of placebo and repetition effects as the patients were tested on the same tests twice, although the fact that emotional faces but not neutral ones were more readily identified would tend to speak against this interpretation. Future large, double blind randomized control trials will need to address these issues.

In conclusion, bumetanide treatment appears to enhance pro-social behavior by improving emotion processing. It bears stressing that to the best of our knowledge, fMRI has not been used in earlier studies to compare the effects of drug treatment in ASD, and that the only fMRI study published on the effect of behavioral therapy only reported an N=2 (Voos et al., 2013). In conclusion, in spite of their intrinsic limitations, our proof-of-concept results combined with the highly promising results of the double blind randomized trial with bumetanide (Lemonnier et al., 2012) converge to call for larger cohorts of participants, of different ages and with different symptom severity to confirm the effect of bumetanide on social processing in autism.

Acknowledgments: We want to warmly thank all the participants and families who took part to this study. Our thanks also go to Carole Burget, Karine Metrailler, Anthony Lissot for their precious logistics help and Juliana Iranpour for the behavioral study stimuli preparation.

Funding: This work was supported by Swiss National Science Foundation PP00P3-130191 to NH; by the Centre d’Imagerie BioMédicale (CIBM) of the University of Lausanne (UNIL), the Swiss Federal Institute of Technology Lausanne (EPFL), as well as the Foundation Rossi Di Montalera. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: All the authors have no conflict of interest, no financial and personal relationships with other people or organizations including employment, consultancies, stock ownership, honoraria, paid expert testimony, and travel grants all during the conduction and termination of the work submitted. An application patent on the use of diuretics has been deposited by the French medical research council on the basis of the clinical results published by our team (Lemonnier et al., 2012). Subsequent to completion of this study, three authors (NH EL and YBA) have become shareholder of a company (Neurochlore, created early 2012) seeking to develop and commercialize bumetanide as a treatment for autism.

References and Notes:

- (CDC) CfDCaP and Baio J. (2012) Prevalence of Autism Spectrum Disorders — Autism and Developmental Disabilities Monitoring Network, 14 Sites, United States, 2008. *Morbidity and Mortality Weekly Report (MMWR)* 61: 1-19.
- Allison T, Puce A and McCarthy G. (2000) Social perception from visual cues: role of the STS region. *Trends Cogn Sci* 4: 267-278.
- American Psychiatric Association T. (2013) *Diagnostic and statistical manual of mental disorders (5th ed)*, Arlington, VA: American Psychiatric Publishing.
- Andari E, Duhamel JR, Zalla T, et al. (2010) Promoting social behavior with oxytocin in high-functioning autism spectrum disorders. *Proceedings of the National Academy of Sciences of the United States of America* 107: 4389-4394.
- APA. (2000) *Diagnostic and Statistical Manual of Mental Disorders, DSM-IV-TR*.
- Ashburner J, Andersson JL and Friston KJ. (2000) Image registration using a symmetric prior--in three dimensions. *Human brain mapping* 9: 212-225.
- Bagby RM, Parker JDA and Taylor GJ. (1994) The twenty-item Toronto Alexithymia Scale-I. Item selection and cross-validation of the factor structure. *Journal of Psychosomatic Research* 38: 23-32.
- Baron-Cohen S, Hoekstra RA, Knickmeyer R, et al. (2006) The Autism-Spectrum Quotient (AQ)--adolescent version. *Journal of autism and developmental disorders* 36: 343-350.
- Baron-Cohen S and Wheelwright S. (2004) The empathy quotient: an investigation of adults with Asperger syndrome or high functioning autism, and normal sex differences. *J Autism Dev Disord* 34: 163-175.
- Bauman M and Kemper TL. (1985) Histoanatomic observations of the brain in early infantile autism. *Neurology* 35: 866-874.
- Bourgeron T. (2009) A synaptic trek to autism. *Curr Opin Neurobiol* 19: 231-234.
- Brown C, Gruber T, Boucher J, et al. (2005) Gamma abnormalities during perception of illusory figures in autism. *Cortex; a journal devoted to the study of the nervous system and behavior* 41: 364-376.
- Buckner RL, Krienen FM, Castellanos A, et al. (2011) The organization of the human cerebellum estimated by intrinsic functional connectivity. *J Neurophysiol* 106: 2322-2345.
- Chao HT, Chen H, Samaco RC, et al. (2010) Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. *Nature* 468: 263-269.
- Cohen I, Navarro V, Clemenceau S, et al. (2002) On the origin of interictal activity in human temporal lobe epilepsy in vitro. *Science* 298: 1418-1421.

- Courchesne E, Mouton PR, Calhoun ME, et al. (2011) Neuron number and size in prefrontal cortex of children with autism. *JAMA : the journal of the American Medical Association* 306: 2001-2010.
- Cox RW. (1996) AFNI: software for analysis and visualization of functional magnetic resonance neuroimages. *Comput Biomed Res* 29: 162-173.
- Croen LA, Connors SL, Matevia M, et al. (2011a) Prenatal exposure to beta2-adrenergic receptor agonists and risk of autism spectrum disorders. *Journal of neurodevelopmental disorders* 3: 307-315.
- Croen LA, Grether JK, Yoshida CK, et al. (2011b) Antidepressant use during pregnancy and childhood autism spectrum disorders. *Arch Gen Psychiatry* 68: 1104-1112.
- Dossche D. (2005) GABA in autism. *International Review of Neurobiology* 71: 1-481.
- Dzhala VI, Talos DM, Sdrulla DA, et al. (2005) NKCC1 transporter facilitates seizures in the developing brain. *Nature medicine* 11: 1205-1213.
- Ekman P and Friesen WV. (1976) *Pictures of facial affects*, Palo Alto: Consulting Psychologists Press.
- Giannandrea M, Bianchi V, Mignogna ML, et al. (2010) Mutations in the small GTPase gene RAB39B are responsible for X-linked mental retardation associated with autism, epilepsy, and macrocephaly. *American journal of human genetics* 86: 185-195.
- Gogolla N, Leblanc JJ, Quast KB, et al. (2009) Common circuit defect of excitatory-inhibitory balance in mouse models of autism. *Journal of neurodevelopmental disorders* 1: 172-181.
- Grice SJ, Spratling MW, Karmiloff-Smith A, et al. (2001) Disordered visual processing and oscillatory brain activity in autism and Williams syndrome. *Neuroreport* 12: 2697-2700.
- Jamain S, Quach H, Betancur C, et al. (2003) Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nature genetics* 34: 27-29.
- Kemper TL and Bauman M. (1998) Neuropathology of infantile autism. *J Neuropathol Exp Neurol* 57: 645-652.
- Lemonnier E, Degrez C, Phelep M, et al. (2012) A randomised controlled trial of bumetanide in the treatment of autism in children. *Transl Psychiatry* 2: e202.
- Lewis DA, Hashimoto T and Volk DW. (2005) Cortical inhibitory neurons and schizophrenia. *Nature reviews. Neuroscience* 6: 312-324.
- Li Y, Cleary R, Kellogg M, et al. (2011) Sensitive isotope dilution liquid chromatography/tandem mass spectrometry method for quantitative analysis of bumetanide in serum and brain tissue. *J Chromatogr B Analyt Technol Biomed Life Sci* 879: 998-1002.
- Lisman J and Buzsaki G. (2008) A neural coding scheme formed by the combined function of gamma and theta oscillations. *Schizophrenia bulletin* 34: 974-980.
- Lisman JE and Idiart MA. (1995) Storage of 7 +/- 2 short-term memories in oscillatory subcycles. *Science* 267: 1512-1515.

- Lord C, Risi S, Lambrecht L, et al. (2000) The autism diagnostic observation schedule-generic: a standard measure of social and communication deficits associated with the spectrum of autism. *J Autism Dev Disord* 30: 205-223.
- Lord C, Rutter M and Le Couteur A. (1994) Autism Diagnostic Interview-Revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. *J Autism Dev Disord* 24: 659-685.
- Marrosu F, Marrosu G, Rachel MG, et al. (1987) Paradoxical reactions elicited by diazepam in children with classic autism. *Funct Neurol* 2: 355-361.
- Mazzuca M, Minlebaev M, Shakirzyanova A, et al. (2011) Newborn Analgesia Mediated by Oxytocin during Delivery. *Frontiers in cellular neuroscience* 5: 3.
- Murakami Y, Kohyama N, Kobayashi Y, et al. (2005) Functional characterization of human monocarboxylate transporter 6 (SLC16A5). *Drug Metab Dispos* 33: 1845-1851.
- Murthy VN and Fetz EE. (1992) Coherent 25- to 35-Hz oscillations in the sensorimotor cortex of awake behaving monkeys. *Proceedings of the National Academy of Sciences of the United States of America* 89: 5670-5674.
- Nardou R, Yamamoto S, Bhar A, et al. (2011a) Phenobarbital but Not Diazepam Reduces AMPA/kainate Receptor Mediated Currents and Exerts Opposite Actions on Initial Seizures in the Neonatal Rat Hippocampus. *Frontiers in cellular neuroscience* 5: 16.
- Nardou R, Yamamoto S, Chazal G, et al. (2011b) Neuronal chloride accumulation and excitatory GABA underlie aggravation of neonatal epileptiform activities by phenobarbital. *Brain* 134: 987-1002.
- Narumoto J, Okada T, Sadato N, et al. (2001) Attention to emotion modulates fMRI activity in human right superior temporal sulcus. *Brain Res Cogn Brain Res* 12: 225-231.
- Patterson PH. (2009) Immune involvement in schizophrenia and autism: etiology, pathology and animal models. *Behavioural Brain Research* 204: 313-321.
- Pizzarelli R and Cherubini E. (2011) Alterations of GABAergic signaling in autism spectrum disorders. *Neural plasticity* 2011: 297153.
- Ploeger A, Raijmakers ME, van der Maas HL, et al. (2010) The association between autism and errors in early embryogenesis: what is the causal mechanism? *Biol Psychiatry* 67: 602-607.
- Schmahmann JD and Sherman JC. (1998) The cerebellar cognitive affective syndrome. *Brain* 121: 561-579.
- Singer W. (1993) Synchronization of cortical activity and its putative role in information processing and learning. *Annual review of physiology* 55: 349-374.
- Smith SM, Jenkinson M, Woolrich MW, et al. (2004) Advances in functional and structural MR image analysis and implementation as FSL. *Neuroimage* 23 Suppl 1: S208-219.
- Sullivan JE, Witte MK, Yamashita TS, et al. (1996) Analysis of the variability in the pharmacokinetics and pharmacodynamics of bumetanide in critically ill infants. *Clinical pharmacology and therapeutics* 60: 414-423.

- Tabuchi K, Blundell J, Etherton MR, et al. (2007) A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. *Science* 318: 71-76.
- Tyzio R, Cossart R, Khalilov I, et al. (2006) Maternal oxytocin triggers a transient inhibitory switch in GABA signaling in the fetal brain during delivery. *Science* 314: 1788-1792.
- Voos AC, Pelphrey KA, Tirrell J, et al. (2013) Neural mechanisms of improvements in social motivation after pivotal response treatment: two case studies. *J Autism Dev Disord* 43: 1-10.
- WASI. (1999) *Wechsler Abbreviated Scale of Intelligence*, San Antonio, TX: The Psychological Corporation.
- Wechsler D and Naglieri J. (2006) *Wechsler Nonverbal Scale of Ability*, San Antonio, TX: PsychCorp Edition, A Brand of Harcourt Assessment.
- Weiss LA. (2009) Autism genetics: emerging data from genome-wide copy-number and single nucleotide polymorphism scans. *Expert review of molecular diagnostics* 9: 795-803.
- Wilson TW, Rojas DC, Reite ML, et al. (2007) Children and adolescents with autism exhibit reduced MEG steady-state gamma responses. *Biological psychiatry* 62: 192-197.
- Woolrich MW, Jbabdi S, Patenaude B, et al. (2009) Bayesian analysis of neuroimaging data in FSL. *Neuroimage* 45: S173-186.
- Worsley K. (2001) Statistical analysis of activation images. In: Jezzard P, Matthews PM and Smith SM (eds) *Functional MRI: An Introduction to Methods*. Oxford: OUP.
- Zhu L, Polley N, Mathews GC, et al. (2008) NKCC1 and KCC2 prevent hyperexcitability in the mouse hippocampus. *Epilepsy Res* 79: 201-212.
- Zurcher N, Donnelly N, Rogier O, et al. (2013) It's All in the Eyes: Subcortical and Cortical Activation during Grotesqueness Perception in Autism. *PLoS One* 8: e54313.

Figure Legends:

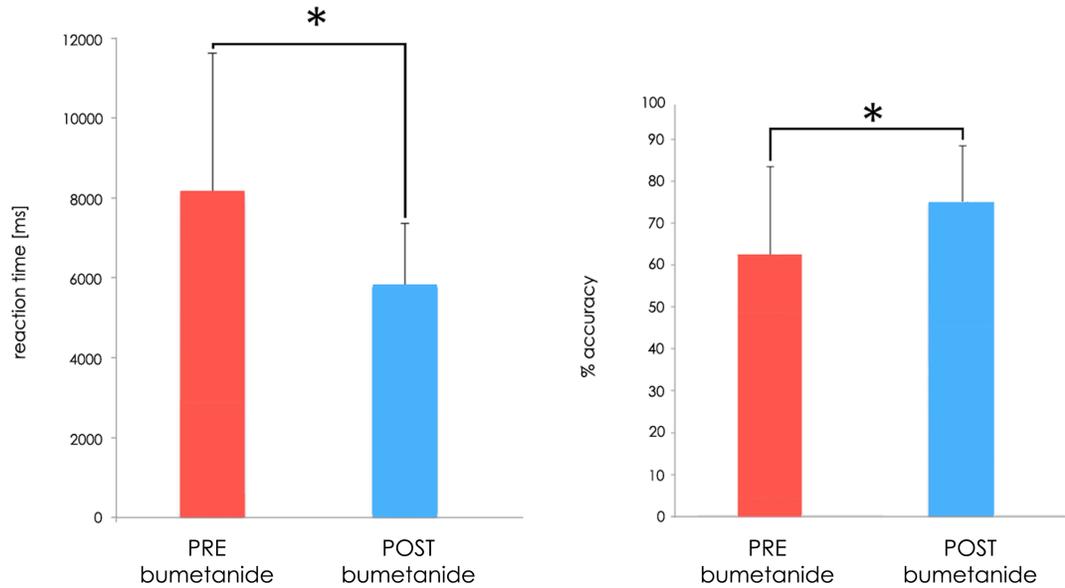


Fig. 1. Results of the behavioral non-verbal emotional matching task in participants before treatment (PRE, red columns) and after treatment (POST, blue columns). Top panel shows reaction times and bottom panel shows % accuracy. Bumetanide treatment significantly reduces reaction time and increases accuracy.

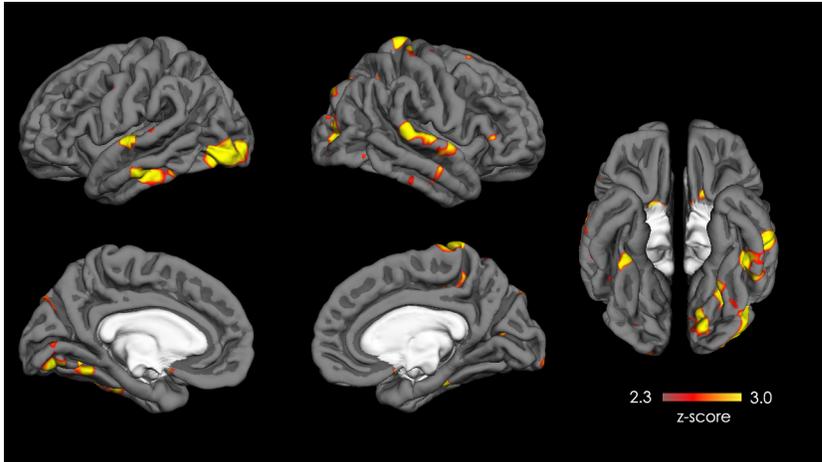


Fig. 2. Statistical maps of differences in fMRI activation for emotional vs. neutral faces, in a POST>PRE treatment comparison. Statistical maps are displayed on the pial cortical surface of the template FreeSurfer brain (fsaverage), on the lateral, medial and ventral views of the right and left hemisphere. Images have been thresholded with $z > 2.3$. The grey mask covers subcortical regions in which activity cannot be expressed in surface rendering.

	Age Scan1	Age Scan2	ADOS S&C	ADI-R S	ADI-R C	AQ	EQ	PIQ
Participants (n=7)	19.3±4.6	20.5±5.1	12.6±3.7	18.6±4.8	13.7±1.1	31.0±6.3	20.3±5.2	101.4±14.5

Table 1: Participant's characteristics. All numbers represent mean±SD. ADOS S&C: score for social and communication at ADOS (module 4); ADI-R S: score for social at ADI-R; ADI-R C: score for communication at ADI-R; AQ: Autism Quotient; EQ: Empathy Quotient; PIQ: performance IQ.

Table 2: clusters ($z > 2.3$, minimum cluster size of 300 voxels) of increased brain activation in response to EMOTIONAL vs. NEUTRAL faces after 10 months of bumetanide treatment

Brain region		Cluster size # of voxels	Z-MAX	MNI Coordinates		
				X	Y	Z
L	Inferior occipital cortex	2615	4.57	-42	-86	-18
L	Lingual cortex		4.50	-26	-62	-6
L	Lateral occipital cortex		4.36	-44	-82	-18
L	Occipital fusiform cortex		4.33	-14	-80	-8
	Cerebellum vermis VI		3.71	-4	-80	-22
L	Cerebellum VI		3.56	-24	-50	-20
L	Cerebellum Crus I		3.41	-20	-86	-24
L	Cerebellum V		3.22	-2	-58	-22
R	Lateral occipital cortex inferior	1516	3.86	28	-86	36
R	Lateral occipital cortex superior		3.84	28	-82	2
R	Occipital fusiform cortex		3.49	24	-82	2
R	Superior parietal lobule		2.84	28	-54	48
R	Temporal fusiform cortex	842	4.75	40	-52	-26
R	Cerebellum VI		4.01	28	-62	-20
R	Occipital fusiform		3.77	30	-62	-16
R	Cerebellum Crus I		3.33	42	-42	-38
R	Inferior temporal cortex		2.83	48	-46	-16
R	Intraparietal sulcus	818	3.55	20	-58	66
R	Posterior cingulate		3.53	10	-26	42
R	Somatosensory cortex		3.51	34	-34	36
R	Precuneus		3.18	6	-46	68
R	Superior parietal lobule		3.06	8	-60	66
R	Inferior occipital cortex	792	4.2	52	-82	-16
L	Inferior temporal cortex	751	4.28	-44	-36	-18
R	Accumbens	459	4.02	12	6	-14
L	Accumbens		3.40	-10	6	-12
L	Orbitofrontal cortex		3.33	-12	12	-14
R	Amygdala		3.32	24	2	-22

R	Temporal pole		3.21	24	-6	-28
R	Superior Temporal cortex	444	4.13	68	-34	8
R	Parietal operculum		2.46	54	-28	14
L	Superior Temporal cortex	400	4.14	-62	-4	-2
L	Central operculum		3.26	-54	-10	-6
L	Precentral cortex		2.56	-62	2	6
L	Supplementary motor area	345	3.48	2	6	66
L	Superior frontal gyrus		3.40	14	8	66
L	Somatosensory cortex	323	4.21	14	-40	74