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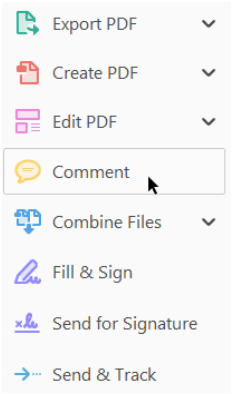

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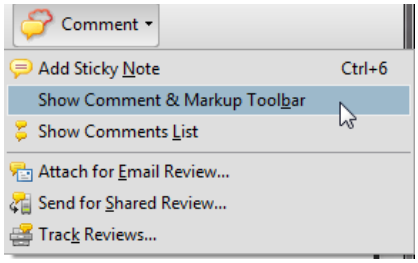

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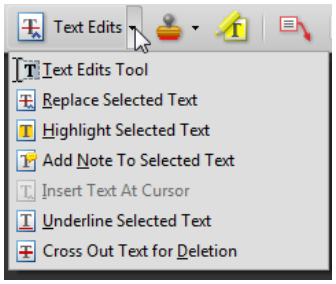

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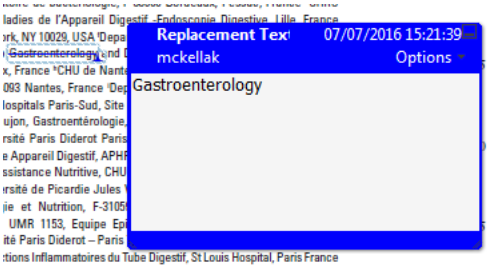

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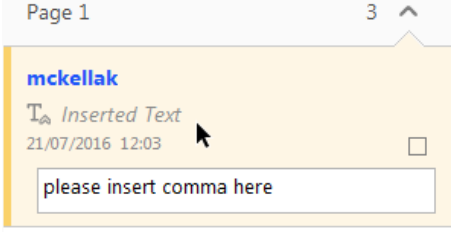

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Mutations affecting glycinergic neurotransmission in hyperekplexia increase pain sensitivity

Pascal Henri Vuilleumier,[1] Raphael Fritsche,[2] Jürg Schliessbach,[1] Bernhard Schmitt,[3] Lars Arendt-Nielsen,[4] Hanns Ulrich Zeilhofer[5] and Michele Curatolo[4,6]

AQ1

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Inhibitory interneurons in the spinal cord use glycine and GABA for fast inhibitory neurotransmission. While there is abundant research on these inhibitory pain pathways in animal models, their relevance in humans remains unclear, largely due to the limited possibility to manipulate selectively these pathways in humans. Hyperekplexia is a rare human disease that is caused by loss-of-function mutations in genes encoding for glycine receptors and glycine transporters. In the present study, we tested whether hyperekplexia patients display altered pain perception or central pain modulation compared with healthy subjects. Seven patients with genetically and clinically confirmed hyperekplexia were compared to 14 healthy age- and sex-matched controls. The following quantitative sensory tests were performed: pressure pain detection threshold (primary outcome), ice water tolerance, single and repeated electrical pain detection thresholds, nociceptive withdrawal reflex threshold, and conditioned pain modulation. Statistical analysis was performed using linear mixed models. Hyperekplexia patients displayed lower pain thresholds than healthy controls for all of the quantitative sensory tests [mean (standard deviation)]: pressure pain detection threshold [273 (170) versus 475 (115) kPa, $P = 0.003$], ice water tolerance [49.2 (36.5) versus 85.7 (35.0) s, $P = 0.015$], electrical single pain detection threshold [5.42 (2.64) versus 7.47 (2.62) mA, $P = 0.012$], electrical repeated pain detection threshold [3.76 (1.41) versus 5.8 (1.73) mA, $P = 0.003$], and nociceptive withdrawal reflex [7.42 (3.63) versus 14.1 (6.9) mA, $P = 0.015$]. Conditioned pain modulation was significantly reduced in hyperekplexia [increase to baseline: 53.2 (63.7) versus 105 (57) kPa, $P = 0.030$]. Our data demonstrate increased pain sensitivity and impaired central pain modulation in hyperekplexia patients, supporting the importance of glycinergic neurotransmission for central pain modulation in humans.

Affiliations are correct

AQ2
1 Department of Anaesthesiology and Pain Medicine, Bern University Hospital, University of Bern, Switzerland
2 Department of Ophthalmology, Canton Hospital of Lucerne, Switzerland
3 Department of Child Neurology, Children's Hospital, University of Zurich, Switzerland
4 Center for Sensory-Motor Interaction, School of Medicine, University of Aalborg, Denmark
5 Institute of Pharmacology and Toxicology, University of Zurich, and Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH) Zurich, Switzerland
6 Department of Anaesthesiology and Pain Medicine, University of Washington, Seattle, USA

Correspondence to: Pascal H. Vuilleumier,
Department of Anaesthesiology and Pain Medicine
Bern University Hospital, CH-3010 Bern, Switzerland
E-mail: pascal.vuilleumier@insel.ch

AQ5 **Keywords:** hyperekplexia; GABA; glycine; startle disease; pain perception; pain modulation

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Introduction

Synaptic inhibition in nociceptive pathways of the spinal cord is mediated by glycine and GABA receptors (Zeilhofer, 2005). Glycine receptors are the major determinants of inhibitory neurotransmission in the retina, spinal cord and brainstem (Lynch, 2004; Chung *et al.*, 2010). Immunofluorescence studies have confirmed abundant glycinergic innervation in the dorsal horn (Zeilhofer *et al.*, 2005), a key site in the classic gate control theory of pain (Melzack and Wall, 1965). Animal studies have shown that non-nociceptive reactions are non-nociceptive only as long as spinal GABAergic and glycinergic inhibition remain intact (Zeilhofer, 2008; Foster *et al.*, 2015). Glycine receptors are members of the pentameric ligand-gated ion channel family, which belongs to the same superfamily of Cys-loop receptors as the $5HT_3$, nicotinic acetylcholine and $GABA_{A/C}$ receptors (Lynch, 2009). Glycine receptors are membrane-embedded proteins that contain an integral chloride-selective pore (Lynch, 2004). GABA and glycine open chloride channels, which hyperpolarize postsynaptic cells and impair the propagation of excitatory signals on dendrites of neurons (Zeilhofer, 2005; Lynch, 2009).

Pharmacological blockade of GABAergic and/or glycinergic neurotransmission in the dorsal horn mimics many symptoms of inflammatory and neuropathic pain (Sivilotti and Woolf, 1994; Sherman and Loomis, 1995; Zeilhofer and Zeilhofer, 2008). Additionally, a loss of synaptic inhibition in the dorsal horn occurs in animal pain models (Coull *et al.*, 2003, 2005; Muller *et al.*, 2003; Harvey *et al.*, 2004*b*). In humans, studies on nociceptive long-term potentiation suggest that loss of inhibitory interneurons in the dorsal horn may play a role in the development of chronic pain (Klein *et al.*, 2004). However, the importance of central inhibitory mechanisms in human pain states is difficult to prove, largely due to the limited possibility to manipulate these pathways in humans.

In humans, impairment of spinal glycine receptors or associated proteins is responsible for hyperekplexia, a rare neurogenetic disease (OMIM #149400). Hyperekplexia, also known as hereditary startle disease or stiff baby syndrome, is a non-epileptic disorder characterized by an exaggerated persistent startle response and neonatal hypertonia to unexpected auditory, somatosensory and visual stimuli (Andermann *et al.*, 1980; Praveen *et al.*, 2001; Zhou *et al.*, 2002). Startle responses and generalized muscle stiffness both gradually subside during the first months of life (Tijssen and Rees, 2007). However, pathological startle responses can remain throughout adulthood, resulting in unprotected falls and injury (Andermann *et al.*, 1980). These features characterize the major form of hyperekplexia.

To date, hereditary hyperekplexia has been identified in >100 pedigrees and >120 sporadic cases (Dreissen and Tijssen, 2012). Most of them are classified as the major form of hyperekplexia. There is a minor form of the disease, described in few families, but this condition may remain under-reported (Bakker *et al.*, 2006).

To date, mutations in five genes encoding for different key elements of inhibitory glycinergic synapses have been associated with hyperekplexia (Shiang *et al.*, 1993; Brune *et al.*, 1996; Humeny *et al.*, 2002; Rees *et al.*, 2002). Two of these genes (*GLRA1* and *GLRB*) encode for glycine receptor subunits. Additionally, defects in the presynaptic glycine transporter gene GLYT2 (*SLC6A5*) have been identified in human hyperekplexia (Eulenburg *et al.*, 2006; Rees *et al.*, 2006). *GPHN*, encoding the glycine receptor clustering molecule gephyrin (Rees *et al.*, 2003), and *ARHGEF9*, an X-linked gene encoding collybistin (Harvey *et al.*, 2004*a*), are each associated with known cases of hyperekplexia (Tijssen and Rees, 2007).

The aim of this study was to evaluate, for the first time in humans, whether symptomatic mutations in the glycinergic system affects central pain processing.

Materials and methods

This study was conducted in accordance with the amended Declaration of Helsinki. The study was approved by the IRB of the Canton Bern, Switzerland (No. 131/11). It was registered in the Clinical Trials Protocol Registration System (NCT01476514). All subjects gave written informed consent.

Design

This was a prospective study of pain thresholds in hyperekplexia patients and a group of sex- and age-matched healthy volunteers. To avoid experimenter bias, quantitative sensory tests of hyperekplexia patients and healthy volunteers were assessed independently by different investigators: R.F. and J.S. tested healthy volunteers and P.V. tested patients with hyperekplexia. P.V. previously trained and extensively supervised R.F. and J.S. on standardized quantitative sensory test measurements, with the aim to maximize inter-observer reliability for investigations that were performed before the present one (Biurrun Manresa *et al.*, 2014; Vuilleumier *et al.*, 2015).

Setting

The experiments were performed at the Department of Anaesthesiology and Pain Medicine, Bern University Hospital, Bern, University of Bern, Switzerland.

Participants

Seven patients with the major form of hyperekplexia and 14 sex- and age-matched healthy volunteers were studied. They received a compensation of 150 Swiss francs for their participation, plus reimbursement for travel expenses. Healthy controls were recruited by advertisement at the Bern University Hospital; patients with hyperekplexia were recruited by contacting neuropaediatricians and neurologists known to care for these patients in Switzerland, Germany, France, the UK, Italy and Washington State, USA. Inclusion criteria for hyperekplexia

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patients were a clinically major form of hyperekplexia diagnosed by a neuropaediatrician or neurologist and a known mutation in one of the following: *GLRA1*, *GLRB*, *SLC6A5* (GlyT2), *GPHN*, glycine receptor clustering molecule gephyrin or *ARHGEF9*. Exclusion criteria were: age below 7 years, pregnancy or breastfeeding, an ongoing treatment with antidepressant drugs, opiates or any analgesic substance during the 10 days before testing, and cognitive deficits, defined as an adult not able to fulfil basic professional activities or a child not able to attend regular school classes. Baseline treatment for hyperekplexia (specified in Table 1) was not discontinued for ethical reasons. Healthy controls were selected to match hyperekplexia patients by gender and age (± 2 years and ± 5 years for patients <18 and $\geqslant 18$ years old, respectively). Exclusion criteria for the control group were any chronic or acute pain, any neuropathy interfering with quantitative sensory measures, intake of any medication known to modulate pain perception or quantitative sensory measures, any drug or substance abuse, pregnancy and breastfeeding.

End-points

The pressure pain detection threshold on the second toe was the primary end-point. This test was chosen because, in previous studies, it discriminated children with growing pain from healthy controls (Hashkes *et al.*, 2004). Additional measures were used as secondary end-points.

General methodological aspects

Participants were positioned in a comfortable supine position with the upper body elevated by 30° in a quiet room dedicated to pain research. Tests were performed on the dominant body side, except for ice-water stimulation, which was applied to the non-dominant body side. Whenever children $\leqslant 12$ years old were tested, one or both of the parents were present during the testing. For subjects between 12 and 17 years of age, parent's presence was discussed in advance and always allowed whenever desired or indicated. Training sessions for the pain tests were performed before beginning data collection; the training lasted until the subjects were familiar with the testing procedures. Participants were not informed about the expected results to avoid conditioning.

Pressure pain detection threshold

The pain detection threshold was measured with an electronic pressure algometer (Algometer, Somedic) applied at the centre of the pulp of the second toe. The probe had a surface area of 1 cm^2. The pressure was increased from 0 at a rate of 30 kPa/s to a maximum pressure of 1000 kPa. The pain detection threshold was defined as the point at which the pressure sensation turned to pain. The subjects were instructed to press a button when these points were reached. The algometer displayed the pressure intensity at which the button was pressed. If the subjects did not press the button at a pressure of 1000 kPa, this value was considered to be the threshold. Three assessments were made for the data analysis.

Thresholds to cutaneous electrical stimulation and nociceptive withdrawal reflex

Cutaneous electrical stimulation was performed through bipolar surface Ag/AgCl-electrodes (Alpine Biomed Adhesive Disposable Surface Electrodes) placed just distal to the lateral malleolus (i.e. innervation area of the sural nerve). EMG reflex responses to electrical stimulation were recorded from the middle of the biceps femoris and the rectus femoris muscles (Ag/AgCl-electrodes). Stimulation and EMG recordings were made by a computer-controlled constant current stimulator (NCS System, Evidence 3102 evo, Neurosoft).

A 25 ms, train-of-five, 1 ms, square-wave impulse (perceived as a single stimulus), was delivered to the skin. The current intensity was increased from 1 mA in steps of 0.5 mA until (i) a biceps femoris reflex with an amplitude exceeding 20 µV for at least 10 ms in the 50–150 ms post-stimulation interval was detected (i.e. single stimulus reflex threshold); and (ii) a pain sensation was evoked (i.e. single stimulus pain threshold).

For repeated (i.e. five stimuli) cutaneous stimulation, the stimulus burst used for single stimulation was repeated five times at 2 Hz, at constant intensity. The current intensity of the five stimuli was increased from 1 mA in steps of 0.5 mA until the subjects felt pain during the last two to three of the five stimuli. Three assessments were made and averaged for data analysis.

Heat and cold pain detection thresholds

For heat stimulation, a 30 × 30 mm thermode was applied to the skin (TSA-2001, Medoc). The test was performed at the

Table 1 Demographics of seven hyperekplexia patients and 14 healthy controls

Hyperekplexia							Healthy controls			
Mutation	Age	Gender	Medication	Weight (kg)	Height (cm)	BMI	Mean Age	Mean weight (kg)	Mean height (cm)	Mean BMI
SLC6A5	10	M	Clonazepam	28	132	16.1	10	36.5	146.5	17.0
GLRA1	34	F	Clonazepam	62	164	23.1	34.5	64	169	22.4
GLRA1	14	M	Clonazepam	56	157	22.7	14.5	51.5	160	20.1
GLRA1	35	F	Desoxyphenobarbital, gabapentin	72	168	25.5	39	61.7	163.5	23.1
GLRA1	28	M	Diazepam	51	163	19.2	27.5	61	167.5	21.7
GLRA1	40	M	Clonazepam	73	179	22.8	41	81	176.5	26
GLRA1	12	M	Valproate, clobazam	50	155	20.8	13.5	52	160.5	20.2

Two matched controls were used for each patient.
BMI = body mass index; F = female; M = male.

lateral aspect of the leg, midway between the knee and the lateral malleolus (Neziri *et al.*, 2011). The temperature of the thermode was continuously increased from 30°C to a maximum of 50.5°C at a rate of 1.0°C/s. The pain detection threshold was defined as it was for pressure stimulation. The subjects were instructed to press a button when the thresholds were reached. At that point, the temperature was recorded and the thermode cooled to 30°C. The thermode also cooled to 30°C even if the detection threshold was not reached at 50.5°C, in which case 50.5°C was considered to be the threshold.

Cold stimulation was performed with the same apparatus and thermode as heat stimulation. The temperature of the thermode was continuously decreased from 30°C to a minimum of 0°C at a rate of 1.0°C/s. The cold pain detection threshold was defined as it was for pressure stimulation. The subjects were instructed to press a button when the threshold was reached. At that point, the temperature was recorded by the software and the thermode heated to 30°C. If the threshold was not reached at 0°C, 0°C was considered to be the threshold. The test was performed at the same site as heat stimulation.

Ice water test

The hand was immersed in ice-saturated water (0.7 ± 1°C). The device consisted of a container separated by a mesh screen into an outer and inner part. The mesh screen prevented direct contact between the ice (placed in the outer part) and the hand of the subject (placed in the inner part). The water was regularly stirred to maintain the temperature in the inner part at 0.7 ± 1°C, as monitored by a digital thermometer (± 0.1°C).

The subjects placed their hands, wide open and submerged up to the wrist, into the container. They were asked to keep their hands in the water until an intolerable sensation of pain was perceived or for a maximum of 2 min. The time from immersion to withdrawal was recorded.

Conditioned pain modulation

Pressure pain applied to the second toe and an ice water test applied to the hand were used as 'test' and 'conditioning' stimuli, respectively. Pressure pain detection threshold was measured at the same time as the subject was withdrawing the hand from the ice water. An increase in the pressure pain detection threshold immediately after the ice water test was considered an indication of functioning conditioned pain modulation (Serrao *et al.*, 2004; Vuilleumier *et al.*, 2013).

Descriptive variables

Age, gender, weight and body mass index were recorded for all participants. Any ongoing medical treatment was recorded for hyperekplexia patients.

Sample size considerations

The sample size was calculated based on previous data on the pressure pain detection threshold in children with growing pains and healthy controls (Hashkes *et al.*, 2004). Assuming a mean value of 579 and 500 kPa in controls and patients, respectively, expecting a standard deviation of 108 kPa, and setting a ratio of 2:1 between the two groups, 45 controls and 23 patients were required to detect a difference in the pain detection threshold between the two groups at a two-sided alpha-level of 5% with a power of 80%. Due to the rarity of the disease, recruitment was extremely difficult. The study was closed after failing to recruit new patients over 2 years.

Statistical analysis

The differences between cases and controls were estimated using linear mixed models with random intercepts for each matched case-control group [i.e. $y_{ij} = \beta_0 + x_{ij}\ \beta_1 + u_i + \varepsilon_{ij}$ for case-control pair i and patient j with $u_i \sim N(0,\tau^2)$ and $\varepsilon_{ij} \sim N(0,\sigma^2)$ *and* $x_{ij} = 0$ for controls and $x_{ij} = 1$ for cases]. The models were estimated by restricted maximum likelihood. The sampling distributions of the test statistics were approximated by a *t*-distribution using the method from Kenward and Roger with the expected information matrix (Kenward and Roger, 1997). One variable was modelled on the log-scale to improve the model fit (ice water tolerance). The group difference is expressed as mean difference or geometric mean ratio (for the variables modelled on log-scale) with a 95% confidence interval (CI) and a *P*-value. The analysis was performed in Stata 14 (StataCorp. 2015, Stata Statistical Software: Release 14. College Station, TX: StataCorp LP).

Results

Of the seven patients enrolled, three were recruited in Switzerland and four in Germany. Six of our patients carried mutations in the *GLRA1* gene, one patient had a mutation in the *SCL6A5* gene. All of them were tested in Bern, Switzerland. All tested hyperekplexia patients were devoid of acute or chronic pain syndromes, as well as devoid of obvious sensory impairments. No startle response was evoked during the quantitative sensory test measures. Table 1 presents the demographics of the hyperekplexia patients and the healthy controls. Table 2 presents the baseline values and the statistical analysis of the performed quantitative sensory tests. Figure 1 illustrates the results of pressure pain detection thresholds (i.e. the primary end-point) and electrical pain detection thresholds as well as the nociceptive withdrawal reflex threshold, the cold pressor test and conditioned pain modulation baseline shifts.

In the analysis of the primary outcome variable, pressure pain detection thresholds were significantly lower in hyperekplexia patients than in controls ($P = 0.003$; mean difference 201 kPa, 95% CI: 82–321).

The cutaneous electrical single-stimulus pain detection threshold in hyperekplexia patients was significantly lower ($P = 0.012$) than in controls, with a mean difference of 2.05 mA (95% CI: 0.54–3.56). Cutaneous electrical repeated-stimulus (temporal summation) pain detection thresholds were also significantly lower in hyperekplexia patients ($P = 0.003$) than in controls, with a mean difference of 2.05 mA (95% CI: 0.85–3.24). The threshold for the nociceptive withdrawal reflex was also significantly decreased in hyperekplexia ($P = 0.024$), with a mean difference from

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Table 2 Results from linear mixed models of pain tests

	Hyperekplexia (n = 7) Mean (SD)	Controls (n = 14) Mean (SD)	Mean difference or GMR (95% CI)	P-value
PPDT (kPa)	273 (170)	475 (115)	201 (82–321)	0.003
CPM (kPa)	53.2 (63.7)	105 (57)	52.1 (6.0–98.3)	0.030
ESPD (mA)	5.42 (2.64)	7.47 (2.62)	2.05 (0.54–3.56)	0.012
ERPD (mA)	3.76 (1.41)	5.80 (1.73)	2.05 (0.85–3.24)	0.003
NWR (mA)[a]	7.42 (3.63)	14.1 (6.9)	6.67 (1.05–12.29)	0.024
IWT (s)[b]	49.2 (36.8)	85.7 (35.0)	1.97 (1.17–3.32)	0.015

[a]One and two patients with missing data in the hyperekplexia and control group, respectively.
[b]Treatment effect expressed as geometric mean ratio (GMR).
CPM = conditioned pain modulation (change from baseline); ERPD = cutaneous electrical repeated-stimulus pain detection threshold; ESPD = cutaneous electrical single-stimulus pain detection threshold; IWT = ice water tolerance time; NWR = nociceptive withdrawal reflex; PPDT = pressure pain detection threshold.

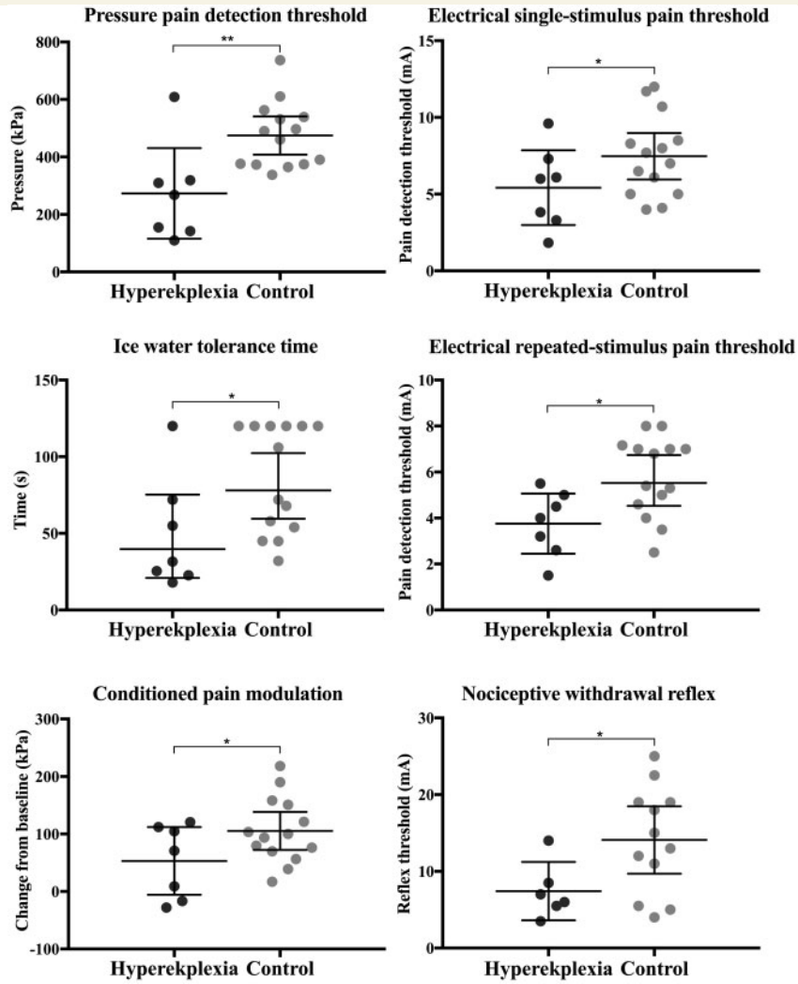

Figure 1 Pain tests. Bars show the mean values with 95% CI or the geometric mean value with 95% CI for the ice water tolerance time. *$P < 0.05$. **$P < 0.005$.

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controls of 6.67 mA (95% CI: 1.05–12.29). In one hyperekplexia patient, the nociceptive withdrawal reflex could not be assessed because the increase in current intensity caused intolerable pain before eliciting a reflex. The nociceptive withdrawal reflex was not assessed in the corresponding controls.

Time tolerated in the ice-water was significantly shorter in hyperekplexia patients ($P = 0.015$) than in healthy

controls, with a mean difference of 1.97 s (95% CI: 1.17–3.32).

The heat pain detection threshold and cold pain detection threshold could not be fit to the statistical model because of a severe violation of the model assumption, and they were not tested for significance. The heat pain detection threshold had a mean value of 45.6°C [standard deviation (SD) = 4.24°C] in hyperekplexia patients and 48.5°C (SD = 2.05°C) in controls. The cold pain detection threshold had a mean value of 7.72°C (SD = 9.57°C) in hyperekplexia patients and 1.62°C in controls (SD = 4.55°C).

Conditioned pain modulation was significantly less effective in hyperekplexia patients ($P = 0.030$), as they displayed a reduced increase in the pressure pain threshold (i.e. test stimulus) from baseline; the mean difference between the two groups was 52.1 kPa (95% CI: 6.0–98.3).

A retrospective sensitivity analysis with additional healthy controls was performed and is presented in the Supplementary material.

Discussion

This study investigated the influence of documented mutations in glycine signalling on pain modulation in humans. Remarkably, very large differences between patients and controls in multiple pain modalities and pain mechanisms (temporal summation and descending pain modulation) were found. The results are highly suggestive of altered central pain processing (gain-of-function) associated with dysfunction in inhibitory glycinergic synaptic transmission.

Many dorsal horn neurons receive both GABAergic and glycinergic synaptic input, hence there is significant overlap in glycinergic and GABAergic neurotransmission in the spinal cord. Fast postsynaptic inhibitory responses mostly exhibit two distinct kinetic patterns: a glycinergic strychnine-sensitive component with fast decay and a slower GABAergic bicuculline-sensitive component. Although it is well established that there is a co-release of GABA and glycine from the same synaptic vesicles, their respective effects on postsynaptic inhibition are not entirely understood (Zeilhofer *et al.*, 2012). Because of the close relationship between GABAergic and glycinergic neurotransmission, the following discussion includes data on pharmacological modulation of both pathways.

Pressure pain

Glycinergic interneurons modulate the processing of mechanical input from low threshold mechanoreceptive afferents to central pain projection neurons (Powell and Todd, 1992; Zeilhofer, 2005). Narikawa *et al.* (2000) detected inhibitory postsynaptic currents (IPSCs) in laminae II of the spinal horn after *in vivo* mechanical stimulation of rodent skin. The glycine antagonist strychnine and the $GABA_A$ antagonist bicuculline abolished these IPSCs. These data may explain our findings, with significantly lower pressure pain detection thresholds in hyperekplexia patients than in healthy controls. Interestingly, pressure pain detection and tolerance thresholds were unaffected by the $GABA_A$ modulators clonazepam or clobazam in a study on healthy volunteers (Vuilleumier *et al.*, 2013), possibly because of low sensitivity of these pain tests in a human pharmacological model, whereby safety considerations limit the doses administered.

Electrical pain and nociceptive reflex thresholds

There is limited literature on glycinergic modulation of pain with cutaneous electrical stimulation. The benzodiazepines clobazam and clonazepam, which enhance GABAergic inhibition, have been tested in 16 healthy volunteers in a cross-over design; while single or repeated cutaneous electrical stimulation failed to detect significant differences between the benzodiazepines and the placebo, pain thresholds with intramuscular electrical stimulation were increased after clobazam and clonazepam administration (Vuilleumier *et al.*, 2013). Another study with healthy volunteers testing clonazepam and clobazam also failed to detect significant differences from placebo in pain thresholds after cutaneous electrical stimulation, as well as with the nociceptive withdrawal reflex (Besson *et al.*, 2015). These results are in contrast with the findings of the present study, again suggesting that the mandatory use of low doses in humans may prevent tests with limited sensitivity to detect analgesic effects. Our study suggests that modulation of painful electrical stimuli is compromised in patients with impaired glycinergic neurotransmission. The lower nociceptive withdrawal reflex in hyperekplexia patients, compared with healthy controls, strongly suggests that the impairment in glycinergic central pain modulation occurs, at least in part, at the spinal level.

Thermal pain thresholds

The ice water test revealed significantly lower cold tolerance in hyperekplexia patients than in healthy controls. In a study performed by our group, there was no significant effect of clobazam and clonazepam on ice water tolerance in healthy volunteers (Vuilleumier *et al.*, 2013). These contradictory findings might be explained by a stronger glycinergic than GABAergic control over ice water tolerance thresholds. Delta9THC is not only an agonist at cannabinoid receptors but also acts as a positive allosteric modulator of glycine receptor (Xiong *et al.*, 2014). In a study of 42 volunteers, smoking cannabis significantly prolonged hand immersion times in ice water (Cooper and Haney, 2016), perhaps partly due to modulation of glycinergic signalling.

Conditioned pain modulation

Conditioned pain modulation is the human counterpart of diffuse inhibitory noxious stimulation, whereby a conditioning stimulus is expected to reduce pain caused by a

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test stimulus (Yarnitsky *et al.*, 2010). It has been postulated that conditioned pain modulation activates descending inhibitory fibre tracts from the subnucleus reticularis dorsalis terminating in the dorsal horn, where serotoninergic and noradrenergic mechanisms contribute to pain modulation (Bouhassira *et al.*, 1992; Bannister *et al.*, 2009). Norepinephrine has been shown to facilitate inhibitory transmission in the adult rat spinal cord through the activation of $\alpha 2$ adrenoceptors (Nabekura *et al.*, 1999). Baba *et al.* (2000) found that norepinephrine dose-dependently increases GABAergic and glycinergic IPSCs in the rodent spinal cord. In an *in vivo* spinal nerve ligation model, in which diffuse noxious inhibitory control is abolished, Bannister *et al.* (2015) were able to restore diffuse inhibitory noxious stimulation with the norepinephrine reuptake inhibitor reboxetine.

The present study shows that conditioned pain modulation is significantly impaired in hyperekplexia patients, whereas a previous study performed by our group showed that classical allosteric modulators of $GABA_A$ receptors did not produce any effect on conditioned pain modulation in healthy volunteers (Vuilleumier *et al.*, 2013), confirming previous results (Kunz *et al.*, 2006). This may suggest that conditioned pain modulation depends more on glycinergic than on GABAergic neurotransmission in humans.

However, a partial answer may stem from the finding in rodents that there is a gradient of inhibitory input in the dorsal horn: glycinergic inhibition is most pronounced in the deep dorsal horn and the inner lamina II; GABAergic inhibition is most pronounced in the outer lamina II to lamina I (Takazawa and MacDermott, 2010). Fibres transmitting low threshold innocuous input terminate in lamina III and lamina II, where glycine is the predominant fast neurotransmitter (Takazawa and MacDermott, 2010; Imlach *et al.*, 2016). This might explain the differential behaviour of conditioned pain modulation/diffuse inhibitory noxious stimulation with respect to glycinergic and GABAergic inhibition: the fibres mediating the conditioning stimulus of conditioned pain modulation/diffuse inhibitory noxious stimulation predominantly end in the inner lamina II region, where glycine receptors dominate fast neuronal inhibition, linking an innocuous sensory input to fast glycinergic hyperpolarization and conditioned pain modulation efficiency. Although co-release of GABA and glycine has been shown (Todd *et al.*, 1996; Zeilhofer *et al.*, 2012), the effects of reductions in glycinergic neurotransmission are indeed delicate in comparison with positive GABAergic stimulation.

Strength and limitations

To our knowledge, no prior study assessed central pain modulation in humans diagnosed with hyperekplexia, a human disease associated with documented glycinergic dysfunction. We have applied a wide spectrum of pain tests. The ability to study human pain pathophysiology in the face of documented defects in glycinergic neurotransmission is an important translational step in clarifying the role of this pathway in human pain conditions. All patients included had a confirmed symptomatic mutation in glycine signalling, and did not suffer from any chronic musculoskeletal or neuropathic pain condition that could have *per se* determined the findings. The main limitation is the small sample size, which may have produced false positive results. However, the measured differences were, quantitatively, large in virtually all tests, and the results were consistent across the different pain modalities, suggesting that the results reflected true group differences, which was confirmed by a sensitivity analysis. Medication was not stopped in hyperekplexia patients for ethical reasons. As baseline medication used in hyperekplexia patients may increase pain thresholds, this may have affected the precision of the estimates and reduced the differences between the two groups. However, the conclusions of the study would not be affected. Anxiety and hypervigilance may be observed in hyperekplexia patients (Kar *et al.*, 2013), and these factors may contribute to altered quantitative sensory tests independent of the presence of hyperekplexia. However, data on the influence of these factors on quantitative sensory tests are not consistent, with several investigations showing no influence on measures of pain sensitivity and spinal nociceptive excitability (Neziri *et al.*, 2010; Rhudy *et al.*, 2011; Terry *et al.*, 2012; Biurrun Manresa *et al.*, 2013; Curatolo *et al.*, 2015). While minimizing observer bias by testing hyperekplexia patients and healthy controls by different investigators, this might have introduced inter-observer variability to the quantitative sensory test measurements. However, several studies have consistently reported satisfactory to excellent inter-observer reliability of quantitative sensory test measures, when performed in a standardized setting by trained investigators (Geber *et al.*, 2011; Nikolajsen *et al.*, 2011; Dyck *et al.*, 2014; O'Neill and O'Neill, 2015; Boland-Freitas *et al.*, 2016; Duffy *et al.*, 2017).

Conclusions

Using a model of human genetic disease, this study provided evidence that glycinergic transmission is important in human pain processing and that loss-of-function mutations in genes encoding for glycine receptors and glycine transporters cause gain-of-function in the pain system.

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Supplementary material

Supplementary material is available at *Brain* online.

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